RUSSIAN ACADEMY OF SCIENCES
SIBERIAN BRANCH

INSTITUTE OF CYTOLOGY AND GENETICS
LABORATORY OF THEORETICAL GENETICS

PROCEEDINGS OF THE THIRD INTERNATIONAL CONFERENCE ON BIOINFORMATICS OF GENOME REGULATION AND STRUCTURE

Volume 1

INTAS SECTION

BGRS' 2002
Novosibirsk, Russia
July 14 - 20, 2002

IC&G, Novosibirsk, 2002

International Program Committee

Nikolay Kolchanov, Institute of Cytology and Genetics, Novosibirsk, Russia (*Chairman of the Conference*)
Ralf Hofestadt, University of Bielefeld, Germany (*Co-Chairman of the Conference*)
Philip Bourne, SDSC, San-Diego, USA (*Co-Chairman of the Conference*)
Nickolai Alexandrov, Ceres Inc., Malibu, USA
Philipp Bucher, Swiss Institute for Experimental Cancer Research, Switzerland
Julio Collado-Vides, National University of Mexico, Mexico
Jim Fickett, AstraZeneca, Boston, USA
Paolo Frasconi, University of Florence, Firenze, Italy
Sergey Goncharov, Sobolev Institute of Mathematics, Novosibirsk, Russia
Igor Goryanin, GlaxoSmithKline, UK
Charlie Hodgman, GlaxoSmithKline, UK
Elza Khusnutdinova, Institute of Biochemistry and Genetics, Ufa Sci. Centre RAS (Ufa), Russia
Lev Kisselev, Engelhardt Institute of Molecular Biology, Moscow, Russia
Boris Kovalerchuk, Central Washington University (Ellensburg), USA
Luciano Milanesi, ITBA, Milan, Italy
John Reinitz, The University at Stony Brook, N.Y., USA
Akinori Sarai, RIKEN Tsukuba Life Science Center, Tsukuba, Japan
Ilya Shindyalov, San Diego Supercomputer Center, USA
Rustem Tchuraev, Institute of Biology, Ufa Sci. Centre RAS, Ufa, Russia
Masaru Tomita, Institute for Advanced Biosciences, Keio University, Japan
Edgar Wingender, GBF, Braunschweig, Germany
Nikolay Yankovsky, Institute of General Genetics, Moscow, Russia
Lev Zhivotovsky, Institute of General Genetics, Moscow, Russia

Local Organizing Committee

Dagmara Furman, Institute of Cytology and Genetics, Novosibirsk,
Nadya Omelianchuk, Institute of Cytology and Genetics, Novosibirsk,
Sergey Lavryushev, Institute of Cytology and Genetics, Novosibirsk,
Galina Kiseleva, Institute of Cytology and Genetics, Novosibirsk,
Elena Borovskikh , Institute of Cytology and Genetics, Novosibirsk,
Nikolay Shkel, Institute of Cytology and Genetics, Novosibirsk,
Andrey Kharkevich, Institute of Cytology and Genetics, Novosibirsk,

The information about the Conference BGRS' 2002 is presented at http://www.bionet.nsc.ru/meeting/bgrs2002/

Our sponsors

Organizers

Institute of Cytology and Genetics, SB RAS

Siberian Branch of the Russian Academy of Sciences

Grants

INTAS Conference Grant

Glaxo Wellcome Inc.

Russian Foundation for Basic Research

Ministry of Industry, Science and Technologies of the Russian Federation

Information sponsors

San Diego Supercomputer Center, United States

RIKEN Tsukuba Institute

Bielefeld University, Faculty of Technology

http://www.karger.com/

In Silico Biology

Others

KWESTA-group: computers, computer accessories, service

CONTENTS

INTRODUCTION

Four volumes of Proceedings of the Third International Conference on Bioinformatics of Genome Regulation and Structure – BGRS' 2002 (Akademgorodok, Novosibirsk, Russia, July 14-20, 2002) incorporate about 180 annotated extended abstracts (short papers) devoted to the actual problems in bioinformatics of genome regulation and structure.

The Conference BGRS' 2002 is organized by the Laboratory of Theoretical Genetics of the Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia. BGRS' 2002 is the third in the series. It will continue the traditions of the previous conferences, BGRS' 98 and BGRS' 2000, which were held in Novosibirsk in August 1998 and 2000, respectively.

As the greatest scientific event within the period between the Conferences BGRS' 2000 and BGRS' 2002, could be undoubtedly viewed the completion of human genome draft sequencing. This event has initiated the beginning of the post-genome era in biology. This era is characterized by sharp increase in research scale in the fields of transcriptomics, proteomics, and systemic biology (gene interaction, gene network functioning, signal transduction pathways), without loosing the fundamental interest to studying structural genome organization.

The structure and regulation of genome are the counterparts of life at molecular level; that is why understanding of fundamental principles of regulatory genomic machinery is impossible unless their structural organization is known, and *vice versa*.

The huge volume of experimental data that has been acquired on genome structure, functioning and gene expression regulation demonstrate the blistering growth. Development of informational-computational technologies of novel generation is a challenging problem of bioinformatics. Bioinformatics has entered that very phase of development, when decisions of the challenging problems determine the realization of large-scale experimental research projects directed to studying genome structure, function, and evolution.

By analyzing the papers submitted for publication in the four-volume issues of the BGRS' 2002, the Program Committee came to a conclusion that participants of the Conference have concentrated their attention at consideration of the hottest items in bioinformatics listed below: (i) regulatory genomic sequences: databases, knowledge bases, computer analysis, modelling and recognition; (ii) large-scale genome analysis and functional annotation; (iii) gene structure finding and prediction; (iv) comparative and evolutionary genomics; (v) computer analysis of genome polymorphism and evolution; computer analysis and modelling of transcription, splicing and translation; structural computational biology - genomic DNA, RNA and protein structural and functional organization; (vi) gene networks, signal transduction pathways and genetically controlled metabolic pathways: databases, knowledge bases, computer analysis, and modelling; principles of organization, functioning, and evolution (vii) data warehousing, Knowledge Discovery and Data Mining; (viii) analysis of fundamental regularities in genome functioning, organization, and evolution.

The researchers working in the fields of experimental biology are also invited to participate in the work of BGRS' 2002 in order to develop a sort of interface between experimental and computer-assisted researches in the fields of genomics, transcriptomics, proteomics, structural and systemic biology, as well as for contributing to promotion of computational biology to experimental research. These results are highlighted in the fourth volume of BGRS' 2002 Proceedings.

All the questions listed above will be suggested to consideration of participants of BGRS' 2002 at plenary lectures, oral communications, poster sessions, Internet computer demonstrations, and round-table discussions.

The Conference is sponsored by Siberian Branch of the Russian Academy of Sciences, by the Institute of Cytology and Genetics SB RAS, by Russian Foundation for Basic Research, by Russian Ministry of Industry, Science and Technologies, by the Company Glaxo Research and Development Limited, by independent International Association formed by the European Community INTAS. The Organizing Committee of the Conference tender thanks to all the sponsors for financial support.

Professor Nikolay Kolchanov
Head of Laboratory of Theoretical Genetics
Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia
Chairman of the Conference

Professor Phil Bourne
SDSC, San-Diego, USA
Co-Chairman of the Conference

Professor Ralf Hofestaedt
Faculty of Technology
Bioinformatics Department
University of Bielefeld, Germany
Co-Chairman of the Conference

Preface

INTAS is an independent International Association formed by the European Community, European Union's Member States and like minded countries acting to preserve and promote the valuable scientific potential of the New Independent States of the former Soviet Union (NIS) and INTAS Member States on the basis of mutual benefit so as to preserve the scientific potential of the NIS by the following activities:

joint research projects and networks covering all scientific fields: exact and natural sciences, economy, social and human sciences;

provision of fellowships to young NIS scientists ;

support to conferences, workshops and meetings;

infrastructure actions, aimed at facilitating research (e.g. the protection and support of unique databases, supply of scientific literature);

project monitoring, dissemination and valorisation of results.

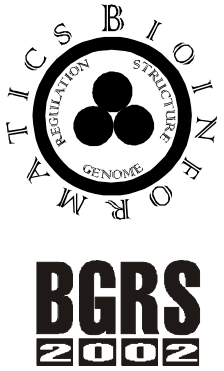
INTAS was one of the principal funding agencies for The Third International Conference on Bioinformatics of Genome Regulation and Structure (BGRS'2002; Novosibirsk, Russia, July 14 – 20, 2002).

A section "Fundamental genetic processes and mechanisms" reporting the latest results of the projects supported by INTAS contains 26 scientific reports on synthetic, physico-chemical and biological studies of sequence-specific conjugates as agents for the regulation of gene expression; enzymes and proteins of DNA replication and repair machinery, including coordination of base excision repair; mechanisms of oxidative stress and age-associated changes in the levels of oxidative stress markers; interactions of nucleic acids with immunoglobulins and other proteins, including cell surface proteins; DNA sequence analysis; molecular aspects of heat stress and adaptation, including hormone control; analysis of karyotype evolution in mammals by comparative chromosome painting; correlations between genetic markers and cultural differences in human populations, and phylogeography of mitochondrial lineages of human populations in former Soviet Union.

Professor Nikolay Kolchanov,
Head of Laboratory of Theoretical Genetics, Vice-Director of the Institute
of Cytology and Genetics, Novosibirsk, Russia

Ingmar Dirk Schmidt
INTAS, Scientific Officer, Belgium

Professor Georgy Nevinsky
Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk, Russia



FUNDAMENTAL GENETIC PROCESSES AND MECHANISMS

SYNTHESIS, PHYSICO-CHEMICAL AND BIOLOGICAL STUDIES OF SEQUENCE-SPECIFIC CONJUGATES AS THE AGENTS FOR REGULATION OF GENE EXPRESSION ON THE LEVEL OF DOUBLE-STRANDED DNA

[1,2]Sinyakov A.N., [3]Arimondo P.B., [3]Bailly C., [3]Sun J.-Sh., [3]Garestier T., [3]Boutorine A.S., [1,2]Ryabinin V.A., [3]Hélène C.

[1] State Research Center for Virology and Biotechnology "Vector", 630559, Kol'tsovo, Novosibirsk Region, Russia, e-mail: sinyakov@niboch.nsc.ru
[2] Institute of Bioorganic Chemistry, SB RAS, Novosibirsk, Russia
[3] Laboratoire de Biophysique, INSERM UR 565, CNRS UMR 8646, Muséum National d'Histoire Naturelle, 43 rue Cuvier, 75231 Paris Cedex 05
*Corresponding author

Key words: *minor groove binders, double-stranded DNA, sequence-specific conjugates*

Resume

The possibility is discussed of stabilizing a DNA triple helix by covalent conjugation to the third strand (through its terminal phosphate) of ligands that have affinity to double and triple helices. Two types ofstabilizers are considered: minor groove binders based on oligopyrroles, and triplex-specific intercalators. As a target, a synthetic 29-mer duplex containing a natural polypurine sequence of the human immunodeficiency provirus was employed. The stabilization with minor groove binders requires several conditions to be respected: a sufficiently long linker capable of reaching the minor groove from the major groove, a specific double-stranded structure of the oligopyrrole fragment, and its in-phase fitness to the target sequence. The best stabilizers of a triplex were novel conjugates in which two parallel molecules containing six pyrrole units each are linked to the same 5'-phosphate of a 16-mer triplex-forming oligonucleotide. The stabilizing properties of these derivatives were comparable to those of benzoindoloquinoline (BIQ) intercalators attached to the terminal phosphate of triple-helix forming oligonucleotides.

Conjugates of MGBs and topoisomerase inhibitor were used by us for construction of artificial nuclease. The two minor-groove binder/camptothecin (MGB-CPT) conjugates were synthesized. The covalent linkage of a MGB to the CPT chromophore exerts a drastic effect on the sequence specificity of DNA cleavage mediated by topoisomerase I. In the presence of MGB-CPT conjugates DNA cleavage was strongly enhanced at one site. Both conjugates are more efficient than free camptothecin.

Introduction

A strategy to design synthetic non-protein molecules that mediate a desirable biological response through its interaction with a specific DNA sequence is very attractive. Double-stranded DNA-sequence specific agents are expected to block transcription and/or replication. The principal advantage of transcription arrest is that transcription represents the first step in gene expression, and, therefore, may provide substantial leverage for drug therapy. Two classes of DNA sequence-specific binders are known actually: minor groove binders and synthetic oligonucleotides.

Minor groove binding ligands (MGB) are small oligomeric molecules that can bind to a minor groove of DNA in a sequence-specific manner. It is the only class of synthetic molecules that recognize predetermined DNA sequences with affinities and specificities comparable to DNA-binding proteins. A number of sequence-specific MGBs that contain N-methylpyrrole or N-methylimidazole units was developed and their DNA binding properties were studied by several laboratories.

The group of P.Dervan have developed hairpin oligocarboxamides with head-to-tail orientation which are capable of binding to mixed A,T,C,G-sequences. Simple rules have been developed to rationally control the sequence specificity of minor-groove binding polyamides containing pyrrole and imidazole amino acids. A pyrrole opposite an imidazole targets a C-G base pair whereas an imidazole opposite a pyrrole targets a G-C base pair. A pyrrole opposite pyrrole binds both A-T and T-A pairs. Addition 1-methyl-3-hydroxypyrrole to the repertoire of monomers allowed the discrimination of A-T from T-A pairs. A hydroxypyrrole opposite pyrrole recognizes a T-A base pair, whereas a pyrrole opposite a hydroxypyrrole binds A-T base pair.

Several groups demonstrated that MGB could inhibit gene expression in cell cultures by binding to the regulatory element, especially to a specific transcription factor-binding site within a promoter for a specific gene. Inhibition of transcription factor binding may interfere with the multiple protein complexes that are responsible for the recruitment of the RNA polymerase. Binding to the coding region, however, do not appear to inhibit gene expression. It seems that in such a case the complex is not stable enough to compete with RNA polymerases. Therefore it appears that more stable complexes of DNA with MGB must be constructed.

Synthetic oligodeoxynucleotides (TFO) have been shown to specifically bind to a major groove of target double-stranded DNA, *via* the formation of triple helices, and modulate in this way gene expression ("anti-gene" or triplex approach). Strategy based on triple helix formation is still limited to polypurine-rich target sequences. With several exceptions, triplexes exist at low pH (<6) and have low dissociation temperature (in majority of cases <37°) and short lifetimes.

Results and Discussion

The aim of this work is to improve the potency and the affinity of different sequence-specific DNA-binding agents by the combination of their capacities through chemical linking. Being covalently linked together, TFO and MGB are able to bind simultaneously to the major and the minor grooves of the target DNA and thus interfere with key cellular processes such as replication and transcription.

Conjugates containing two parallel or antiparallel MGB attached to the same terminal phosphate of one oligonucleotide strand were synthesized. It was demonstrated that the conjugates interact with their target DNA much stronger than individual components. Interaction of conjugated MGB with DNA was characterized on the model of short oligonucleotide duplexes with one strand containing either one hairpin or two single-stranded oligocarboxamide residues attached to the same terminal phosphate. Approximately the same level of stabilization was observed both due to attachment of one classic hairpin MGB and two parallel or antiparallel single-stranded MGB moieties: thermal denaturation temperature of the modified duplexes was about 40°C higher compared to that of unmodified ones. The role of structural factors such as the length of carboxamide moiety, pyrrole/imidazole contents, size of linker and presence of a terminal positive charge on the MGB residue was elucidated. The stabilization of duplex formation was sequence-specific.

Attachment of MGB to TFO increases affinity of the construction for double-stranded DNA and makes the interaction much less sensitive to pH, polypurine-polypyrimidine sequence of the target DNA and temperature. Our principal model was a 29-mer polypurine sequence of HIV proviral DNA from genes *nef* and *pol* and 16-mer TFO $T_4CT_4C_6T$. It was demonstrated that, in case of 1:1 TFO:MGB conjugate, interaction between conjugate and double-stranded target is sequence-specific both for MGB and TFO components, whereas for 1:2 TFO:MGB conjugate the DNA-MGB interaction plays a crucial role. Stability of complexes between target DNA and 1:2 conjugates is surprisingly high at elevated temperatures (>50°C) and high pH (8,3).

Conjugates of MGBs and topoisomerase inhibitor were used by us for construction of artificial nuclease.

Topoisomerase I unwinds DNA molecules by cleaving one DNA strands following by religation. In the presence of inhibitors, such as camptothecine, rebeccamycin, etoposide, the reaction could be stopped on the stage of cleavage. The main advantage of these inhibitors is that they are inert to other biological substances. They recruit intracellular enzyme topoisomerase that cuts the DNA molecule in their presence. In general, topoisomerase inhibitors are highly toxic because of their low specificity. Attachment of these molecules to highly sequence-specific DNA-binding agents would transform them into highly specific artificial nucleases that might be directed only to selected genes.

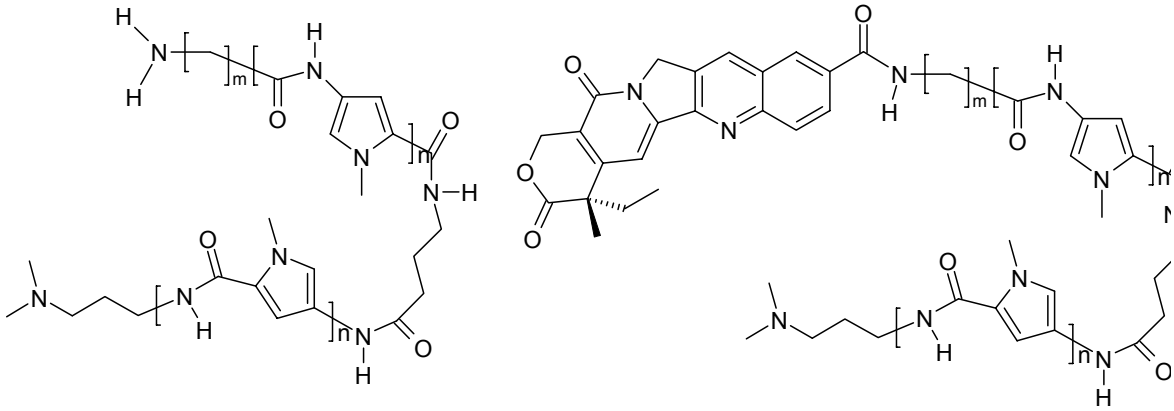
DNA cleavage by topoisomerase I can be targeted to specific sequences by the linkage of topoisomerase inhibitors to sequence-recognition elements, such as as oligonucleotides that form triple helices or small molecules related to the antibiotics netropsin and distamycin. The limited sequence-recognition properties of these antibiotics and analogues that form 1:1 drug:DNA complexes restrict their use as DNA-cleaving delivery systems. No sequence specificity was reported for cleavage by conjugates with the camptothecin derivatives. Here we present an alternative approach to direct camptothecin to specific sites in DNA and to introduce DNA breaks at a precise location by making use of the recognition of the target sequence by hairpin polyamide ligands that bind to the minor groove of DNA in sequence-specific manner. We have compared these conjugates to camptothecin that is covalently linked to triplex-forming oligonucleotides that bind in the major groove at oligopyrimidine-oligopurine sequences.

Two hairpin polyamides, each containing two series of three or four N-methylpyrrolecarboxamide units that form antiparallel dimers, were synthesized. Compounds 1 and 3 were linked to 10-carboxycamptothecin through aminoalkyl side chains of different lengths. The two minor-groove binder/camptothecin (MGB-CPT) conjugates 2 and 4 contain the same dimethylaminopropilamino cationic side chain on the C-terminal pyrrole heterocycle and an identical γ-aminobutyrate residue between the two oligopyrrole branches, but they differ in number of N-methylated pyrrole units (2 x 3 or 2 x 4 pyrrole rings) and the nature of alkyl connector (propyl in 2 or pentyl in 4) between the MGB moiety and the topoisomerase I inhibitor.

A quantative analysis of obtained data revealed that at low concentrations the hybride 4 was more efficient than hybrid 2 at directing cleavage to this site. This result consistent with the higher DNA binding affinity of the dimer of tetrapyrrole rings relative to the dimer of tripyrrole rings. Both conjugates 2 and 4 are more efficient than free camptothecin. The results reported here parallel those recently obtained with a CPT-conjugate of a triplex-forming oligonucleotide that bind to the

major groove of DNA and was shown to direct DNA cleavage mediated by topoisomerase I to the same site. So both approaches provide a suitable route to guide a cytotoxic agent to a selected sequence in DNA. The main advantage of MGB-CPT over TFO-CPT conjugates is that the binding of MGBs is not restricted to oligopyrimidine-oligopurine DNA sequences as observed with TFOs. In addition, MGBs are expected to be more cell permeable than TFOs and, therefore, might be more suitable for cell studies.

In summary, the covalent linkage of a hairpin polyamide to camptothecin provide an efficient system to direct DNA cleavage mediated by topoisomerase I to specific sites. Hairpin polyamide/ camptothecin conjugates represent a new category of single-strand-breaking artificial nucleases that could provide a bases for potential therapeutic applications.



1 n = 3; m = 3
3 n = 4; m =5

2 n = 3; m = 3
4 n = 4; m = 5

Fig. 1. Structure of hairpin polyamides and their conjugates with 10-carboxycamptothecin. The MGB-CPT conjugates were incubated with a 324 base pair ^{32}P-labeled DNA fragment prior to the addition of topoisomerase I to initiate the DNA clevage reaction. With camptothecin alone, the position of seven cleavage sites were determined. The covalent linkage of a MGB to the CPT chromophore exerts a drastic effect on the sequence specificity of DNA cleavage mediated by topoisomerase I. In the presence of MGB-CPT 2 or 4, DNA cleavage was strongly enhanced at one site, whereas cleavage at all other CPT-induced sites were strongly decreased.

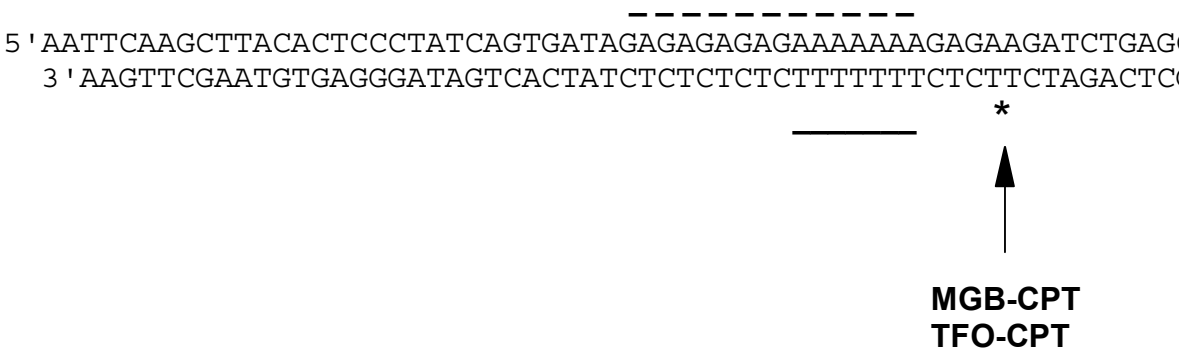


Fig. 2. Partial sequence of the DNA fragment used to locate the topoisomerase I cutting sites. The site selected by MGB-CPT and TFO-CPT conjugates is indicated by arrow. The positions of the MGB binding site was inferred from DNAase I footprinting experiments. The solid line indicate the binding site of MGB, the dotted line indicate the binding site of TFO.

Acknowledgements

The work was supported by the INTAS grant № 01-0638.

INTERACTION BETWEEN FLAP ENDONUCLEASE-1 AND REPLICATION PROTEIN A

Khodyreva S.N., Nazarkina J.K., Petruseva I.O., Safronov I.V., Lavrik O.I.

Novosibirsk Institute of Bioorganic Chemistry SB RAS, 630090, Novosibirsk, Russia, e-mail: svetakh@niboch.nsc.ru
*Corresponding author.

Key words: *flap endonuclease-1; replication protein A; base excision repair*

Resume

Motivation: Various DNA base damages are corrected by base excision repair (BER) pathway. Although interaction of proteins taking part in BER have been extensively investigated for the last decade the details of their interactions remain to be elucidated. In the present study we have investigated the effect of replication protein A (RPA) on the interaction of flap endonuclease 1 (FEN-1) with DNA containing flap-structures.

Results: The influence of RPA on the activity of FEN-1 was shown to depend on the flap length. The inhibitory effect of RPA was only revealed with the flap-length of 21 nucleotides but not with flap-length of 4- or 8-nucleotides. The data of photoaffinity modification and gel-retardation methods have demonstrated that RPA can form complex only with that flap-structure, single stranded part of which, is 21 nucleotides. Competition of RPA with FEN-1 for flap substrate appears to be the main cause of inhibition of FEN-1 activity.

Introduction

Various DNA-damaging agents produce modified bases in DNA that are repaired by the base excision repair (BER) pathway (Parikh et al., 1999). DNA glycosylases recognize the damaged bases and remove them. The repair of the resulting apurinic/apyrimidinic site (AP-site) involves the replacement of either a single nucleotide (short-patch BER) or several nucleotides (long-patch BER). AP endonucleases incise sugar-phosphate backbone immediately 5' to the AP site, generating a 3'-hydroxyl terminus and 5'-terminus with a deoxyribose phosphate (dRP) group. In long patch pathway resynthesis of DNA by DNA polymerase β or DNA polymerases δ or ε in complex with PCNA can displace dRP-containing strand to create a 5' flap. Flap structures are also intermediates of DNA replication and recombination. FEN-1 is the major enzyme of flap structure processing; it removes unannealed 5' flap as intact segment. Then DNA ligase joins resulting ends. The question about details of interactions between proteins acting in the long-patch pathway is still opened.

In the present study we have investigated the influence of RPA on FEN-1 activity. RPA, an abundant nuclear protein, is known to participate in DNA replication, repair and recombination interacting with single-stranded DNA. Data about influence of RPA on long-patch BER are contradictory. DeMott and colleagues reported [DeMott et al., 1998] that RPA stimulates long-patch BER in reconstituted system containing DNA polymerase β, DNA ligase and FEN-1. RPA stimulation of PCNA-dependent repair of AP sites in cell extract was also shown (Dianov et al., 1999). On the other side, it was reported (Stucki et al., 1998) that there was no effect of RPA on BER. If RPA does stimulate long patch BER, the mechanism of this stimulation is not clear. In any case, in these investigations the influence of RPA was analyzed on the level of the whole process and did not concern given enzyme.

Results and Discussion

It is known (Murante et al., 1995), that free 5'-strand of flap structure is required for the efficient recognition of a flap substrate by FEN-1. Indeed, the addition of SSB-protein to a system reduced the FEN-1 activity. We have investigated FEN-1 activity in the presence of RPA, eukaryotic single-stranded binding protein. RPA is known to associate with single-stranded DNA by two different ways (Blackwell et al., 1997). The so-called "8 nucleotides" binding type results in formation of DNA-protein complexes with the low affinity. In the second way molecule of RPA changing its conformation associates with approximately 30 nucleotides with the high affinity.

We proposed that influence of RPA on FEN-1 activity would depend on flap length since RPA could efficiently bind flap structures with the long single-stranded chain. We have used flap structures with different length of unannealed 5'-end. Using structure with the flap length of 21 nucleotides, we have found that increase of RPA concentration resulted in decreasing FEN-1 activity more than on 20% (Fig. 1, lanes 11-15). This effect appears to be a consequence of competition between RPA and FEN-1 for binding of single-stranded portion of flap structure. Indeed, according to the data of gel-retardation assay in the presence of 0.4 μM RPA the most of flap substrate is in the complex with RPA (Fig. 2, lane 4). As was shown (Biswas et al., 1997) yeast RPA could inhibit activity of RTH1 (homolog of FEN-1) by melting or destabilizing the DNA duplex. Our data obtained by polyacrylamide gel electrophoresis under nondenaturing conditions indicate that melting DNA duplex in our conditions does not occur.

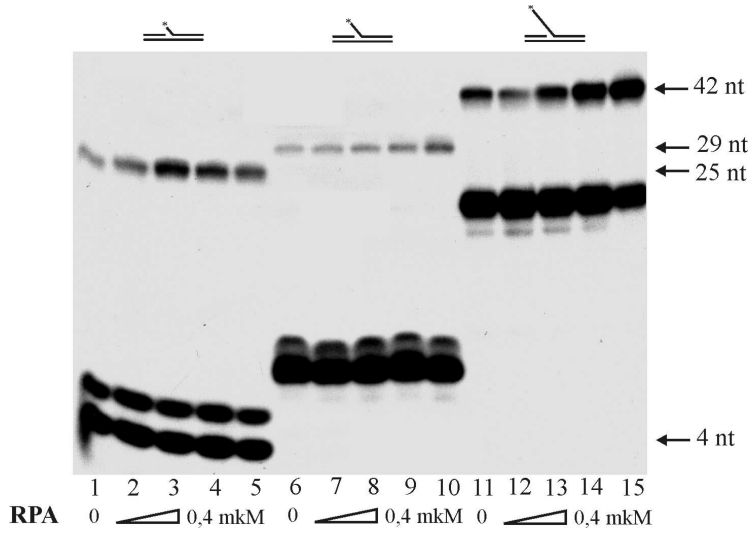


Fig. 1. The influence of RPA on the FEN-1 activity.

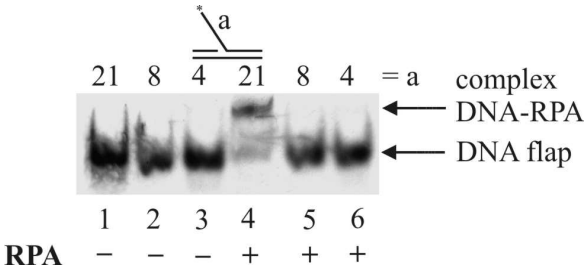


Fig. 2. Association RPA with DNA flap.

The analogous experiments were carried out using substrates with flap length of 8 and 4 nucleotides. Affinity of RPA to these structures must be low. The gel-retardation assay did not detect any complexes of DNA-RPA in that case. Inhibition of FEN-1 activity in the presence of RPA did not also occur for these substrates (Fig. 1, lanes 1-10). Therefore, RPA efficiently associates only with flap structures containing long single-stranded portion inhibiting the substrate cleavage by FEN-1. This selectivity in inhibitory action of RPA appears to be the determining factor of the length of patch in long-patch BER. An average patch-size was shown to be 6.6 nucleotides (Frosina et al., 1996). Displaced during DNA resynthesis the single-stranded chain of this length can be efficiently cleaved by FEN-1 whereas RPA cannot yet bind effectively and inhibit the cleavage.

To investigate the interactions of EN-1 and other proteins acting in long-patch BER pathway by the method of affinity modification flap structures containing photoreactive groups were designed. These structures contain the photoreactive groups in elbow point and has different size of flap. FEN-1 binds substrates with the highest stability to point of cleavage (Murante et al., 1995). DNA polymerases catalyzing stand-displacement synthesis interact with DNA near the point of cleavage.

The data of affinity labelling of RPA by photoreactive structures with different flap length demonstrate that efficiently RPA interacts only with 21 nucleotide-flap (Fig. 3, lanes 1, 4).

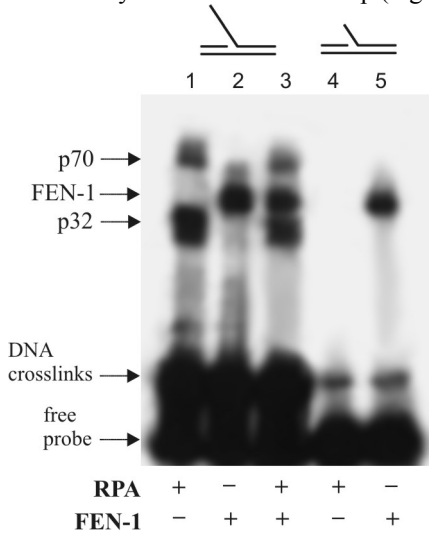


Fig. 3. Photoaffinity labelling FEN-1 and RPA.

No RPA labelling was observed with 8 nucleotide-flap structure. These data well agree with data obtained by gel-retardation assay (Fig. 2). FEN-1 was labeled by both 8- and 21 nucleotide-flap structures (Fig. 3, lanes 2, 5). When reaction mixture contains both RPA and FEN-1, decreasing intensities of appropriate bands have been observed, in comparison with labelling RPA or FEN-1 only. These results are evidence of competition RPA and FEN-1 for 21 nucleotides flap binding.

Obtained photoreactive flap structure being analogs of intermediates of long-patch base excision repair were shown to effective reagents for photoaffinity labelling of AP endonuclease, DNA polymerase β, poly(ADP-ribose) polymerase. We have used them to investigate interactions between proteins acting in the long-patch pathway.

Acknowledgements

This work was partially supported by RFBR grants № 01-04-48895, 02-04-48404, 01-04-48854 and by INTAS.

References

1. Biswas E.E., Zhu F.X., Biswas S.B. (1997) Stimulation of RTH1 nuclease of yeast Saccharomyces cerevisiae by replication protein A. Biochemistry. 36, 5955-5962.
2. Blackwell L.J., Borowiec J.A., Mastrangelo I.A. (1997) Single-stranded-DNA binding alters human replication protein A structure and facilitates interaction with DNA dependent protein kinase. Mol. Cell. Biol. 16(9), 4798-4807.
3. DeMott M.S., Zigman S., Bambara R.A. (1998) Replication protein A stimulates long patch DNA base excision repair. J. Biol. Chem. 42, 27492-27498.
4. Dianov G.L., Bente R.J., Kenny M.K., Bohr V.A. (1999) Replication protein A stimulates proliferating cell nuclear antigen-dependent repair of abasic sites in DNA by human cell extracts. Biochemistry. **38**, 11021-11025.
5. Frosina G., Fortini P., Rossi O., Carrozzino F., Raspaglio G., Cox L.S., Lane D.P., Abbondandolo A., Dogliotti E. (1996) Two pathways for base excision repair in mammalian cells. J. Biol. Chem. 16, 9573-9578.
6. Murante R.S., Rust L., Bambara R.A. (1995) Calf 5' to 3' exo/endonuclease must slide from a 5' end of the substrate to perform structure-specific cleavage. J. Biol. Chem. 270, 30377-30383.
7. Parikh S.S., Mol C.D., Hosfield D.J., Tainer J.A. (1999) Envisioning the molecular choreography of DNA base excision repair. Current option in structural biology. 9, 37-47.
8. Stucki M., Pascucci B., Parlanti E., Fortini P., Wilson S.N., Hubscher U., Dogliotti E. (1998) Oncogene. 17, 835-843.

AN ENZYMATIC SYNTHESIS OF 5'-END SUBSTITUTED OLIGONUCLEOTIDES USING T4 POLYNUCLEOTIDE KINASE AND γ-AMIDES OF ATP, BEARING PHOTOREACTIVE GROUPS

Petrousseva I.O., Safronov I.V., Komarova N.I., Kamynina T.P., Lavrik O.I., Khodyreva S.N.

Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia, e-mail: irapetru@niboch.nsc.ru
*Corresponding author

Key words: *T4 polynucleotide kinase; photoreactive ATP derivatives; 5'-end substituted photoreactive oligonucleotides, photoaffinity labelling*

Resume

Photoreactive derivatives of oligonucleotides containing arylazido groups at 5'-phosphate introduced by chemical synthesis extensively are used for the investigation of the components of DNA replication/repair systems by the methods of photoaffinity modification. We proposed that ATP derivatives bearing photoreactive substitutions at γ-phosphate might turn out substrates for T4 polynucleotide kinase (T4 PNK) and this allows introducing different groups into the 5'-OH end of oligonucleotides. A set of γ- phosphoamides of ATP was synthesized and shown to be substrates of this enzyme. Photoreactive oligonucleotide conjugate obtained by using of the γ-N- [-(4-azido-2,3,5,6-tetrafluorobenzoyl)-aminoethyl] amide ATP and 17-mer deoxyribooligonucleotide as substrates has been used for the photoaffinity labelling of human replication protein A (RPA) and flap endonuclease 1 (FEN 1). It can be proved that tested set of the γ-amides of ATP can be applied for the realization of the new enzymatic approach synthesis of 5'-end-phosphate substituted derivatives of ribo- and deoxyribooligonucleotides.

Introduction

It was shown for some enzymes of nucleic-acid-metabolism that substitution at γ-phosphate of don't (NTP) didn't suppress substrate properties of nucleoside triphosphate (Grachev et al., 1980; Arzumanov et al., 1996) Adenosine-5'-O- (3-thiophosphate) was successfully used for thiophosphorylation of 5'-OH-group of oligonucleotide using T4 PNK (Oshevski, 1982). We proposed that ATP derivatives bearing other substitutions at γ-phosphate might also turn out substrates for this enzyme. Our interest in such a tool is based on necessity to have a wide range of 5'-end modified oligonucleotides for investigation of the interaction of enzymes and factors of DNA replication and DNA repair with the targeted DNA structures.

Results and Discussion

γ-Phosphamides of ATP I-IV (Fig. 1) were shown to be substrates of T4 PNK.

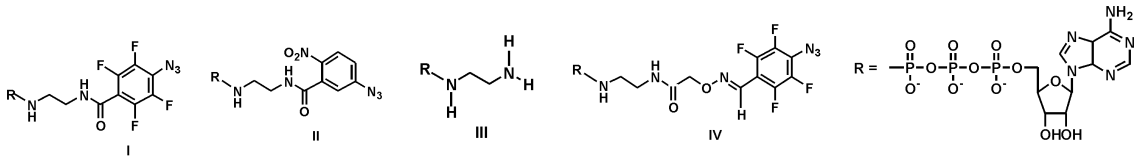


Fig. 1. Structural formulas of γ-amides ATP. I - γ-FAB-ATP, II - γ-NAB-ATP, III - γ- EDA-ATP, IV - γ-FABO-ATP.

A pattern of transfer of phosphoryl groups with different substitutions to 14-mer ribooligonucleotide is shown in Fig. 2. The choice of the concentrations of ATP derivatives and oligonucleotides for preliminary trials of new compounds in the T4 PNK catalyzed reaction is based on the known K_m values: 65 μM for ATP and 6.5 μM for 5'-OH-polynucleotide substrate (Sano, 1976; Nicols et al., 1978). The efficiency of transfer was shown to depend on the nature of modifying group and required different concentration of ATP analogs to achieve comparable levels of transfer.

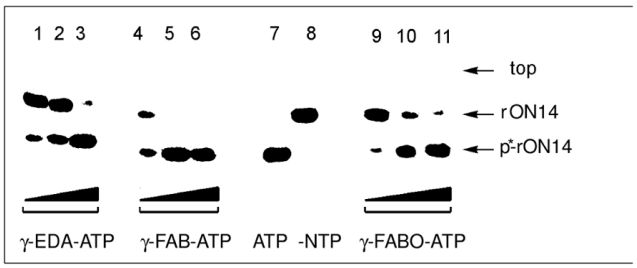


Fig. 2. Substrate properties of γ-amides of ATP in phosphorylation of rON14 catalyzed by T4 PNK. All reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ (buff. A), and 15 μM rON14. T4 PNK 2 U/μL. Lanes 1, 2, 3 contained 194 μM, 772 μM, 1544 μM γ-EDA-ATP; lanes 4, 5, 6 - 90 μM, 180 μM, 900 μM γ- FAB-ATP; lane 7 - 75 μM ATP; lane 8 - without NTP; lanes 9, 10, 11 - 33.3 μM, 166 μM and 332 μM γ-FABO-ATP. p*-rON14 designates phosphorylation product of rON14, which corresponds to the phosphoryl residue donor has been used. Reaction mixtures were incubated at 37°C for 30 min and analyzed by PAG electrophoresis under denaturing conditions followed by staining with "Stains all".

Panel A of Fig. 3 represents an example of chromatographic separation the reaction products on the column using multiwave detection and Panel B demonstrates the dependence of phosphorylation level of ON12 on the concentration γ-NAB-ATP.

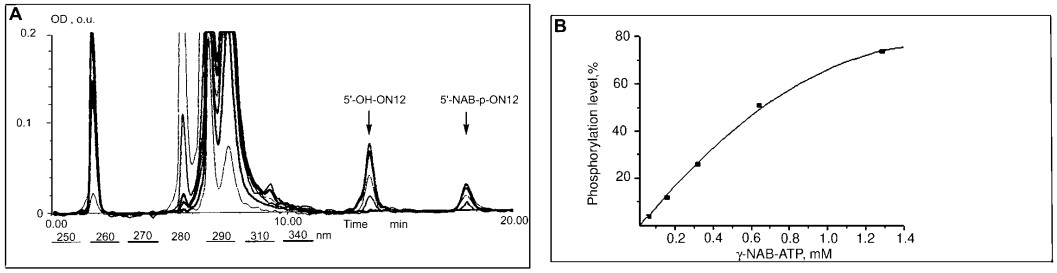


Fig. 3. Analysis of the T4 PNK-dependent phosphorylation product yield as a function of concentration of γ-NAB-ATP. (A) MLC separation of the reaction mixture containing T4PNK - 1.0 U/μL, 15μM ON12, 0.3 mM γ-NAB-ATP and T4PNK - 1.0 U/μL, in buf A after 30 min incubation. Conditions of chromatography: 70 μL Polysil SA column, linear gradient of potassium phosphate 0.0 - 0.3 M in 30% CH_3OH, pH 7.5 as eluent; rate 100 μL/min; multiwave detection. (B) Plot of the phosphorylation level of the ON12 versus the γ-NAB-ATP concentration. T4PNK - 1.0 U/μL.

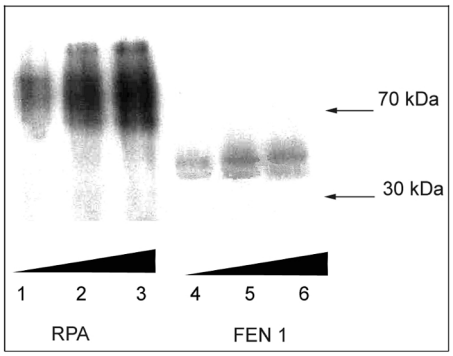


Fig. 4. Photoaffinity labelling of RPA and FEN 1 by 5'-FAB-p-ON17. Reaction mixture in buf. A contained 1.3 μM 3'-[^{32}P] photoreactive primer-template. (Radioactive labelling of photoreactive oligonucleotides was performed via incorporation of [^{32}P]dCMP into 3'-end of the primer by pol β. The reaction mixture in buf. A contained: 10 μM α^{32}[P] dCTP, 1.3 μM primer-template and 0.4 μM pol β. Reaction was continued at 37° C for 30-60 min afterwards reaction mixture was heated at 90°C for 10 min for denaturation of pol β, and then allowed to cool slowly to room temperature; denatured protein was discarded by centrifugation). RPA or FEN-1 were added to the reaction mixtures to final concentration 0.1 - 0.5 μM. Reaction mixtures were placed on ice and then irradiated for 15 min (λ> 280 nm). The products of UV-crosslinking were separated by SDS-PAGE and visualized by autoradiography. The positions of unmodified proteins (according to Coomassie staining) are indicated on the right.

Efficient transfer of modified phosphoryl groups requires higher concentrations of T4PNK than conventionally used with ATP. Bulky substituents at phosphate group may influence both K_m and V_{max} values. The radioactive photoreactive 5'-FAB-p-derivative of 17-mer oligonucleotide ON17 was used (in the form of duplex) as reagent for photoaffinity modification of two proteins involved in the DNA metabolism, human RPA and FEN 1. The fact of photoinduced labelling of the proteins by enzymatically synthesized 5'-derivative of oligonucleotide (Fig. 4) confirms the transfer of modified phosphoryl group and the integrity of photoreactive arylazido group after the purification procedure used. The difference in apparent molecular masses of the proteins products of their modification is about 8 kDa that corresponds the molecular mass of crosslinked oligonucleotide. RPA, the nuclear single-stranded DNA binding protein, is involved in DNA replication, nucleotide excision repair (NER) and homologous recombination. It is a stable heterotrimer consisting of subunits with molecular masses of 70, 32 and 14 kDa (p70, p32 and p14, respectively (Wold, 1997; Iftode et al., 1999). Using for affinity modification Of RPA realized in the labelling only subunit of the heterotrimer protein RPA (Fig. 4, lanes 1-3) lake the cases when chemically synthesized 5'-photoreactive oligonucleotides were used (Kolpashchikov et al., 2001; Khlimankov et al., 2001a) The higher the concentration of RPA in the reaction mixture the more intensive labelling was (compare lanes 1-3, Fig. 4). In contrast to pattern of RPA labelling the amount of FEN 1 modification product practically was not increased at higher FEN 1 concentrations (compare lanes 4-6, Fig. 4). The peculiarity of FEN 1 labelling appears to reflect faster removing the photoreactive nucleotide from 5'-end of the reagent via exonucleolytical degradation (Lieber, 1997) under the

increased enzyme concentration like the case described in (Khlimankov et al., 2001b) for the chemically synthesized 5'-derivative. The easiness, high yield of the goal product and the absence of the side products are the obvious advantages of the developed approach in comparison with chemical synthesis, especially in those cases when wide range of photoreactive oligonucleotides with different sequences is necessary.

Acknowledgements

This work was supported in part by a grant INTAS-96-1778, RFBR № 01-04-48895, 01-03-32439 and NATO Collaborative Linkage Grant-No 978233.

References

1. Arzumanov A.A., Semizarov D.G., Victorova L.S., Dyatkina N.B., Krayevsky A.A. (1996) Gamma-phosphate-substituted 2'-deoxynucleoside 5'-triphosphates as substrates for DNA polymerases. J. Biol. Chem. 271, 24389-24394.
2. Grachev M.A., Mustaev A.A., Oshevski S.I. (1980) A route to RNA with an alkylating group at the 5'-triphosphate residue. Nucl. Acids Res. 8, 3413-3426.
3. Iftode C., Daniely Y., Borowiec J.A. (1999) Replication protein A (RPA): the eukaryotic SSB. Crit. Rev. Biochem. Mol. Biol. 34, 141-180.
4. Khlimankov D.Iu., Rechkunova N.I., Kolpashchikov D.M., Petruseva I.O., Khodyreva S.N, Favre A., Lavrik O.I. (2001a) Interaction of human replication protein A with DNA-duplexes, containing gaps of varying sizes. Mol. Biol. (Mosk). 35, 827-835.
5. Khlimankov D.Y., Rechkunova N.I., Kolpashchikov D.M., Khodyreva S.N., Lavrik O.I. (2001b) Affinity labelling of flap-endonuclease FEN-1 by photoreactive DNAs. Biochemistry (Mosc.). 66, 733-739.
6. Kolpashchikov D.M., Khodyreva S.N., Khlimankov D.Y., Wold M.S., Favre A., Lavrik O.I. (2001) Polarity of human replication protein A binding to DNA. Nucl. Acids Res. 29, 373-379.
7. Lieber M.R. (1997) The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays. 19, 233-240.
8. Nicols B.P., Lindell T.D., Stellwagen E., Donelson J.E. (1978). A rapid purification of T4 polynucleotide kinase using Blue Dextran-Sepharose chromatography. Biochim. Biophys. Acta. 526, 410-417.
9. Oshevski S.I. (1982) Enzymatic transfer of ATP gamma S thiophosphate onto the 5'-hydroxyl of an oligonucleotide as a route to reactive oligonucleotide derivatives. FEBS Lett. 143, 119-223.
10. Sano H. (1976) Kinetic studies on the reaction catalyzed by polynucleotide kinase Wold M.S. (1997) Replication protein A: a heterometric, single-srande DNA-binding protein required for eukaryotic DNA metabolism. Annu. Rev. Biochem. 66, 61-92.

INTERACTIONS OF RPA AND FEN-1 WITH DNA STRUCTURES APPEARED IN DNA REPLICATION AND REPAIR

*[1]Khlimankov D.Yu., [1]Rechkunova N.I., [1,2]Khodyreva S.N., [1]Petruseva I.O., [2]Nazarkina J.K., [1]Belousova E.A., [1,2]Lavrik O.I.

[1] Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia
[2] Novosibirsk State University, 630090, Novosibirsk, Russia
e-mail: denship@niboch.nsc.ru
*Corresponding author

Key words: *affinity labelling, protein-DNA interaction, replication protein A, flap endonuclease-1, DNA replication and repair*

Resume

Motivation: To unravel the role of replication factor A (RPA) and flap-endonuclease-1 (FEN-1) in the various processes of DNA metabolism, we have investigated the interaction of RPA and FEN-1 with DNA structures, which mimic repair and replication intermediates likely to be found *in vivo*. Photocrosslinking has been used to analyse the interaction of RPA and FEN-1 subunits with the upstream-primer of nicked and flapped DNA.

Results: We examined interaction of RPA and FEN-1 with DNA structures containing nicks and flaps and influence of these proteins on the interaction of DNA polymerase β (Pol β) with DNAs. UV crosslinking and subsequent analysis showed that the p70 subunit mainly interacts with 3′-end of nick and flap. Intensity of protein labelling also depends on the kind of DNA intermediate including the presence of 5'-phosphate or 5'-tetrahydrofuran within nick or flap. DNA polymerase β labelling by the photoreactive DNA structures containing nick and flap was increased (up to three fold) in the presence of RPA and FEN-1. We also examined influence of RPA and FEN-1 on long patch BER strand-displacement synthesis catalyzed by Pol β. RPA alone and taking together with FEN-1 does not influence on strand-displacement synthesis, in contrast to FEN-1.

Introduction

The human single-stranded DNA (ssDNA) binding protein, replication protein A, is a stable heterotrimer (p70, p32, and p14) required during the initiation and elongation stages of DNA replication and it is involved in both excision DNA repair and homologous recombination processes. Mammalian flap endonuclease-1 is a structure-specific metalloenzyme that acts in processing of both the Okazaki fragments during lagging strand DNA synthesis and flap intermediates during DNA damage repair. Earlier photocrosslinking has been used to analyse the interaction of RPA and FEN-1 with the primer of a partial duplex DNA (Kolpashchikov et al., 2001, Khlimankov et al., 2001). In summary, the pattern of RPA subunit labelling in DNA structures with gap or template extension is determined by the polar RPA binding to ssDNA. Recently the same method has been used to analyse the interaction of RPA and FEN-1 with nicked and flapped DNA structures. We found that p70 subunit RPA predominantly interacts with 3'-end of nick and flap. But intensity strongly depend on the kind of DNA intermediate including the presence of 5'-phosphate or 5'-tetrahydrofuran within nick or flap.

Results and Discussion

To unravel the role of RPA and FEN-1 in the various processes of DNA metabolism, we have investigated interaction these proteins with DNA structures, which mimic intermediates likely to be found *in vivo*. Nicks and flaps being intermediates of many processes of DNA metabolism, including replication and repair. Spontaneous damages of DNA induced by different factors are improved by the base excision repair system (BER). The mammalian cells having two sub-pathways of BER: single nucleotide resynthesis depends on Pol β (short patch BER), and 2-15 nucleotide repair (long patch BER) processed by Pol β and (or) other DNA polymerases (Frosina et al., 1996).

We used FAP-dUTP containing N-(3-chlorine-4-azido-2,5-difluoropyridine (FAP) moiety) as photoreactive analogue (Fig. 1D). This dNTP analogue are shown to be good substrate for DNA polymerases (Kolpashchikov et al., 1999) including DNA polymerase β. Photoreactive moiety was introduced into the 3′-end of the nick and flap by the activity of Pol β (Fig. 1A, B). Photoreactive nicks and flaps were used to examined interaction of RPA and FEN-1 with DNA structures arising at different steps of long patch BER and influence of these proteins on the interaction of Pol β with DNAs. UV crosslinking and subsequent analysis showed that the p70 subunit mainly interacts with 3′-end of DNA nick and flap. Intensity of protein labelling depends on the kind of DNA intermediate including the presence of 5'-phosphate or 5'-tetrahydrofuran within nick or flap (Fig. 2A, 3A). DNA polymerase β labelling by the photoreactive DNA structures containing nick and flap was increased (up to three fold) in the presence of RPA and FEN-1 (Fig. 2B) In contrast to these

data, affinity labelling of RPA, FEN-1 and Pol β by the structures containing gaps was shown earlier to demonstrate competitive character (Kolpashchikov et al., 2001, Khlimankov et al., 2001).

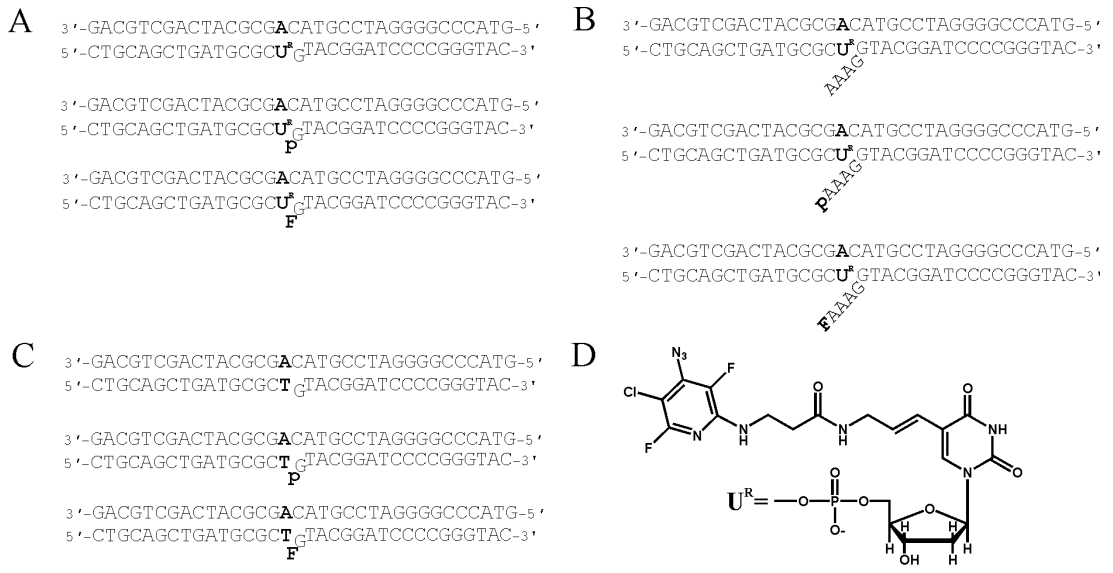


Fig. 1. Structure of the photoreactive dNTP analog and oligonucleotide DNAs.

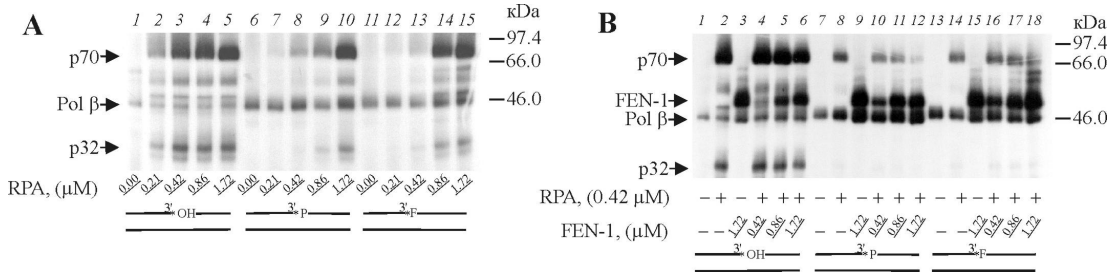


Fig. 2. Affinity labelling RPA, Pol β (A) and RPA, Pol β and FEN-1 (B) by 3'-photoreactive nicks.

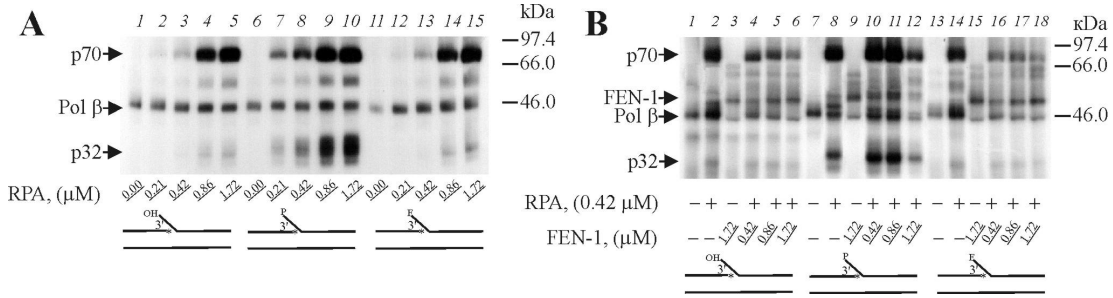


Fig. 3. Affinity labelling RPA, Pol β (A) and RPA, Pol β and FEN-1 (B) by 3'-photoreactive flaps.

Apparently, increasing affinity labelling of Pol β in presence RPA and FEN-1, indicates a functional cooperation between these proteins. To further address the role Pol β in long-patch BER and it potential combined action with RPA and FEN-1, we used oligonucleotide DNA substrates (Fig. 1C) to reconstitute system of BER. We examined influence of RPA and FEN-1 on long patch BER strand-displacement synthesis catalyzed by Pol β. RPA alone and taking together with FEN-1 does not influence on strand-displacement synthesis, in contrast to FEN-1 (Fig. 4).

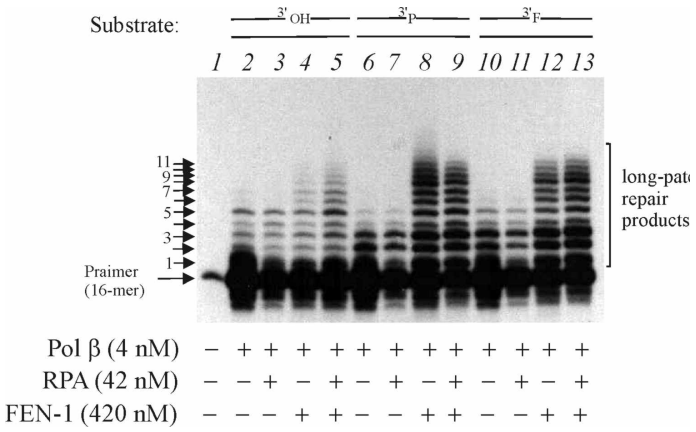


Fig. 4. Influence of RPA and FEN-1 on strand-displacement synthesis catalyzed by Pol β.

Base on the date obtained we propose that RPA and FEN-1 interacts with structures arising during processes replication and excision repair. At the same time RPA was not stimulate Pol β strand-displacement synthesis DNA in the course of long-patch BER. Consequently RPA do not play crucial role in this process and rather do not influence on repair resynthesis DNA induced by activity of DNA polymerase β. Since replication factor A is main factor of DNA replication it is possible to propose when replication process is blocked DNA repair runs independently. What the function of RPA in Pol β-dependent repair steps and mechanism of RPA and its subunits interaction with the BER proteins demand following study.

Acknowledgements

This work was supported by grant INTAS (96-1778).

References

1. Frosina G., Fortini P., Rossi O., Carrozzino F., Raspaglio G., Cox L.S., Lane D.P., Abbondandolo A., Dogliotti E. (1996) Two pathways for base excision repair in mammalian cells. J. Biol. Chem. 271, 9573-9578.
2. Khlimankov D.Yu., Rechkunova N.I., Kolpashchikov D.M, Khodyreva S.N., Lavrik O.I. (2001) Affinity labelling of flap-endonuclease FEN-1 by photoreactive DNAs. Biochemistry (Mosk). 66, 905-912.
3. Kolpashchikov D.M., Khodyreva S.N., Khlimankov D.Yu., Wold M.S., Favre A., Lavrik O.I. (2001) Polarity of human replication protein A binding to DNA. Nucl. Acids Res. 29, 373-379.
4. Kolpashchikov D.M., Zakharenko A.L., Dezhurov S.V., Rechkunova N.I., Khodyreva S.N., Degtiarev S.Kh., Litvak V.V., Lavrik O.I. (1999) New reagents for affinity modification of biopolymers. Photoaffinity modification of Tte-DNA polymerase. Bioorg Khim. 25, 129-136.

SITE-SPECIFIC PHOTOMODIFICATION OF MAMMALIAN POLY(ADP-RIBOSE) POLYMERASE-1 WITH PHOTOREACTIVE DNA BASE EXCISION REPAIR INTERMEDIATE

Sukhanova M.V., Lavrik O.I., Safronov I.V., Khodyreva S.N.

Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia, e-mail: Mary@niboch.nsc.ru
*Corresponding author

Key words: *poly(ADP-ribose) polymerase, photoaffinity modification, BER*

Resume

Motivation: Poly(ADP-ribose) polymerase-1 (PARP-1) is a zinc-finger DNA binding protein that detects and signals DNA strand breaks generated directly or indirectly by genotoxity agents. In response to these lesions, the immediate poly(ADP-ribosyl)ation of nuclear proteins convents DNA interruption into intracellular signals that activate DNA repair. Under physiological conditions and limited DNA damage, PARP-1 plays a role as a survival factor allowing the activation of DNA repair pathways through recruitment of the base excision repair (BER) complex. In this study we examined the interaction between PARP-1 and BER intermediate DNA.

Results: Site-specific photomodification of the nicked DNA-PARP-1 complexes has been carried out with photoreactive DNA probe *in situ*.

Introduction

Base excision repair (BER) is major pathway to repair abasic (AP) sites, which can be generated by spontaneous and induced loss of bases or as a result of removing a specifically modified base by DNA-N-glycosylases. Recent studies indicate that AP site repair in higher eukaryotes may proceed by either one of two alternative pathways: "short patch" and "long patch" BER pathway (Frosina et al., 1996). The first step in both pathways is the incision of the phosphate backbone immediately 5' to the AP site by AP endonuclease (APE), generating a 3'-hydroxyl terminus and 5'-terminus with a deoxyribose phosphate (dRP) group. In short patch BER pathway DNA polymerase β (β-pol) fills the single nucleotide gap and excises the 5' dRP residue. In mammalian cells, repair of methylated bases, oxidized bases, and abasic sites, appears to occur predominantly by this sub-pathway (Dianov et al., 1999). In other cases, for example when the sugar of abasic sites is removed not efficiently, the "long patch" BER sub-pathway mediates repair (Prasad et al., 2001). This sub-way involves limited strand displacement and DNA synthesis to replace 2 to 15 nucleotides in the damage strand. The DNA synthesis step of "long patch" BER can be conducted by β-pol in cooperation with FEN-1 or other DNA polymerase δ/ε (PCNA-dependent). The various steps in single nucleotide and long patch BER may be coordinated via protein-protein and DNA–protein interactions. Bimolecular complexes have been observed for X-ray cross-complementing factor (XRCC1) and DNA ligase III and for DNA polymerase β with following components: APE, PARP-1 and DNA ligase I (Dantzer et al., 1999). PARP-1 was proposed to be a DNA-damage signaling enzyme involved in BER. By which mechanism is PARP-1 involved in BER? PARP-1 has been supposed to play a role of nick sensor in this repair complex which in turn recruits BER proteins to the site of damage to facilitate the repair process, or as a factor acting directly on the activities of these repair enzymes through poly(ADP-ribosyl)ation (D'Amours et al., 1999).

Methods

To examine the interaction of PARP-1 with DNA intermediates formed upon BER, we used photoaffinity labelling DNA probe. The probe was formed *in situ*, using an end-labeled oligonucleotide containing a synthetic abasic site. This site was incised by apurinic/apyrimidinic endonuclease (APE) creating a nick with 3'-hydroxyl and 5'-sugar phosphate groups at the ends, and then dCTP derivatives bearing in the *exo-N*-position of cytosine a photoreactive group was added to the 3'-hydroxyl group. The photoaffinity labelling reaction mixture contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.01 μM [^{32}P]-labeled photoreactive DNA, 0.1 μM PARP-1, 1 μM DNA polymerase β, and 0.1 mM NAD^+ (when needed).

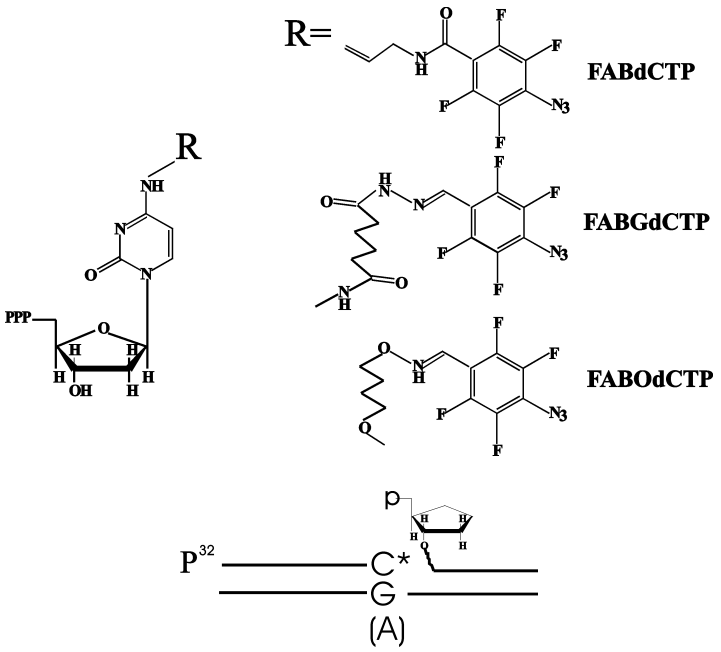


Fig. 1. Structure of the photoreactive dCTP analogs (FAB-dCTP, FABO-dCTP, FABG-dCTP) and photoreactive DNA probe.

Site-specific photomodification within PARP-1 – photoreactive DNA complexes was carried out when exposed to UV irradiation at 303-312 nm. The UV cross-linked products were separated by SDS-PAGE and visualized by autoradiography. The photocrosslinking efficiency of PARP-1 was estimated for each photoreactive DNAs.

Results and Discussion

Interaction of PARP-1 with DNA duplexes carrying single-stranded breaks was investigated in cellular extracts and in reconstituted system (Lavrik et al., 2001). To determine the interaction of PARP-1 with BER intermediate we used photoreactive DNAs. The structure of the photoreactive nicked DNA is shown in Fig. 1. The photoreactive dCTP analogs (FAB-dCTP, FABO-dCTP, FABG-dCTP) and [^{32} P]-5'-end-labeled 34-base pair oligonucleotide containing a synthetic abasic site 3-hydroxy-2-hydroxymethyltetrahydrofuran (THF) 5'-phosphate opposite dG or dA, were used to produce the photoreactive BER intermediate by a system reconstituted of purified APE and DNA polymerase β. The arylazido moiety in the photoreactive group can be activated with near (>300 nm) UV light. The abasic-site containing DNA was cleaved by APE and photoreactive FAB-dCMP (FABO-dCMP, FABG-dCMP) residues were introduced into the resulting 3'-hydroxyl group by DNA polymerase β *in vitro*. This process resulted in formation of photoreactive nick containing the THF-phosphate moiety at the 5'-end and the dCMP analog at the 3'-end. Replacement of natural deoxyribose by THF leads to inability of β-pol to cleave dRP-group (Lavrik et al., 2001) that allows to regard this photoreactive DNAs as intermediates of "long patch" BER.

Figure 2 shows efficiency of photocrosslinking of PARP-1 with photoreactive DNAs. The photoreactive DNA probe with FABO-dCMP provided the maximum photocrosslinking of DNA to PARP-1 (4.5%). The lowest efficiency of photocrosslinking was observed for the photoreactive DNA with FABG-dCMP (1.5%). Efficiency of photomodification of PARP-1 increased by 1-1.5% in the presence of DNA polymerase β for photoreactive nicked DNA containing dCMP analogs opposite dG and by 0.5% for dCMP analogs opposite dA. Pre-incubation photoaffinity labelling reaction mixture with NAD^+ before the UV light resulted in disappearance the cross-linking product of PARP-1 and decrease the labelling of DNA polymerase β. PARP-1 as well as DNA-polymerase β becomes poly(ADP-ribozyl)ated and the enzymatic activity of the last is inhibited.

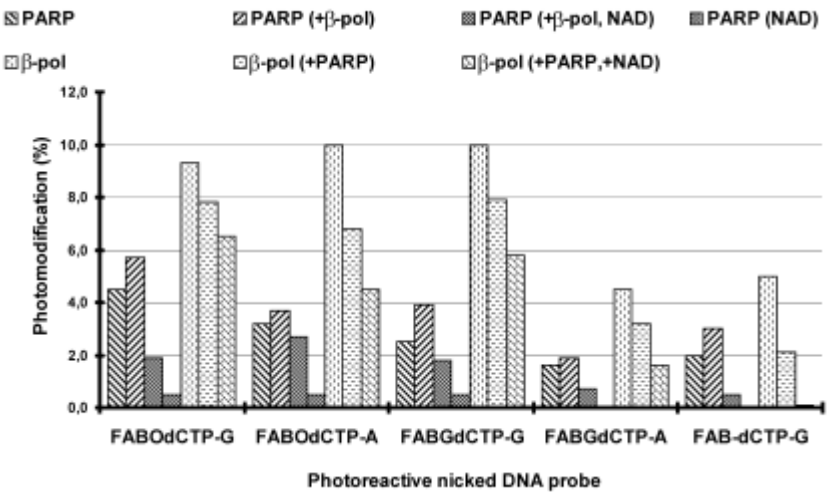


Fig. 2. Photocrosslinking efficiency (%) of PARP-1 and DNA polymerase β with photoreactive nicked DNA.

The function of PARP-1 in base excision repair was not obvious for a long time. In response to DNA damage PARP-1 binds to DNA strand breaks and catalyzes the transfer of ADP-ribose moiety from its substrate NAD^+ to a limited number of protein acceptors involved in chromatin architecture and DNA metabolism (replication, reparation) including the enzyme itself (D'Amours et al., 1999). ADP-ribosylated proteins, mainly DNA-binding proteins lose their affinity for DNA and consequently are rapidly inactivated. Therefore, poly(ADP-ribosyl)ation is an immediate post-translation modification of nuclear DNA-binding proteins, induced by DNA damaging agents. Thus PARP-1 could be involved in several processes: facilitating or accelerating the repair process, either by recruiting the repair enzyme (DNA polymerase β) and/or by acting directly on their activities (XRCC1 and DNA polymerase β are poly(ADP-ribosyl)ated *in vitro* (Masson et al., 1998; Ohash et al., 1986). DNA polymerase β was shown to be required for the repair of AP sites via "long patch" sub-way conducting strand displacement DNA synthesis (Dianov et al., 1999). The fact that "long patch" repair is affected in the absence PARP-1 (Dantzer et al., 2000) suggests that the protein could also be involved in strand displacement especially when gap filling is performed by the low fidelity DNA polymerase β.

Acknowledgements

This work was partially supported by RFBR grants № 01-04-48895, 02-04-48404, 01-03-32439, and by INTAS.

References

1. D'Amours D., Desnoyers S., Poirier G.G. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear function. Biochem. J. 342, 249-268.
2. Dantzer F., Schreiber V., Niedergant C., Trusso C., Flatter E., de la Rubia G., Oliver J., Rolli V., Menissier-de Murcia J., de Murcia G. (1999) Involvement of poly(ADP-ribose) polymerase in base excision repair. Biochimie. 81, 69-75.
3. Dantzer F., de la Rubia G, Menissier-de Murcia J., Hostomsky Z., de Murcia G., Schreiber V. (2000) Base excision repair is impaired in mammalian cells lacking poly(ADP-ribose) polymerase-1. Biochemistry. 39, 3559-7569.
4. Dianov G.L., Prasad R., Wilson S.H., Bohr V.A. (1999) Role of DNA polymerase β in the excision step of long patch mammalian base excision repair. J. Biol. Chem. 274, 13741-13743.
5. Frosina G., Fortini P., Rossit O., Raspaglion G., Cox L.S., Lane D.P., Abbondanolo A., Dogliotti E. (1996) Two pathways for base excision repair in mammalian cells. J. Biol. Chem. 271, 9573-9578.
6. Lavrik O.I., Prasad R., Sobol R.W., Horton J.K., Ackerman E.J., Wilson S.H. (2001) Photoaffinity labelling of mouse fibroblast enzymes by a base excision repair intermediate. J. Biol. Chem. 276, 25541-25548.
7. Masson M., Niedergang C., Schreiber V., Muller S., Menissier-de Murcia J., de Murcia G. (1998) XRCC1 Is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. Mol. Cell. Biol. 18, 3563-3571.
8. Ohashi Y., Itaya A., Tanaka Y., Yashihara K., Kamiya T., Matsukage A. (1986) Poly(ADP-ribosyl)ation of DNA polymerase β *in vitro.* Biochem. Biophys. Res. Commun. 140, 666-673.
9. Prasad R., Lavrik O.I., Kim S-J., Kedar P., Yang X-P., Berg B.J.V., Wilson S.H. (2001) DNA polymerase β – mediated long patch base excision repair. J. Biol. Chem. 86, 32411-32414.

BINARY SYSTEM OF PHOTOAFFINITY REAGENTS FOR SELECTIVE LABELLING OF DNA POLYMERASES IN NUCLEAR EXTRACT

[1,2]Lebedeva N.A., [1]Rechkunova N.I., [1]Khodyreva S.N., [2]Favre A., [1,2]Lavrik O.I.

[1] Institute of Bioorganic Chemistry SB RAS, 630090, Novosibirsk, Russia, e-mail: nataleb@niboch.nsc.ru
[2] Institut Jacques Monod, Paris, France
*Corresponding author

Key words: *affinity labelling, DNA polymerase, dNTP analogs, base excision repair*

Resume

Motivation: Application a new method of selective labelling of DNA polymerases interacting with the branch point intermediate of BER in crude systems such as nuclear and cellular extracts is necessary to understand of mechanism of protein-DNA and protein-protein interactions inside DNA replicative and DNA repair machinery.

Results: A binary system of photoaffinity reagents for selective affinity labelling of DNA polymerases has been developed. The photoreactive probe was formed in bovine testis nuclear extract, using an end-labeled oligonucleotide containing a synthetic abasic site. The photoreactive group was then activated, either directly (UV light exposure at 320 nm) or through energy transfer from a pyrene residue in the presence of a dTTP analog containing a pyrene group (Pyr-dUTP) under UV light exposure (λ = 365 nm). DNA polymerase β was the main target crosslinked by photoreactive BER intermediates in this nuclear extract. In contrast, several proteins were labelled under the conditions of direct activation of arylazidogroup.

Introduction

Affinity labelling method is useful for studying of molecular organization of protein ensembles (Meisenheimer et al., 1997, Knorre et al., 1998). This method has been used recently to identify proteins interacting with photoreactive base excision repair intermediate in a cellular extract of mouse embryonic fibroblasts (Lavrik et al., 2001). In view of the expanding discovery of new DNA polymerases (Grachev et al., 1996), it seems useful to have approach to identify DNA polymerases among other proteins in a crude cellular or nuclear extracts. One way to do so is through selective affinity labelling of proteins. Recently a binary system of photoaffinity reagents was developed and used for selective labelling of DNA polymerases in a reconstituted system (Kolpashchikov et al., 1999; Rechkunova et al., 2000).

Here we are adopting it to carry out selective labelling of DNA polymerases involved in BER in mammalian nuclear extracts. In the present work, we examined the question of whether proteins in nuclear extract can be selectively labeled by a photoaffinity DNA probes representing intermediates in long patch BER. We found that only a few proteins in the extract were strongly labeled by this BER probe. Then photoreactive BER intermediates were combined with Pyr-dUTP as photosensitizer. DNA polymerase β was specifically crosslinked in bovine testis nuclear extract using this binary photolabelling system.

Results and Discussion

We used FAB-dCTP and FAB-ddUTP containing (2,3,5,6-tetrafluoro-4-azidobenzoyl (FAB) moiety) as photoreactive analogues (Fig. 1). A photoreactive moiety was introduced into the 3'-margin of a gap by DNA polymerases of the nuclear extract, thus creating the photoreactive BER intermediate.

Activation of the arylazido group carried by DNA intermediates by UV-light (λ= 320 nm) caused several proteins to be crosslinked in nuclear extract. However crosslinking pattern shows already remarkable specificity. For example, only five crosslinked products have been found in nuclear extract. Therefore, nuclear proteins interacting preferentially with a photoreactive BER intermediate can be selected from the proteins in the crude nuclear extract.

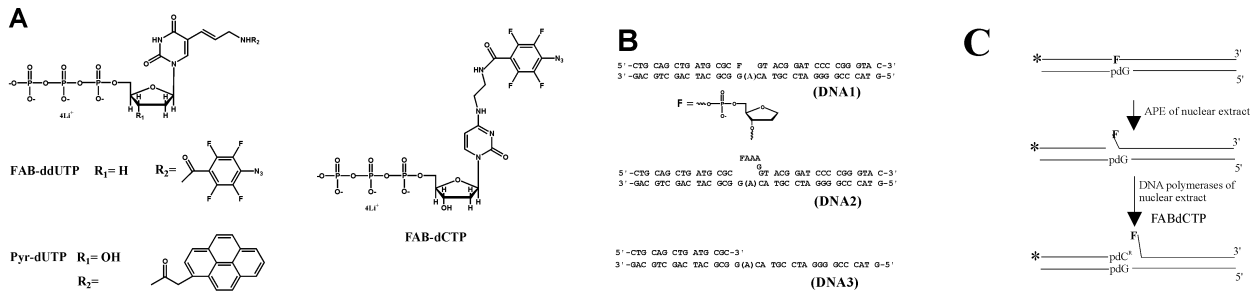


Fig. 1. Structure of the photoreactive dNTP analogs and oligonucleotide DNAs. A, The photoreactive dNTP analogs. B, 34-base pair oligonucleotide containing a synthetic abasic site (3-hydroxy-2-hydroxymethyltetrahydrofuran (THF) 5'-phosphate) opposite G or A (DNA1), 34 mer DNA duplex containing flap structure (DNA2) and DNA duplex with the 3'-protruding template strand (DNA3). **C,** Scheme of photoreactive moiety introduction into the nick raised in DNA repair process. The synthetic abasic site of DNA1 (THF) is incised by AP-endonuclease (APE). DNA polymerases in the nuclear extract incorporate a FAB-dCMP moiety opposite dG or a FAB-ddUMP moiety opposite dA.

To identify DNA polymerases interacting with photoreactive BER intermediates in bovine testis nuclear extract we have applied the binary system of photoaffinity reagents (Fig. 2). When reaction mixtures containing nick-DNA and flap-DNA and photoreactive dNTP analogues were supplemented with Pyr-dUTP and irradiated with light (365 nm), the predominant crosslinked product migrates as a band with molecular mass around 46 kDa and corresponds to β-pol. This probably reflects the high efficiency of β-pol interaction with photoreactive BER intermediates in nuclear extract. In the absence of Pyr-dUTP or in the presence pyrenebutyric acid under irradiation by UV light (λ= 365 nm) crosslinking products were poorly detected that proves the specificity of crosslinking of DNA polymerases sensitized by Pyr-dUTP.

BER proteins interacting with the similar photoreactive BER intermediates in cellular extract of mouse embryonic fibroblasts were identified recently by immunoprecipitation. Poly(ADP-ribose) polymerase (PARP-1), DNA polymerase β, flap endonuclease-1 and apurinic/apirimidinic endonuclease were identified as proteins crosslinked to photoreactive BER intermediate (Lavrik et al., 2001).

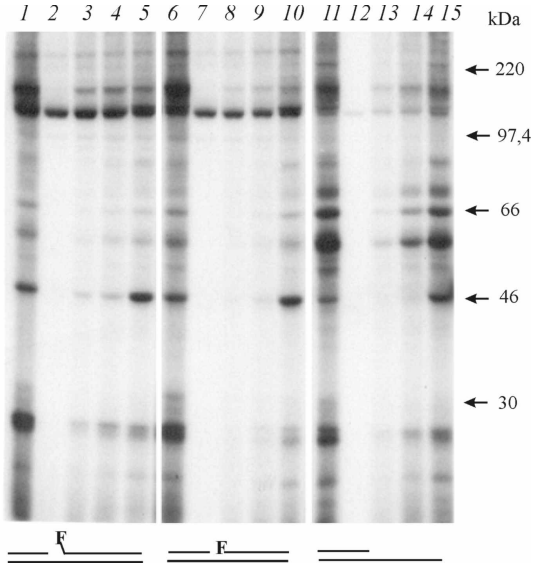


Fig. 2. Photoaffinity labelling of nuclear proteins by different photoreactive DNAs. Photoreactive ddUMP moiety was introduced into flap-DNA1 (lanes 1-5), nick-DNA2 (lanes 6-10) and DNA3 (lanes11-15) by the activity of nuclear DNA polymerase. Lanes 2, 7, 12 demonstrates control reaction without FAB-ddUTP. Reaction mixtures were irradiated by UV-light 320 nm (direct labelling lanes 1, 2, 6, 7, 11, 12), and 365 nm in the presence of Pyr-dUTP (sensitized labelling lanes 5, 10, 15) or in absence of Pyr-dUTP (lanes 3, 8, 13), or in the presence of 1-pyrenbutiric acid (lanes 4, 9, 14). The UV-crosslinked products were separated by SDS-PAGE and visualized by autoradiography. The positions of identified proteins and protein markers are indicated.

These data speak in favor of significant selectivity of photoaffinity labelling procedure used in the experiments. Therefore we could suggest that the similar proteins could interact with this BER intermediate in bovine testis nuclear extract. Purified recombinant β-pol, APE, FEN-1 and PARP-1 were extra added to nuclear extract after primer elongation before UV-irradiation (λ=320 nm). An increase in labelling was observed for products migrating at 42, 45, 52 and 120 kDa, respectively. Overall, these results indicate that FEN-1, APE, β-pol and PARP-1 can be labeled by the probes when added to the nuclear extract individually and that these purified proteins can bind the probe and compete with other proteins in the extract. The identity of β-pol was proved by immunoprecipitation experiments using specific antibodies. Another proteins of DNA repair machinery (RPA, DNA ligase I) were also added to the reaction mixture. However observed mobility of the crosslinked products in that case was remarkably different from the mobility of products identified in nuclear extracts.

Next, we conducted experiments to prove identification of the protein responsible for the higher molecular mass product, which was the major labelling product. A likely candidate is PARP-1. This notion was further examined by adding NAD^+ to

the reaction mixture. The premise of this experiment was that in the presence of NAD^+, PARP-1 will become auto-poly(ADP-rybosyl)ated. Therefore the gel mobility of PARP-1 will change. When reaction mixtures were supplemented with NAD^+ before the UV light crosslinking, this higher molecular mass labeled product was not observed, and a significant amount of labeled protein failed to enter the gel. Labelling of FEN-1, APE and β-pol was not altered by the addition of NAD^+. Thus, the protein responsible for the higher molecular mass product appeared to be poly(ADP-ribosyl)ated. The identity of this crosslinked product and PARP-1 was proved by using specific antibodies against PARP-1. It was shown that PARP-1 was a main target of labelling by the photoreactive BER intermediate used as a probe in the experiments with cellullar extracts of mouse embryonic fibroblasts (Lavrik et al., 2001) and PARP-1 acts as activating factor for long-patch BER synthesis (Prasad et al., 2001).

Taken together all results demonstrate that only few proteins can be crosslinked to photoreactive BER intermediates in nuclear extracts of bovine testis under irradiation by UV-light (λ=320 nm) and β-pol is one of the main crosslinking target for photoreactive BER intermediate under irradiation by UV light (λ=365 nm) in the presence of Pyr-dUTP.

Acknowledgements

We thank Dmitry Kolpashchikov and Igor Safronov for synthesis of photoreactive dNTP derivatives. This work was supported by grants from the Russian Foundation for Basic Research (00-04-49309, 00-04-22002, 02-04-48404, 01-04-48895) and by INTAS grant YSF-00-83.

References

1. Grachev M.A., Mustaev A.A., Zaychikov E.F. (1996) Highly selective Affinity labelling of RNA polymerases: A way to study functional topography of the active site. Chemical Modification of Enzymes (Kurganov V.I., Nagradova N.K., Lavrik O.I. ed.). 309-346, Nova Science Publishers, New York.
2. Knorre D.G., Godovikova T.S. (1998) Photoaffinity labelling as an approach to study supramolecular nucleoprotein complexes. FEBS Lett. 433, 9-14.
3. Kolpashchikov D.M., Rechkunova N.I., Dobrikov M.I., Khodyreva S.N., Lebedeva N.A., Lavrik O.I. (1999) Sensitized photomodification of mammalian DNA polymerase beta. A new approach for highly selective affinity labelling of polymerases. FEBS Lett. 448, 141-144.
4. Lavrik O.I., Prasad R., Sobol R.W., Horton J.K. Ackerman E.J., Wilson S. (2001) Photoaffinity labelling of mouse fibroblast enzymes by a base excision repair intermediate. Evidence for the role of poly(ADP-ribose) polymerase-1 in DNA repair. J. Biol. Chem. 276**,** 25541-25548.
5. Meisenheimer K.M., Koch T.H. (1997) Photocross-linking of nucleic acids to associated proteins. Crit. Rev. Biochem. Mol. Biol. 32, 101-140.
6. Prasad R., Lavrik O.I., Kim S.J., Kedar P., Yang X.P., Vande Berg B.J., Wilson S.H. (2001) DNA polymerase beta -mediated long patch base excision repair. Poly(ADP-ribose)polymerase-1 stimulates strand displacement DNA synthesis. J. Biol. Chem. 276, 32411-32414.
7. Rechkunova N.I., Kolpashchikov D.M, Lebedeva N.A., Petruseva I.O., Dobrikov M.I., Degtyarev S.K., Lavrik O.I. (2000) Highly selective affinity labelling of DNA-polymerase from Thermus thermophilus B35 by a binary system of photoreactive agents. Biochemistry (Mosc.). 65, 244-249.

DOMAINS OF HUMAN REPLICATION PROTEIN A CONTACTING THE 3'-END OF THE PRIMER

[1]Pestryakov P., [2]Weisshart K., [1]Khodyreva S.N., [2]Nasheuer H.-P., [1]Lavrik O.I.*

[1] Novosibirsk Institute of Bioorganic Chemistry SB RAS, 630090, Novosibirsk, Russia
[2] Department of Biochemistry, Institute of Molecular Biotechnology, Jena, Germany
e-mail: pavel@rav.sscc.ru
*Corresponding author

Key words: *DNA replication, photoaffinity labelling, replication protein A*

Resume

In addition to single-stranded DNA (ssDNA) human replication protein A (RPA) is able to bind other physiologically relevant DNA structures such as double-stranded DNA, UV- and cis-platinum damaged DNA, partial DNA-duplexes. In this work we studied involvement of different structural domains of RPA in interactions with partial DNA-duplexes. Results indicate that conformation of RPA bound to partial duplex containing 5'-protruding template strand seems to be different from that of both free RPA and protein bound to the ssDNA. Photoaffinity labelling of RPA and further identification of protein domains modified by photoreactive 3'-end of the primer showed that domains located in central part of RPA32 subunit (amino acids 43-171) and C-terminal part of RPA70 subunit (amino acids 432-616) are involved in specific interactions the 3'-end of the primer.

Introduction

The human single-stranded DNA-binding protein, replication protein A, is a stable heterotrimer consisting of three subunits (RPA70, RPA32 and RPA14). RPA is one of the key players in most of processes of DNA metabolism (Iftode et al., 1999). Its function is associated with DNA-binding activity. Total of three DNA-binding sites are now distinguished in RPA70 subunit. DNA-binding domains A, B (DBD-A, DBD-B) lies in the central part of this subunit, DNA-binding domain C (DBD-C) in the C-terminal part of RPA70. The fourth DNA-binding domain D (DBD-D) resides in central part of RPA32 subunit. ssDNA-binding is performed in multistep polar pathway resulting in final elongated conformation with occluded binding size of 30 nucleotides (Bastin-Shanower, Brill, 2001; Bochkareva et al., 2001).

Apparently partial DNA-duplexes are more likely to be found as *in vivo* intermediates. Earlier photoaffinity labelling studies using partial photoreactive DNA-duplexes showed that RPA32 and RPA70 can interact with or be in close proximity to 3'-end of the primer (Kolpashchikov et al., 2000; Lavrik et al., 1999). Recently we investigated conformational changes, which accompany binding of RPA to partial duplexes, and further attempts were made to identify protein domains involved in such interaction.

Results and Discussion

To clarify whether RPA binding to partial duplexes containing 5'-protruding end has some peculiarities in comparison to ssDNA-binding we used limited proteolysis technique. Proteolysis was performed using trypsin as a protease and RPA was either free in solution or bound to DNA-hairpins with ssDNA-tails of various lengths. We used automated N-terminal sequencing and immunoblotting using monoclonal antibodies raised against different parts of RPA to precisely identify the products (Fig. 1A, Fig. 4).

No new cleavage sites in case of the hairpin with 33 nt ssDNA-tail were found in comparison to the results of partial proteolysis of RPA bound to $(dT)_{30}$ oligonucleotide reported earlier (Fig. 2; Fig. 4; Gomes et al., 1996). We also noticed the same decrease in the rate of RPA70 proteolytical degradation upon binding to the hairpin with long protruding tail as was observed for the RPA binding single-stranded oligonucleotide. However, rate of RPA32 degradation in our experiments was not dependent on the length of ssDNA and proteolysis was performed as if RPA was free in solution (Fig. 2).

This result is in contrast to the significant increase in RPA32 proteolytical sensitivity upon binding 30nt ssDNA oligonucleotide (Gomes et al., 1996). It is assumed that when RPA interacts with ssDNA stretches more than 30 nt long all four DBDs are bound to ssDNA (Bastin-Shanower, Brill, 2001). Plausible explanation of differences observed in our experiments is that RPA bound to DNA hairpin exists in distinctive conformation. We suspected that in this conformation DBD-D found in RPA32 and low-affinity binding domain DBD-C from RPA70 are interacting with primer-template junction.

Indeed both RPA70 and RPA32 could be crosslinked to the growing primer in the replicating SV40 chromosomes (Mass G. et al., 2001) and to the photoreactive residue incorporated in the 3'-end of the primer in partial DNA-duplex containing 5'-protruding ssDNA-tail (Fig. 3; Lavrik et al., 1999). Combination of photolabelling technique and limited proteolysis was used to tag and identify individual structural domains of RPA which are involved in interaction with the primer-template junction.

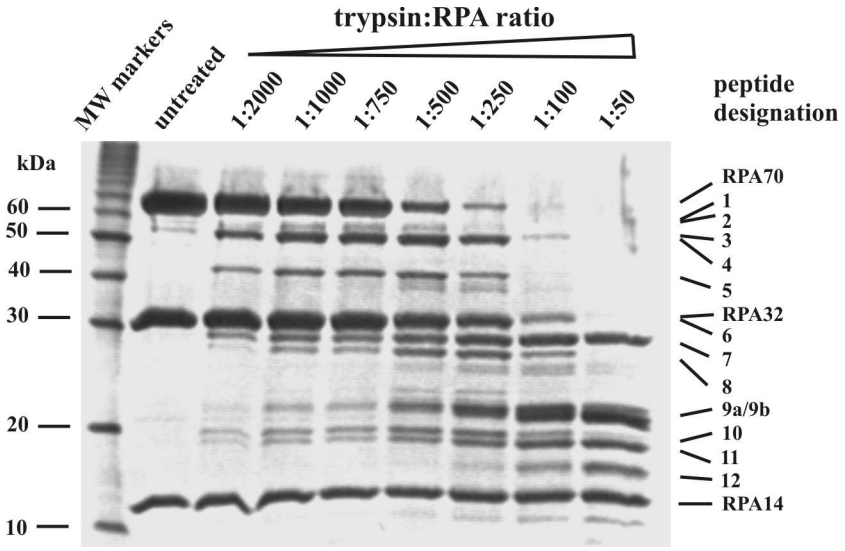


Fig. 1. Limited proteolysis of RPA with trypsin (Coomassie-stained).

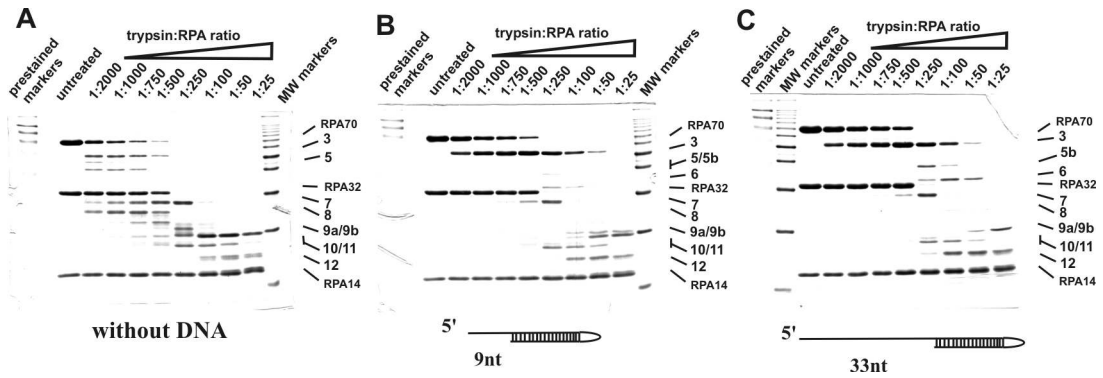


Fig. 2. Limited proteolysis of RPA bound to partial DNA-hairpins (Coomassie-stained) (RPA and DNA were in equimolar concentrations).

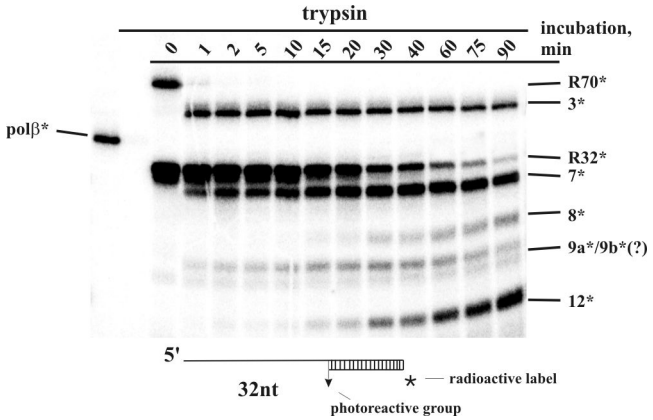


Fig. 3. Limited proteolysis of RPA-DNA crosslinking products (autoradiogram) (RPA and DNA were in equimolar concentrations).

We used DNA structures containing $(dT)_{30}$ 5'-protruding tail and photoreacive 3'-terminal nucleotide in the primer strand. After UV-induced crosslinking DNA-protein conjugates were subjected to trypsin for partial digestion and analyzed. Only limited number of partially digested peptides was DNA-crosslinked (Fig. 3). Out of them we are able to assign three RPA70-derived products that corresponds to tryptic peptides containing C-terminal part, and two RPA32-derived products containing the central part of the subunit. At the same time we were not able to detect UV-crosslinked products corresponding to peptides representing exactly N-terminal and central part from RPA70 (Fig. 4).

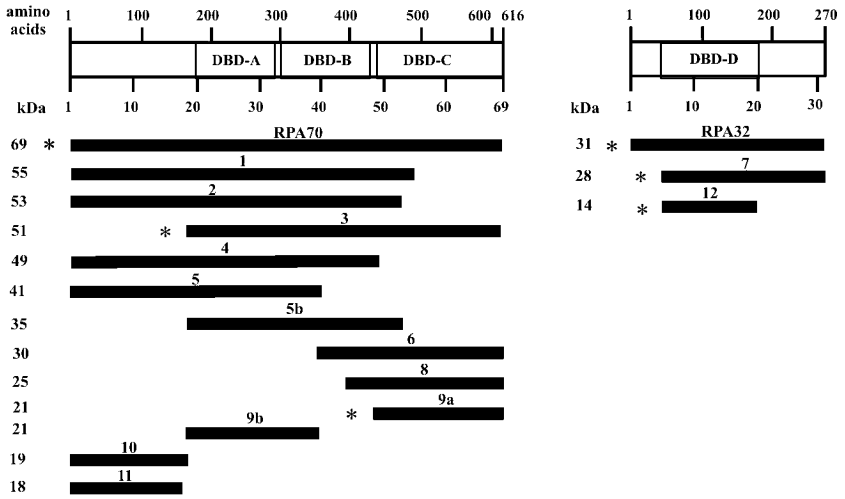


Fig. 4. Peptide map of RPA70 and RPA32 digested by trypsin. (Peptides that were labeled after RPA crosslinking to the 3'-photoreactive primer are indicated with asterisk).

These findings suggest that RPA trimer interacts with partial DNA-duplexes in the following manner. High-affinity DBD-A and DBD-B domains are bound to the ssDNA tail of the hairpin and located distantly from the 3'-end of the primer. C-terminal RPA70 structural domain that bear DBD-C is either bound to template strand in close proximity to the 3'-end or interacts with 3'-end of the primer whereas DBD-D is directly interacting with primer strand.

Acknowledgements

This work was supported in part by INTAS grant and grants from the Russian Foundation for Basic Research (00-04-22002, 01-04-48854 и 02-04-48404).

References

1. Bastin-Shanower S.A., Brill S.J. (2001) Functional analysis of the four DNA binding domains of replication protein A. The role of RPA2 in ssDNA binding. J. Biol. Chem. 276, 36446-36453.
2. Bochkareva E., Belegu V., Korolev S., Bochkarev A. (2001) Structure of the major single-stranded DNA-binding domain of replication protein A suggests a dynamic mechanism for DNA binding. Embo J. 20, 612-618.
3. Gomes X.V., Henricksen L.A., Wold M.S. (1996) Proteolytic mapping of human replication protein A: evidence for multiple structural domains and a conformational change upon interaction with single-stranded DNA. Biochemistry. 35, 5586-5595.
4. Iftode C., Daniely Y., Borowiec J.A. (1999) Replication protein A (RPA): the eukaryotic SSB. Crit. Rev. Biochem. Mol. Biol. 34, 141-180.
5. Kolpashchikov D.M., Khodyreva S.N., Khlimankov D.Y., Wold M.S., Favre A., Lavrik O.I. (2001) Polarity of human replication protein A binding to DNA. Nucl. Acids Res. 29, 373-379.
6. Kolpashchikov D.M., Pestryakov P.E., Wlassoff W.A., Khodyreva S.N., Lavrik O.I. (2000) Study of interaction of human replication factor A with DNA using new photoreactive analogs of dTTP. Biochemistry (Mosc.). 65, 160-163.
7. Lavrik O.I., Kolpashchikov D.M., Weisshart K., Nasheuer H.P., Khodyreva S.N., Favre A. (1999) RPA subunit arrangement near the 3'-end of the primer is modulated by the length of the template strand and cooperative protein interactions. Nucl. Acids Res. 27, 4235-4240.
8. Mass G., Nethanel T., Lavrik O.I., Wold M.S., Kaufmann G. (2001) Replication protein A modulates its interface with the primed DNA template during RNA-DNA primer elongation in replicating SV40 chromosomes. Nucl. Acids Res. 29, 3892-3899.

AGE-ASSOCIATED CHANGES IN OXIDATIVE DAMAGE IN OXYS RATS WITH INCREASED SENSITIVITY TO FREE RADICALS AND PROTECTION OF THE RATS FROM OXIDATIVE DAMAGE BY MIRTILENE FORTE AND ADRUSEN ZINCO

Krysanova J.S.[1*], Sinitsyna O.I.[1], Ishchenko A.A.[2], Kolosova N.G.[1], Vasyunina E.A.[1], Nevinsky G.A.[1,2*]

[1] Institute of Cytology and Genetics SB RAS, 630090, Novosibirsk, Russia
[2] Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia
e-mail: Nevinsky@niboch.nsc.ru; evas@niboch.nsc.ru
*Corresponding authors

Key words: *OXYS rat strain, reactive oxygen species (ROS), aging, mitochondria, bilberry extract, vitamin E*

Resume

Motivation: Oxygen free radicals (oxidative stress) have been hypothesized to play an important role in the etiology of a variety of degenerative diseases and in the process of aging. Searching for new dietary antioxidants is an important challenge in nutrition research.

Results: To investigate a correlation between oxidative stress and accumulation of different kinds of oxidative damage we compared age-dependent levels of protein carbonyl groups and activities of an antioxidant enzyme, catalase, in nuclear and mitochondrial extracts of liver cells from Wistar rats and OXYS rats, the latter strain characterized by increased sensitivity to free radicals. Faster age-dependent increase in the level of protein carbonyl groups was found in OXYS as compared with Wistar rats. A complicated pattern of age-dependent changes in the activity of catalase was observed in cytosol and mitochondrial extracts. Long-term uptake of dietary supplements Mirtilene Forte (*Vaccinum myrtillus* extract) or Adrusen Zinco (vitamin E complex with zinc, copper, selenium and ω-3 polyunsaturated fatty acids) sharply decreased the level of protein oxidation in cytosol and mitochondrial extracts of hepatocytes of Wistar and of OXYS rats. Both dietary supplements increased activity of catalase in liver mitochondria of OXYS rats.

Introduction

All higher organisms generate energy by aerobic respiration, a process that involves a stepwise four-electron reduction of molecular oxygen to water. The partially reduced species that are produced as intermediates and by-products of aerobic respiration, including $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$, are potent oxidants attacking different cellular components including DNA, proteins, lipids of mitochondria and nuclei. These oxidants are also generated in cells through exposure to ionizing radiation and other agents that produce free radicals. A major target of reactive oxygen species (ROS) is the cellular genome, and oxidative stress can lead to DNA lesions, resulting in numerous genotoxic adducts, DNA strand breaks and mutations. Oxidative damage to cells is ongoing and has been regarded as a significant factor in carcinogenesis and aging. Therefore, special attention had been focused on understanding the mechanism of oxidative damage. The OXYS strain of rats appears to be useful as a model for studies of ROS *in vivo* action, since this strain is characterized by accelerated senescence and mitochondrial dysfunction, and chronic aging pathologies such as cataracts, brain disorders, cardiomyopathy, etc. (Salganik et al., 1994). This study examines the effects of dietary supplements such as Mirtilene forte and Adrusen zinco on protein oxidation and catalase activity in cytosol and mitochondrial extracts of rat liver.

Methods

Animals. Male white Wistar and OXYS rats from the RAS Institute of Cytology and Genetics (Novosibirsk, Russia) at 3, 6 and 12 months of age were used in this study. The second group of Wistar and OXYS rats (12 months of age), consisting of three subgroups (each of 13 rats), was used for three dietary treatment. Diet 1 was the basal diet used for the control group of rats. Diets 2 and 3 were the same as the basal diet with the addition of 230 mg Mirtilene forte/kg weight or 1 g Adrusen zinco/kg weight (340 mg proteins, 260 mg carbohydrates, 350 mg lipids, 23 mg zinc, 1 mg copper, 88 μg selenium and 24 mg vitamin E), respectively. Mirtilene forte is an extract of bilberry shrub and is normalized to 25% anthocyanosides. Adrusen zinco contains zinc, copper, selenium, vitamin E and polyunsaturated fatty acids.

Rat liver mitochondria were isolated using a combination of differential and gradient centrifugation in sucrose (Weinbach, 1951). Liver mitochondrial extracts were prepared by suspending the mitochondria in the lysis buffer (20 mM HEPES-NaOH, pH 7.5, 0.4 M NaCl, 5 mM EDTA, 3 mM DTT, 5% glycerol, 0.3% Nonidet P-40) and clarified by centrifugation for 1 h at 100,000 *g*.

Protein carbonyl content was measured after reaction with 2,4-dinitrophenylhydrazide by quantifying the resulting protein hydrazone derivatives spectrophotometrically at 370 nm (extinction coefficient, $22 \cdot 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$). The concentration of carbonyl groups was expressed as nmol carbonyl per mg protein.

Catalase dependent decomposition of H_2O_2 (10 mM) was followed directly by decrease in the absorbance at 240 nm. The difference in absorbance per second was calculated. The CAT activity was expressed as k (s^{-1}) per mg/ml of the analyzed protein sample.

The results are reported as mean ± standard error of at least 3 different experiments for each mitochondrial extract or cytosol sample of each animal, averaged over at least 5 different animals. The differences between samples were analyzed by the Student's *t*-test, $P < 0.05$ was considered statistically significant.

Results and Discussion

We have examined whether there was age-dependent oxidative damage to proteins in liver cell cytosol (microsomal fraction) and mitochondria of OXYS and Wistar rats. We have determined the carbonyl group content in proteins of OXYS and Wistar rats at 3, 6, and 12 months of age. The oxidative damage to proteins in mitochondria was ~1.5-2-fold higher than in the cytosol. The protein carbonyl content in cytosol and mitochondrial extracts of OXYS and Wistar rats changed in a complicated manner. In the liver cytosol from both strains it increased ~1.2-fold at 6 months compared to 3 months, and then decreased at 12 months. On the contrary, oxidation of mitochondrial proteins decreased between 3 and 6 months by a factor of 1.6-2.4 and then increased in both strains at 12 months. Interestingly, nearly the same reproducible difference (1.23-fold) between the two strains in the protein carbonyl content in both cytosolic fraction and mitochondrial extracts was observed in 12-month-old animals.

Adrusen zinco had a significant effect on the levels of protein oxidation products in cytosol and mitochondria (Fig. 1). The mitochondria of the control group of rats had 1.4-fold (P<0,05) higher level of protein carbonyls than that of the supplemented group.

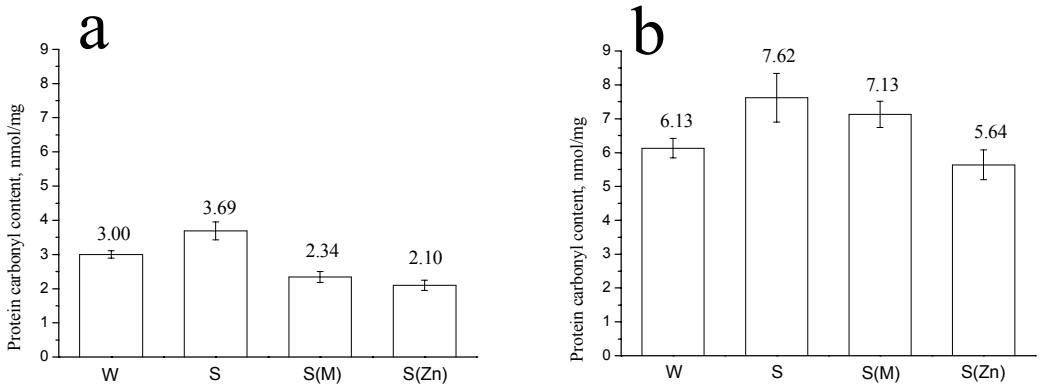


Fig. 1. Effect of Adrusen zinco (Zn) and Mirtilene forte (M) on the level of protein carbonyl group in the cytosol (a) and mitochondria (b) of OXYS (S) rats. W - corresponds to the level of protein carbonyl group of control Wistar rats before their treatment with antioxidants.

Similarly to the mitochondrial extracts, the protein carbonyl content in cytosolic fraction of OXYS rats fed Adrusen zinco decreased ~2-fold (*P*<0,001) and became lower than in the Wistar control group. We found that treatment with Mirtilene forte was effective in preventing the age-related increase in oxidative damage to proteins in cytosol (Fig. 1). At the same time, there was no significant effect on carbonyl content in the mitochondria of OXYS rats treated with Mirtilene forte.

An antioxidant enzyme, catalase (CAT), can play a critical role in cell protection against oxidative DNA damage. We observed nearly the same level of CAT activity in the cytosol of rats at 3 and 6 months, and its remarkable increase at 12 months of age. The age-associated pattern of the CAT activity in the mitochondrial extracts was quite different from that in the cytosol fraction. The mitochondrial extracts of OXYS demonstrated a high level of the activity at 3 months that significantly decreased in the 6 months old rats. Interestingly, the CAT activity changed little in Wistar rats from 6 to 12 months, but there was a significant increase in this activity in the mitochondrial extracts of OXYS rats. Finally, the CAT activity at 12 months was ~1.7-fold (statistically significant) higher in OXYS than in Wistar rats. More pronounced increase in the CAT activity in OXYS compared to Wistar rats suggests its accelerated induction during the process of aging in OXYS rats.

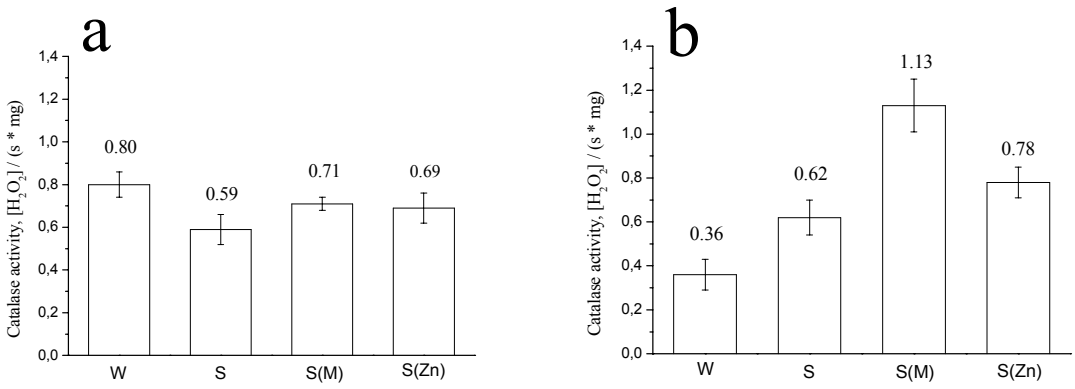


Fig. 2. Effect of Adrusen zinco (Zn) and Mirtilene forte (M) on the level of catalase activity in the cytosol (a) and mitochondria (b) of OXYS (S) rats. W, the level of CAT in Wistar rats before their treatment with antioxidants.

Fig. 2 illustrates effects of Adrusen zinco and Mirtilene forte on the level of liver CAT activity. Mirtilene forte significantly (1.8-fold) enhanced the CAT activity in mitochondrial extracts, whereas it had no effect on the CAT activity in cytosol. Treatment with Adrusen zinco did not alter the CAT activity in both mitochondrial and cytosol extracts of OXYS rats hepatocytes.

Interestingly, despite Adrusen zinco affected the protein carbonyl content, no remarkable changes in the CAT activity were found. Since the components of this supplement are lipid-soluble, the effect of Adrusen zinco may to be mediated through inhibition of oxidation of lipids and membrane proteins. The catalase mainly acts in water phase, thus, the effect of Adrusen zinco may not be significant in this case.

Many polyphenols, including anthocyanins, have marked antioxidative activity (Cao et al, 1999). The antioxidant effect may reflect the ability of bilberry extract to chelate transition metal ions involved in radical-forming processes such as Fenton reactions (Ramirez-Tortosa et al., 2001). Thus, an improvement in antioxidant status of liver hepatocytes was expected as a result of consumption of Mirtilene forte.

In summary, this study demonstrates that both investigated antioxidants decrease the oxidative damage to proteins in the liver of 12-month old OXYS rats and at the same time enhance the antioxidant defense system, exemplified by catalase activity in the mitochondria.

Acknowledgements

The research was supported in part by grants from INTAS (97-1645), Russian Foundation for Basic Research (01-04-48892; 02-04-49889), and by a SD RAS grant for young scientists.

References

1. Cao G., Shukitt-Hale B., Bickford P.C., Joseph J.A., McEwen J., Prior R.L. (1999) Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants. J. Appl. Physiol. 86, 1817-1822.
2. Ramirez-Tortosa C., Andersen Q.M., Gardner P.T., Morrice P.C., Wood S.G., Duthie S.J., Collins A.R., Duthie G.G. (2001) Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Radic. Biol. Med. 31, 1033-1037.
3. Salganik R.I., Solovyova N.A., Dikalov S.I., Grishaeva O.N., Semenova L.A., Popovsky A.V. (1994) Inherited enhancement of hydroxyl radical generation and lipid peroxidation in the S strain rats results in DNA rearrangements degenerative diseases and premature aging. Biochem. Biophys. Res. Commun. 199, 726-733.
4. Weinbach E.C. (1951) A procedure of isolating stable mitochondria from rat and kidney. Anal. Biochem. 2, 334-335.

AGE-ASSOCIATED CHANGES IN THE LEVELS OF OXIDATIVE STRESS MARKERS IN THE BRAIN OF OXYS RATS

[*]***Shcheglova T.V., Kolosova N.G., Sinitsina O.I., Vasunina E.A., Loskutova L.V.***

Institute of Cytology and Genetics SB RAS, 630090, Novosibirsk, Russia, e-mail: scheglov@niboch.nsc.ru
[*] Corresponding author

Key words*: OXYS rat strain, oxidative stress, brain aging*

Resume

Motivation: Oxygen free radicals have been hypothesized to play an important role in aging and a number of pathologic processes. Free radicals are formed in the brain as part of normal metabolic processes such as mitochondrial respiration. High oxygen uptake and low antioxidant defense increase vulnerability of the brain to oxidative damage. Behavior of the young OXYS rats, characterized by accelerated senescence, is similar to that of old Wistar rats. OXYS rats have lowered response to novel stimuli, higher anxiety, and impaired capability of one-trial learning. Therefore, the OXYS strain could be a good model to study human degenerative diseases and the mechanism of oxidative protein and DNA damage and repair in mammals.

Results: Here we have analyzed for the first time age-dependent and interstrain differences in oxidative stress markers and antioxidant enzymes in the brain of OXYS and Wistar rats. We have shown that the learning and memory impairments usually can be registered in the OXYS rats at 3-4 months of age and that these symptoms significantly increase with age. At the same time, the level of protein carbonyl groups in the OXYS rats was elevated (≥1.4-fold), compared with that in Wistar, only for the ages of 12-14 months and older. The level of an antioxidant enzyme superoxide dismutase was 1.8-fold higher in the brain of young OXYS rats. Thus, cognitive impairment in the OXYS rats precedes detectable changes in these oxidative stress markers. Our data are consistent with the view of oxidative stress as a mosaic process, dependent on damage of DNA, proteins and lipids in the brain.

Introduction

Aging is influenced by various environmental factors, of which free radicals are thought to be the most important, as proposed by Harman in 1956. Accumulation of oxidative damage in different components of brain membranes with age leads to their depolarization, changes in neuron sensitivity, deviations from optimal conditions of functioning of receptors, channels, enzymes, and modulators, as well as to change in a number of membrane receptors and their affinity to neuromediators. This ultimately leads to functional brain impairment.

OXYS rats show some chronic aging pathologies such as short lifespan, accelerated degeneration of thymus, liver, myocardium, bone, and development of cataracts. The OXYS strain is argued to be a model for studying human degenerative diseases. One of the potential causes of age-related neuronal damage is reactive oxygen species. The objective of this study was to investigate relationships between brain functioning and changes in the oxidative stress proxies in the brain of OXYS (from 3 to 24 month of age), as compared to control Wistar rats.

Methods

Male white Wistar and OXYS rats from the Institute of Cytology and Genetics of RAS (Novosibirsk, Russia) from 3 to 24 months of age were used in this study. The animals were housed in colonies under standard conditions. The rats were sacrificed by decapitation, and the brains were frozen at -70°C.

Protein carbonyl content was measured after reaction with 2,4-dinitrophenylhydrazide and quantifying the resulting protein hydrazone derivatives spectrophotometrically at 370 nm (extinction coefficient, 22×10^3 $l \cdot mol^{-1} \cdot cm^{-1}$). The concentration of carbonyl groups was expressed as nmol carbonyl per mg protein (Reznick, Packer, 1994). The protein content was determined by Bradford assay, using bovine serum albumin as a standard.

Lipid peroxidation (LPO) was determined using conjugated diene (CD) (Steinbrecher, 1990).

To assay for SOD activity in brain extracts, xanthine/xanthine oxidase-mediated ferricytochrome *c* reduction assay (Flohe, Otting, 1984) was used. Samples (40 μl) were added to 550 μl of the reaction mixture containing 0.025 μmol of xanthine in 0.1 mM NaOH, and 0.01 μmol of cytochrome *c* in 50 mM phosphate buffer, pH 7.8, supplemented with 0.1 mM EDTA and 10 mM sodium azide. The reaction was initiated by adding 10 μl of the xanthine oxidase solution (0.5 U/ml in 0.1 mM EDTA). The absorbance change was monitored for 3 min at 25°C. SOD activity was calculated from the absorbance at 550 nm using a concurrently run standard curve and expressed as units of activity per mg protein. One unit of SOD activity is defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction under the conditions specified by 50%.

Total levels of reduced glutathione (GSH) in brain extracts were measured spectrophotometrically at 412 nm by method of Tietz (1969) using 2-nitro-5-thiobenzoic acid. The concentration of thiols was expressed as nmol thiol per mg protein.

Statistical analysis. The results were presented as mean ± Er of at least 3 different experiments for each brain extract sample of each rat. The results are an average of brain extracts from at least 5-8 different animals. All data assessed by the one-way or two-factor analysis of variance (ANOVA).

Results and Discussion

Impairments of cognitive functions (lowered response to novelty, higher anxiety, and impairment ability to one-trial learning) are usually the earliest symptoms of worsening of memory in senescent humans and animals (Loskutova, Kolosova, 2000).

Learning and memory impairments can usually be noticed in OXYS rats at 3-4-month of age; these symptoms become progressively worse with age. In order to find a possible biochemical explanation of this phenomenon, we have analyzed relationships between several physiological indices of brain impairment, changes in the levels of oxidative damage of proteins and lipids, and others oxidative stress proxies in the brain of OXYS (from 3 to 24 month of age) in comparison with the same parameters of control Wistar rats.

First, we have examined whether there was age-dependent oxidative damage to proteins and lipids in brain tissues of OXYS and Wistar rats. We have determined protein groups and lipid peroxides content in OXYS and Wistar rats at 3, 10, and 14 and 24 months of age. Fig. 1 demonstrates that the level of lipid peroxides and protein carbonyl content increased with age in the brain of rats from both strains (Fig. 1). Interestingly, a significant difference between OXYS and Wistar rats can be revealed only for 12-14-months old or older rats; ~1.4-fold (statistically significant) higher level of these parameters in brain of OXYS as compared with Wistar rats was revealed. The relative amount of protein carbonyl groups for OXYS and Wistar rats at 24 month of age, in comparison 4-month old rats, was increased by a factor of 3.3 and 2.3, respectively, while the content of lipid peroxides increased 2.7- and 1.8-fold.

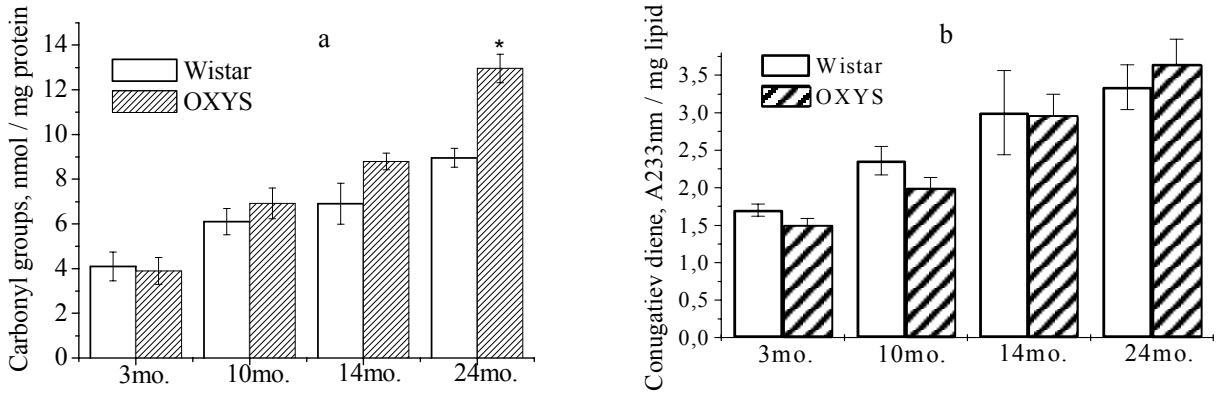


Fig. 1. Comparison of a relative content of protein carbonyl groups (a) and lipid peroxides (b) in the brain of OXYS and Wistar rats of different age.

Under normal circumstances, nuclear and mitochondrial oxygen radicals may be detoxified by superoxide dismutase (SOD), decreasing the levels of protein, lipid, and DNA modifications. In order to estimate possible differences in such detoxification in OXYS and Wistar rats we have compared SOD activities in the brain of rats as well as age-related changes in SOD and non-enzymatic ROS scavengers' vitamin E and glutathione. The activity of SOD in brain of OXYS rat at 24 months of age was ~1.7-fold (statistically significant) lower than in Wistar rats of the same age.

However, there was no significant difference in protein carbonyl groups in the brain of 3-4-month-old OXYS and Wistar rats, even though young OXYS rats already demonstrate pronounced learning and memory impairment. At the same time, we have observed a significant difference in the level of superoxide dismutase between young OXYS and Wistar rats; SOD activity was higher 1.8-fold (Fig. 2).

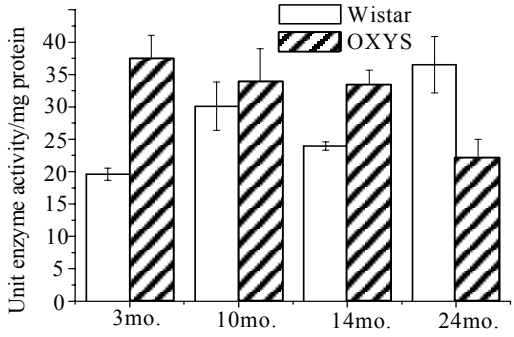


Fig. 2. Comparison of relative levels of superoxide dismutase in the brain tissues of OXYS and Wistar rats of different age.

At the same time, the level of reduced glutathione in OXYS was 1.3-fold lower than in Wistar rats. Vitamin E is a natural antioxidant protector of mammalian organisms. However, there was no detectable difference in the level of vitamin E for two analyzed rat strains. Since SOD is inducible, increased levels of ROS in OXYS can lead to elevated SOD activity in response to increased oxidative damage.

Thus, our results suggest that accumulation of detectable levels of oxidative stress markers (oxidized proteins and lipids) with age occurs slower than manifestation of cognitive impairment in the OXYS rats. In order to uncover a possible reason for this, we have analyzed relative levels of LPO in different regions of the 4-month-old OXYS and Wistar rat brain (cortex, hippocampus, midbrain and cerebellum). No pronounced interstrain difference was found. At the same time, two-way ANOVA analysis revealed statistically significant differences in the levels of LPO for specific regions of rat brains; both OXYS and Wistar rats demonstrated interstructural differences according to Fisher indices (F) ($F_{3,33}$ = 5,8, p = 0,003 for Wistar and $F_{3,26}$ = 4,8, p = 0,008 for OXYS rats). The LPO levels were higher in the cortex of Wistar and in the hippocampus of OXYS rats. In addition, according to our preliminary data, DNA damage with formation of etheno derivatives of adenine and cytosine is more extensive in OXYS than in Wistar rats. Interestingly, etheno DNA lesions are not distributed uniformly in the brain, but there exist specific brain areas accumulating more lesions, leading to a mosaic picture of brain damage. Thus, our results and related studies indicate that the changes in brain function may be related to progressive oxidation of critical brain proteins and lipids. An intriguing possibility is that damage of some critical regions in the brain, leading to cognitive impairment in OXYS rats, may occur in earlier age than more extensive damage of the brain as a whole, which can be registered using analysis of oxidative stress markers.

Acknowledgements

The research was supported in part by grants from INTAS (97-1645), Russian Foundation for Basic Research (02-04-48317 and 02-04-49889).

References

1. Flohe L., Otting F. (1984) Superoxide dismutase assays. Methods in Enzymology. 105, 93-104.
2. Herman D. (1956) Aging, a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298-300.
3. Loskutova L., Kolosova N. (2000) Emotional State and One-Trial Learning in OXYS Rats with Hereditarily Elevated Production of Oxygen Radicals. Bull. of Experimental Biol. and Med. 130, N 8, 746-749.
4. Reznick A., Packer L. (1994) Oxidative to proteins: spectrophotometric method for carbonyl assay. Methods in Enzymology. 233, 357–363.
5. Steinbrecher U. (1990) Free Radicals, Lipoproteins, and Membrane Lipids. Ed. L.Douste-Blazy, Paoleti. Plenum, N.-Y., 193 p.
6. Tietz F. (1969) Enzymatic method for the quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. Anal. Biochem. 27, 502-522.

REPAIR OF 8-OXOGUANINE IN DNA OF PROKARYOTES, WISTAR RATS AND OXYS RATS WITH OVERGENERATION OF FREE RADICALS

Nevinsky G.A.[1,*], Ishchenko A.A.[1], Sinitsina O.I.[2], Vasunina E.A.[2], Koval V.V.[1], Vasilenko N.L.[1], Saparbaev M.[3], Fedorova O.S.[1]

[1] Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia, e-mail: nevinsky@niboch.nsc.ru.
[2] Institute of Cytology and Genetics, SB RAS, 630090, Novosibirsk, Russia
[3] Group de Reparation des Lesions Radio et Chimio-Induites, UMR 8532, Paris, France
*Corresponding author

Key words: *Escherichia coli, Wistar and OXYS rats, enzymes removing 8-oxoguanine, mechanism of action, nuclear and mitochondrial extracts*

Resume

Motivation: Oxidative damage to cells, suggested as a significant factor in carcinogenesis and aging, leads to formation of premutagenic modifications of DNA bases, including 8-oxoguanine (oxoG), thymine glycols, FapyGua and FapyAde (imidazole ring-opened guanine and adenine, respectively). Many of these are miscoding and mutagenic or cytotoxic. *E. coli* 8-oxoguanine-DNA glycosylase (Fpg) and its mammalian analogs remove a number of purine lesions from DNA, but oxoG is believed to be the major physiological substrate for these enzymes. Study of enzymes removing oxidative lesions is important from scientific and applied point of view.

Results: To quantitatively elucidate structural determinants of substrate specificity and a mechanism of action of Fpg, we have investigated the interaction of Fpg with a series of single- and double-stranded (ss and ds) nonspecific and specific oligodeoxynucleotides (ONs) using steady-state and pre-steady-state kinetics. The results are analyzed using a thermodynamic model of non-specific and specific DNA recognition by Fpg.

To investigate the correlation between oxidative stress and accumulation of DNA damage, we have determined age-dependent levels of activities removing oxoG from DNA in liver cells from OXYS rats, which are characterized by inherited overgeneration of free radicals, in comparison with those of control Wistar rats. OxoG DNA glycosylase/AP lyase activity in mitochondrial extracts from OXYS was remarkably higher than that in Wistar old rats. Our results are in agreement with shorter life-span of OXYS and with mitochondrial theory of aging that postulates accumulation of DNA damage in mitochondrial genomes as the cause of mitochondrial dysfunction and accelerated aging.

Introduction

Significant progress has been achieved in the study of interactions of various enzymes with DNA, mostly due to accumulation of a wealth of data on three-dimensional structures of their complexes with DNA (reviewed in Bugreev, Nevinsky, 1999). However, structural analysis of interactions with DNA does not provide a quantitative estimate of the relative importance of molecular contacts, or of the relative contributions of strong and weak, specific and non-specific contacts to the total affinity of an enzyme for DNA and led to the concept, wrong to some extent, that specific contacts such as pseudo-Watson-Crick interactions, provide high affinity for specific DNA sequences, and thus result in high specificity and high efficiency in catalysis. We have shown that interactions between enzymes and long DNAs at the molecular level can be successfully analyzed by a method of stepwise increase in ligand complexity (SILC) (reviewed in Bugreev, Nevinsky, 1999). In the present work we have analyzed *E. coli* Fpg by SILC and pre-steady state kinetics with fluorescence detection. In addition, we have analyzed a difference in specificity of mitochondrial and nuclear glycosylase activity of Wistar and OXYS rats.

Materials and Methods

Methods used for preparing homogeneous *E. coli* Fpg and different ONs, the enzyme activity assay, and measurements of thermodynamic and kinetic parameters using SILC and pre-steady-state techniques were described earlier (Ishchenko et al., 1999; Fedorova et al., 2002). K_M and V_{max} or K_I were estimated using non-linear regression analysis; errors in the values were within 10-30%.

Results and Discussion

We have found that any short specific or non-specific, ss- or ds ON can inhibit the Fpg reaction competitively; orthophosphate and dNMPs are minimal ligands for the enzyme. To assess the additivity of Fpg interactions with ONs of different length, the logarithmic dependencies of K_I for $d(pN)_n$ on the number of mononucleotide units (n) were analyzed.

The linear log-dependencies for ss $d(pN)_n$ ($0 \le n \le 10$, n = 0 corresponds to Pi) provide evidence for additivity of the Gibbs energy (ΔG^o) for the interaction of ten individual dNMP units with Fpg (Fig. 1).

Values of f (1.56), the increase in Fpg affinity for various ss $d(pN)_n$ for a one-unit increase in their length, were calculated from the slopes of the linear parts of these curves. The monotonous increases in K_d, reflecting interactions between the enzyme and one unit of ss DNA, are equal to the reciprocals of these factors ($K_1 = 1/f = 0.64$ M). The interaction of Fpg with all units of $(pN)_n$ is additive and the K_d (K_1) values for any ON can be obtained by multiplying K_d for the minimal ligand (P_i) by $K_d = 1/f$ for each of the mononucleotide units, according to a power function: $K_d[(pN)_n] = K_d[(P_i)] \times [1/f]^n = K_d[(dNMP)] \times [1/f]^{n-1}$ ($1 \le n \le 10$). We conclude that Fpg does essentially not contact with nine of the ten DNA bases, but mainly interacts with the sugar-phosphate backbone of the ligand. Linear increase in log K_1 for duplexes held up to $n = 14$ (Fig. 1). The affinity of Fpg for duplexes is about two orders of magnitude higher than for ss ONs. The change in affinity for the $d(pT)_n \bullet d(pA)_n$ duplex ($n \ge 8$) is described formally by the same function as for ss ONs (see above), but the factor f increases to 2.22. We estimated the contribution of interactions of a nonspecific (pG) nucleotide (~ -3.4 kcal/mole) and its structural elements with the Fpg lesion pocket: sugar (-0.12 kcal/mole), base (-0.54 kcal/mole), Pi (-2.84 kcal/mole). All contacts of Fpg with the damaged DNA strand provide $\Delta G^0 = -6.8$ kcal/mole. The contribution of the complementary strand nucleotide units into the affinity for ds DNA may be estimated as $\Delta G^0 \approx -2.8$ kcal/mole. Fpg can partially melt DNA creating a sharp kink in the substrate. All types of interaction of Fpg with nonspecific DNA can be described approximately using the thermodynamic model in Fig. 2.

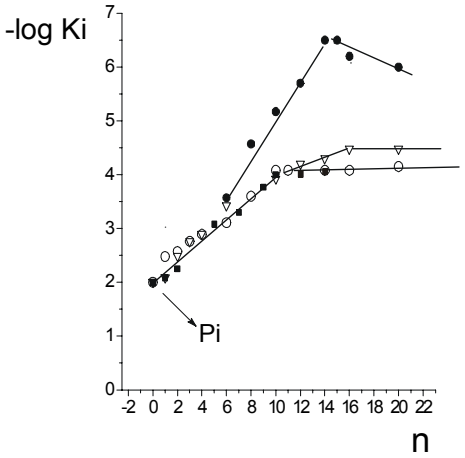


Fig. 1. Logarithmic dependencies of K_d values for ss and ds $d(pN)_n$ versus number (n) of nucleotide units: $d(pT)_n$ (Δ);$d(pA)_n$ (O); $d(pC)_n$ (■);$d(pT)_n$+$d(pA)_n$ (●).

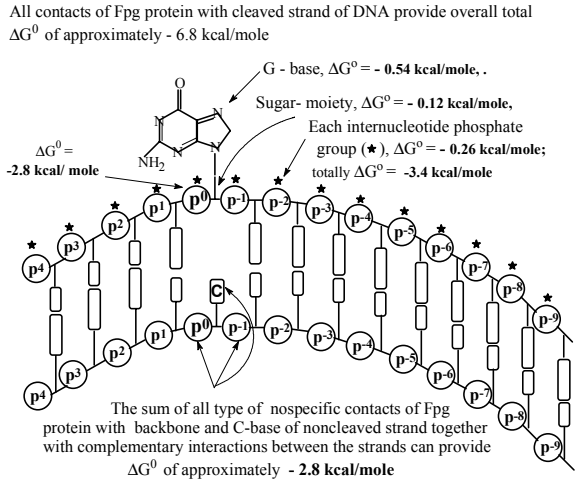


Fig. 2. Thermodynamic model of nonspecific DNA recognition by Fpg.

As the interaction energies of different structural elements (base, phosphate and sugar) of various dNMPs with the lesion recognition center of Fpg are nearly additive, the ratio (4.7) of the K_d values for dGMP and oxo-dGMP ($Kd \approx 0.21$ M; $\Delta\Delta G^0 = -0.90$ kcal/mole) gives an estimate of the relative contribution of the oxoG base into specific interactions of DNA with Fpg. The $\Delta G^0 = -0.8$ kcal/mole reflects strengthening of previously formed weak additive contacts, or formation of new stronger contacts, between Fpg and internucleotide phosphate groups of ss ONs. The maximal increase in the affinity of specific ds DNA compared with that for nonspecific ds DNA is characterized by $\Delta\Delta G^0 \approx -2.8$ kcal/mole. Therefore, an additional increase in affinity for specific DNA due to the presence of complementary strand may be characterized by $\Delta\Delta G^0 = -(2.8\text{-}0.9\text{-}0.8)$ kcal/mole = -1.1 kcal/mole. This affinity increase may be caused by several factors, including strengthening of the contacts formed between Fpg and ss ONs in the absence of the second strand, a change in conformation of both strands, or even formation of additional contacts of Fpg with both strands of ds DNA. Overall, the Fpg interactions with specific ds ONs may be approximately described by the thermodynamic model shown on Fig. 3. The efficiency of specific contact formation between Fpg and specific DNA does not exceed two orders of the total affinity; the relative contribution of nonspecific interactions to the total affinity is ~6-7 orders of magnitude. Formation of the Fpg-DNA complex cannot explain high specificity of the enzyme action. Non-modified bases can be removed from DNA by Fpg, but only at high enzyme concentrations and longer incubation times (by a factor of 10^4–10^7) than for specific DNA containing the oxoG base. Fpg cannot excise bases from nonspecific DNA with noticeable efficiency. The catalytic stage appears significantly more sensitive to the DNA structure than the stage of formation of the complex between the enzyme and DNA. The specificity of Fpg action is provided by the stages of (i) enzyme-induced DNA fitting to the optimal conformation, and (ii) catalysis: k_{cat} increases by 7-8 orders upon the transition from non-specific to specific DNA. In order to reveal individual steps of Fpg-DNA complex formation, its conformational change along the reaction pathway, and to detect

transient intermediates, we have used pre-steady-state fluorescence methods (Fedorova et al., 2002). We found that the bimolecular encounter of DNA with Fpg is very fast, and is accompanied by a fast conformational change of the enzyme. Subsequent steps of fitting and catalysis were much slower. The obtained results provide evidence for specific conformational changes of Fpg protein and DNA during the catalytic reaction, providing high rate and specificity of Fpg protein.

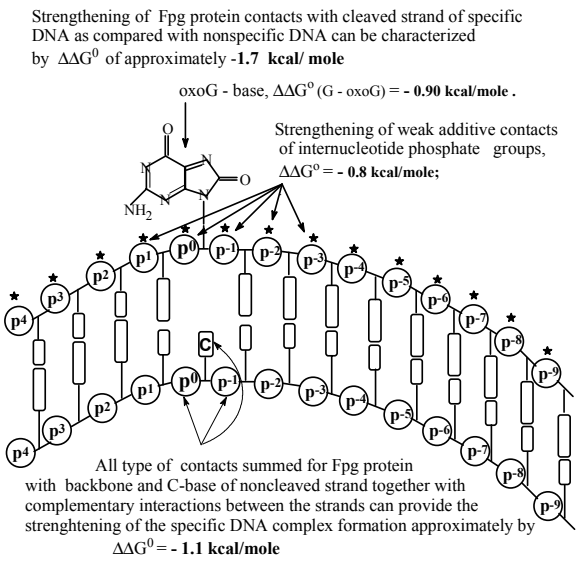


Fig. 3. Thermodynamic model of specific DNA recognition by Fpg.

In the present work we have developed a method for determination of the level of oxoG-, hypoxanthine-, and uracil-DNA glycosylase activities in nuclear and mitochondrial lysates of hepatocytes from Wistar rats and rats with inherited overgeneration of free radicals (OXYS strain). The pronounced difference in specificity of mitochondrial and nuclear oxoG-DNA glycosylase/AP lyase activities were revealed for both rat strains. Our results suggest an induction of oxoG-, uracil- and hypoxanthine-specific repair pathway with age in both types of rats. The levels of oxoG-DNA glycosylase/AP lyase activities in nuclear extracts from both strains of rats were comparable and approximately tenfold higher than in mitochondrial extracts. On the contrary, oxoG-DNA glycosylase/AP lyase activity in mitochondrial extracts of OXYS was remarkably higher than that of Wistar old rats, and a significant increase in this activity occurs earlier in OXYS than in Wistar rats. Our results are in agreement with a shorter life-span of OXYS and with the mitochondrial theory of aging that postulates that accumulation of DNA damage in mitochondrial genomes leads to mitochondrial dysfunction and accelerates the process of aging.

Acknowledgement

The research was made possible in part by grants from INTAS (97-1645), the Wellcome Trust (U.K.), Russian Fund of Fundamental Investigations (01-04-48892; 99-04-49744), and from the Siberian Division of the Russian Academy of Sciences.

References

1. Bugreev D.V., Nevinsky G.A. (1999) Possibilities of the method of step-by-step complication of ligand structure in studies of protein-nucleic acid interactions: mechanisms of functioning of some replication, repair, topoisomerization, and restriction enzymes. Biochemistry (Mosc). 64, 237-49.
2. Fedorova O.S., Nevinsky G.A. Koval V.V., Ishchenko A.A., Douglas K.T. (2002) Stopped flow kinetic studies of interaction between *E. coli* Fpg protein and DNA substrates. Biochemistry. 41, 1520-1528.
3. Ishchenko A.A., Koval V.V., Fedorova O.S., Douglas K.T., Nevinsky G.A. (1999) Structural requirements of double and single stranded DNA substrates and inhibitors, including a photoaffinity label, of Fpg protein from *E. coli*. J. Biomol. Struct. Dynam. 17, 301-310.

COMPUTATIONAL PREDICTION AND EXPERIMENTAL ANALYSIS OF THE CURVED DNAs AS THE HOT SPOTS OF RECOMBINATION

Timchenko T.V., Vasyunina E.A., Scheglova T.V., Rogozin I.B., Sinitsyna O.I.*

Institute of Cytology and Genetics SB RAS, 630090, Novosibirsk, Russia, e-mail: evas@niboch.nsc.ru
*Corresponding authors

***Key words**: recombination, curved DNA, double strand breaks, DNA rearrangements*

Resume

Motivation: The genetical recombination (GR) is a fundamental mutational process shaping genomes and providing an unvalued source for the evolutionary process. Investigation of molecular mechanisms responsible for recombinations and their inducing factors is a challenge to direct genomic rearrangements in either pro- or eukaryotes. It is very important for successful modelling of process of GR to use the combination of theoretical and experimental approach.

Results: To investigate a correlation between a local curvature of DNA and GR we have analyzed series of AT-rich DNA fragments (of 42 BP) with different curvatures of the sugar-phosphate backbone. Conformational deviations of the DNA fragments were revealed by means of computational analysis and the two-dimensional electrophoresis and then classified in accordance with their electrophoretic mobility. The fragments were cloned into plasmids what allowed us to reveal and quantify the induction of recombination. It was shown that the capability of AT-rich regions in DNA to stimulate recombinations at the flanking direct repeats correlates with some features of their local conformation.

Introduction

The curved DNA is one of the most important features of DNA studied by various theoretical and experimental methods (reviewed by Gabrielian, Bolshoy, 1999). Despite that the curved (bent) DNA structures are known for a number of years, their biological functions are not well understood (reviewed by Hagerman, 1990). The curved (bent) DNA is the sequence-directed curvature of the helix axis of double-stranded DNA, which arises from sequences containing tracts of oligo(dA)-oligo(dT). The anti-bent DNA is known as a succession of bends which cancel one another leading thereby to a straight DNA structure (Eckdal, Anderson, 1990). There are data supporting the suggestion concerning the participation of bent and/or anti-bent DNA elements in the genomic rearrangements (reviewed by Meuth, 1989). An important finding in favour of this notion which was reported recently is the discovery of the fact that in all junctions resulting from various types of chromosomal rearrangements in mammalian cells the intrinsically bent DNA elements are present (Milot et al., 1992; Stary, Sarasin, 1992). There are of convincing data showing that the bent DNA elements interact specifically with some functionally important proteins such the IHF and the HU protein (Pontiggia et al., 1993) It was found that the bent DNA structures are recognized by nicking enzymes (Howard et al., 1991). A number of studies demonstrate that DNA ends are needed for the GR (Roth, Wilson, 1988). We suggest that the bent DNA tracts are the sites where the double-strand DNA breaks (**dsb**) occur while as it was shown earlier the intramolecular recombinations are provided by the interaction of direct repeats flanking the broken ends (Salganik, Dianov, 1992). This study examines the effects of local curvature of DNA on recombination between the direct repeats flanking the sites of DNA bending.

Methods

Computational analysis. For bending and curvature calculation the program "BEND_TRI" (Goodsell, Dickerson, 1994) has been used. It was shown that this program produces a good approximation of bending and curvature values (Gabrielian et al., 2000). This program calculates the magnitude of local bending B(i) and macroscopic curvature C(i) at each position i along a sequence. This program is using DNA structure model consistent with both solution data from gel retardation, cyclization kinetics and structural data from x-ray crystallography (Goodsell, Dickerson, 1994). Bending of DNA described the tendency for successive base pairs to be non-parallel in an additive manner over several base pair steps. Curvature of DNA represents the tendency of the helix axis to follow a non-linear pathway over an appreciable length (Goodsell, Dickerson, 1994).

E.coli strains and plasmids. The *E. coli* strains used are listed in Table 1. Plasmids series on the base of pBR327 plasmid where the *tet* gene was disrupted by a complementary insertion of its own sequences forming direct repeats with length 165 or 21 bp were used (Dianov et al., 1991b; Mazin et al., 1991).

Media and growth conditions. The bacterial culture were grown in LB broth according to (Dianov et al., 1991b).

Recombinant DNA methods. Molecular cloning, purification of plasmid DNA, agarose and polyacrylamid gel electrophoresis, transformation and nucleotide sequence analysis were all performed according to standard procedure (Maniatis et al., 1982).

Table 1. Characteristics of *Escherichia coli* stains and plasmid used.

Strain	Relevant genotype[a]	Reference
AB1157	Rec^+	Bachmann (1972)
JC10287	d (srlR-recA)304	Czonka and Clark (1979)
JC5571	recB21recC22sbcB (RecF)	Horii and Clark(1973)
JC5589	recB21recC22sbcA (RecE)	Horii and Clark(1973)

[a]In all cases, other mutations were: thr-1 ara-14 leuB6 del(gpt-proA062 lacY1tsx-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1.

Measurement of deletion frequencies. The deletions mediated by direct repeats restore the structure of tet gene and, as a consequence, a reversion to tetracycline resistance occurred. The procedure used for measuring deletion frequencies was according to (Dianov et al., 1991b).

Results and Discussion

We have analyzed series of DNA fragments (of 42 bp) with different AT-rich structure.

Results of computational analysis of these fragments allowed to select three ones with different curvature:

A = GATCCATATTTAAAATTTATGGGTCTATATTTATTATTAAAA

C = AGAGATTTTTTAGAGATTTTTAGAGATTTTTTAGAGATTTTT

N = TCTAATCTCTCTCTAATCTCTTCTAATCTCTCTCTAATCTCT

where **A** is anticurved DNA fragment, **C** is curved DNA fragment, **N** is normal DNA fragment. Results of computational analysis of these fragments are shown in Table 2.

Table 2. Curvature and bending measures for 3 oligonucleotides.

Oligonucleotide	Mean value of C(i)	Maximum value of C(i)	Mean value of B(i)	Maximum value of B(i)
A	5.7	18.1	12.6	26.9
C	12.3	30.5	11.7	26.9
N	7.6	17.7	13.9	26.9

Both mean and maximum values of C(i) for the C-fragment were significantly higher compared to A- and N-fragments while no differences were observed for B(i) values. Conformational deviations of the DNA fragments were also analyzed by means of the two-dimensional electrophoresis and then classified in accordance with their electrophoretic mobility. A-fragments showed a pretty high electrophoretic mobility. This allowed us to consider them anticurved. C-fragments showed low electrophoretic mobility, so they were considered as curved. And the control N-fragments did not deviate from the marker's edge.

The fragments were cloned between the direct repeats into plasmids what allowed us to reveal and quantify the induction of recombination (Fig. 1). The repeats were separated by a polylinker containing unique restriction sites (we used *Sma1* site) what allowed to insert arbitrary DNA fragments and thus to define their contribution to recombination. The recombination at direct repeats leads to the deletion of one repeat altogether with the region between them. This regenerates the *tet* gene function and ensures the quantification of recombination in a simple phenotype test (Dianov et al., 1991b). It was been shown that the capability of analyzed AT-rich regions in DNA to stimulate recombinations at the flanking direct repeats correlates with some features of their local conformation. Anticurved **(A)** and curved **(C)** DNA fragments stimulated the recombination frequency at direct repeats flanking sites of curvature of DNA in different strains of *E. coli* in contrast to normal **(N)** DNA fragment. The level of recombination induction does not depend on the length of the repeats flanking the site of curvature. Also, it does not depend on the recombination path within *E. coli* cells.

Structural analysis of deletion plasmids has revealed all spontaneous recombinants as dimers and the recombinants induced by the conformational alterations were present as monomers (Fig. 2). We analyzed 50 tetracycline-resistant clones for each experiment.

So, the data obtained evidence in favour of the fact that AT-rich DNA regions with computational predicted either curved or anticurved sugar-phosphate backbone may be considered as sites of specific DNA incision thus ensuring their potential "hot spot" functioning during the homologous recombination.

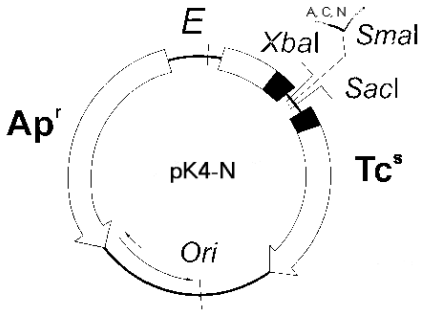


Fig. 1. A scheme of the construction of DNA plasmids containing A, C, N – DNA fragments: direct repeats are painted black. *N* is length of repeats.

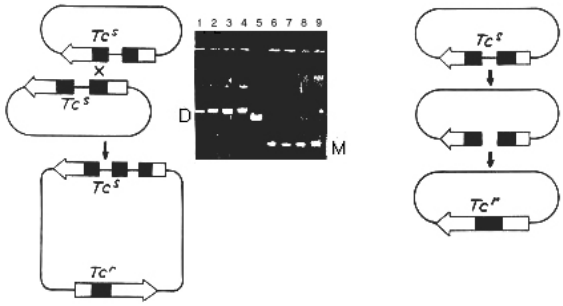


Fig. 2. Analysis of deleted plasmids by 1% agarose gel electrophoresis.

D, dimers; M, monomers. Lanes 1-4, pK4-165N plasmids from tetracycline-resistant clones; lane 5, marker of dimer of pK4-165; lines 6-8, pK4-165C plasmids from tetracycline-resistant clones; lane 9, marker of monomer of pK4-165.

Acknowledgements

The research was supported in part by grants from Russian Foundation for Basic Research (02-04-49889 and 02-04-48342).

References

1. Anderson J.N. (1986) Nucl. Acids Res. 14, 18513-8533.
2. Birnboim H.C., Doly J. (1979) Nucl. Acid Res. 7, 1513-1522.
3. Dianov G.L., Timchenko T.V., Sinitsina O.I., Kuzminov A.V., Medvedev O.A., Salganik R.I. (1991a) Mol. Gen. Genet. 225, 448-452.
4. Dianov G.L., Kuzminov A.V., Mazin A.V., Salganik R.I. (1991b) Mol. Gen. Genet. 228, 153-159.
5. Eckdahl T.T., Anderson J.N. (1990) Nucl. Acids Res. 18, 1609-1612.
6. Ehrlich S.D. (1989) In Berg D.E., Howe M.M. (eds). Mobile DNA. American Society for Microbiology, Washington DC, 797-830.
7. Gabrielian A., Bolshoy A. (1999) Comput. Chem. 23, 263-274.
8. Gabrielian A.E., Landsman D., Bolshoy A. (2000) In Silico Biol. 1, 183-196.
9. Goodsell D.S., Dickerson R.E. (1994) Nucl. Acids Res. 22, 5497-5503.
10. Hagerman P.J. (1990) Ann. Rev. Biochem. 59, 755-781.
11. Howard M.T., Maxwell P.L., Hsich T., Griffith J.D. (1991) J. Mol. Biol. 217, 53-62.
12. Maniatis T., Fritsch E.F., Sambrook J. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
13. Mazin A.V., Kuzminov A.V., Dianov G.L., Salganik R.I. (1991) Mol. Gen. Genet. 228, 209-214.
14. Meuth M. (1989) In Berg D.E., Howe M.M. (eds). Mobile DNA. American Society for Microbiology, Washington DC, 797-830.
15. Milot E., Belmaaza A., Wallenburg J.C., Gusew N., Bradley W.E.C., Chartrand P. (1992) EMBO J. 11, 5063-5070.
16. Pontiggia A., Negri A., Beltrame M., Bianchi M.E. (1993) Mol. Microbiology. 7, 343-350.
17. Roth D., Wilson J. (1988) In Kucherlapati R., Smith R. (eds). Genetic recombination. American Society for Microbiology, Washington DC, 621-653.
18. Salganik R.I., Dianov G.L. (1992) Mutation Res. 266, 163-170.
19. Stary A., Sarasin A. (1992) Nucl. Acid Res. 20, 4269-4274.

CO-ORDINATION OF BASE EXCISION REPAIR

Allinson S.L., Dianov G.L.*

MRC Radiation and Genome Stability Unit, Oxfordshire, U.K, e-mail: g.dianov@har.mrc.ac.uk

Key words: *DNA damage/base excision repair/repair pathways*

Resume

Simple base damages are repaired through the short-patch base excision repair (BER) pathway when only one damaged nucleotide is removed and replaced. BER is initiated by DNA glycosylase, which removes the damaged base. AP endonuclease cleaves the phosphodiester bond adjacent to the AP site and then DNA polymerase and DNA ligase complete the repair. During long-patch repair DNA polymerase adds several nucleotides and displaces the 5'-sugar phosphate as a part of an oligonucleotide flap that is subsequently removed by a specific endonuclease. Recent data indicate that these processes are co-ordinated by multiple protein-protein interactions. We will discuss the molecular mechanisms involved in directing DNA damage processing.

Introduction

DNA is an inherently unstable molecule and is subject to chemical degradation through processes such as hydrolysis, oxidation and alkylation (*1*). This instability of the primary structure of DNA may lead to a loss or corruption of the information held within the genome with potentially deleterious effects for the organism. The aqueous environment of the cell is itself a source of endogenous DNA damage, with many of the chemical bonds of DNA being labile under physiological conditions. For example, it has been estimated that the instability of the N-glycosylic bond of purines to hydrolysis may result in the formation of abasic (AP) sites at a rate of approximately 10 000 per human genome per day (*2*). The accumulation of unrepaired AP sites can both block DNA synthesis and promote mutations (*3*). In addition to the lability of the N-glycosylic bond, the exocyclic amino groups of the DNA bases are subject to hydrolytic deamination. In the case of cytosine, this results in the formation of uracil which, if not repaired, can directly mispair with adenine, generating C to T transition mutations (*4*). Oxidative damage to DNA by reactive oxygen species, which arise as a result of normal oxidative metabolism, is also an important source of endogenous damage. For example, reactive oxygen species can be formed as a result of exposure to ionising radiation (*5*). Oxidation of DNA bases occurs predominately through radical addition to the π-bonds of the purine or pyrimidine rings (*6*). The products of such reactions, e.g. 8-oxoguanine, formamidopyrimidines and ring saturated pyrimidines such as thymine glycol, are often mutagenic or cytotoxic (*7*). The ubiquity of DNA damaging agents and the need to ensure the integrity of the genome has necessitated the evolution of a system of DNA repair mechanisms which are able to reverse the effects of DNA damage. The majority of the simpler, non-bulky base lesions discussed above are repaired via Base Excision Repair (BER) pathways. BER has been functionally conserved throughout evolution and is present in all forms of life from bacteria to the higher mammals (*8*). The crucial role BER plays in maintaining genome stability is signified by the embryonic lethal phenotype of mouse knockout models deficient in key enzymes of BER such as APE1 (*9*), Pol β (*10*) and DNA ligase I (*11*). The basic enzymology of BER has been reasonably well elucidated and the repair pathways have been reconstituted *in vitro* using purified enzymes (*12-14*). BER is a multistep process initiated by the activity of a class of enzymes, termed glycosylases, which specifically catalyse the removal of the damaged base, leaving an abasic site (AP site). Subsequent processing of the AP site is then proposed to proceed via one of a number of possible sub-pathways depending on the nature of the initiating glycosylase and the abasic site. These sub-pathways are the two short-patch (single-nucleotide replacement) and the long-patch pathway (Fig.). These pathways share a commonality of mechanism in their requirement for an AP endonuclease, a DNA polymerase and a DNA ligase, with a number of accessory enzymes playing an additional role. However, it is yet to be determined how the repair machinery is directed towards one sub-pathway rather than another. Glycosylases can be broadly divided into two different subgroups, the monofunctional, or pure, glycosylases and the bifunctional glycosylases which have an associated AP lyase activity (*5*). In the former case, of which uracil-DNA glycosylase (UDG), is a classic example, the base is removed through nucleophilic attack of the N-glycosylic bond by an activated water molecule. Repair then proceeds via a highly co-ordinated series of concerted reactions. The second class of glycosylases, the bifunctional glycosylases, include the oxidative damage processing enzymes hOGG1, which removes oxidised purines, and hNTH1, which removes oxidised pyrimidines. Bifunctional glycosylases remove damaged bases through nucleophilic attack by an active site lysine residue, forming a Schiff base with C1' of the deoxyribose during the course of the excision reaction. This covalent complex can either hydrolyse, leaving an intact AP site, or can undergo β-elimination, cleaving the phosphodiester backbone 3' to the AP site, leaving a 3'-α,β-unsaturated aldehyde residue and a 5'-phosphate (*15*). The blocking unsaturated aldehyde residue can then be excised by APE1 (*16*), although the 3'-phosphodiesterase activity of APE1 is significantly (100 fold) weaker than its AP

* Corresponding author.

endonuclease activity (*17*). The product of sequential AP lyase and 3'-phosphodiesterase processing of an AP site is a one-nucleotide gap with flanking 3'-OH and 5'-phosphate terminii. Repair could then be completed simply by insertion of a single nucleotide followed by ligation and indeed, short patch BER predominates in the repair of the oxidative lesions, 8-oxoguanine and thymine glycol (*18-19*). Although several pathways are involved in BER, the mechanism directing and co-ordinating these pathways is unclear.

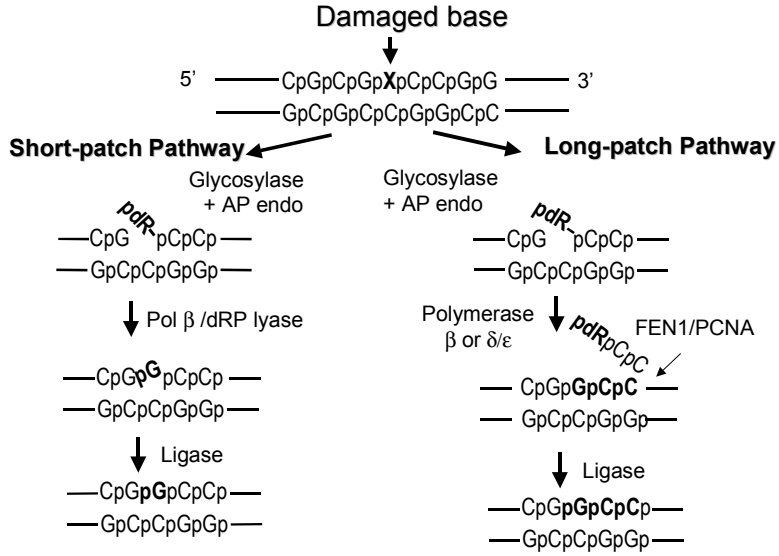


Fig. Base excision repair pathways.

Results and Discussion

Recent work carried out in our laboratory has found that repair by whole cell extracts of a single 8-oxoguanine residue site-specifically incorporated into closed circular substrate DNA proceeds via a Pol β-dependent short-patch mechanism. In the absence of Pol β, i.e. in Pol β null mouse cell extracts, repair is only able to proceed through the long-patch pathway. Complementation of Pol β null extracts with the dRP lyase-deficient Pol β K72A mutant did not restore single nucleotide patch repair. We therefore concluded that a dRP residue remained at the 5' terminus following processing of 8-oxoguanine by OGG1 and APE1 with the implication that, under our experimental conditions, repair proceeded via the same pathway as that for repair initiated by monofunctional glycosylases (*20*). It is tempting to speculate that the majority of short-patch base excision repair proceeds through a single general mechanism regardless of the AP lyase activity of the initiating glycosylase and the alternative pathway is relatively minor. In this model the observed AP lyase activity of the bifunctional glycosylases might be an artefactual consequence of Schiff base formation during the glycosylase reaction, which may serve some other function *in vivo*. It is possible that the formation of a covalent complex between the glycosylase and the AP site serves to protect the AP site from degradation by the cellular milieu.

As discussed above, the dRP lyase activity of polymerase β must remove the dRP residue from the 5'-terminus of the nicked BER intermediate before ligation and therefore completion of the short patch repair pathway can occur. However, the dRP lyase activity of Pol β proceeds via a β-elimination mechanism and is therefore exquisitely sensitive to chemical modification of the dRP. Both oxidised and reduced AP sites are refractory to β-elimination and repair of these sites must occur by an alternative mechanism. This repair is accomplished through the long-patch BER pathway in which between 2 and 8 nucleotides are incorporated into the repair gap. The dRP is displaced as part of an oligonucleotide 'flap' which is subsequently removed by flap endonuclease (FEN1) *(14-2)*. DNA ligase is then free to complete the repair process by rejoining the free ends of the DNA. The long-patch pathway has been reconstituted in vitro using purified proteins by a number of laboratories and it has been demonstrated that under such conditions both Pol β and Pol δ/ε are able to support repair. Long-patch repair was found to be proliferating cell nuclear antigen (PCNA) dependent and a model was proposed whereby Pol δ, the activity of which is PCNA dependent, was responsible for the strand resynthesis step of long-patch BER (*21*). This model, however, prompts the question: how does the cellular repair machinery direct repair through one or other pathway before the dRP intermediate is reached?. We hypothesised that BER is initiated by Pol β regardless of the repair patch size. We subsequently incorporated the polymerase inhibitors aphidicolin and dideoxynucleotide triphosphates in repair reactions catalysed by human whole cell extracts and demonstrated that repair of reduced AP sites by mammalian cell extracts was insensitive to aphidicolin (*22*). These data demonstrated that long-patch BER was initiated by Pol β and we were able to propose a model by which both short- and long-patch BER proceed via a unified mechanism up to insertion of the first nucleotide.

Conclusions

There is increasing evidence that BER proceeds through a highly orchestrated mechanism with each step co-ordinated with the next through protein-protein interactions. The co-ordination of repair in this manner is likely to have evolved to protect the genome from the potentially deleterious effects of exposing repair intermediates to the cellular milieu. We have proposed a model whereby BER proceeds through a single mechanism up to the point immediately following insertion of the first nucleotide. In this model, the signal for direction of repair through either of the short- patch or long-patch repair pathways would be the lability of the dRP residue to β-elimination by the dRP lyase activity of Pol β. This provides a logical solution to the problem of the cell having to 'know' which pathway to proceed along before commencing repair.

References

1. Lindahl T. (1993) Nature. 362, 709-715.
2. Lindahl T., Nyberg B. (1972) Biochemistry. 11, 3610-3618.
3. Loeb L.A., Preston B.D. (1986) Ann. Rev. Genet. 20, 201-230.
4. Duncan B.K., Miller J. (1980) Nature (London). 287, 560-561.
5. Demple B., Harrison L. (1994) Annu. Rev. Biochem. 63, 915-948.
6. Burrows C.J., Miller J.G. (1998) Chemical Reviews. 98, 1109-1151.
7. Wallace S.S. (1998) Radiat. Res. 150, S60-79.
8. Lindahl T., Wood R.D. (1999) Science. 286, 1897-1905.
9. Xanthoudakis S., Smeyne R.J., Wallace J.D., Curran T. (1996) Proc. Natl Acad. Sci. USA. 93, 8919-8923.
10. Gu H., Marth J.D., Orban P.C., Mossman H., Rajewsky K. (1994) Science. 265, 103-106.
11. Bentley D., Selfridge J., Millar J.K., Samuel K., Hole N., Ansell D.W., Melton D. W. (1996) Nat. Genet. 13, 489-491.
12. Dianov G., Lindahl T. (1994) Curr. Biol. 4, 1069-1076.
13. Kubota Y., Nash R.A., Klungland A., Schar P., Barnes D., Lindahl T. (1996) EMBO J. 15, 6662-6670.
14. Klungland A., Lindahl T. (1997) EMBO J. 16, 3341-3348.
15. Mol C.D., Izumi T., Mitra S., Tainer J.A. (2000) Nature. 403, 451-454.
16. McCullough A.K., Dodson M.L., Lloyd R.S. (1999) Annu. Rev. Biochem. 68, 255-285.
17. Klungland A., Hoss M., Gunz D., Constantinou A., Clarkson S.G., Doetsch P.W., Bolton P.H., Wood R.D., Lindahl T. (1999) Mol. Cell. 3, 33-42.
18. Wilson D.M. III, Barsky D. (2001) Mutat. Res. 485, 283-307.
19. Dianov G., Bischoff C., Piotrowski J., Bohr V.A. (1998) J. Biol. Chem. 273, 33811-33816.
20. Dianov G.L., Thybo T., Dianova I.I., Lipinski L.J., Bohr V.A. (2000) J. Biol. Chem. 275, 11809-11813.
21. Allinson S.A., Dianova I.I., Dianov G.L. (2001) EMBO J. 20, 6919-6926.
22. Matsumoto Y., Kim K., Bogenhagen D.F. (1994) Mol. Cell. Biol. 14, 6187-6197.
23. Podlutsky A.Ja., Dianova I.I, Podust V.N., Bohr V.A., Dianov G.L. EMBO J. 20, 1477-1482.

SUBSTRATE SPECIFICITY OF DNA-HYDROLYZING ANTIBODIES FROM BLOOD OF PATIENTS WITH MULTIPLE SCLEROSIS

Baranovskii A.G.[1,*], Buneva V.N.[1,2], Doronin B.M.[3], Mogelnitskii A.S.[4], Favorova O.O.[5], Nevinsky G.A.[1,2]

[1] Institute of Bioorganic Chemistry SB RAS, 630090, Novosibirsk, Russia, e-mail: baranoff@niboch.nsc.ru
[2] Novosibirsk State University, 630090, Novosibirsk, Russia
[3] Novosibirsk Medical University, Novosibirsk, Russia
[4] Novosibirsk Army Hospital, Novosibirsk, Russia
[5] Russian State Medical University, Moscow, Russia
[*]Corresponding author

Key words: *DNA-hydrolyzing antibodies, substrate specificity, multiple sclerosis*

Resume

*Motivation***:** DNA-hydrolyzing antibodies (Abs, DNA-abzymes) were shown to be good biochemical markers of some autoimmune diseases such as systemic lupus erythematosus (SLE) and multiple sclerosis (MS). Therefore, the detailed study of specificity of substrate hydrolysis by the DNA-abzymes, comparison of enzymic properties of Abs with those for enzymes possessing the same activity, and the study of optimal conditions for DNA hydrolysis may be considered as an additional effective way for MS diagnostics and for a evaluation of deterioration or improvement of the clinical state of the patients.

Results: The specificity of homo- and heterooligonucleotides hydrolysis by various preparations of DNA-abzymes from the blood of patients with MS was compared with that for known human DNases. It was shown that polyclonal MS-Abs can contain from one to a few types of DNase activities activated or not by metal ions and characterized by different substrate specificities**.** The findings speak in favor of the generation of either a relatively small or an extremely large pool of polyclonal catalytic IgG by the immune system of individual patients.

Introduction

DNA- and RNA-hydrolyzing antibodies (AT) were detected in the sera of patients with several autoimmune and viral diseases: SLE, rheumatoid arthritis, sclerodermia, Hashimoto's thyroiditis, polyarthritis, multiple sclerosis, viral hepatitis, and AIDS (Vlassov et al., 1998; Baranovskii et al., 1998; reviewed in Nevinsky et al., 2000, 2002). We have shown also that the milk of normal human mothers contains sIgA and IgG Abs hydrolyzing DNA and RNA. At the same time, we did not detect DNase or RNase activities of Abs from the sera of 50 normal humans (men and women) and in sera of patients with influenza, pneumonia, tuberculosis, tonsillitis, duodenal ulcer, and some types of cancer. The nuclease activities were shown to be an intrinsic property of auto-Abs in all the above cases.

All the data obtained demonstrate an extremely pronounced heterogeneity of polyclonal nuclease Abs from sera of autoimmune patients concerning all their kinetic characteristics, substrate specificities, and relative specific activities.

Methods

IgGs were isolated from blood serum of MS-patients or healthy donors using a method previously described by us for serum Ab purification on protein A-Sepharose under conditions which remove nonspecifically bound proteins and lead to essentially homogeneous preparations of IgG (Baranovskii et al., 1998).

Deoxyribooligonucleotides (ON) cleavage was determined by incubation of various 5'-[^{32}P]-labeled ON (5 nM) with IgG (0.2-0.6 μM) from different patients using reaction mixtures (20 μl) containing 20 mM Tris-Hepes, pH 7.4, and some other components (*see* below) at 37^{o}C for 2 h. The reaction products were separated in 25% PAG containing 7 M urea in 50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.3 (50 V/cm, 6 h), and analyzed by autoradiography.

Results

Here we present for the first time the data for substrate specificity study of DNA-hydrolyzing antibodies from blood of patients with MS (in hydrolysis of different single stranded oligonucleotides) and the comparison of enzymic properties of MS-Abs with human nucleases.

As shown previously using the plasmid DNA cleavage assay, DNase activity of IgGs was detected in ~90% patients with MS (Baranovskii et al., 1998). However, the relative activities of IgGs from the sera varied markedly from patient to patient. In order for more detailed analysis of MS-abzymes a comparison of substrate specificity of Abs of several patients was carried out using different ON.

It should be noted that the ON cleavage pattern was strongly dependent on the patient (Abz1-Abz5). Some Abs demonstrated sequence-independent hydrolysis of ON. For example, Abz1 and Abz2 hydrolyzes all internucleoside bonds of homo- and heteroON with comparable efficiency (Fig. 1, lane 4, 6, and Fig. 2, lane 7, 9).

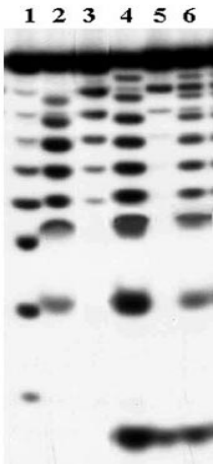


Fig. 1. Comparison of abzyme- and DNase-catalyzed oligonucleotide $d(T_{10})$ cleavage patterns. The 5`-[32P]-labeled ON (5 nM) was digested with DNase II (lane 1; 50 μg/ml, 50 mM sodium acetate, pH 5.0, 0.8 mM $MgCl_2$), DNase I (lane 2; 50 μg/ml, 20 mM Tris-Hepes, pH 7.4, 4.8 mM $MgCl_2$, 0.5 mM $CaCl_2$), and with Abs from various patients (lanes 3-6; 30-90 μg/ml, 20 mM Tris-Hepes, pH 7.4, 5 mM $MgCl_2$). Lane 3 – Abz4, lane 4 – Abz1, lane 5 – Abz5, lane 6 – Abz2.

Sometimes (for example, Abz2) Abs produces in parallel both 5'-phosphate terminated products (similar to those of DNase I; lane 2) and 3'-phosphate terminated ONs (doublet bands of Fig. 1, lane 6), which are typical for DNase II (lane 1). Fig. 2a demonstrates that Abz3 hydrolyzes heteroON in a sequence-dependent manner. In contrast to other Abs, Abz4 possessed only 3'-exonuclease activity (Fig. 1, lane 3), while Abz5-dependent products correspond to both 3'- and 5'-exonuclease activities (Fig. 1, lane 5). We have revealed that, in principle, there may be different MS-Abs demonstrating all possible combinations of endo- and exonuclease activities.

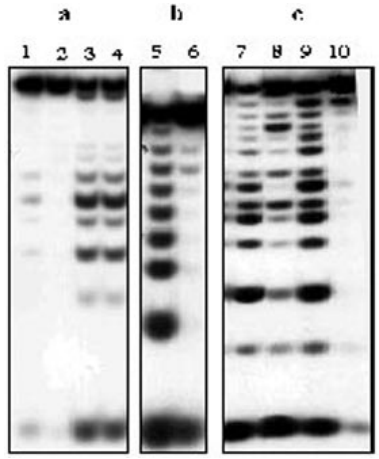


Fig. 2. Influence of some compounds on the hydrolysis of 5`-[^{32}P]-labeled ONs (**a** – dAGCAGTGGCGCCCGA; **b** - $d(pT)_{10}$; **c** - dCCAGTCACGACGTT) with different MS-Abs (1-4 - Abz3; 5-8 - Abz1; 9,10 - Abz2) in 20 mM in Tris-Hepes, pH 7.4, containing (with the exception of lane 10) 5 mM $MgCl_2$ and: lane 1 – 10 nM DNA of a Bluescript plasmid; lane 2 – 0.2 μM none-labeled 5`-phosphorylated ON of the same sequence; lane 3, 5, 7, 9 – no additions; lane 4 - 100 mM NaCl; lane 6 – 2 mM potassium phosphate; lane 8 – 0.3 mM EGTA; lane 10 – 1 mM EDTA.

It was shown that hydrolysis of ONs by different Abs may be strongly dependent on the conditions used. Thus, comparison of lanes 7 and 8 (Fig. 2) demonstrates only a change of site-specificity of Abz1 after addition of EGTA to reaction mixture containing $MgCl_2$. At the same time in the absence of $MgCl_2$ Abz2 can not display its endonuclease and 5'-exonuclease activities (compare lanes 9 and 10, Fig. 2), while Me^{2+} ions do not influence on Abs 3'-exonuclease activity. Increase of NaCl concentration up to 100 mM (Fig. 2, compare lanes 3 and 4) did not effect on the cleavage of ON by Abz3, while addition of potassium phosphate (up to 3 mM) strongly inhibited endonuclease activity of Abz1 (Fig. 2, compare lanes 5, 6). Inhibition experiments indicate that in contrast to known DNases, MS-Abs show very high affinity for both double stranded DNA (Kd <10 nM, Fig. 2, lane 1) and single stranded ON (Kd <100 nM, Fig. 2, lane 2).

Discussion

Polyclonal MS-Abs were found to be capable of hydrolyzing of both single and double stranded DNA and there is a strong variation of the cleavage patterns of substrates from patient to patient. In addition, high concentration of NaCl did not effect on cleavage of ONs, but 50 mM NaCl inhibited hydrolysis of plasmid DNA by a factor of 2 (data are not shown). On the contrary, potassium phosphate can inhibit hydrolysis of ON. According to influence of Me^{2+} ions investigated MS-abzymes may be divided into three groups: only Mg^{2+} dependent Abs; Abs dependent upon other Me^{2+} or Mg^{2+} simultaneously with other metal ions; and metal independent Abs.

All the data obtained clearly show that preparations of polyclonal abzymes can consist of a mixture of different number of subfractions of Abs which can hydrolyze DNA at various conditions and in according to differing mechanisms. The findings speak in favor of the generation of either a relatively small or an extremely large pool of polyclonal catalytic Abs by the immune system of individual patients. Most probably that the fraction of catalytic MS-Abs more often contains a specific major subfraction due to which all of them share common properties in the hydrolysis of classical double-stranded DNA substrates.

According to the literature data only one nuclease cleaving ON is presented at human sera: PDase I - phosphodiesterase giving 5'-nucleotides, and it is unselective 3'-exonuclease dependent on Mg^{2+} or Ca^{2+} ions. In spite of some similarity, the specificities of catalytic Abs and known human DNase I, DNase II, and phosphodiesterase are quite different and the observed spectra of catalytic Ab substrate specificity differs remarkably from patient to patient. Therefore, the observed multiplicity of abzymes activities is impossible to explain by changes in the sets of DNases in different individuals. Taken together it is clear that polyclonal catalytic antibodies of each MS patient can contain extremely different repertories of DNA-hydrolyzing IgG subfractions.

Acknowledgements

This work was supported by grants from INTAS, the Russian Foundation for Basic Research (№ 01-04-49759, 01-04-49761), Russian Ministry of Education («Foundational Researches in Technical Sciences» (№ TOO-9.3-2074) and grant from the 6-th Competition-Examination for Young Scientists of RAS on Fundamental and Applied Researches (№ 234).

References

1. Baranovskii A.G., Kanyshkova T.G., Mogelnitskii A.S., Naumov V.A., Buneva V.N., Gusev E.I., Boiko A.N., Zargarova T.A., Favorova O.O., Nevinsky G.A. (1998) Polyclonal antibodies from blood and cerebrospinal fluid of patients with multiple sclerosis effectively hydrolyze RNA and DNA. Biochemistry (Moscow). 63, 1459-1469.
2. Nevinsky G.A., Kanyshkova T.G., Buneva V.N. (2000) Natural catalytic antibodies (abzymes) in normalcy and pathology. Biochemistry (Moscow). 65, 1245.
3. Nevinsky G.A., Favorova O.O., Buneva V.N. (2002) Natural Catalytic Antibodies - New Characters in the Protein Repertoire. In: Protein-protein interactions; a molecular cloning manual, E. Golemis eds. Cold. Spring Harbor, 523-534.
4. Vlassov A.V., Baranovskii A.G., Kanyshkova T.G., Prints A.V., Zabara V.G., Naumov V.A., Breusov A.A., Giege R., Buneva V.N., Nevinsky G.A. (1998) Substrate specificity of serum DNA- and RNA-hydrolyzing antibodies of patients with polyarthritis and autoimmune thyroiditis. Mol. Biol. (Moscow). 32, 463-470.

BONE MARROW HEMOPOIESIS IN IMMUNOPATHOGENESIS OF AUTOIMMUNE-PRONE MRL/MPJ*LPR* MICE

Orlovskaya I.A.*, Dubrovskaya V.V.[1], Toporkova L.B., Chernykh E.R., Tikhonova M.A., Sakhno L.V., Buneva V.N.[1], Kozlov V.A., Nevinsky G.A.[1]

Research Institute of Clinical Immunology, SB RAMS, Novosibirsk, Russia
[1] Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia
*e-mail: irorl@mail.ru

Key words: *MRLMpJlpr mice, autoimmunity, cell colony formation, lymphocyte proliferation, apoptosis*

Resume

Motivation: The study of hematopoietic disturbances in autoimmune MRLMpJ*lpr* mice may be very useful for understanding of mechanism of autoimmune disease development and searching for new strategies of autoimmune disorder correction.

Results: Lymphocyte proliferation and apoptosis at different stages of the autoimmune condition in MRLMpJ*lpr* mice was studied. Hematopoietic progenitors colony formation in the course of the disease was characterized. A detectable difference at the level of lymphocyte proliferation, cell apoptosis, and the relative amount of BFU-E and CFU-GEMM cell colonies was revealed between healthy young mice and animals spontaneously developing pronounced symptoms of the autoimmune disorder.

Introduction

MRLMpJ*lpr* mice develop an autoimmune (AI) disorder characterized by marked hypergammaglobulinemia, production of numerous autoantibodies, and severe lymphadenopathy (lupus-like autoimmune disease). *Lpr* is known to correspond to a mutation within genes encoding Fas ligand, a death factor that binds to its receptor and induces apoptosis (Nagata, Suda, 1995). Polyclonal TCR^+ cells lacking CD4 and CD8 (double negative T cells) accumulate in lymph nodes and other organs of MRL*lpr*/*lpr* mice due to lack of Fas-induced apoptosis (Watanabe-Fukunada et al., 1992).

Undoubtedly, there is an intimate correlation between hematopoietic and immune systems. Hematopoietic processes participate heavily in organ-specific and systemic AI diseases. A perpetually growing number of observations suggest that AI diseases originate from defects in hematopoietic stem cells (HSCs) (Ikehara et al., 1990). Increased numbers of actively proliferating HSCs has been found in NZB mice characterized by spontaneous development of systemic lupus erythematosus (SLE) (Morton et al., 1978). MRL*lpr*/*lpr* mice have radioresistant abnormal stem cells: an age-dependent increase in splenic colony forming units (CFUs) in *lpr*/*lpr* has been described (Ishida et al., 1994). We have earlier shown abnormal bone marrow stem cell differentiation (numbers of bone marrow-committed precursors) in NZB mice: augmentation of erythroid differentiation is accompanied by a decline in myeloid precursors (Orlovskaya et al., 2001).

Materials and Methods

The onset of autoimmune conditions in MRLMpJ*lpr* mice was monitored by proteinuria (>2,5+). To study the development of the disease we estimated lymphocyte apoptosis and proliferation in thymus, spleen, bone marrow and lymph nodes. Bone marrow hematopoiesis (CFU-GM, BFU-E and CFU-GEMM colonies) was also evaluated. We studied three groups of mice: 1, young mice (4-5 weeks of age) without proteinuria (-); 2, pre-diseased mice (24 weeks of age), proteinuria (-); 3, spontaneously diseased mice (24 weeks of age) demonstrating pronounced proteinuria (++).

Quantitation of DNA fragmentation. Cells ($1x10^5$/ml) were washed in phosphate-buffered saline containing 0.02% EDTA and 0.1% NaN_3. Cells were fixed in 1 ml of 1% paraformaldehyde for 1 h at 4^oC. Fixed cells were incubated in 1 ml propidium iodide (PI) solution (50 μg/ml PI and 20 μg/ml RNase A) at 20^oC. PI fluorescence of individual nuclei was measured using an Epics Profile flow cytometer. A minimum of 10,000 events was counted per sample. Results are reported as the percentage of hypodiploid (fragmented) nuclei reflecting the relative proportion of apoptotic cells (Nicoletti et al., 1991).

Lymphocyte proliferation. All in vitro assays were performed in complete RPMI (50 μM β-mercaptoethanol, 100 UI/ml penicillin) supplemented with 10% FCS. Cells were isolated and cultured ($1x10^5$ cells) in 0,15 ml medium with or without 2 μg/ml concanavalin A. Proliferation assays were performed for three days, ^3H incorporation was measured over the last 18 h. of culture.

In vitro culture of bone marrow progenitor cells. Bone marrow was flushed out of femurs of mice. To assess bone marrow cells colony formation, $2x10^4$ cells/dish were cultured in methylcellulose-based medium for murine cells M 3434 (Stem Cell Technology, Canada) containing SCF, EPO, IL-3, IL-6. Colonies (CFU-GM, BFU-E, CFU-GEMM) were scored after 14 days of incubation at 37^0 C and 5% CO_2 in a humidified incubator.

Results

The lymph node cells of young mice (group 1) were characterized with low mitogen responsiveness, which was not associated with increased cell death, because spontaneous and activated lymphocyte apoptosis was not increased (Table). The level of spontaneous and stimulated lymphocyte proliferation in pre-diseased mice without proteinuria (group 2) was increased, especially for LN Con A-stimulated cells ($P<0.05$, as compared with group 1). Similar to young mice, it was not accompanied by enhanced cell death. Thus, the level of cell apoptosis in both mice groups did not differ.

Table. Lymphocyte proliferation and apoptosis.

Parameter	Thymus		Lymph nodes (LN)		Spleen	
Proliferation	0[ξ]	ConA	0	ConA		ConA
Group 1	557±95	10541±2268	1204±730	2473±1637	1366±541	25559±5902
Group 2	1011±374	17913±5287	1747±304	15692±6327*	3863±2176	40550±10943
Group 3	1424±536	4993±2633**	1401±424	14209±5174	4288±1222	50991±15720
Apoptosis (24 h)						
Group 1	12±2	23±3	11.5±0,5	18.5±0,5	10±1	13±3
Group 2	8,7±1.2	24.3±3,2	12.7±2	18.5±2.5	17±2.1	21.3±3
Group 3	26±3.2	27±4.4*,**	14,2±1,3	16,3±1.9	17.2±3.1	19±2.8

[ξ] without mitogen; * $p<0,05$ as compared with group 1 (W-M-U test); ** $p<0,05$ as compared with group2 (W-M-U test).

The proliferative response level of thymocytes of diseased mice with pronounced proteinuria was significantly decreased while the level of thymocyte spontaneous apoptosis was elevated ($P<0,05$) in comparison with groups 1 and 2. It should be noted that splenocyte proliferation in mice with nephritis was 2-fold higher than in young mice. Our results are supported by well-known data that autoimmune MRLMpJ*lpr* mice show a dramatic thymic involution with age. It is possible that autoimmune process development is associated with the depletion of CD4+CD25+ T cells that can control the population of autoreactive T cells (Pacholczyk et al., 2002).

We have revealed the normal distribution of committed progenitors in bone marrow of young mice, in the same manner as in normal young (DBAxC57Bl/6)F1-hybrids (Fig.). The quantity of BFU-E and CFU-GEMM colonies was remarkably increased in aged MRLMpJ*lpr* mice without clinical manifestation of disease (proteinuria). An elevated number of CFU-GEMM was accompanied by a striking increase in their size (CFU-GEMM colonies area was about equal to the area of the others). Age-dependent rise of HSC proliferation seems to be one of the causes of the active hematopoiesis we have observed in bone marrow of pre-diseased mice.

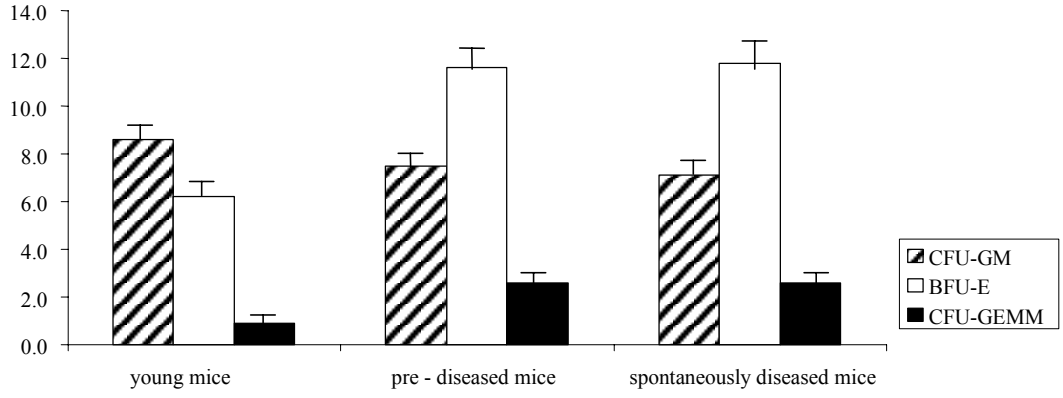


Fig. Bone marrow hemopoietic progenitors nambers in MRL-MpJ lpr mice of different groups.

As for the diseased (proteinuria, ++) mice we have revealed the same frequency of every investigated progenitor as in pre-diseased mice, but the size of colonies looked normal.

The augmentation of HSC proliferation accompanied by the increase in the number of BFU-E and CFU-GEMM colonies as a consequence of disturbances in the regulation of proliferation and differentiation of HSC may contribute to the formation of the immunopathological status.

Morphological investigations (Warner, 1971) has been shown that bone marrow hematopoiesis depends on the stage of autoimmune disease: severe forms were associated with bone marrow hypoplasia, whereas the light forms of autoimmune disorders were accompanied by bone marrow hyperplasia. It was demonstrated that expressed bone marrow hypoplasia in autoimmune hemolytic anemia and others AI diseases is caused by anti-erythrocyte autoantibodies that can interact with hemopoietic precursors.

Our continuing studies are aimed to determine a possible influence of immune disturbances following the development of AI conditions on hematopoiesis in MRLMpJ*lpr* mice.

Acknowledgements

This work was supported by a grant from INTAS, RFBR (№ 02-04-49350), and a grant for young scientists from Siberian division of RAS.

References

1. Ikehara S., Kawamura M., Takao F. et al. (1990) Organ-specific and systemic autoimmune diseases originate from defects in hematopoietic stem cells. Proc. Natl Acad. Sci. USA. 87, 8341-8344.
2. Ishida T., Inaba M., Hisha H. et al. (1994) Requirement of donor-derived stromal cells in the bone marrow for successful allogenic bone marrow transplantation. J. of Immunol. 152, 3119-3123.
3. Morton J.I., Siegel B.V. (1978) Transplantation of autoimmune potential. III. Immunological hyper-responsiveness and elevated endogenous spleen colony formation in lethally irradiated recipients of NZB bone marrow cells. Immunology. 5, 863-868.
4. Nagata S., Suda T. (1995) Fas and Fas ligand: *lpr* and *gld* mutations. Immunol. Today. 16**,** 39-43.
5. Nicoletti G., Migliorati M.C., Pagliacci et al. (1991) A rapid and simple method forme as during thymocyte apoptosis by propidium iodide staining and flow cytometry. J. of Immunol. Methods. 139, 271-275.
6. Orlovskaya I.A., Kozlov V.A. (2001) The interaction of hemopoiesis and immunogenesis in immunopathology development. Russ. J. of Immunology. 6, 2, 168-175.
7. Pacholczyk R., Kraj P., Ignatowicz L. (2002) Peptide specificity of thymic selection of CD4+CD25+ T cells. J. of Immunol. 168, 613-619.
8. Warner N.L., Moor M.A.S. (1971) Defect in hematopoietic differentiation in NZB and NZC mice. J. of Exp. Med. 2, 313-334.
9. Watanabe-Fukunada R., Brannan C.I., Copeland N.G. et al. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature. 356, 314-317.

ISOLATION AND IDENTIFICATION OF CELL SURFACE NUCLEIC ACIDS-BINDING PROTEINS

Chelobanov B.P., Laktionov P.P., Kharkova M.V., Rykova E.Yu., Pyshnyi D.V., Pyshnaya I.A., [1]Kim A.A., [2]Sczakiel G., Vlassov V.V.

Novosibirsk Institute of Bioorganic Chemistry SB RAS, 630090, Novosibirsk, Russia, e-mail: chelobanov@niboch.nsc.ru
[1] Institute of Nuclear Physics, Tashkent, Uzbekistan
[2] Institute of Molecular Medicine, Luebeck, Germany
*Corresponding author

Key words: *oligonucleotides, ODN-binding proteins, nucleic acids receptor, affinity modifications, isolation of oligonucleotide-binding proteins*

Resume

Motivation: Investigation of endogenic extracellular nucleic acids (exNAs) demonstrate that exNAs are not intact molecules, but could influence functioning of cells (Garcia-Olmo et al., 2000). To realize their potencies nucleic acids should interact with cell surface receptors or penetrate into cells. Binding and penetration of nucleic acids into cells was shown to depend from interaction with cell surface proteins (Laktionov et al., 1999). Thus these interactions are important in respect to functioning of extracellular nucleic acids. In practical aspects, these interactions determinate delivery and the efficiency of antisence oligonucleotides (ODN) and efficiency of transfection.

Results: It was shown that similar cell surface proteins bind single and double stranded DNA, few of these proteins were separated and identified by MS/MS sequencing.

Introduction

Penetration of nucleic acids and oligonucleotides into cells and proteins involved in this process were extensively investigated. Few protein candidates that can be involved in penetration of ODNs were described (Diesbach et al., 2000; Griffoni et al., 2001; Siess et al., 2000; Geselowitz, Neckers, 1995; Benimetscaya et al., 1997; Kimura et al., 1994). Keratinocytes are known as the only one cell type readily absorbing oligonucleotides and accumulating compounds in the nuclei (Noonberg et al., 1993), but the proteins that participate in binding and penetretion of NAs into keratinocites are not identified.

Earlier we have investigated interaction of different ODNs with keratinocytes by affinity modification of proteins of cell surface with ^{32}P-labeled reactive oligonucleotide conjugates. Binding of ODNs with proteins does not depend from sequence of oligonucleotide whereas patterns of affinity labeled oligonucleotide-binding proteins and the extent of modification of individual proteins depend on the type of reactive group of the conjugate. It was clearly demonstrated that 68 kDa oligonucleotide-binding proteins are exposed out of the cells and participate in binding of oligonucleotides.

The objectives of this work were investigation of affinity modification of cell surface proteins with ss- and ds-deoxyribooligonucleotides, development of the method of separation of nucleic acids binding proteins, separation and identification of the proteins responsible for binding of nucleic acids with cell surface.

Materials and Methods

Oligonucleotide pCAGTAAATATCTAGGA ($p(N)_{16}$) was synthesized in a synthesizer ASM-700 (BioSet, Russia) by the phosphorimidite method. Oligonucleotide pCAGTAAATATCTAGGA-deg-rU ($p(N)_{16}$degU) was synthesized as referred (Duran et al., 1990). The 5' end was labeled with ^{32}P using T4 polynucleotide kinase. Oligonucleotide $p(N)_{16}$ was conjugated to alkylating reagent 4-[(N-2-chloroethyl-N-methyl)amino]benzylamine (ClR-) through its 5'-phosphate according to (Mishenina et al., 1979). Fluorescein isothiocyanate was conjugated with ODN-rU after modification of 5'-phosphate with diaminopentane (DAP) in 0,1 M Na_2CO_3 for 4 hours at room temperature as described earlier (Haralambidis et al., 1987).

Cells of human epidermoid carcinoma cell line A431 were grown in DMEM medium with 10% heat inactivated fetal bovine serum at 37^0C, 5% CO_2. In affinity modification experiments cells were incubated with 1 μM reactive oligonucleotides for 1h at 37^0C, separated into membrane cytosole (MC), cytosole (CF) and nuclear fractions (NF) and analyzed by SDS-PAGE as described (Laktionov et al., 1999).

The modified proteins from A431 cells were affinity purified on Ultrogel A2–antifluorescein antibodies. The ODN-binding proteins eluted with Glycin/HCl pH 2.5 were separated by SDS-PAGE. The protein 68 kDa band was identified by autoradiography, Coomassie staining of the gel and immunochemistry staining of the nitrocellulose blot and sequenced by MS/MS sequencing.

Results and Discussion

Interaction of cell surface proteins with ss- and ds deoxyribonucleic acids were investigated by affinity modification of cellular proteins with reactive ss- and ds ODNs (Fig. 1). It was shown that reactive ds ODNs (lines 1, 2) modified similar sets of cell surface proteins as the ss ODNs (lines 3-5). Proteins with molecular masses about 68 kDa that were shown earlier to be responsible for binding of oligonucleotides with cell surface bind ss- and ds ODNs and seems to be universal for binding of ss and ds nucleic acids.

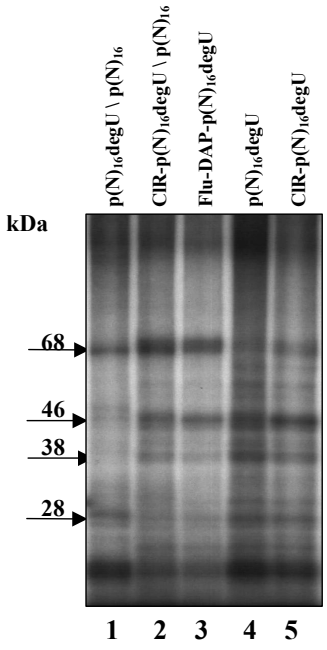


Fig. 1. Affinity modification of MC proteins with reactive ODN conjugates in DMEM medium. Autoradiograph of 10-20% SDS-PAGE.

In order to work out method of separation of these proteins we investigate affinity modification of A431 cells and cellular fractions of A431 cells with [^{32}P]ClRp(N)$_{16}$. In contrast to living cells, incubation of cytosolic, membrane-cytosolic or nuclear fractions with the affinity reagents resulted in labelling of a great number of proteins. The 68 kDa proteins were present in this mixture as minor components (Fig. 2). The data obtained demonstrate that affinity modification of cell surface proteins of living cells with ODN bearing specific ligands in contrast to other approaches enable to reveal and separate the proteins that are important for ODN binding with living cells.

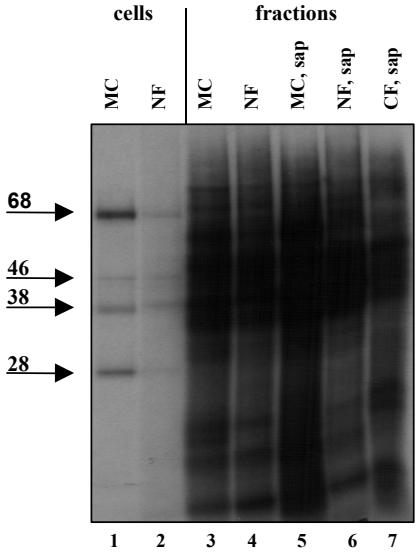


Fig. 2. Affinity modification of cellular proteins with [^{32}P]ClRp(N)$_{16}$. Cells or cell fractions were incubated with 1μM [^{32}P]ClRp(N)$_{16}$ at 37°C for 1 h. The modified proteins were analysed by 10-20 % SDS-PAGE followed by autoradiography.

1, 2 - MC and NF of A431 cells modified with [^{32}P]ClRp(N)$_{16}$ in DMEM medium (control). 3, 4 - MC and NF of A431 cells modified with [^{32}P]ClRp(N)$_{16}$. 5, 6, 7 - modification of MC, NF, and CF of A431 cells treated with saponin.

5'-Fluorescein-labeled oligonucleotide conjugate with uridine bound to the 3'-end through an diethylene glycol (deg) linker was used as the affinity reagent for isolation of 68 kDa cell surface proteins after oxidation of the 3' ribose moiety with sodium periodate. The modified with ^{32}P-labeled Flu-DAP-p(N)$_{16}$degU proteins from 1x10^9 A431 cells were affinity

purified on Ultrogel A2–antifluorescein antibodies. The ODN-binding proteins eluted with Glycin/HCl pH 2.5 were concentrated with ultrafiltration and separated by SDS-PAGE. Specific protein bands were revealed after immunostaining with anti-Flu antibodies and silver staining of NC blot, autoradiography of the gel (Fig. 3.) and with Coomassie staining.

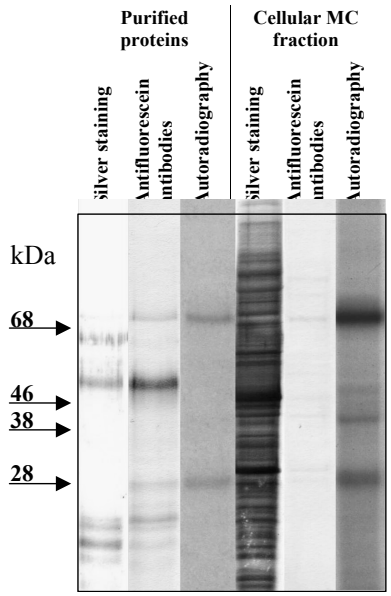


Fig. 3. Separation of ODN-binding proteins of A431 cells. 10^9 cells were incubated with 1μM [^{32}P] Flu-DAP- $p(N)_{16}$degU (oxidized with sodium periodate) in DMEM medium at 37°C, 5% CO_2 for 2 hours. The MC fraction was prepared as described above and applied to the UltrogelA2-rabbit anti-fluorescein antibodies. After elution with Glycin/HCl pH 2.5 and subsequent electrophoretic separation, the ODN-binding proteins were transferred to nitrocellulose and autoradiographed or stained with colloidal silver or anti-fluorescein antibodies.

Proteins were sequenced by MS/MS sequencing and identified with data bank search. It was found that 68 kDa band contains few ODN-binding proteins: albumin and keratin 1 among them. Cytokeratin 1 was shown earlier to be express onto cell surface and revealed with antibodies on a surface of living cells (Mahdi et al., 2001). This data confirm possibility of this protein to bind extracellular nucleic acids and its importance for interactions of nucleic acids with cells.

Acknowledgements

The work was supported by the grant INTAS 99-00630, CRDF REC 008, RFBR 01-04-49862 and grants for young scientists from the RAS № 224.

References

1. Benimetscaya L., Loike J.D., Khakled Z., Loike G., Silverstein S.C., Cao L., Elkhoury R.Y., Cai J., Stein C.A. (1997) Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. Nature Med. 2, 414-420.
2. Diesbach P., Derens C., N′Kull F., Monsigny M., Sonveaux E., Wattiez R., Courtoy P.J. (2000) Identification, purification and partial characterization of an oligonucleotide receptor in membranes of Hep G2 cell. Nucl. Acids Res. 28, 868-874.
3. Duran M., Chevrie K., Chassignol M., Thuong N.T., Maurizot J.C. (1990) Nucl. Acids Res. 18, 6353.
4. Garcia-Olmo D., Garcia-Olmo D.C., Ontanon J., Martinez E. (2000) Horizontal transfer of DNA and the "genometastasis hypothesis. Blood. 95(2), 724-725.
5. Geselowitz D.A., Neckers L.M. (1995) Bovine serum albumin is a major oligonucleotide-binding protein found on the surface of cultured cells. Antisense Res. Dev. 5, 213-217.
6. Griffoni C., Laktionov P.P., Rykova E.Y., Spisni E., Riccio M., Santi S., Bryksin A., Volodko N., Kraft R., Vlassov V., Tomasi V. (2001) The Rossmann fold of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a nuclear docking site for antisense oligonucleotides containing a TAAAT motif. Biochim. Biophys. Acta: Mol. Cell. Biol. Lipids. 1530(1), 32-46.
7. Haralambidis J., Chai M., Tregear G. W. (1987) Nucl. Acids Res. 15, 4857.
8. Kimura Y., Sonehara K., Kuramoto E., Makino T., Yamamoto S., Yamamoto T., Kataoka T., Tokunaga T. (1994) Binding of oligoguanylate to scavenger receptor is required fo oligonucleotides to augment NK cell activity and induce IFN. J. Biochem. 116, 991-994.
9. Laktionov P.P., Dazard J-E., Vives E., Rykova E.Yu., Piette J., Vlassov V.V., Lebleu B. (1999) Characterisation of membrane oligonucleotide-binding proteins and oligonucleotide uptake in keratinocytes. Nucl. Acid Res. 27(11), 2315-2324.
10. Mahdi F., Shariat-Madar Z., Todd R.F. 3rd, Figueroa C.D., Schmaier A.H. (2001) Expression and colocalization of cytokeratin 1 and urokinase plasminogen activator receptor on endothelial cells. Blood Apr. 97(8), 2342-2350.
11. Mishenina G.F., Samukov B.B., Shubina T.N. (1979) Biochemistry (USSR). 5, 886.
12. Noonberg S.B., Garovoy M.R., Hunt C.A. (1993) Characteristics of oligonucleotide uptake in human keratinocyte cultures. J. of Investigative Dermatology. 101, 727-731.
13. Siess D.C., Vedder C.T., Merkens L.S., Tanaka T., Freed A.C., McCoy S.L., Heinrich M.C., Deffebach M.E., Bennett R.M., Hefeneider S.H. (2000) A human gene coding for a membrane-associated nucleic acid-binding protein. J. Biol. Chem. 275, 33655-33662.

COMPUTER ANALYSIS OF SUGARBEET COMPLETE MTDNA SEQUENCE HELPS TO SELECT PRIMERS FOR EFFECTIVE PCR FINGERPRINTING

Ivanov M.K., Revenko A.S., Kabilov M.R., Dymshits G.M.

Institute of Cytology and Genetics, SB RAS, 630090, Novosibirsk, Russia, e-mail: ivanov@niboch.nsc.ru

Key words: *mtDNA, polymorphism, degenerated primers, RAPD, DNA fingerprinting, transcription pattern, CMS*

Resume

Motivation: Approaches, commonly used in the search for molecular markers of plant mitochondrial genes and including RFLP-analysis or cDNA synthesis, are small-productive, require multistep time-consuming protocols and expensive enzymes. Approaches based on PCR lack these shortcomings. The question arises how to select the primers for effective PCR fingerprinting of plant mitochondrial genomes. The methods of primer selection which are in use for nuclear DNA fingerprinting are unapplicable in case of mitochondria. There is no published information about successful mtDNA fingerprinting in plants.

Results: In our work we have used the results of computer analysis of complete mitochondrial DNA (mtDNA) (Kubo et al., 2000), sequence of sugarbeet to select the set of degenerated primers for PCR. These primers were used in PCR-fingerprinting, combined with northern-blot hybridization, in cytoplasm typing and the search for open reading frames involved in the forming of cytoplasmic male sterility (CMS) trait in sugarbeet.

By using this approach we received the range of molecular markers and proved this technique to be effective and our results to be correct. We also performed computer analysis of three sequenced plant mt-genomes and revealed the range of motifs, that can be used to design the universal primer set for PCR fingerprinting of mtDNA from different plant species.

Availability: The computer program used in this work is available on request from the authors.

Introduction

In all higher plant species studied so far mtDNA of CMS plants (S) differs from normal (N) mtDNA by the structural rearrangements, which may affect coding parts of the genes or their localization and lead to the changes of transcription patterns (Schnable, Wise, 1998). Mitochondrial CMS determinants in sugar beet are not identified until now despite long years of investigation.

Unlike nuclear genomes, higher plant mt-genomes contain few repeated sequences and don't contain microsatellite DNA at all; up to 40% of their DNA is represented by genes and open reading frames. So that the ways of primer selection, which are used for PCR-fingerprinting of nuclear genomes, are unefficient for mitochondrial genome analysis. There are no published examples of successful PCR fingerprinting of plant mt-genomes, notwithstanding the existing need for effective fingerprinting technique usable for the investigation of plant mt-genome rearrangements (Lorenz et al., 1997).

Methods and Algorithms

In our work we have used 6 different sugarbeet lines: 3 with N-cytoplasm and 3 with S-cytoplasm. The length of an unchanging motif (6 b.) was selected, proceeding from the size of higher plant mt-genome (about 250-2000 kbps). The motifs used in primer synthesis were selected by computer analysis of the full sequence of sugarbeet N-type mt-genome. We have designed the computer program which could count occurrence of any (of possible 4098) hexamer in the genome (total number of repeats divided by genome size) and the standard deviation of distances between the two adjacent repeats. The GC-content of hexamers was also taken into account when designing the primers.

As hexanucleotides have low temperature of effective hybridization (25-35^{0}C), when unspecific annealing is very possible, we have used the primers, including, besides the hexamer, an extra degenerated motif of 2-4 b.p. at its 5'-end. This allows to produce reproducible and informative PCR-fingerprints at the annealing temperature of up to 50 ^{0}C. As each degenerated primer actually represents the set of 4^2-4^4 different sequences (the degree depending on the length of degenerated tail), we used 16-80 μM primers instead of standard 2.5-5 μM. With standard concentrations of these primers, no visible PCR patterns were produced.

Extraction of nucleic acids, cloning procedures, southern and northern blot analyses were performed according to standard procedures.

Results and Discussion

Twelve 5'- degenerated primers were used in PCR analysis. With 7 of them the polymorphism of PCR fingerprints between N- and S-type of mtDNA was shown and 15 reproducible polymorphic bands were produced. The structure of primers which generated polymorphisms and occurrence of the hexamer motifs from these primers are given at the Table 1.

Table 1. Structure of degenerated primers which reveal polymorphisms between mtDNA from N- and S-cytoplasms of sugarbeet.

Primer structure	Number of conserved hexamer in sugarbeet mt-genome
5' NNNNGCCCCTT 3'	227
5' NNNNGGAAAG 3'	315
5' NNNNCTTTCC 3'	269
5' NNNNCTTTCG 3'	198
5' NNNNGCTGCT 3'	152
5' NNNNAAGGGG 3'	166
5' NNNNCTCTTC 3'	269

An example of such the polymorphism is given at (Fig. 1).

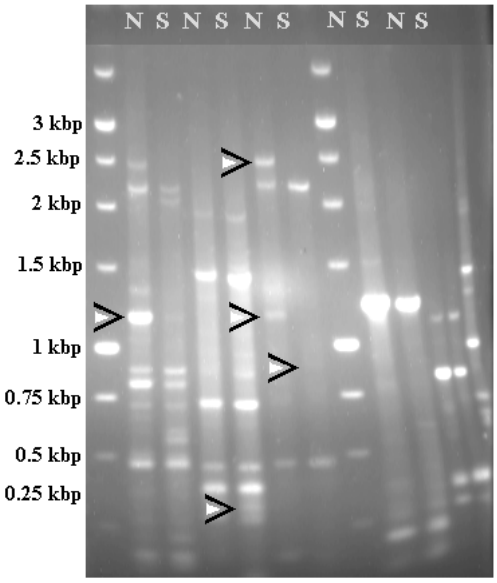


Fig. 1. Polymorphism between sugarbeet mtDNA from normal (N) and male-sterile (S) cytoplasm revealed by PCR with different degenerated primers. Bands marked by arrows were further used as probes in Northern hybridization (see below).

Polymorphic PCR products, specific for N- or S-cytoplasm, were used as templates for synthesis of the labeled probes. The probes were hybridized to electrophoresed mtRNA samples from both N- and S- cytoplasms. 4 of 6 polymorphic PCR products hybridized to corresponding transcripts in either N- and S-cytoplasm. In all cases the differences in transcription patterns between N- and S-cytoplasm were revealed. An example of such the differences is presented at (Fig. 2).

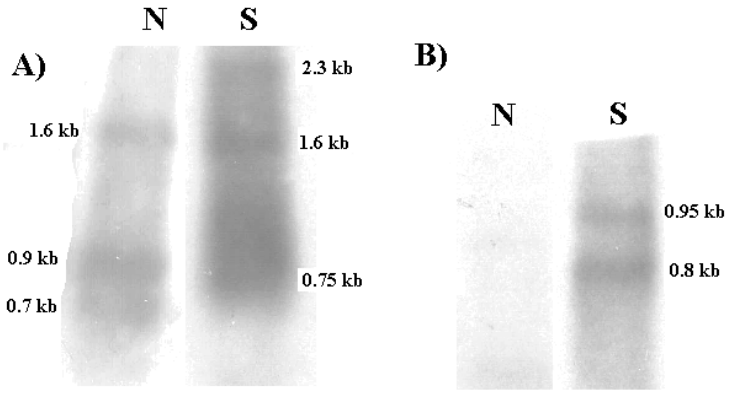


Fig. 2. Northern-blot hybridization with polymorphic PCR products from degenerated primers reveals the differences in transcription patterns between N and S cytoplasms in sugarbeet. PCR products from 5'NNNNGCCCTT primer with the length of about 1200 bp (A) and 1000 bp (B) were used as hybridization probes.

The two polymorphic PCR products appeared to correspond to parts of mitochondrial *orf324* and *atpA* genes. For these genes the CMS-specific rearrangements have been described earlier (Kubo, Mikami, 1996; Senda et al., 1993). Two other polymorphic PCR products, homologous to expressed sequences in the mt-genome, didn't correspond to certain mt-genes or ORFs, which transcription in sugarbeet had been investigated earlier during CMS trait investigations. So, use of 12 primers, selected and designed as described, produced not less than 4 molecular markers of transcribed CMS-specific genes, two of them being new.

There was also performed the comparative analysis of occurrence and distribution of all possible hexanucleotide motifs among the mitochondrial genomes of sugarbeet, arabidopsis and marchantia by using the same computer program. For each of possible 4098 motifs the occurrence in the genome and the standard deviation of distances between the two adjacent repeats was count. The groups of motifs were revealed, which distribution in all three mt-genomes is almost identical. General idea resulting from this analysis is that the same set of primers may be applied to mtDNA analysis in different plant species, including the ones which complete mtDNA sequence is still undefined. This may have great importance, i.e. in the studies of cytoplasmic inheritance in distant plant hybrids.

An identical computer analysis of distribution of both hexa- and pentanucleotide motifs among human and rat mt-genomes (which both have far smaller sizes, about 16 kbps) revealed no motifs which could be used in designing the universal primer set for PCR fingerprinting of animal mtDNA.

Acknowledgements

The work was supported by Russian Foundations for Basics Research, grant № 01-04-48971.

References

1. Kubo T., Mikami T. (1996) A duplicated sequence in sugarbeet mitochondrial transcripts is differentially edited: analysis of orfB and its derivative orf324 mRNAs. Biochimica et Biophysica Acta. 1307, 259-262.
2. Kubo T., Nishizawa S., Sugawara A., Itchoda N., Estiati A., Mikami T. (2000) The complete nucleotide sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for $tRNA^{Cys}$(GCA). Nucl. Acids Res. 28(13) 2571-2576.
3. Lorenz M., Weihe A., Borner T. (1997) Cloning and sequencing of RAPD fragments amplified from mitochondrial DNA of male-sterile and male-fertile cytoplasm of sugar beet (*Beta vulgaris* L.). Theor. Appl. Genet. 94, 273-278.
4. Schnable P.S., Wise R.P. (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends In Plant Sci. 3(5), 175-183.
5. Senda M., Mikami T., Kinoshita T. (1993) The sugar beet mitochondrial gene for the ATPase alpha-subunit: sequence, transcription and rearrangements in cytoplasmic male-sterile plants. Curr. Genet. 24, 164-170.

NONRADIOACTIVE TS-OLA TEST SYSTEMS FOR SNP GENOTYPING AND IDENTIFICATION OF KNOWN POINT MUTATIONS IN TARGET NUCLEIC ACID SEQUENCES

[1]Ivanova E.M., [1]Pyshnyi D.V., [2]Skobeltsyna L.M., [2]Dymshits G.M., [3]Stepanov V.A., [3]Odinokova O.N., [3]Puzyrev V.P., [1]Zarytova V.F.

[1] Novosibirsk Institute of Bioorganic Chemistry, SB RAS, 630090, Novosibirsk, Russia
[2] Institute of Cytology and Genetics, SB RAS, 630090, Novosibirsk, Russia
[3] Institute of Medical Genetics, Tomsk, 634050, Russia
e-mail: pyshnyi@niboch.nsc.ru

Key words: *single-nucleotide polymorphism, point mutation, DNA ligase, oligonucleotides, phenylketonuria, HCV, Y-chromosome, test*

Resume

Development of inexpensive, reliable and universal tests for single-nucleotide substitutions in DNA is of great importance for human genome investigations. A new OLA approach for single nucleotide polymorphism (SNP) genotyping and point mutations testing - TSOLA - has been developed. The approach is based on ligation of a tandem containing three short oligonucleotides (octamer-tetramer-octamer), complementary to target DNA sequence, with T4 DNA ligase. The TSOLA test systems enable to identify a mismatch in any nucleotide position in tetramer hybridization site of target DNA. Such the test systems can be applied to the analysis of any prokaryotic or eukaryotic genome and discriminate homozygotes from heterozygotes. We have demonstrated the advantages of TSOLA by designing and testing three different TSOLA test systems: for hepatitis C virus, for point mutation R408W in human PAH gene, causing phenylketonuria, and for human Y-chromosome Tat SNP genotyping.

Introduction

More than 1.5 million of single-nucleotide polymorphisms (SNPs) in human genome are distributed by clusters almost evenly. These are of great interest as universal genetic markers, which may be used as a tool in human population genetics, in genotype-phenotype studies *etc*. Point mutations result in many well-known human inherited diseases. So the investigations, directed on creation of effective techniques for SNP identification, are of great importance. A range of such the techniques has been developed yet (Carlson et al., 2001), but none of these is universal, therefore the search of new approaches to optimal techniques development still goes on. First of all, such techniques are necessary for diagnostics of human inherited pathologies, which are caused by known point mutations. The test systems must also reveal the mutant alleles in healthy heterozygous carriers, be inexpensive and easy to manufacture. Recently, a new variant of OLA (oligonucleotide ligation assay) for single-nucleotide substitution analysis was developed (Koulikova-Zarytova et al., 2000; Skobeltsyna et al., 2000). The new approach, TSOLA, differs from OLA by the use of three short oligonucleotides in the tandem hybridization and consequent ligation on DNA templates instead of two longer oligonucleotides. The selectivity of such TSOLA in respect to the mismatch is >20 times greater that of OLA (Pyshnyi et al., 2000). Below several modifications of TSOLA are described.

Results and Discussion

The TSOLA colorimetric test system contains three short synthetic oligodeoxyribonucleotides: octanucleotide **B**~pN_8, immobilized on polymer beads (**B**), soluble tetranucleotide pN_4 and octanucleotide pN_8***Bio** which carries a biotin residue at its 3'- end phosphate. The sequences of these oligonucleotides, arranged in order pN_8 - pN_4 - pN_8*, generate the sequence which is fully complementary to 20mer of interest in target DNA template. In the presence of both T4 DNA ligase and complementary template the oligonucleotides form the tandem, **B**~pN_8 + pN_4 + pN_8***Bio,** which is efficiently ligated. The final product of this ligation is biotinylated **B**~pN_{20}**Bio** 20mer oligonucleotide immobilized on polymer beads. In this case, biotin residues remain covalently bound on the bead surface. After washing off the beads, the bound biotin residues can be detected by standard procedure *via* streptavidine-AP conjugate inducing the accumulation of insoluble chromogenic products and staining the beads. If the DNA template has any single-base substitution in the tetranucleotide binding site, then the tandem instability, on the one hand, and a mismatch location in the active center of ligase, on the other hand, will inhibit the tandem ligation and hence the immobilization of biotin residues to the beads. Such the beads wouldn't be stained. The character of unknown base substitution in the tetranucleotide binding site can be determined by the set of tests with different tetranucleotide sequences.

The described approach was verified in the range of test systems.

TatY TSOLA test system allows one to discriminate C/T single-nucleotide polymorphism in the Tat locus of human Y-chromosome. The Y-chromosomal SNP markers are intensively employed in population genetic studies. The advantage of using these markers is that these usually biallelic hemizygous markers make it possible to follow paternal lineages. The results of the analysis of 10 PCR samples in TatY TSOLA system are represented on Fig. 1. It can be seen from these examples, that the signals are alternative in each case. The positive signal after ligation with certain tetramer variant determines the allele type in the DNA sample: samples 2,5,7-9 - C allele; samples 1,3,4,6,10 - T allele.

R408W TSOLA system (Fig. 2) has been developed to test the recessive point mutation $C_{1444} \rightarrow T_{1444}$ in codon 408 of phenylalanine hydroxylase (PAH) gene, followed by Arg → Trp amino acid replacement, which results in human inherent phenylketonuria (PKU). This mutation is designated as R408W. In medical practice there exists the simple RFLP test for R408W mutation. Therefore this mutation was chosen to demonstrate the correspondence between the results of RFLP and TSOLA methods of testing.

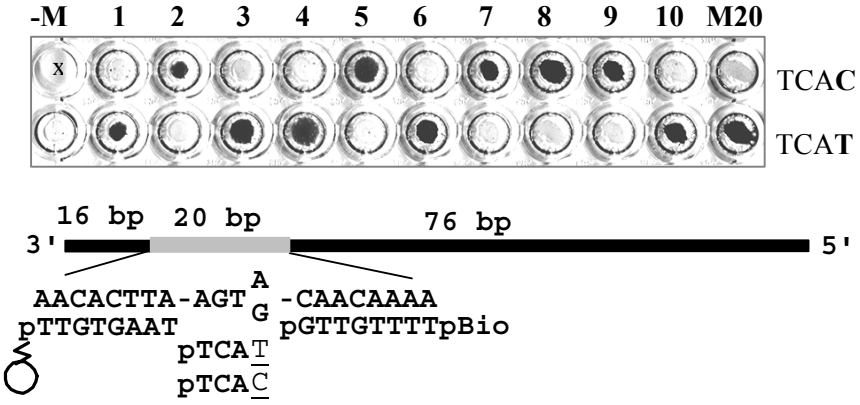


Fig. 1. SNP typing of human TatY locus by Tat-Y TSOLA.

1-10 - 112-bp DNA samples as ligation template; **M20** – single-stranded 20-bases Tat-specific sequence as ligation template; **M** – no template; X – empty cell.

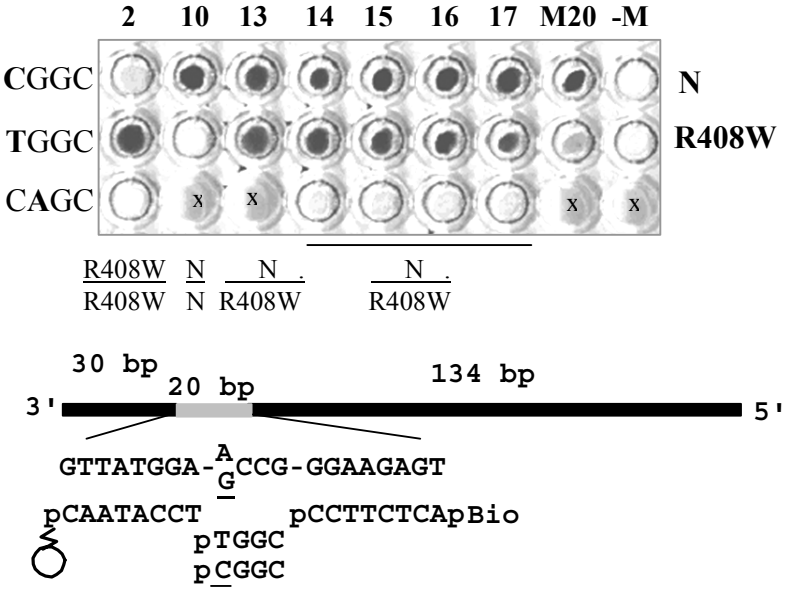


Fig. 2. Human PAH gene point mutation R408W testing by TSOLA technique.

184-bp DNA samples as ligation template (RFLP data):

2– R408W/R408W (PKU); **10** – N/N (healthy); **13** – N/R408W (healthy); **14 -17 - ?**/R408W (PKU); **M20** – single-stranded 20-bases ligation template; **M** – no template; X – empty cells.

Unlike the previous example with Y-chromosome DNA, these DNA samples are diploid. 17 clinical PCR samples of DNA from donors with different phenotypes - either persons with PKU or healthy ones - were tested to show the presence of normal and mutant alleles. Also the third tetramer variant (C**A**GC) was taken as the control. The results of R408W testing by TSOLA demonstrated absolute agreement with the results of RFLP test.

It is seen from Fig. 2 that both types of homozygote, mutant R408W/R408W (samples 1-7; 2 is represented) with PKU phenotype and normal N/N (samples 8-10, 10 is represented) with normal phenotype generate positive signal with only one tetranucleotide variant. Heterozygous samples (samples 11-17; 13-17 are represented) provide positive signals with each of the two allelic tetranucleotide variants. No visible signal was produced when using the control tetramer C**A**GC.

RFLP test shows the one allele from the heterozygous samples to be sensitive to StyI restriction endonuclease and hence to be authentic R408W. The other allele is not sensitive, that is characteristic of the normal (N) allele but also any unknown third one. It is possible to authenticate the StyI-resistant allele as N from RFLP data if only taking account of the healthy phenotype.

As it is seen from Fig. 2, R408W TSOLA test, unlike the RFLP test, allows the phenotype-independent precise definition of the allele of interest by the positive signal in answer to the corresponding tetramer. The positive response testifies the strict complementarity of the tetranucleotide and DNA sample. In all heterozygous samples the both alleles of this locus are defined precisely - N /R408W.

The feature of the third example is the possibility of TSOLA test system to identify not only the individual SNP alleles, but also, in case of need, all nucleotide sequences of interest, as the oligonucleotides will ligate only in the presence of the complementary template. Thus, TSOLA can be useful in infectious disease diagnostics. Until now, the viral infections are diagnosed by primer-specific PCR with consequent analysis of PCR products. However, the danger of unspecific pseudopositive response always exists. The received PCR product may be not virus-specific. TsOLA test-system assures reliable determination of PCR product specificity.

An example of testing hepatitis C virus in four RT-PCR samples from people suffering from hepatitis of unidentified etiology is represented on Fig. 3. HCV RNA, extracted from supposed virus carriers, were amplified and tested by HCV TSOLA system with different variants of a tetranucleotide. All four tests demonstrated the positive signal when HCV-1b variant of tetranucleotide was used, suggesting the viral etiology of hepatitis in these patients.

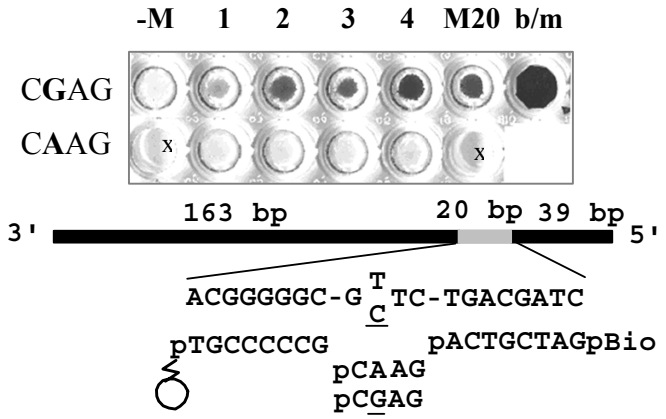


Fig. 3. HCV TSOLA test of hepatitis C virus cDNA sequence after HCV primer-specific RT-PCR of sera from four donors with hepatitis.

1,2,3,4 – 222-bp cDNA samples as ligation template; **M20** – single-stranded 20-bases HCV-specific sequence as ligation template; **-M** – no template; **b/m** – black marker; X – empty cells.

On the whole, several hundreds of samples were analyzed by using different test systems based on TSOLA. In no case the inadequate answer was received. Unlike the majority of other SNP-genotyping methods, TSOLA is universal for any known DNA sequence, allows one to discriminate homozygote from heterozygote and may be used in both hand testing, which doesn't require special apparatus, and (potentially) in "microchip" polygenic technique. TSOLA method may also reveal other changes in the 20mer genomic sequence of interest, i.e. small insertions or deletions. Only several microliters of blood (i.e. from fingertip) is needed for analysis. On the present level of TSOLA development the testing may be performed within one day.

Acknowledgements

This work was partially supported by Russian Foundation for Basic Research (grant № 00-04-48506 to V.S), by Wenner-Gren Foundation for Anthropological Research (grant № 6801 to V.S.), by INTAS, and by the Program for Projects of Young Researchers, Russian Academy of Sciences (grant № 230, 6th competitive evaluation, 1999).

References

1. Carlson C.S., Newman T.L., Nickerson D.A. (2001) SNPing in the human genome. Current Opinion in Chem.Biol. 5, 78-85.
2. Koulikova-Zarytova V.F., Ivanova E.M., Pyshnyi D.V. et al. (2000) Procede de detection d'une sequence d'acide nucleique a analyser. Patent FR № 9704290.
3. Pyshnyi D.V., Skobeltsyna L.M. et al. (2000) Detection of single-base substitutions in amplified fragments *via* ligation of a tandem of short oligonucleotides in solution and on a solid carrier. Mol. Biol. (Mosk.). Engl. Tr. 34, 840-851.
4. Skobeltsyna L.M., Pyshnyi D.V. et al. (2000) Development of a colorimetric test system for detection of point mutations *via* ligation of a tandem of short oligonucleotides on methacrylate beads. Mol. Biol. (Mosk.). Engl. Tr. 34, 321-327.

CLONING OF *SUPPRESSOR OF UNDERREPLICATION* GENE FROM *D. ERECTA*

Yurlova A.A., Belyakin S.N., Makunin I.V., Zhimulev I.F.

Institute of Cytology and Genetics, SB RAS, 630090, Novosibirsk, Russia,
Phone: (3832) 33-39-12; Fax: (3832) 33-39-12; e-mail: annush@bionet.nsc.ru

Key words: *SuUR, Drosophila*

Resume

Motivation: The *SuUR* mutation leads to disappearance of breaks in intercalary heterochromatin (IH) regions of *D. melanogaster* polytene chromosomes due to suppression of DNA underreplication in these regions. It seems that in *SuUR* mutants the DNA in IH regions finishes replication earlier but the mechanism of SuUR action remains obscure despite the protein structure became available after the gene has been cloned. Neither known motifs nor domains were found in SuUR protein besides nuclear localization signals and a region with moderate similarity to N-terminal part of SNF2/SWI2 proteins. In order to find functional domains in SuUR protein we applied comparative approach. It is known that very often functional protein modules are highly conservative. We decided to analyze the *SuUR* gene in different *Drosophila* species.

Results: We analyzed divergence of *SuUR* gene in different *Drosophila* species (*D. erecta, D. teissieri, D. yakuba, D. mauritiana, D. takahashii, D. eugracilis, D. virilis*) by Southern-blot hybridization. The strong signals were observed only within *melanogaster* subgroup. No signal was presented when DNA from distantly related species as *D. virilis* was probed with *SuUR* gene DNA. We amplified by PCR and cloned genomic DNA corresponding to *SuUR* gene from *D. erecta*. The sequence of whole gene was determined. Comparison of *D. erecta* and *D. melanogaster* putative SuUR proteins showed remarkably low level of substitutions in N terminal part which possesses similarity to SNF2/SWI2 family of proteins. Negatively charged cluster separates SNF2/SWI2-like region and highly variable positively charged middle part of protein. Several substitutions are present in the negatively charged region in *D. erecta* SuUR homolog, but none of them introduces positively charged residue. Data obtained suggest possible functional role of N-terminal part of SuUR protein.

Introduction

In salivary gland polytene nuclei new S-phase starts without completion of previous round of DNA replication and as a result some late replicated regions become underrepresented in comparison to the bulk of euchromatic DNA. Besides of pericentric heterochromatin some euchromatic regions are also underreplicated. Underreplication in such regions (named Intercalary Heterochromatin) of salivary gland polytene chromosomes results in visible constrictions and chromosomal breaks. The *Su*ppressor of *U*nder*r*eplication *(SuUR)* mutation leads to DNA underreplication suppression in pericentric IH regions of polytene chromosomes (Belyaeva et al., 1998; Moshkin et al., 2001), which have no breaks or constrictions at all. The *SuUR* gene was cloned (Makunin et al., 2002) but mechanisms of SuUR action on late replication regions still remain obscure. Analysis of protein failed to reveal any significant matches with known domains except for the moderate similarity to N-terminal part of SNF2/SWI2 proteins and the presence of two bipartite nuclear localization signals. Being difficult to trace the *SuUR* phenotype hampers employment of traditional genetic manipulations, such as a mutagenesis, in identification of functional domains in gene.

It is known that functional modules often retain their structure during evolution. No SuUR orthologs could be identified in protein databases by BLAST search. To find conservative regions within SuUR we analyzed *SuUR* gene in different *Drosophila* species and cloned the *SuUR* ortholog from *D. erecta*, the most distant species among melanogaster subgroup.

Results and Discussion

We estimated divergence of *SuUR* gene in Southern-blot hybridization, by probing genomic DNA from different *Drosophila* species with *SuUR* probe. Strong unique signals were observed only with species from melanogaster subgroup whose divergence started about 17-22 million years ago (Ashburner, 1989). Distantly related species, such as *D. virilis* (40-60 Myr divergence time from *D. melanogaster*) gave no or very weak signals. It could indicate that *SuUR* belongs to a fraction of fast evolving genes in drosophilids (Schmid, Tautz, 1997).

D. melanogaster and *D. erecta* are most divergent species in *melanogaster* subgroup. *D. erecta* genomic DNA was amplified using several pairs of primers for *D. melanogaster SuUR* gene, cloned in *pBluescript* and sequenced. The *D. erecta SuUR* retains exon-intron boundaries, but has 200 bp insertion in first intron. Third intron and 3' UTR have numerous substitutions that make alignment in these regions almost impossible. Putative transcription start site and promoter region have very few replacements.

D. erecta SuUR protein was deduced from genomic sequence taking into consideration conservative splicing sites. Its length of 962 amino acid residues is the same as for *D. melanogaster* SuUR. Comparison of proteins revealed 849 identities (87% identities) and 8 gaps. This low level of similarity places *SuUR* among the most fast evolving genes in drosophilids (Schmid, Tautz 1997). Despite too high number of substitutions, *D. erecta SuUR* has characteristics similar to those of *D. melanogaster SuUR*. Proteins have identical molecular weight - 107,5 kDa, equal amount of positive residues KR=148, and almost equal net charge: +45 for *D. melanogaster* SuUR and +46 for *D. erecta* SuUR.

Aminoacid substitutions in *D. erecta* as compared to *D. melanogaster* are distributed non-randomly along the protein sequence (Fig.). Virtually no replacements are present in N-terminal part, especially in the region with similarity to SNF2/SWI2 and in the region with weak similarity to AT-hook (Fig.). High level of substitutions is found in positively charged middle part of the protein. Substitutions in the middle part of the protein do not affect Nuclear Localization Signals (not shown). Statistically significant large spacing between positively charged residues is conserved in *D. erecta* protein despite of presence of several replacements in this region (Fig.). Such large spacing indicates clustering effects in the protein. Negatively charged regions could interact with positively charged proteins, such as histones. C-terminal part of the protein has fewer replacements than the middle part but still more than the N-terminal part has.

Data obtained indicate that N-terminal part of SuUR (region with similarity to SNF2/SWI2 proteins and negatively charged region) could have some functional role in SuUR protein. This comes alongside with the observation that the 360 aa long peptide from N-terminal part of SuUR could compete with the intact SuUR protein (Volkova E.I., Kolesnikova T., Makunin I.V., unpublished).

```
1    MYHFVSEQTPEVRLTDEALVTSHVTQYLKSFQLDAVRFVYDRLAKREFCILNDESGLGKVATVAALLSALPPAKKTLVVL
     ..........L..SA......R.....................................................

81   QNDEQLLTGWRFHLDTLTDLQVYIIQGVQDTTDSPHSVYLAKWSQLRSIGDLSRLKFDYIMVDNRGHSLNNSFCTSMLLK
     ............................H.....................................................

161  QFEGRVNVLISSVDVTSDVRLLYNVLRLGGRLEHQYKSFASFDRKFHLPDPKEVFSKRIDLEEYYKQRGFLSEYIKDFRL
     ..............I....K.........D.........SR...................................

241  RRFRHQFDKSLPLVAPEQYKHNLNLWLASKNSQSTISGSDVCSTIASIDNNPAQQNKTGLFEETDRLSEHSVDDV-AMSP
     .........T.........................L...E....V...E...P...E.V.V..S..I........V....

321  LIFEYSESDDEPLTVEPDADQNPVLVVSSDDCEIVTPPSTPQNRTPSLNESPRTKSKKKFSKKTSPRKKADLTDSEEDDE
     ....S.......I..D.V.NE.................N..P....LV.K.........C...P..C.E.......K...

401  ATDNMPPRKRTRAATVHLTPKTRRLNVRILRVSLDTLSTPPPSRTTTAIVTPKTEPTARRKNLKKRTVSPVDVGRPATRG
     GLT-.....S.......F..........L......A....T..GA....I......S.....---..KQ.M.........

481  MQRLTRSAETKINSKYLKHRSLDDVKRSFPRRVKLEGNQTPRSSKQIVKQEPK--SKVGQEKKQKTVDVPAQGTAKRKPG
     .........S.........HV...A..N....I.A......KT..R.....S.AKA.PE.K..I....K...E.P.....

561  RPRKCQTKTEDLGKTKTKPNSKHLPPTPQVLSGSSLSSEYMQCAQRIPDNLDAIESPAFRVPFTPQQTPMLLTLPSTHNL
     .....K.L..T...S.......P.........................D.............................

641  LNDSEVVSIPLYKDPVETVVINSSHDECSPQDPSQSRRTKALKRKRKPVTSVNSSFGGGLGLPPAKRSA-NKSPDLFSIS
     .......A...F..Q............S.....................DAP............Q.....T..........

721  SEHSQIPLAQPRPSSPFEGFKIFGSEVKQFQQQLAKVNISVPKKKRDRSCLDILEQMFEPRQQQSAKTSPKVLPTLPLTQ
     .DL.............................H...T.PA.....................L...S............I.

801  KDDAESTITQRRRTLLEDDFFEITNNGQFGSRMRLNSSGEVSPVQPDQQSVRPSQANKITNYLIGSGITQERTQPSNGNR
     ....TT.F....G.......................A.........Q.......T............V.............

881  NSILASLRKSPKSPKQGAKSTQATKLTRWFGSVFGGGASQTSSVESVSAPSTPVNSSTSAAACQTRSARSGGASGPTKRK
     ...V...........H..RT.................................P..........T......T.......

961  RLELFK
     ......
```

Fig. Comparison of *D. melanogaster* SuUR (upper line) and *D. erecta* SuUR (lower line). Identical residues are shown as a dots, dashes corresponds to gaps. Region with similarity to SNF2/SWI2 is underlined by solid line, region devoid from positive residues underlined by dashed line, amino acids with similarity to AT-hook are shown in bold and underlined by dots.

Acknowledgements

This work was supported by grants from the Russian Foundation for Basic Research (02-04-48222 and 00-15-97984), the Russian State Program «Frontiers in Genetics» (2-02) and INTAS grant (99-01088).

References

1. Ashburner M. (1989) *Drosophila:* A Laboratory Manual. Vol 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Belyaeva E.S., Zhimulev I.F., Volkova E.I., Alekseyenko A.A., Moshkin Y.M. et al. (1998) *Su(UR)ES*: a gene suppressing DNA underreplication in intercalary and pericentric heterochromatin of *Drosophila melanogaster* polytene chromosomes. Proc. Natl Acad. Sci. USA. 95, 7532-7537.
3. Makunin I.V., Volkova E.I., Belyaeva E.S., Nabirochkina E.N., Pirrotta V., Zhimulev I.F. (2002) The *Drosophila* Suppressor of Underreplication protein binds to late-replicating regions of polytene chromosomes. Genetics. 160, 1023-1034.
4. Moshkin Y.M., Alekseyenko A.A., Semeshin V.F., Spierer A., Spierer P. et al. (2001) The *bithorax* complex of *Drosophila melanogaster*: Underreplication and morphology in polytene chromosomes. Proc. Natl Acad. Sci. USA. 98**,** 570-574.

5. Schmid K.J., Tautz D. (1997) A screen for fast evolving genes from Drosophila. Proc. Natl Acad. Sci. USA. 94(18), 9746-9750.

SIGNAL TRANSDUCTION PATHWAYS INITIATED VIA CELL SURFACE RECEPTOR CD150

Sidorenko S.P., Mikhalap S.V., Shlapatska L.M., Yurchenko M.Yu., Akimov Yu.M., Chekhun V.F.

Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology National Academy of Sciences of Ukraine, Kyiv, Ukraine, e-mail: svetasid@onconet.kiev.ua
*Corresponding author

Key words: *signal transduction pathways, CD150, SH2-containing proteins, docking of proteins, computer modelling*

Resume

Motivation: Cell surface receptor CD150 represents a novel group of receptors that could transmit both activating and inhibiting signals, which regulate cell fate. The CD150 subfamily members are involved in the development and pathogenesis of severe immunodeficiencies, and CD150 serves as a cell receptor for measles virus. That is why the CD150-initiated signal transduction pathways are of great interest both for theoretical biology and practical medicine.

Results: Using different experimental and computational biology approaches we identified the molecules that are interacting with cytoplasmic tail of CD150 (CD150ct), their binding sites and possible combinations of binding. We found that ligation of CD150 activates ERK via Ras-pathway, and also results in Akt phosphorylation. An experimental system based on knockout cell lines allowed us to clarify the mechanisms of regulation of CD150-initiated signal transduction pathways. The model of initiation and regulation of CD150-mediated signal transduction pathways is proposed.

Introduction

CD150 cell surface receptor of activated T and B lymphocytes, dendritic cells and monocytes belongs to the coreceptor molecules that could modulate cell fate. CD150 is a member of immunoglobulin superfamily and shares homology with CD2, CD48, CD84, CD229, CD244, 19A, Ly108 and NTB-A (Sidorenko, Clark, 1993; Wang et al., 2001). CD150 serves as a receptor for measles virus and other morbilliviruses (Tatsuo et al., 2000), and is involved in pathogenesis of X-linked lymphoproliferative disorder, B cell non-Hodgkin's lymphoma and familial hemophagocytic lymphohistiocytosis (Morra, 2001). Divergent functions of CD150 are linked with unique structure of CD150 cytoplasmic tail, especially with the presence of paired immunoreceptor tyrosine-based switch motif (ITSM) TxYxxV/I (Shlapatska et al., 2001). This motif could bind different subsets of signal transduction molecules. However, it is not known to date how binding of these molecules is regulated, and what signal transduction pathways are initiated via CD150.

Methods

Experimental systems for studies of protein-protein interactions. For identification of molecules interacting with CD150 and signal transduction pathways initiated via this cell surface receptor we used three different experimental systems.

Immunoprecipitation of CD150 with monoclonal antibody IPO-3 followed by biochemical identification of proteins that coprecipitates with CD150 (Mikhalap et al., 1999).

GST-fusion proteins of CD150ct (GST-CD150ct) in non-phosphorylated and tyrosine-phosphorylated forms. Using PCR-based site directed mutagenesis, we made constructs of GST-CD150ct fusion proteins with phenylalanine (F) replacements at tyrosines Y269, Y281, Y307, Y327, and Y281+Y327. These mutant fusion proteins were used in pull-down experiments for typing of binding sited in CD150ct (Shlapatska et al., 2001).

To clarify the signal transduction pathways that are initiated via CD150 we developed and experimental system based on DT40 chicken B lymphoma cell line and sublines deficient in Lyn, Syk, Btk, SHIP and SHP-2. These sublines were transfected with CD150 alone or together with the adaptor protein SH2D1A. CD150 was cloned into vector pApuro and SH2D1A cDNA – into pcDNA3 vector.

Computer modelling. The structural models of CD150 cytoplasmic tail were constructed on 2gn5-template of bacteriophage fd RNA-binding protein by threading method at FUGUE (http://www-cryst.bioc.cam.ac.uk/~fugue/prfsearch.html) and SAUSAGE (http://www.embl-heidelberg.de/predictprotein/submit_meta.html) servers. Substitution of matrix residues, phosphorylation and energy optimization were made in HyperChem 6.0 package (http://www.hyper.com/products/default.htm). Studies of CD150ct-SH2-domains interactions were obtained by macromolecular docking method (Hex 2.4 program, http://www.biochem.abdn.ac.uk/hex/). For predicting protein interface interactions we used Protein-Protein Interaction Server (http://www.biochem.ucl.ac.uk/ bsm/PP/server/index.html) and for phosphorylation sites prediction we used NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/).

Results and Discussion

Using different experimental approaches we identified the molecules that are able to interact with cell surface receptor CD150 in B lymphocytes. CD150 coprecipitated with cell surface receptor CD45 (220 kDa) that has two intrinsic tyrosine phosphatase domains. Also it associated with Src-family kinases Lyn and Fgr, SH2-containing protein tyrosine phosphatase SHP-2, SH2-containing inositol phosphatase SHIP, and the adaptor protein SH2D1A. We found that in B cell lines CD150 was differentially associated with tyrosine phosphatase SHP-2 versus the inositol phosphatase SHIP and the adaptor protein SH2D1A. Using mutational analysis we found that both Y281 and Y237 in TxYxxI/V motif in CD150 cytoplasmic tail are essential for binding of SHP-2 as well as SHIP. Apparently, SH2D1A may function as a regulator of alternative interactions of CD150 with SHP-2 or SHIP via a TxYxxV/I motif. Mutations in SH2D1A are found in patients with X-linked lymphoproliferative syndrome (XLP), common variable immunodeficiency (CVID) and familial hemophagocytic (Sayos et al., 1998; Morra et al., 2001). That is why, enlightening the place of SH2D1A in regulation of signal transduction pathways will help to understand pathogenesis of these severe immunodeficiencies and design the effective approaches for therapy. Multiple sequence alignments showed the presence of ITSM TxYxxV/I motif not only in CD2 subfamily members, but also in the cytoplasmic domains of the members of the SHPS, Siglec, CEA, and LIR families. However, association of SH2D1A with ITSM motifs was found only in CD150, CD84, CD229, CD244, SF2000 and NTB-A.

To analyze the possible combinations of interactions of SH2-containing proteins with CD150ct and to clarify the regulatory role of the adaptor protein SH2D1A, we used computational biology methods. The 3D-model of CD150ct was built using threading approach on 2gn5-template of bacteriophage fd RNA-binding protein. This model was used to clarify the mechanism of SH2-containing molecules binding to the CD150ct. Docking studies revealed that binding of SH2D1A to Y281 in CD150ct changed the conformation of CD150ct and exposed Y307 for phosphorylation. Also we found that both SH2D1A and SHP-2 could bind Y281 and Y327 in CD150ct, but with preferential binding of SH2D1A to Y281, and SHP-2 to Y327. Results of experimental analysis of SH2-domains binding sites in CD150ct and computer modelling of proteins docking demonstrated a differential binding of the adaptor protein SH2D1A simultaneously with SHIP versus SHP-2 to CD150ct.

To find out what signal transduction pathways are linked to CD150 and are regulated by SH2D1A we used DT40 cell sublines transfected with CD150 alone or together with SH2D1A. DT40 sublines deficient in key components of signaling cascades helped us to clarify the mechanisms of regulation CD150-initiated signaling. We found that:

1) Signaling via CD150 activates ERK1 (Ras pathway), but did not affect JNK and p38 MAPK (Rac pathway).

2) CD150-mediated Erk1 activation is SHIP dependent and possibly depends on SHP-2.

3) CD150-induced Akt activation that is regulated by the adaptor protein SH2D1A.

4) CD150-mediated Akt activation is SHIP independent.

5) CD150-mediated Akt activation is regulated by tyrosine kinases Lyn (-), Syk (+), Btk (-), and tyrosine phosphatase SHP-2 (-).

Results of experimental studies and computer modelling allowed us to build models of CD150 interactions with different components of signaling cascades (Fig. 1), CD150-initiated ERK-1 activation (Fig.1) and also regulation of CD150-initiated Akt phosphorylation by the adaptor protein SH2D1A (Fig. 2).

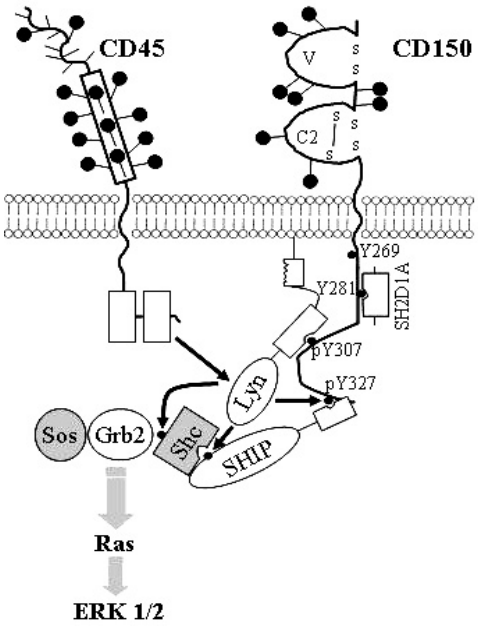


Fig. 1. The model of CD150 interactions with components of signal transduction pathways and CD150-initiated ERK activation. SH2D1A binding to Y281 in CD150ct changes conformation of CD150ct and makes Y307 available for phosphorylation by Lyn tyrosine kinase. SH2-domain of Lyn binds to pY307, which enhance its activity. Then Lyn phosphorylates Y327 that serves a binding site for SH2-domain of SHIP. SHIP also is a substrate of Lyn and upon phosphory-lation attracts PTB-domain of the adaptor protein Shc. Tyrosine phosphorylated Shc form complex with Grb2 and Sos that leads to Ras pathway activation and results in ERK1/2 phosphorylation.

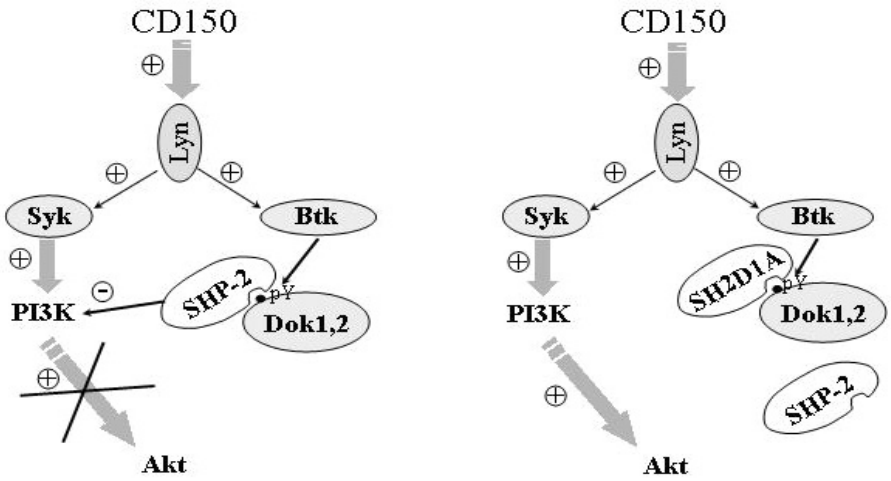


Fig. 2. Regulation of CD150-initiated Akt activation. Signal via CD150 activates Lyn tyrosine kinase, which is known to be a positive regulator of tyrosine kinases Syk and Btk. Our experiment showed that Syk is positive regulator of CD150-mediated Akt phosphorylation. Negative regulatory role of Btk in this cascade presumably is mediated via SHP-2 (left panel). In the presence of the adaptor protein SH2D1A, which competes with SHP-2 for the binding sites on the adaptor proteins Dok1,2, the negative roles of Btk and SHP-2 are blocked, that results in CD150-mediated Akt activation.

Acknowlegements

This work was supported by HHMI grant 76195-548101, INTAS grant 96-1493 (to S.S.P.), CRDF grants UB-531 (to S.S.P.) and USB-383 (to S.L.M.).

References

1. Mikhalap S.V., Shlapatska L.M., Berdova A.G., Law C.L., Clark E.A., Sidorenko S.P. (1999) CDw150 associates with src-homology 2-containing inositol phosphatase and modulates CD95-mediated apoptosis. J. Immunol. 162, 5719-5727.
2. Morra M., Silander O., Calpe S., Choi M., Oettgen H., Myers L., Etzioni A., Buckley R., Terhorst C. (2001) Alterations of the X-linked lymphoproliferative disease gene SH2D1A in common variable immunodeficiency syndrome. Blood. 98, 1321-1325.
3. Sayos J., Wu C., Morra M., Wang N., Zhang X., Allen D., De Vries S.E., Aversa G., Terhorst C. (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature. 395, 462-469.
4. Shlapatska L.M., Mikhalap S.V., Berdova A.G., Zelensky O.M., Yun T.J., Nichols K.E., Clark E.A., Sidorenko S.P. (2001) CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J. Immunol. 166, 5480-5487.
5. Sidorenko S.P., Clark E.A. (1993) Characterization of cell surface glycoprotein IPO-3, expressed on activated human B and T lymphocytes. J. Immunol. 151, 4614-4624.
6. Tatsuo H., Ono N., Tanaka K., Yanagi Y. (2000) SLAM (CDw150) is a cellular receptor for measles virus. Nature. 406, 893-897.
7. Wang N., Morra M., Wu C., Gullo C., Howie D., Coyle T., Engel P., Terhorst C. (2001) CD150 is a member of a family of genes that encode glycoproteins on the surface of hematopoietic cells. Immunogenetics. 53, 382-394.

MOLECULAR APPROACHES TO STUDY STRESS ADAPTATION, BIOACTIVITY, AND PHYLOGENETIC RELATIONSHIPS WITHIN THE PORIFERA

Schröder H.C., Müller W.E.G.

Institut für Physiologische Chemie, Universität Mainz, Duesbergweg 6, D-55099 Mainz, Germany and German Center of Excellence *BIOTEC*marin (Co-ordinator: W.E.G. Müller), e-mail: hschroed@mail.uni-mainz.de
*Corresponding author

Key words: *heat shock protein, stress response, evolution, sponges, Porifera, Lake Baikal*

Resume

Motivation: There are two reasons for the increasing interest in sponges (Porifera). First, these simplest multicellular animals are key organisms to understand molecular evolution of Metazoa. Second, sponges are the richest source for bioactive compounds among all metazoan phyla. In addition, these animals turned out to be good bioindicators in environmental monitoring studies. A genome project (expression of functional sponge genome; http://spongebase.uni-mainz.de/cgi-bin/blast/blastserver.cgi) has been started.

Results: The data presented and gathered in frame of an INTAS project show that there is evidence for a monophyletic origin of the freshwater sponges in Lake Baikal. The expression of heat shock proteins was used as a bioindicator in endemic Baikalian sponges to detect stress by pollutants. Strategies for the sustainable use of (marine) sponges for the production of pharmacologically valuable compounds are reported.

Availability: http://www.biotecmarin.de.

Introduction

Sponges (Porifera) are the simplest multicellular animals. Recent results revealed that the genes critical for multicellularity are already present in these organisms. The elucidation of cDNA and genome structure has pushed this phylum into the center of current research activities in molecular evolution of Metazoa. Based on molecular sequence data it became overt that all metazoan animals are monophyletic (Müller, 1998). It turned out that phylogenetically conserved sponge proteins can be very helpful for the evaluation of differences in evolutionary rates in different animal lineages. Therefore sponges may be used as reference animals in molecular evolutionary studies of Metazoa.

There is only little solid knowledge about the molecular evolution of freshwater sponges. Endemic freshwater sponges from ancient lakes, such as Lake Baikal (Siberia) and Lake Ochrid (Macedonia), are an attractive object for studies on evolution. Lake Baikal is most unique in its fauna. More than 1500 endemic animal species are found in this lake. The Baikalian sponges constitute one group singled out as an independent family, Lubomirskiidae. The endemic family Lubomirskiidae has approximately 10 species belonging to 3 genera: *Lubomirskia* (Fig. 1A: *Lubomirskia abietina*), *Baikalospongia* and *Swartschewskia*. Studies on embryogenesis, larvae structure and data of 18S rDNA indicate a close relationships between Lubomirskiidae and Spongillidae, but the question about which Spongillidae genera gave rise to Baikalian sponges has not yet been decided. Some authors point out that Lubomirskiidae sponges are remotely reminiscent of the Caspian sponge *Metschnikowia* and the Ohrid sponge *Ochridaspongia rotunda* (Fig. 1B).

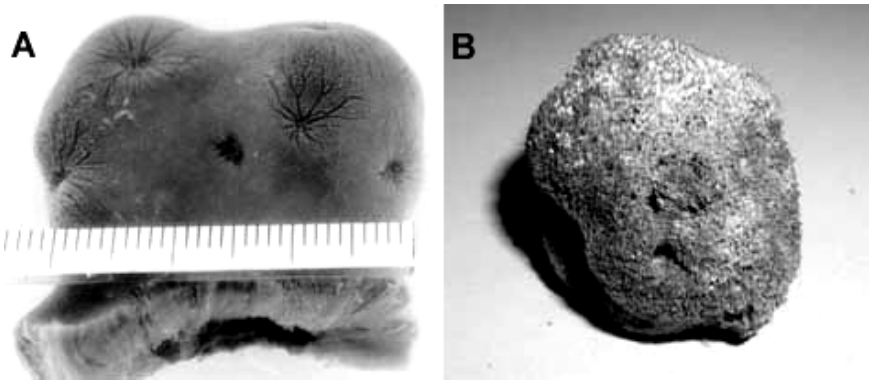


Fig. 1. Endemic freshwater sponges from ancient lakes.
A. *Lubomirskia abietina* collected at 35 m depth in Lake Baikal (Efremova et al., 2002).

B. *Ochridaspongia rotunda* collected at 30-40 m depths in Lake Ochrid (photo: W.E.G. Müller).

Methods

The endemic Baikalian freshwater sponges *Lubomirskia baicalensis* (Pallas), *Baikalospongia bacillifera* (Dybowsky), *Baikalospongia intermedia* (Dybowsky), *Baikalospongia recta* (to be published by S.M.Efremova) and *Swartschewskia papyracea* (Dybowsky) as well as the cosmopolitan freshwater sponge *Spongilla lacustris* (Linnaeus) were collected by SCUBA diving during expeditions to Lake Baikal. The marine sponge *Suberites domuncula* (Olivi) was collected from the Northern Adriatic, near Rovinj (Croatia). The source of the reagents and the methods applied have been described (Efremova et al., 2002a, b; Schröder et al., 2002).

Results and Discussion

Response of sponges to environmental stressors

There are many reasons to use sponges as bioindicators. Sponges are sessile filter-feeders which are able to accumulate pollutants from the filtered water. They are equipped with effective defense systems to pollutants and express a number of sensitive stress proteins (biomarkers), allowing the use of these animals as bioindicators to monitor pollutional stress before irreversible damage occurs. The protective mechanisms of sponges against stress comprise the expression of heat-shock proteins (HSPs), the induction of the multixenobiotic resistance (MXR) mechanism, and apoptotic elimination of cells. Over the last years, various genes involved in the stress response of sponges have been cloned and characterized. In frame of project INTAS-96-1787, a computer database on heat shock gene expression ("Heat shock knowledge base"), available through the Internet (http://wwwmgs.bionet.nsc.ru/mgs/dbases/heatshock/) was developed, including a description of environmental effects on HSP expression (Stepanenko et al., 2000).

The expressions of heat shock protein HSP70 was determined in the endemic Baikalian sponges B. intermedia, L. abietina and Lubomirskia fusifera (Efremova et al., 2002a, b). Tissue cubes of B. intermedia and dissociated cells of *L. fusifera* and *L. abietina* responded to temperature stress (10-16°C above ambient temperature) with an increase in expression of HSP70. Studies on the effects of model pollutants on HSP70 expression revealed that lead and zinc but not copper cause a strong induction of HSP70 in *B. intermedia*. A dose-dependent rise in HSP70 expression was also found after exposure of this sponge to waste water from the final refinement and aeration pond of the Baikalsk pulp and paper mill, one potential source of pollution in the southern basin of Lake Baikal.

Production of sponge secondary metabolites

Environmental pollution may affect chemical defense mechanisms of sponges. Marine sponges are known to produce a large variety of secondary metabolites. Bioactive compounds from sponges have also attracted attention in medicine due to their antibacterial, antiviral, antitumoral, antifungal, or antiinflammatory activities. Despite the large number of compounds and the high variety of structurally different natural products, only a very limited number of these secondary metabolites have been tested in clinical trials. Limited availability of larger quantities of a particular sponge species as starting material for extraction of the compounds is one of the major causes for the low attractiveness of such secondary metabolites for commercial development. It is the aim of the German Center of Excellence "BIOTECmarin" to follow suitable routes to obtain larger quantities of sponge secondary metabolites. These routes comprise: sustainable cultivation of sponges on biobatteries and ARCON structures; development of 3D cell culture systems and bioreactor units for sponges; production of bioactive sponge secondary metabolites using the Primmorph/Fragmorph systems; cultivation and characterisation of sponge associated microorganisms and their role in bioactive metabolite production; cloning of a functional sponge genome and isolation of gene clusters for bioactive metabolite biosynthesis; and isolation, chemical and biological characterisation of bioactive compounds. More information on the participating groups and the subprojects are available at http://www.biotecmarin.de.

Evolution of endemic sponge species

The phylogeny of the endemic freshwater sponges in Lake Baikal is not well understood. Molecular sequence data using 18S rDNA revealed only a low degree of discrimination between endemic Lubomirskiidae and cosmopolitan Spongillidae. Therefore the following approaches have been undertaken; (i) partial cloning and sequence anlysis of the mitochondrial cytochrome oxidase subunit I (COI) gene, which encodes one subunit of the cytochrome oxidase; and (ii) cloning and analysis of one selected intron of the tubulin gene (Schröder et al., 2002). The following endemic Baikalian sponges (family Lubomirskiidae) were studied; *L. baicalensis, B. intermedia, B. recta, B. bacillifera* and *S. papyracea*. Sequence comparisons were performed with the cosmopolitan freshwater sponge *S. lacustris* (family Spongillidae) and with the marine sponge *S. domuncula*. The sequence comparison of the mitochondrial COI gene revealed that the Baikalian sponges derived from one common ancestor from which also *S. lacustris* derived. The sequences of the COI gene from *B. recta, B. intermedia, B. bacillifera* and *L. baicalensis* were identical and separated from that of *S. lacustris* and *S. papyracea*. A further resolution between the different sponge species was not possible using this gene segment. In a second approach the exon/intron sequences framing the intron-2 of the sponge tubulin gene were chosen for the phylogenetic analysis. The intron sequences were aligned and the phylogenetic tree was constructed. This analysis revealed again a monophyletic grouping with *S. lacustris* as the closest related species to the common ancestor (Fig. 2A, B). From these data we conclude

that the Baikalian sponges studied here, are monophyletic and originated from a common ancestor together with *S. lacustris*. Furthermore, it can be deduced that *S. papyracea* branches off first among the different endemic Baikalian sponge species.

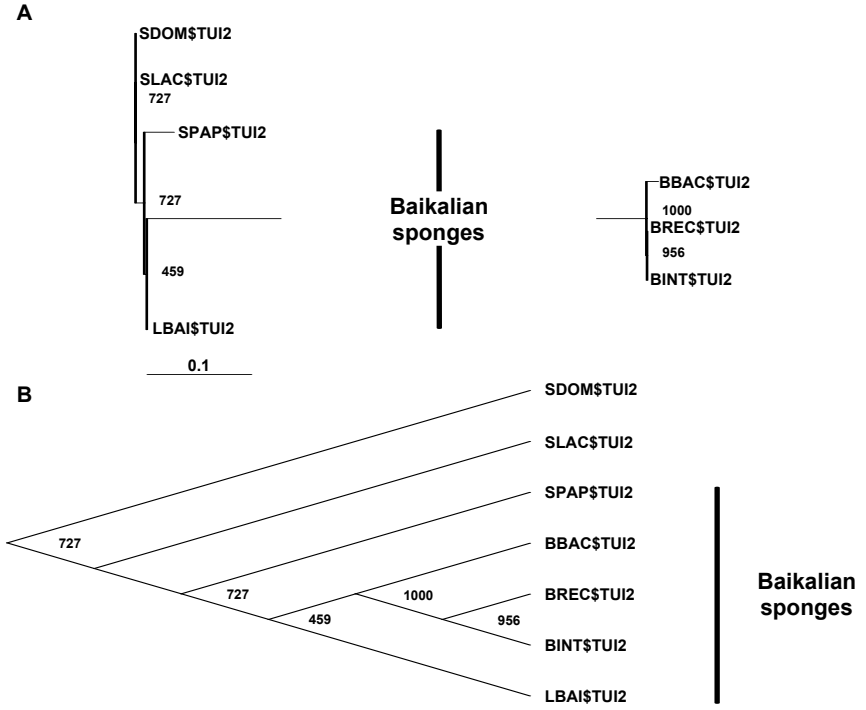


Fig. 2. Phylogenetic relationship of the intron-2 sequences from tubulin of the Baikalian freshwater sponges *S. lacustris* (SLAC$TUI2), *L. baicalensis* (LBAI$TUI2), *B. intermedia* (BINT$TUI2), *B. bacillifera* (BBAC$TUI2), *B. recta* (BREC$TUI2) and *S. papyracea* (SPAP$TUI2) in relation to the marine sponge *S. domuncula* (SDOM$TUI2). **A.** Rooted phylogenetic tree; **B.** cladogram (slanted), constructed after alignment of the intron sequences. (Schröder et al., 2002).

Acknowledgements

This work was supported by the BMBF (Center of Excellence on Molecular Biotechnology of Marine Sponges "*BIOTEC*marin"; F0345) and INTAS grant 96-1787.

References

1. Efremova S.M., Itskovich V.B., Parfenova V., Drucker V.V., Müller W.E.G., Schröder H.C. (2002a) Lake Baikal: a unique place to study evolution of sponges and their stress response in an environment nearly unimpaired by anthropogenic perturbation. Cell. Mol. Biol. in press.
2. Efremova S.M., Margulis B.A., Guzhova I.V., Itskovich V.B., Lauenroth S., Müller W.E.G., Schröder H.C. (2002b) Heat shock protein Hsp70 expression and DNA damage in Baikalian sponges exposed to model pollutants and waste water from Baikalsk Pulp and Paper Plant. Aquatic Toxicology. 57, 267-280.
3. Müller W.E.G. (1998) Origin of Metazoa: sponges as living fossils. Naturwiss. 85, 11-25.
4. Schröder H.C., Efremova S.M., Itskovich V.B., Krasko A., Müller I.M., Müller W.E.G. (2002) Molecular phylogeny of the freshwater sponges in the Lake Baikal. J. Zool. Syst. Evol. Res. in press.
5. Stepanenko I.L., Levitsky V.G., Schröder H.C. (2000) Heat shock knowledge base. Proc. of the Second Intern. Conf. on Bioinformatics of Genome Regulation and Structure (BGRS-2000). 3, 59-61.

COMPUTER ANALYSIS OF D-LOOP OF MITOCHONDRIAL DNA VARIATION IN ASIAN HORSE BREEDS

Udina I.G.

N.I.Vavilov Institute of General Genetics, RAS, Moscow, Russia, e-mail: udi@vigg.ru

Key words: *Asian horse breeds, genetic diversity, mtDNA, D-loop, phylogeny*

Resume

Motivation: To conserve the biological variety of aboriginal breeds, their genetic diversity must be estimated. Therefore, the goal of our work was to estimate genetic diversity of Asian horse breeds by variation of D-loop of mtDNA, including comparative analysis of D-loop variation in horse breeds originating from other geographical regions. We intended to prove also, that high genetic diversity was characteristic for Asian horse breeds.

Results: Asian horse breeds (Mongolian, Tuvinian, Akhaltekin, Przewalski, Yunan, Japanese and Cheju horses) were studied by the variation of D-loop of mtDNA and compared with thoroughbred horses and Arabian horses. According to mtDNA D-loop variation, the time of differentiation of Mongolian breed is 0.36 Mya, thus, suggesting that accumulation of heterogeneity has begun long before the domestication of horses. Gene pool of Asian breeds, which can be considered as the main source of horse breed origin was shown to include various types of D-loop, but no geographic affiliation of particular D-loop types was demonstrated.

Introduction

At present, intensive research of world horse diversity is performed including goals of estimation of genetic diversity of breeds and population diversity and search of breed specific genetic markers. The total number of horses has reduced greatly in our country and many breeds have disappeared during last years. To conserve the biological variety of Russian breeds, their genetic diversity must be estimated.

Therefore, the goal of our work was to estimate genetic diversity of Asian horse breeds by computer analysis of variation of D-loop of mtDNA, including comparative analysis of D-loop variation in other horse breeds. Special aim was to determine the level of genetic diversity of Asian horse breeds. Earlier, it was shown, that present living Przewalski horses are not diverse and, therefore, it can be argued, whether they can be the wild ancestor of all horse breeds. Thus, we intended to estimate the level of diversity of Mongolian and Tuvinian breeds, which are believed to be originated from wild Przewalski horses, in order, to prove, that original gene pool of wild horses was substantially diverse.

Methods and Algorithms

DNA was isolated from total blood samples from Akhaltekin horse (provided by L.Khrabrova and A.Zaitsev, All-Russian Research Institute of horse breeding, Ryazanskaya oblast) and from two Mongolian horses (provided by T.Tsedev, Institute for Biological Sciences of the Mongolian Academy of Sciences, Ulan-Bator, Mongolia)

by standard techniques. Sequence variation of mtDNA D-loop region of breeds was studied in the 336-bp fragment localized between gene $tRNA^{Pro}$ and the first conservative block of D-loop analogous (Ishida et al., 1995). Automatic direct sequencing of PCR-products was performed. NCBI database (http://www.ncbi.nlm.nih.gov/); (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome) was used to search nucleotide sequences of D-loop of Asian horse breeds and from other breeds. The Internet server http://www.searchlauncher.bcm.tmc.edu/ was used for multiple alignment and formatting of nucleotide sequences of D-loop. To construct matrices of genetic distances and phylogenetic trees software MEGA (Kumar et al., 1993) was used. We constructed several phylogenetic trees for 49 nucleotide sequences of mtDNA D-loop of Asian horses (Tuvinian, Mongolian, Akhaltekin, Yunnan, Japanese, Cheju and Przhevalskii) from Genbank (including our nucleotide sequences) and the mtDNA D-loop of *Equus asinus* (Genbank, AF220938) was used as an outgroup. Later on, an analysis of the same pool of sequences was performed along with mtDNA D-loop sequences from Thoroughbred horses and Arabian horses from Genbank.

We constructed also a tree of selected mtDNA D-loop sequences of seven Cheju (C1-C7) (Kim et al., 1999), seven Mongolian: (M1-M4) (Kim et al., 1999), M5 and M6 (our data), M7 (Ishida et al., 1995), one Akhaltekin (A1 (our data), two Yunnan (Y1 and Y2) and one Przewalski (P1) (Kim et al., 1999), one Japanese (J1)(Ishida et al., 1995) and three thoroughbred (T1-T3) horses (Ishida et al., 1994) was constructed. Tamura-Nei distances were used for the neighbor-joining method. The same mtDNA D-loop of *Equus asinus* (Genbank, AF220938) was used as an outgroup.

Implementation and Results

The computer analysis of mtDNA D-loop sequences revealed the absence of geographical affiliation of particular D-loop types to any breed or geographic region. However, our analysis demonstrated high nucleotide diversity of Mongolian breed compared with Tuvianian breed, thus, indicating its gene pool obviously originated from wild horses (close to modern Przewalski horses).

The variation of mtDNA D-loop for Mongolian horses (M5 and M6, our data) and one Akhaltekin horse (A1, our data) and in 19 corresponding mtDNA D-loop sequences (see Materials and Algorithms) from databases and papers is demonstrated by phylogenetic tree shown in Fig. The tree in total is similar with trees constructed for 49 mtDNA sequences from Asian horses and for the same number of Asian and Thoroughbred and Arabian horses. There are several clusters on the tree, which united mtDNA sequences from various breeds. Thoroughbred horses D-loop sequences occurred in two different clusters, this is in agreement with the fact of its mixed origin (the breed has Asian and European routes). The Cheju horses (Korean) are also of mixed origin as they were proved to include ancient routes from Mongolian horses and respectively recent admixture of thoroughbred horses (Kim et al., 1999). Mongolian horses D-loop sequences are present in all clusters that demonstrate high diversity of the breed (which also is characteristic of Cheju horses). Therefore, presently observed reduced diversity of Przewalski horses is the result of inbreeding and bottle neck effect for the population (or particular lineages). Local ancient horse breeds, such as Mongolian, Tuvinian and Cheju, were shown to have high nucleotide diversity of mtDNA D-loop. The gene pool of these horse breeds originated from wild horses (Przewalski horse). Thus, we proved, that modern horse breeds inevitable could have originated from wild Przewalski horse.

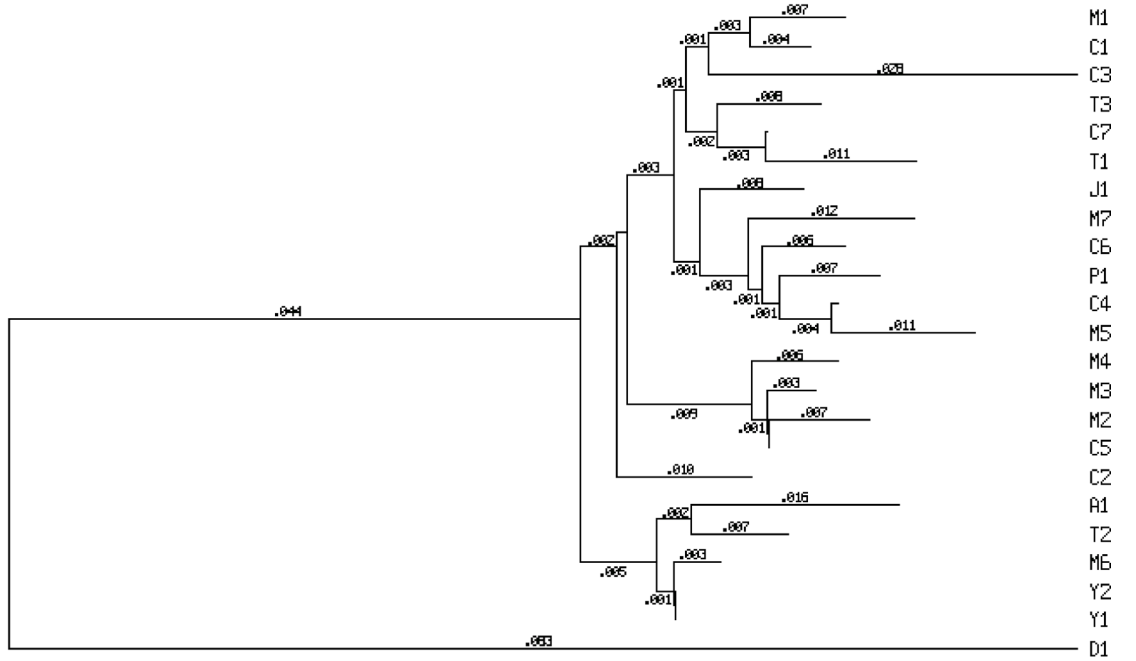


Fig. Neighbor-joining phylogenetic tree of 22 horses including Mongolian (M1-M7), Cheju (C1-C6), Yunnan (Y1 and Y2), Przewalskii (P1), Japanese (J1) and thoroughbred horses (T1-T3) (see Materials and Algorithms) based on Tamura-Nei distance. Corresponding values of genetic distances are shown in figures. D1 - *Equus asinus* (Genbank, AF220938).

Discussion

The time of divergence of horse breeds (basing on total D-loop variation) is 0.5 Mya (Kim et al., 1999). By the data of these authors, we have estimated the time of differentiation of Mongolian horses as 0.36 Mya. Domestication of horses was believed to be several thousand years ago.

Therefore, obtained data demonstrate high level of differentiation, obviously, accumulated by gene pool of wild horse populations before horse domestication. Mongolian horses (which are considered as domesticated descendants of wild horses called Przewalski) are characterized by high level of heterozygosity. Probably, gene pool of the founders of Mongolian breed was one of the main sources of Asian horse breed origination, as they include high variety of mtDNA types, which are similar with mtDNA types from the spectrum of horse breeds of different origin.

Acknowledgements

The work was supported by the Federal Scientific Technical Program, contract № 43.073.1.1.1505, and RAS Program "Regulator mechanisms of functioning and development". The author is grateful to M.Kostyuchenko for the help in laboratory work.

References

1. Ishida N., Hasegawa T., Takeda K., Sakagami M., Onishi A., Inumaru S., Komatsu M., Mukoyama H. (1994) Polymorphic sequence in the D-loop region of equine mitochondrial DNA. Anim. Genet. 25, 215-221.
2. Ishida N., Oyunsuren T., Mashima S., Mukoyama H., Saitou N. (1995) Mitochondrial DNA sequences of various species of the genus Equus with special reference to the phylogenetic relationship between Przewalskii's wild horse and domestic horse. J. Mol. Evol. 41, 180-188.
3. Kim K.I., Yang Y.H., Lee S.S., Park C., Ma R., Bouzat J.L., Lewin H.A. (1999) Phylogenetic relationships of Cheju horses to other horse breeds as determined by mtDNA D-loop sequence polymorphism. Anim. Genet. 30, 102-108.
4. Kumar S., Tamura K., Nei M. (1993) Molecular evolutionary genetic analysis. Version 1.0. The Pennsylvania State University. University Park. PA 16802.

DOPAMINE AND JUVENILE HORMONE IN THE CONTROL OF DROSOPHILA REPRODUCTION UNDER NORMAL CONDITIONS AND HEAT STRESS

Gruntenko N.E., Adonjeva N.V., Karpova E.K., Chentsova N.A., Faddeeva N.V., Rauschenbach I.Yu.

Institute of Cytology and Genetics SB RAS, 630090, Novosibirsk, Russia, e-mail: nataly@bionet.nsc.ru
*Corresponding author

Key words: *Drosophila, fertility, juvenile hormone, dopamine, heat stress*

Resume

Motivation: Juvenile hormone (JH) is well known to control the reproductive function in insect (*Drosophila* in particular) females under normal environmental conditions. In turn, dopamine (DA) was shown to play a regulating role in both synthesis and degradation of JH in some insect species. Therefore, JH and DA interactions are of great interest with relation to insect reproduction under adverse conditions, taking into account their crucial role in stress reaction.

Results: Heat stress results in dose-dependent decreases of JH esterase (JHE) activity and fertility in wild type *Drosophila virilis* females. Application of exogenous JH shows a similar effect on both JH degradation and fecundity, suggesting that heat stress elicits a rise of endogenic JH titre. In a mutant *D. virilis* strain differing from wild type by an increased DA level and lower JHE activity exogenous JH application causes even more prolonged reduction of fertility. That suggests that the mutant has a constantly increased JH titer. In wild type flies exogenous JH causes a decrease of DA level, probably as a feed back response.

Introduction

Earlier we showed that females of *D. melanogaster* strain carrying *ebony* mutation resulting in a double increase of DA content had a sharp reduction of fertility and strong alterations in JH degradation activity compared to wild type under normal conditions (Rauschenbach et al., 2001). JH-hydrolysing activity is decreased in young and increased in mature mutant females. Such ontogenetic differences in the control of JH synthesis were also demonstrated for DA in females of *Blattella germanica*: the amine stimulates JH production and increases oocyte growth on days 1 and 2 of the first ovarian cycle and causes the opposite effect on days 6 and 7 (Pastor et al., 1991). According to this and the data of Altaratz et al. (1991) on JH synthesis in *D. melanogaster*, we suggest an existence of interaction mechanism for DA and JH and the coordinate regulation of JH synthesis and degradation in the control of insect reproduction. Here we examine the effect of exogenous JH on DA and fertility levels in *D. virilis* females.

Materials and Methods

D. virilis strain 101 is wild type whereas strain 147 carries three visible mutations and a larval temperature-sensitive lethal on chromosome 6 (Rauschenbach et al., 1993). For applications 2 μg of JH-III ("Fluka", Germany) in 1 μl of acetone per female were used. DA content was measured using a slightly modified method of Maickel et al. (1968). JH hydrolyzing activity was measured by the assay of Hammock and Sparks (1977). Fertility analysis was designed as described (Rauschenbach et al., 1996).

Results and Discussion

We performed the application of JH to wild type *D. virilis* females at the very beginning of egg laying when, according to our conception, DA stimulates JH synthesis and increases JH titer (Fig. 1). Control females were treated with pure acetone. The application results in a decrease of DA level, which can be possibly interpreted as feed back response to a rise in JH titer. This corresponds well with our idea of an interaction between DA and JH.

The influence of JH application on fertility of mutant (147) females of *D. virilis* is shown in Fig. 2. 147 strain has an increased DA level, low fertility, delay in oviposition onset and decreased JHE activity (Rauschenbach et al., 1993, 1996). JH degradation in 147 females reaches the level typical of wild type only on the 4th day after hatching, and only then they start to oviposit. If the reduced JH degradation in young 147 females is, as we assume, coordinate with an increase of JH synthesis, application of exogenous hormone should result in a further rise of the hormone titer and in severe consequences for oogenesis. Data of Fig. 2 show that exogenous JH causes a reduction of fecundity for 6 days after application. This agrees with our conception of a coordinate regulation of JH synthesis and degradation in *Drosophila.* Figure 2. The effect of exogenous JH on fertility in females of wild type (101) and mutant (147) strains of *D. virilis* (application on the 3rd day - shown by arrow).

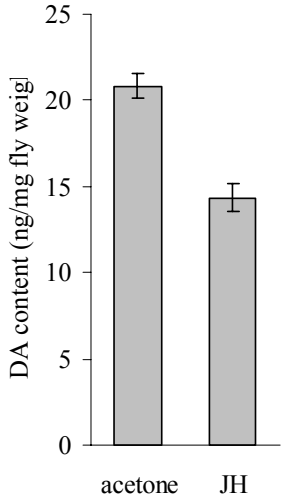


Fig. 1. The effect of exogenous JH on DA level in wild type *D. virilis* females.

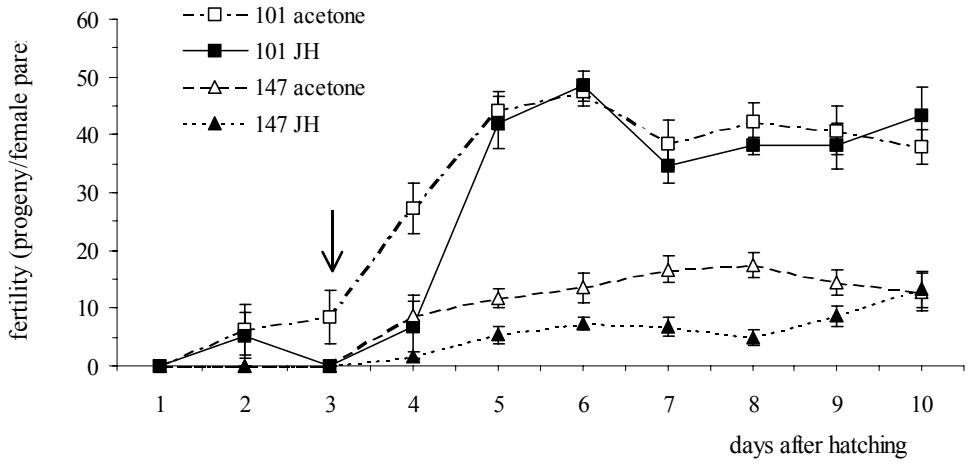


Fig. 2. The effect of exogenous JH on fertility in females of wild type (101) and mutant (147) strains of *D. virilis* (application on the 3rd day - shown by arrow).

Another possibility to check if the coordinate regulation of JH synthesis and degradation really exists was JH hydrolysis measurement in *D. virilis* females treated with exogenous JH. Data in Table clearly demonstrate that JH application results in a sharp decrease of JHE activity in both strains under study. (Flies treated with acetone and untreated ones were used as control).

Table. JHE activity in 4-day-old *D. virilis* females 5 h after JH application.

Group	JH hydrolysis (pmol/min/fly)	
	strain 101	strain 147
Control: intact	11.23±0.39	12.71±0.58
acetone	12.28±1.14	11.53±0.38
Juvenile hormone	5.51±0.76	4.97±0.40

As it is clear from Fig. 2, after JH application wild type females stop oviposition for 1 day but then restore it. Investigation of JHE activity dynamics in females after application (Fig. 3) reveals that the oviposition resumption occurs simultaneously with the normalisation of endogenic metabolism of JH. Thus, the oviposition delay is a result of JH level increase.

Earlier we showed that under heat stress in wild type *Drosophila* females DA level was going up, JH degradation level - going down and oviposition was delayed (Rauschenbach et al., 1993, 1996). If it is JH titer increase that elicits the egg laying delay, the correlation between changes in JH metabolism and fecundity should exist. In order to check this assumption we studied the effect of heat stresses of different duration on fertility and JH degradation in wild type *D. virilis* females. Comparison of Figs. 4 and 5 clearly shows that such a correlation does exist.

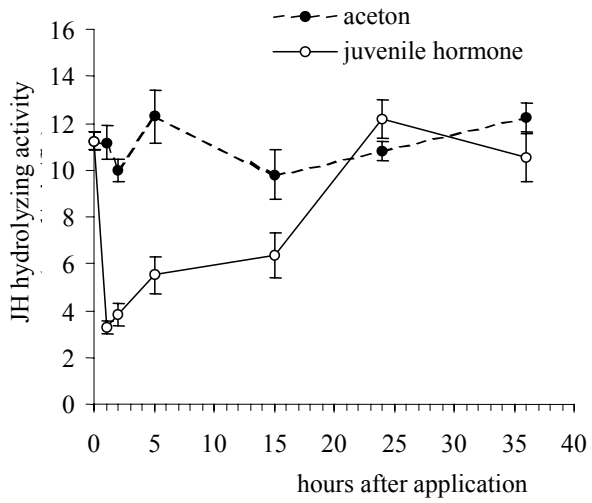


Fig. 3. The effect of exogenous JH on JHE activity in wild type *D. virilis* females.

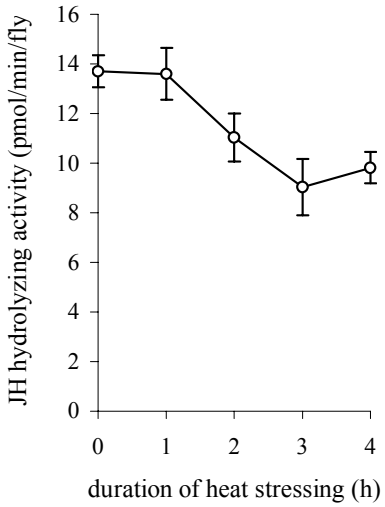


Fig. 4. Heat stress effect on different duration on JHE activity in wild type *D. virilis* females.

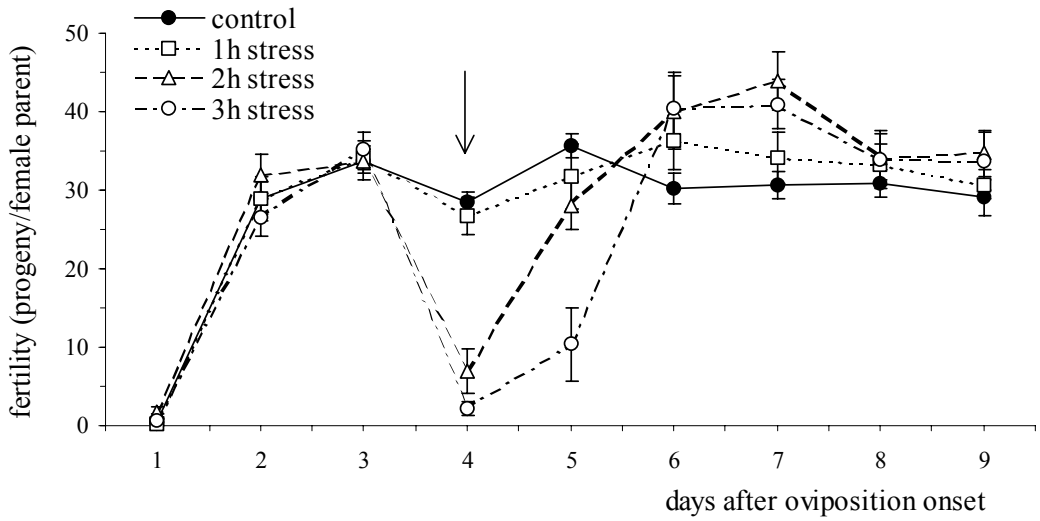


Fig. 5. The effect of heat stress of different duration on fertility of wild type *D. virilis* females (stress on the 4th day - shown by arrow).

Acknowledgements

This study was supported by INTAS Fellowship grant for Young Scientists YSF 01/1-0010 and by grants № 01-04-48905 and 00-15-97970 from the Russian Fundamental Research Foundation.

References

1. Altaratz M., Applebaum Sh.W., Richard D.S., Gilbert L.I., Segal D. (1991) Regulation of juvenile hormone synthethis in wild-type and apterous mutant *Drosophila.* Mol. Cell. Endocrinol. 81, 205-216.
2. Hammock B.D., Sparks T.C. (1977) A rapid assay for insect juvenile hormone esterase activity. Analyt. Biochem. 82, 573-579.
3. Maickel P., Cox R.H., Saillant J., Miller F.P. (1968) A method for the determination of serotonin and noradrenaline in discrete areas of rat brain. Int. J. Neuropharmacol. **7,** 275-278.
4. Pastor D., Piulachs M.D., Cassier P., Andre M., Belles X. (1991) *In vivo* and *in vitro* study of the action of dopamine on oocyte growth and juvenile hormone production in *Blattella germanica*. CR Acad. Sci. III, Paris. 313, 207-212.
5. Rauschenbach I.Yu., Gruntenko N.E., Khlebodarova T.M., Mazurov M.M., Grenback L.G., Sukhanova M.Jh., Shumnaja L.V., Zakharov I.K., Hammock B.D. (1996) The role of the degradation system of the juvenile hormone in the reproduction of Drosophila under stress. J. Insect Physiol. 42, 735-742.
6. Rauschenbach I.Yu., Vasenkova I.A.,Gruntenko N.E., Khlebodarova T.M., Sukhanova M.Zh., Korochkin L.I. (2001) On some regularities of genetic control of *Drosophila* stress reaction. Russ. J. of Genet. 37, 73-80.
7. Rauschenbach I.Y., Serova L.I., Timochina I.S., Chentsova N.A., Schumnaja L.V. 1993. Analysis of differences in dopamine content between two lines of *Drosophila virilis* in response to heat stress. J. Insect Physiol. 39, 761-767.

THE GENOMIC ORGANIZATION AND EVOLUTION OF *AEGILOPS SPELTOIDES* SUBTELOMERIC REGIONS

Adonina I., Vatolina T., Scherban A., Salina E.A.*

Institute of Cytology and Genetics, SB RAS, 630090, Novosibirsk, Russia, e-mail: salina@bionet.nsc.ru
* Corresponding author

Key words*: subtelomeric regions, tandem repeat,* Aegilops speltoides, *polymorphism, genome specific sequences*

Resume

Motivation. The aim of this study is to obtain the knowledge about the structural organization of the Spelt1 and Spelt52 repeats in the subtelomeric chromosome regions of the different accessions of *Aegilops speltoides.*

Results. The genomic organization and evolution of two tandemly repetitive sequences, Spelt1 and Spelt52, located in the subtelomeric regions of the *Ae. speltoides* chromosomes were studied. Analysis of primary structure established a monomer length of 178 bp for Spelt1; this family is highly conserved, judging from the 98% homology shown among six studied monomers. The Spelt52 family contains monomers of two types, Spelt52.1 and Spelt52.2, which share a conserved stretch of 283 bp with nonhomologous regions of 92 bp and 106 bp, respectively. The abundance and the composition of Spelt1 and Spelt52 were shown to differ greatly within seven studied *Ae. speltoides* lines in the subtelomeric chromosome regions.

Introduction

The organization of the telomeric and subtelomeric chromosome regions has acquired a special status in studies of the genome organization. It has been repeatedly demonstrated that these chromosome regions are responsible for chromosome positioning in the interphase nucleus, that they affect the duration of mitosis and meiosis, and allow complete replication of the chromosomal ends. In the past years, the detailed organization and function of the telomere repeats (TTTAGGG)n has been clarified. However, much less information on the DNA regions adjacent to telomeric repeats, namely the subtelomeric chromosome regions, is available. In a previous paper, we have described the family of species-specific subtelomeric repeat, Spelt1, from *Aegilops speltoides,* a putative B genome donor of polyploid wheats (Salina et al., 1998). Another tandemly repeated sequence, Spelt52 (pAesKB52), was shown to be localized at the subtelomeric chromosome regions of some of the *Triticeae* S-genome species (*Ae. speltoides, Aegilops sharonensis* and *Aegilops longissima*) (Anamthawat-Jonsson, Heslop-Harrison, 1993; Salina et al., 2000). However, the available evidence is obviously insufficient for making inferences about the genomic organization and evolution of these DNA sequences and of the subtelomeric chromosome regions possessing repeated sequences. The aim of this study is to obtain the knowledge about the structural organization of the Spelt1 and Spelt52 repeats in the subtelomeric chromosome regions of the different accessions of *Ae. speltoides.*

Materials and Methods

Plants

The *Ae. speltoides* lines were obtained from Prof. M.Feldman (The Weizmann Institute of Science, Israel).

DNA probes

A 150-bp *Spelt1* sequence was previously isolated (Salina et al., 1998). The DNA region from the duplicated region of pAesKB52 sequence (Anamthawat-Jonsson and Heslop-Harrison, 1993) was previously cloned and designated as Spelt52 (Salina et al., 2000).

Dot- and Southern hybridization

The inserted fragments of recombinant plasmids were labeled with α^{32}P-dATP (Amersham Pharmacia Biotech, UK) using Klenow fragment and M13 primers. Dot and blot hybridization was carried out according to the conventional Amersham protocol.

PCR, DNA cloning and nucleotide sequencing

The PCR conditions were as follows: 2 min at 94^0C; 30 cycles of 1 min at 94^0C, 1 min at the appropriate annealing temperature, and 2 min at 72^0C, followed by a final 5 min at 72^0C. The PCR fragments of interest were excised from the gel using Chip for DNA Recovery (RECOCHIP; Takara, Japan). Isolated DNAs were subcloned into pCR 2.1 of TOPO TA Cloning system for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector (Invitrogen, USA). Nucleotide sequencing was performed by the dideoxy method using an ABI 377 DNA sequencer (Applied Biosystems Inc. USA). Nucleotide sequences were searched for homology to all the sequences in the DDBJ, GenBank and EMBL databases using the BLAST algorithm (Altschul et al., 1990). For sequence analysis DNASIS (Hitachi Software Engineering, Japan) software was used.

Results

Organization of Spelt1 and Spelt52 repeat sequences in the genomes of seven lines in Aegilops speltoides

Dot hybridization was done to compare the amount and composition of the Spelt1 and Spelt52 repeats in the seven lines of *Ae. speltoides.* This shows that the seven *Ae. speltoides* lines, selected from different sources, varied in amount of the two repeats. The Spelt52 repeat was predominantly present in the line TS01, whereas Spelt1 repeat was much more abundant in all other studied lines. The finding that the *Ae. speltoides* accessions varied considerably in the amount of two subtelomeric repeats, Spelt1 and Spelt52, required further examination about their repetitious situation in the genome. The DNAs from different lines were digested with restriction endonucleases, which allow detecting a ladder of tandemly repeated monomeric unit in the subtelomeric families. No differences in the tandemly repeated organization were found among the analyzed *Ae. speltoides* lines (data not shown).

Primary structure of Spelt1 and Spelt52 monomeric units and their adjacent regions

Four primers were chosen on the basis of the data available for the primary structure of *Ae. speltoides* subtelomeric regions (Table 1). The specific PCR products, which contained Spelt1 or Spelt52 sequences in some primer combinations (Table 2), were then advanced to cloning for characterization of their structure. Analysis of the primary structure of clones containing only Spelt1 demonstrated that all the three sequences have very similar structures showing 99% homology, although different primer pairs of Sp1L-Sp1R and Sp1R were used in PCR (Table 2). The cloned sequence Ae07 is arranged in tandem arrays with a repeated monomeric unit, which is 178 bp long and 98% homologous to the 150 bp Spelt1 sequence we cloned earlier using *Taq*1 genomic digest (Salina et al., 1998).

In the case of the Spelt52 repeats, the DNA sequences comprising of the repeat itself and adjacent regions were cloned. It was shown, the Spelt52 repeat family is composed of two types of monomers (Table 2). One type containing the conserved part and additional 106 bp nonhomologous sequence upstream of 5'-TC(-/C)T(G/A)TTAGTT-3' was designated Spelt52.1 (Ae70); the other, which contains the conserved part and additional other nonhomologous sequence of 92 bp long located upstream of 5'-TC(-/A)TT(G/C)TT(-/A)GTT-3', was designated Spelt52.2 (Ae71).

Analysis of the sequences obtained by cloning the PCR products with the primer pair Sp1L-Sp52R revealed two types of sequences (Table 2). Ae069 is completely homologous to the Spelt1 sequence, while the region of homology to Spelt52 is restricted only by primer size. Ae99 consists of two regions following one another. The first 56 bp region shows 100% homology to Spelt1 was followed by the second 83 bp with 94% homology to Spelt52 (Table 2).

Table 1. List of primers and their design.

Primer name	Sequence (5'-3')	Source of sequences for primer design	
		accession number in databases	reference
Sp1L (left) Sp1R (right)	tccaaaccatccccgtcaagcg aagttcttctggccgtgccata	YO9217	Salina et al., 1998
Sp52L (left) Sp52R (right)	gcacacaaacccggagaaagt tccccgtttcttctctagcct	Z21644	Anamthawat-Jonsson, Heslop-Harrison, 1993

Table 2. Description of cloned sequences.

Cloned sequences	Primers combinations	Length bp	Acc. number	Homology to../ length (bp)/ %
Ae07	Sp1R-Sp1L	498	*	*Spelt1* / 498 / 98%
Ae26 Ae27	Sp1R	498 498		Ae07 /498 / 99% Ae07 / 498 / 99%
Ae70 Ae71	Sp52R-Sp52L	600 591	* *	*Spelt52* /223_271/ 92%_92% *Spelt52* /226_273/ 95%_90%
Ae069 Ae99	Sp1L-Sp52R	176 139		*Spelt1* / 163 / 92% *Spelt1* & *Spelt52* / 56&83 / 100%&94%

(_) – insertion between region showing homology
(&) – homologous regions following each other

Discussion

Divergence in the major cereals has been often accompanied by amplification of DNA sequences of the subtelomeric region of chromosomes creating species-specific tandemly arranged arrays of the sequences. As early as in 1979, Flavell et al. (1979) have demonstrated that *Triticum monococcum, Ae. speltoides* and *Triticum taushii*, the putative donors of the A, B and D genomes of polyploid wheats, respectively, have different fractions of repetitive DNA; those repetitive DNA sequences represent more than 2-3% of the DNA in the *Ae. speltoides* genome, but are not detectable in their counterparts of the *T. monococcum* and *T. taushii*. Spelt1 and Spelt52 are absent from species with the A and D genomes (Anamthawat-Jonsson, Heslop-Harrison, 1993; Salina et al., 1998), and precisely these repeats we implicate here as the possible causes of the differences in repetitive DNA fractions previously found among the putative donors of the A,B, and D genomes (Flavell et al., 1979). Both PCR analysis and Southern hybridization revealed the presence of these repeat families arranged in identical tandem arrays in all the lines of *Ae. speltoides*, however, the repetitive sequences differ greatly within lines in

abundance (data not shown). Spelt1 and Spelt52 families differ by monomeric unit length and divergence level. It is worthy to note that monomer length of Spelt1 (178 bp) and Spelt52 (370-390 bp) is equal to the length that wraps around a nucleosome core with linker (170-180 bp or double), which is consistent with the monomer length of tandem repeats of many species. The Spelt1 and Spelt52 repeats can be at different distances from each other in the subtelomeric regions. The two repeat families can cluster as blocks at a distance apart (Salina et al. unpublished). When the Sp1 - Sp52 primer pair was used, PCR revealed that there were at least some regions in the *Ae. speltoides* genome in which Spelt1 - Spelt52 adjoin (Table 2). Spelt52 seems to be evolutionarily more ancient than Spelt1. This is supported by the higher divergence of Spelt52, namely, the presence of Spelt52.1 and Spelt52.2 families. Furthermore, the Spelt52 repeat family is present in the *Ae. speltoides, Ae. longissima* and *Ae. sharonensis* species (Anamthawat-Jonsson, Heslop-Harrison, 1993), which are three of the five belonging to the *Sitopsis* section. As for the Spelt1 repeats, they to arose during the speciation of *Ae. speltoides* (Salina et al., 1998). Analysis of the organization and variability of Spelt1 and Spelt52 families on a broader basis of lines of *Aegilops* species and their amphidiploids, together with knowledge of previous the genomic organization in wheats, would be helpful in resolving challenging issues of the evolution of species of the *Sitopsis* section and of polyploid wheat origin. More light would be shed on the role of the subtelomeric repeats in the evolution of diploid species and in the formation of the complex amphidiploid nucleus.

Acknowledgements

The research was supported in part by the INTAS , grant № 01-0537.

References

1. Altschul S.F. et al. (1990). Basic local alignment search tool. J. Biol. 215, 403-410.
2. Anamthawat-Jonsson K., Heslop-Harrison J.S. (1993) Isolation and characterization of genome-specific DNA sequences in *Triticeae* species. Mol. Gen. Genet. 240, 151-158.
3. Flavell R.B. et al. (1979) Repeated sequence DNA comparisons between Triticum and Aegilops species. Heredity. 42, 309-322.
4. Salina E.A., Ozkan H., Feldman M., Shumny V.K. (2000) Subtelomeric repeat reorganization in synthesized amphiploids of wheat. Proc. Conf. "Biodiversity and Dinamics of Ecosystems in North Eurasia", Novosibirsk, 102-105.
5. Salina E.A., Pestsova E.G., Adonina I.G., Vershinin A.V. (1998) Identification of a new family of tandem repeats in *Triticeae* genomes. Euphytica. 100, 231-237.

KARYOTYPE EVOLUTION IN MAMMALS: A REAPPRAISAL BY COMPARATIVE CHROMOSOME PAINTING

[1]Graphodatsky A.*, [1]Perelman P., [1]Alkalaeva E., [1]Trifonov V., [1]Shtukkert A., [1]Serdukova N., [1]Biltueva L., [1]Vorobieva N., [2]Ferguson-Smith M.A., [2]Yang F., [2]O'Brien P., [3]Robinson T.J., [4]Suwattana D., [5]Sharipov I.K.

[1] Institute of Cytology and Genetics, SB RAS, 630090, Novosibirsk, Russia
[2] University of Cambridge, UK
[3] University of Stellenbosch, SAR
[4] Chulalongkorn University, Bangkok, Thailand
[5] Institute of Genetics and Cytology, Alma-Ata, Kazakhstan
*Corresponding author: graf@bionet.nsc.ru

Key words: *karyotype evolution, mammals, chromosome painting*

Resume

Motivation: Painting technology permits to make a revision of data and theories on karyotype evolution available from routine cytogenetics. We apply Zoo-FISH technology to study major tendencies of karyotype evolution across mammalian orders.
Results: Human chromosome specific probes were used to track the way of karyotype changes in some mammalian orders. Painting probes localization allowed us to analyze karyotype and genome evolution and produce complete integrative maps for human and particular species. Available from painting studies data were analyzed and two modes of karyotype evolutional changes (slow and drastic) were demonstrated.

Availability: http://www.bionet.nsc.ru/chromosomes

Introduction

Two main tendencies of mammalian karyotype evolution were already supposed in comparative banding studies: slow (karyotype evolution) and catastrophic (karyotype revolution) (Graphodatsky, Biltueva, 1987). Appearance of new technologies in comparative cytogenetics such chromosome sorting and painting, and improvements in molecular methods (DOP-PCR, heterological fluorescence in situ hybridisation) has lead to formation of new field of comparative genomics. Newly obtained data confirmed the concept of different karyotype evolution rates (Murphy et al., 2001). Sorting chromosomes derived from species with conservative karyotypes permit to reveal large chromosomal rearrangements and roughly describe a whole picture of karyotype evolution (e.g. human, American mink chromosomal sets) (O'Brien et al., 1999, Hameister et al., 1997). Whereas probes from species with rearranged genomes allows tracing of fine changes in karyotypes (Graphodatsky et al., 2000). In the past decade 11 mammalian orders were studied using human paints, and evolutionary conserved chromosome segments were revealed across wide range of species. The number of such segments reflects degree of karyotype conservation and indicates rate of their evolution. Here we analyze our and published data from standpoint of mammalian karyotype evolution trends.

Materials and Methods

Metaphase preparations were made from primary fibroblast cultures and bone marrow established from Siberian chipmunk (*Tamias sibirica*), Indian elephant (*Elephas maximus*), and bactrian camel (*Camelus bactrianus*). Tissue culturing, metaphase preparation, and G-banding followed the method described in Graphodatsky et al., 1995. *Human chromosome-specific probes* were supplied by Centre for Veterinary Science, Department of Clinical Veterinary Medicine, University of Cambridge, UK. *Chromosome painting* was performed as previously described (Yang et al., 1995).

Results and Discussion

On the basis of available for today painting data one could choose three taxa with extremely rearranged karyotypes: Hylobatidae in Primates, Canidae in Carnivora and Myomopha in Rodentia (Stanyon et al., 1995, Yang et al., 1999, Copeland et al., 1993, Grutzner et al., 1999). Perhaps, at the moment of these taxa formation the rate of chromosomal rearrangements has been increased. Such events display karyotype revolution tendency. These taxa are characterised by the large number of conserved segments revealed using human paints or gene mapping. Indeed, mouse (2n=40, Myomorpha) has about 180 conservative blocks, dog (2n=78, Canidae) – 73, and gibbon (2n=52, Hylobatidae) – 65 segments. On the contrary, other species from the same orders chimpanzee (2n=48, Primates) and American mink (2n=30, Carnivora) have 23 and 32 conservative segments respectively (Stanyon et al., 1995, Hameister et al., 1997).

In taxa characterising by karyotype revolution tendency, there are species with additional variable elements of genome, so-called B-chromosomes. We have studied these elements using a microdissection (Fig. 1) (Trifonov et al., 2002).

Accordingly to our results, B-chromosomes mainly consist of repeated sequences and are very supple, i.e. easily change their content and morphology. This phenomenon could reflect general tendency of such genomes to rapid karyotype changes.

By contrast, there are species with utterly conserved karyotypes. In the previous study we used American mink-specific painting probe set to hybridize with 8 Mustelidae species (Graphodatsky et al., 2002). Almost equal number conserved syntenic segments (about 21) were identified in all these species using mink probes. We have shown that Mustelidae karyotypes retain the same banding pattern, and differ by minor changes, thus follow karyotype evolution tendency.

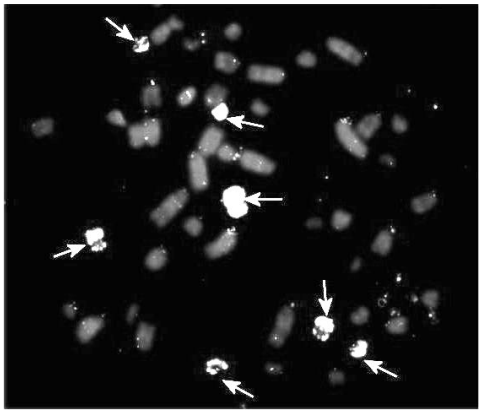


Fig. 1. FISH with B-chromosome specific prob onto metaphase chromosome of Asian wood mouse *Apodemus peninsulae.* Arrows indicate hybridization signals on B-chromosome of different morphology and size.

In present investigation, we proceed from the guess that species with extremely rearranged genomes appear in taxa along with conservative ones. To supply our assumption, we have painted with human probes representative of suborder Scuiromorpha, Rodentia – chipmunk (*Tamias sibiricus*, 2n=36). 32 segments of conserved synteny common to the human and chipmunk genomes were identified (Fig. 2). Our results indicate that chipmunk has even less rearranged genome than species from other mammalian orders with slow rate of karyotype evolution. Consequently, during Rodentia evolution there was critical turning point, when rate of karyotype rearrangements was abruptly increased leading to formation of myomorpha genomes.

It is important to emphasize that high diploid number not imply highly rearranged karyotype. Painting with human probes the Bactrian camel (2n=78, Artyodactyla) and Indian elephant (2n=56, Proboscidea) genomes revealed 44 conserved elements in both species (Fig. 3). Such values are in line with 32 conserved blocks found between human and cat (Wienberg et al., 1997), the 47 blocks for human and pig (Rettenberger et al., 1995) and 50 blocks between human and cow (Solinas-Toldo et al., 1995). So general events leaded to formation camel and elephant karyotypes were multiple fissions of conserved elements without considerable reshuffling between conserved syntenic groups.

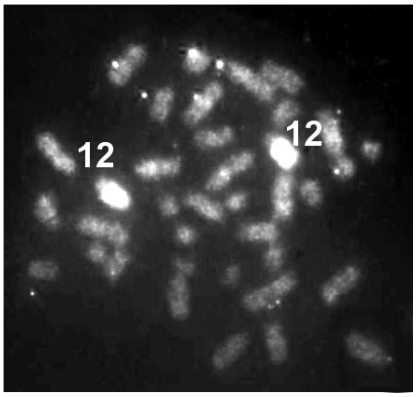


Fig. 2. Localization of human chromosome 7 paint onto metaphase chromosomes of Siberian chipmunk.

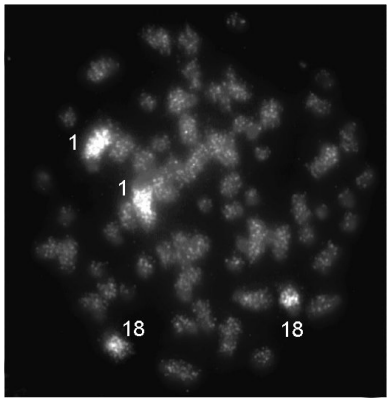


Fig. 3. Localization of human chromosome 3 paint onto metaphase chromosomes of bactrian camel.

Painting probes of species with utterly rearranged genomes are very useful instrument for fine comparative mapping (Graphodatsky et al., 2000). The number of conserved segments between dog and human karyotypes about twice times bigger then for other species with conserved chromosomal sets (Yang et al., 1999). Unique set of dog chromosome-specific paints permits identification of inversions that separate karyotypes of different species. In addition, dog-specific paints allow to deduce ancestral pattern of segment order on particular chromosome when outgroup species is used. In the previous research, we used dog paints localization to trace chromosomal changes in karyotypes considered as highly conserved like

human and American mink. Some cryptic inversions were identified (Graphodatsky et al., 2000). This kind of rearrangements inside stable syntenic blocks accompanies divergence of conservative karyotypes.

Thus, our results confirm the hypothesis of two major patterns of genome reconstruction across mammalian species.

Acknowledegments

This work is supported by INTAS 02_2163 grant and research grants from the Russian Fund for Basic Research.

Reference

1. Copeland N.G., Jenkins N.A., Gilbert D.J., Eppig J.T., Maltais L.J., Miller J.C., Dietrich W.F., Weaver A., Lincoln S.E., Steen R.G. et al. (1993) A genetic linkage map of the mouse: current applications and future prospects. Science. 262, 5130, 57-66.
2. Graphodatsky A.S., Biltueva L.S. (1987) Homology of G-banding patterns of mammalian chromosomes. Genetika. 23, 93-103 [Russian].
3. Graphodatsky A.S., Beklemisheva V.R., Dolf G. (1995) High-resolution GTG-banding patterns of dog and silver fox chromosomes: description and comparative analysis. Cytogenet Cell Genet. 69, (3-4), 226-231.
4. Graphodatsky A.S., Yang F., Serdukova N., Perelman P., Zhdanova N., Ferguson-Smith M.A. (2000) Dog chromosome-specific paints reveal evolutionary inter- and intrachromosomal rearrangements in the American mink and human. Cytogenet Cell Genet. 90, 275-278.
5. Graphodatsky A.S., Yang F., Perelman P., O'Brien P.C.M., Serdukova N., Milne B.S., Biltueva L.S., Fu B., Vorobieva N.V., Kawada S.I., Robinson T.J., Ferguson-Smith M.A. (2002) Comparative molecular cytogenetic studies in the order Carnivora: mapping chromosomal rearrangements onto the phylogenetic tree. Cytogenet Genome Res. (In press).
6. Grutzner P., Himmelbauer H., Paulsen M., Ropers H.H., Haaf T. (1999) Comparative mapping of mouse and rat chromosomes by fluorescence in situ hybridization. Genomics. 55, 306-13.
7. Hameister H., Klett Ch., Bruch J., Dixkens Ch., Vogel W., Christensen K. (1997) Zoo-FISH analysis: the American mink (*Mustela vison*) closely resembles the cat karyotype. Chromosome Res. 5, 5-11.
8. Murphy W.J., Stanyon R., O'Brien S.J. (2001) Evolution of mammalian genome organization inferred from comparative gene mapping. Genome Biol. 2, 1-8.
9. O'Brien S.J., Menotti-Raymond M., Murphy W.J. et al. (1999) The promise of comparative genomics in mammals. Science. 286, 458-481.
10. Solinas-Toldo S., Lengauer C., Fries R. (1995) Comparative genome map of human and cattle. Genomics. 25, 489-496.
11. Stanyon R., Arnold N., Koehler U., Bigoni F., Wienberg J. (1995) Chromosomal painting shows that "marked chromosomes" in lesser apes and Old World monkeys are not homologous and evolved by convergence. Cytogenet Cell Genet. 68, 1-2, 74-78.
12. Trifonov V.A., Perelman P.L., Kawada S.-I., Iwasa M.A., Oda S.-I., Graphodatsky A.S. (2002) Complex structure of B-chromosomes in two mammalian species: *Apodemus peninsulae* (Rodentia) and *Nyctereutes procyonides* (Carnivora). Chromosome Res. 10, 109-116.
13. Yang F., Carter N.P., Shi L., Ferguson-Smith M.A. (1995) A comparative study of karyotypes of muntjacs by chromosome painting. Chromosoma. 103, 642-652.
14. Yang F., O`Brien P., Milne B., Graphodatsky A.S., Solanky N., Trifonov V., Rens W., Sargan D., Ferguson-Smith M. (1999) A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. Genomics. 62, 189-202.

CORRELATIONS BETWEEN GENETIC AND CULTURAL TRAITS IN POPULATIONS OF HUMANS

***Borinskaya S.A.*[1], *Korotaev A.B.*[2]**

[1] Vavilov Institute of General Genetics, RAS, Moscow, Russia, e-mail: borinskaya@vigg.ru
[2] Russian State University for the Humanities, Moscow, Russia

Key words: *human populations, genomics, adaptation, ethnography, culture*

Resume

Motivation: New fields of interaction between natural and humanitarian sciences may be opened through correlation studies between human population allele frequency data and the data from formalized description of ethnographic traits (Murdock, 1967; Murdock et al., 1999).

Results: Up to now we have found at least two interesting, statistically reliable and meaningful correlations.

i) Higher frequency of ApoE e3 allele correlates positively with intensity of agriculture in the populations that may be connected with the spreading of agriculture in human history. Higher frequency of ApoE e4 allele correlates positively with the level of input of hunter-gathering in food.

ii) Higher allele frequency of an HIV protective allele of SDF1 gene correlates positively with human population density.

Such correlations may be an evidence of genetic influence on forming human cultural and social traits or an evidence of the phenomenon that human social and cultural environment may be selective factors in human evolution. That extends our view on the spectrum and levels of genetic control of life.

Introduction

Each human population can be described in terms of frequencies of genetic as well as culural/social traits. The two different types of traits are scored quantitatively and stored in two different types of databases. Population frequencies of biochemical and molecular-genetic markers (alleles) are openly available from an electronic database called ALFRED (http://alfred.med.yale.edu/alfred/index.asp). Population frequencies of many social and cultural traits are openly available from a database called Ethnographic Atlas that being published by Peter Murdock since 1967 (Murdock, 1967; Murdock et al., 1999). Currently the Atlas contains formalized description of 1267 human populations worldwide for more than 150 ethnographic traits. Only few of the 150 traits from the Atlas (e.g. geographic location, altitude, temperature) have already been related to genetic traits by human population geneticists. The over majority of the ethnographic traits (such as language type, economy type, marriage and relations type, social type, cultural type, diet etc.) were mainly out of consideration by geneticists yet. Comparison of the genetic and ethnographic databases is possible and interesting to undertake even without any a priori hypothesis to be trailed. Should any correlation is revealed we have a basis to think over its meaning. If meaningful we could start to study molecular mechanisms behind the correlation between the genotypic and phenotypic features of populations.

Materials and Methods

Population allele frequencies have been taken from ALFRED database (http://alfred.med.yale.edu/alfred/index.asp) and from articles mentioned in the next chapter. We have entered the allele frequency data into the Ethnographic Atlas. A set of statistical programs called SPSS was used to calculate the correlation parameters.

Implementation and Results

Level of adaptiveness of different ApoE alleles may depend on the type of social and economic structure of population

Human ApoE gene is located in 19q13.2 region of the human genome. The gene codes for a protein called apolipoprotein E. The protein involved in lipid metabolism and some other important cell functions. Alleles e2, e3, and e4 are the most spread in humans. ApoE e4 allele is considered to be the ancestral allele for humans (Rogaev et al., personal communication) and still spread broadly in hunter-gatherer populations. The population frequencies of e4 allele correspond to 35% for some populations in Africa and Australia and to 5-15% in different populations in Europe (Mahley, Rall, 2000). It is known that e4 allele is a risk factor for Alzheimer disease development. There was a suggestion on a relation between e4 allele frequency and general food supply in the population (Corbo, Scacchi, 1999).

We have screened for available information on ApoE allele frequencies and found the data for 20 populations from different parts of the world (Gelernter et al., 1998; Corbo, Scacchi, 1999; Sheehan et al., 2000; Jaramillo-Correa et al., 2001; Mustaphina et al., 2001). The data have been entered into the Ethnographic Atlas.

We have found that e3 allele frequency correlates positively with the intensity of agriculture (Pirson correlation index r=0.6, p=0.01) but e4 allele frequency correlates negatively with the intensity of agriculture (r= – 0.68, p=0.001). From the data the agriculture intensity may be considered as a culture specific factor of positive selection for some gene alleles (ApoE e3 allele in the case) in humans.

ApoE e4 allele frequency positively with the input of both hunting (r=0.5, p=0.02) and gathering (r=0.5, p=0.04) in the population food supply. It correspond to published data on a higher frequency of ApoE e4 allele at hunter-gatherer tribes (Jaramillo-Correa et al., 2001; Corbo, Scacchi, 1999; Sandholzer et al., 1995). It was shown that in case of hunter-gatherers the e4 allele frequency is associated neither with Alzheimer disease nor with cholesterol blood level (Corbo, Scacchi, 1999) in contrast to the populations based on agricultural food supply. The difference in the diets is supposed to be the reason behind the difference in the associations. Furthermore, it was suggested that higher level of e4 allele frequency may be explained by a positive selection because the allele allow more effective cholesterol absorption for the carrier which is adaptive at a low cholesterol diet (Scacchi et al., 1997). It was shown that physical activity may modulate effects of ApoE genotype on lipid profile (Bernstein et al., 2002). It may be another reason behind the adaptiveness of e4 allele in case of nomadic tribes.

We have also found ApoE allele correlations with population densities (connected probably with agriculture), as well as correlations with climate conditions and periodical starvation in the populations. From our analysis we have also independently confirmed some regional maximums for ApoE allele frequencies.

It seems probable from our data that ApoE allele frequencies for a population may be resulted not only from genetic drift but reflect a process of natural selection with some factors specific for humans, because different ApoE alleles seems to be adaptive at different types of socio-economic structure of the population.

HIV resistance allele frequencies correlations with ethnographic traits

We have studied correlations of ethnographic traits frequencies with population frequencies of mutations rendering humans with resistance to Human Immunodeficit Virus (HIV) infection and to development of Acquired Immune Deficit Syndrome (AIDS). Known HIV resistance alleles in three different human genes have been studied. Those are two chemokin receptor genes CCR2 and CCR5 as well as a gene called SDF1 coding for a stromal-derived factor, a ligand of CXCR4 coreceptor.

We have screened for available information on HIV protective allele CCR2-64I population frequency and found the data for 22 populations from different parts of the world. The data have been entered into the Ethnographic Atlas. We have found that CCR2-64I allele population frequency correlates positively with patricentric type of social structure (r=0,6, p=0,001). Taking into consideration that the demic diffusion is the model of the transmission of family-relation social organization in human societies (Guglielmino et al., 1995), we suggest that the positive correlation may reflect the process of spreading of CCR2-64I allele preferably by male carriers from the territory of the mutation origin.

We have found that the HIV protective SDF1 allele population frequency correlates positively with human population density. Formally it means that the adaptive value of the allele is higher at higher population densities. The allele might be positively selected since the time of human population expansion. At the time when the allele start spreading, the selective factor might be an infection agent similar to HIV on the step of interaction with SDF1 gene product.

Discussion

Genetic background behind our social and cultural traits is implicitly obvious. But there were no tool to messier and evaluate such kind of relations. Here we suggest the correlation between frequencies of genetic and ethnographic traits as the instrument to approach such kind of studies.

An intrinsic weakness of the correlation study reliability is that the populations described by geneticist and a humanitarian scientist may not be quite the same group of individuals but rather an overlapping group according to the described features (location, ethnic group, language etc). Though such kind a drawback is rather typical for many kind of population studies that are traditional for both genetic and ethnographic research.

Only few percent of human populations have already a "combined" description from the humanitarian and genetic point of view. Though the scoring of ethnographic traits have a longtime history and well developed, the DNA typing of polymorphisms in the human genome has started rather recently and we do not understand much about the connection between the polymorphisms and the human phenotypic traits, social traits included. Only few hundreds out of millions of known human polymorphisms are being typed now, but we may be pretty sure that the millions polymorphism will be typed for million individuals within one human generation time. At the time point the ample data on our genetic traits will complement the ethnographic data quite properly. From the cooperation of natural and humanitarian sciences we may expect to get a new much higher level of understanding of genetic basis of human population existence and development and the understanding of the human nature in general.

Acknowledgements

The work was supported by a grant from the state program "Russian Foundation for Humanitarian Research".

References

1. Mustaphina O.E., Tuktarova I.A., Bikmeeva A.M., Nasibullin T.R., Khusnutdinova E.K. (2001) Study of apolipoprotein E gene polymorphism in Volga-Urals region population. Genetics. 37:558-562.
2. Bernstein M.S., Costanza M.C., James R.W., Morris M.A., Cambien F., Raoux S., Morabia A. (2002) Physical activity may modulate effects of ApoE genotype on lipid profile. Arterioscler Thromb Vasc Biol. 22:133-40.
3. Corbo R.M., Scacchi R. (1999) Apolipoprotein E (APOE) allele distribution in the world. Is APOE*4 a 'thrifty' allele? Ann. Hum. Genet. 63: 301-310
4. Gelernter J., Kranzler H., Lacobelle J. (1998) Population studies of polymorphisms at loci of neuropsychiatric interest (tryptophan hydroxylase (TPH), dopamine transporter protein (SLC6A3), D3 dopamine receptor (DRD3), apolipoprotein E (APOE), mu opioid receptor (OPRM1), and ciliary neurotrophic factor (CNTF). Genomics. 52:289-297.
5. Guglielmino C.R., Viganotti C., Hewlett B., Cavalli-Sforza L.L. (1995) Cultural variation in Africa: role of mechanisms of transmission and adaptation. Proc. Natl Acad. Sci. USA. 92, 7585-7589.
6. Jaramillo-Correa J.P., Keyex G., Ruiz-Garcia M., Rodas G., Bernal J. Population genetic analysis of the genes APOE, APOB (3'VNTR) and ACE in some black and amerindian communities from Columbia. Hum. Hered. 2001, 52:14-33.
7. Mahley R.W., Rall S.C. (2000) Apolipoprotein E: more than a lipid transport protein. Ann. Rev. Genomics Hum. Genet. 1, 507-537.
8. Murdock G.P. Ethnographic Atlas. Pittsburgh: Pittsburgh Univ. Press, 1967.
9. Murdock G.P., Textor R., Barry H. (III), White D.R., Gray J.P., Divale W. (1999–2000). Ethnographic Atlas. World Cultures. 10(1), 24–136. F. AT01–09.SAV.
10. Sandholzer C., Delport R., Vermaak H., Utermann G. High frequency of the apo epsilon 4 allele in Khoi San from South Africa. Hum Genet. 1995, 95(1):46-8.
11. Scacchi R., Corbo R.M., Rickards O., Mantuano E., Guevara A., De Stefano G.F. Apolipoprotein B and E genetic polymorphisms in the Cayapa Indians of Ecuador. Hum. Biol. 1997, 69(3):375-82.
12. Sheehan D., Bennett T., Cashman K. (2000) Apolipoprotein E gene polymorphisms and serum cholesterol in healthy Irish adults: a proposed genetic marker for coronary artery disease risk. Ir. J. Med. Sci. 169, 50-54.

PHYLOGEOGRAPHY OF MITOCHONDRIAL LINEAGES OF TURKIC AND URALIC – SPEAKING PEOPLE

[1]*Khusnutdinova E., [1]Bermisheva M., [1]Victorova T.,[1]Korshunova T., [1]Salimova A., [3]Svyatova G., [2]Tambets K., [2]Willems R.

[1] Institute of Biochemistry and Genetics of the Ufa Scientific Center of RAS, 450054, Ufa, Russia,
Fax: 35-61-00, e-mail: ekkh@anrb.ru
[2] Dept. Evolutionary Biology, Tartu University, 51010, Tartu, Estonia
[3] Republican centre of health of mother and child, Kazakhstan
*To whom correspondence should be addressed

Key words: *mtDNA, polymorphism, populations*

Resume

Phylogeography of the non-recombining maternally inherited mitochondrial DNA (mtDNA) have been broadly used to shed light on different aspects of demographic history of human populations.

We have analyzed about 2000 mtDNA of native populations from the Russian Pacific to the Atlantic coast of France using the sequencing of the first hypervariable segment on mtDNA control region and combined it with RFLP typing of informative coding region polymorphisms. Specific attention was paid to the people speaking Uralic languages (Maris, Komis, Mordvins, Udmurts) and to the Altaic language family speakers of Turkic (Tatars, Bashkirs, Chuvashis, Kazakhs, Uzbeks, Nogays, Uighurs) and Tungusic (Oroks) branches. Except Udmurts, the Volga basin populations encompass only a limited share of mtDNA haplogroups typical for eastern Asians and native Siberians, their frequency being somewhat higher (10%-11%) among Turkic speakers than among Finno-Ugric speaking populations. In contrast, among Uralic-speaking Udmurts their share is 27%. Surprisingly, the majority of mtDNA of Nogays, who are supposed to descent from Mongoloid Golden Horde inhabitants, belong also to western Eurasian variants of maternal lineages (~63%), testifying about an extensive admixture within about the last 750 years. Further eastwards (Kazakhs and Uzbeks), the frequency of eastern Asian mtDNA haplotypes start to exceed those typical for western Eurasia, reaching more than 60% among Kazakhs.

Thus, with only a little variation, there is a clear east-west gradient of the "Mongoloid" mtDNA lineages alongside the Steppe Belt and linguistic affinities of populations play a lesser role.

Introduction

Maternally inherited mtDNA is a useful and informative genetic marker for investigating human population history. It became apparent that large continental areas such as western, eastern and southern Eurasia differ profoundly in their mtDNA variety with only limited zones of admixture, eg in Central Asia (Torroni, Wallace, 1994; Wallace et al., 1995; Comas et al., 1998). Thus, the major eastern Eurasian-specific mtDNA haplogroups A, B, F and M are only rarely found among western Eurasian populations whereas the dominant European haplogroup H is virtually absent in China and Japan. Hence, any deviation from this general finding deserves special attention as it may prove to be particularly informative. However, to date only limited information is available concerning mtDNA diversity in the Central Asia, Caucasus and Volga-Ural region of Russia. We therefore undertook a comprehensive analysis of mtDNA diversity in 13 populations.

Methods and Algorithms

The blood samples were collected in 13 locations from Volga-Ural region, North Caucasus, Central Asia, Sahalin island and consisted about 2000 unrelated individuals. Eight ethnic groups belong to Altaic language family: Bashkirs, Tatars, Chuvashis, Kazakhs, Uzbeks, Nogays, Uighurs represent the Turkic branch, Oroks belong to Tungusic branch. Four populations belong to Uralic language family: Maris, Mordva, Udmurts, Komi.

Hypervariable segment I (HVSI) of control region of mtDNA was amplified with primers A15909 and B16498 followed by sequencing of the segment between nps 16024 and 16383 by use of DYEnamic^{TM} ET terminator cycle sequencing premix kit. The fluorescently labeled extension products were analysed on an Applied Biosystems Model on a 377 DNA sequencer. A selection of 26 RELP sites diagnostic for main European and Asian mtDNA haplogroups, was screened.

Result and Discussion

Analysis of mtDNA polymorphism in Volga-Ural region revealed that most of mtDNA types of the populations studed belong to clusters (H, U, T, S, W, I, R, N1) specific for people of West Eurasia. Most frequent of them are haplogroup H (12-14%) and U (18-44%). On the whole, among the populations studed by us frequency of European types of the mtDNA appeared to be the highest among Mordvins, Maris, Komi-Zirians. On the other hand, the level of spreading lineages of

mtDNA, specific for East Eurasia also reaches larger, it was not shown before for the West and Central Europe. The presence of gaplogroup G, D, C, Z and F with high frequency in some ethnic groups such as Turkic (Bashkirs) and Finno-Ugric Udmurts, Komi-Permiaks indicates substantial sharing of Siberian and Central-asians components in ethnogenesis populations of Volga-Ural region. Analysis of geographic spreading of mtDNA lineages for populations of Volga-Ural region shows, that in populations situated on the border between Europe and Asia, there is a remarkable increase both of frequency, and of the levels variety of mtDNA types for the populations of Siberia and Central Asia. Thus, in spreading of mtDNA types for people of Volga-Ural region leading role is played by the factors of geographic nearness or distance, but not by linguistic barriers.

This means, that Finno-Ugric people of Volga-Ural regions by maternal lineages have greater similarity with their immediate Turkic neighbours, than Balto-Finns people have linguistic affinity.

Surprisingly, the majority of mtDNA of Nogays, who are supposed to descent from Mongoloid Golden Horde inhabitants, belong also to western Eurasian variants of maternal lineages (~63%), testifying about an extensive admixture within about the last 750 years. East Asian-specific lineages of mtDNAs were observed in 38,2%. The same value for Bashkirs is quite close – 60% and the two populations are also more similar at the level of individual mtDNA lineages. So, the study of a ratio of European and Mongoloid components in the mitochondrial gene pool of Nogays, by data on polymorphism of HVSI mtDNA has allowed find a large proportion of Asian component in comparison with other populations from Caucasus (Ossets, Adygs, Georgians, Armenians) (Tambets, 1999). Besides, for Nogays were founded mtDNAs lineages (U7), coinciding with people of India. In ethnic history of Nogays there is explanation of this fact. Nogays Horde consist of both Turkic-language tribes and Indo-Irano-languges people Usuns. Further eastwards (Kazakhs and Uzbeks), the frequency of eastern Asian mtDNA haplotypes start to exceed those typical for western Eurasia, reaching more than 64% among Kazakhs. Supercluster M was found with most high frequency (50%). Besides, lineage of China also are detected. The modern populations of Central Asia is very non-uniform in its ethnic structure. Territory of Kazakhstan during a historically long period has been a place of interaction of many ethnic layers: in the formation of Kazakhs participated Turkic-speaking populations from Siberia, Altai, Indo-Iranians from Near East, Mongolian tribes and later Slavs of Eastern Europe. Thus, it is possible to explain a high level of genetic variability of mtDNA by a complicated ethnic history of the given people. Analysis mtDNA's variability in ethnic group of Oroks, presence native population of West Siberian is shown, that more of mtDNA's types get in four haplogroups M, S, D, U, specific for people East Eurasia. Character spreading mtDNA's lineages for Oroks and level them genetics variability points on founder effect, in historic past. From literature it is known that the frequency of eastern Asian mtDNA haplotypes for Turkic-speaking Yakuts is 90% or higher and among Turkish Turks as low as about 5-6%. Furthermore the same for the Baltic Uralic-speaking Estonians is below 1%. Thus, with only a little variation, there is a clear east-west gradient of the "Mongoloid" mtDNA linages alongside the Steppe Belt and linguistic affinities of populations play a lesser role.

Acknowledgements

This work is supported by the Russian Foundation for Basic Research grants № 01-04-63086, 01-04-48487 and by INTAS 759 grant.

References

1. Comas D., Calafell F., Mateu E. et al. (1998) Trading genes along the Silk Road: mtDNA sequences and the origin of Central Asian populations. Amer. J. Hum. Genet. 63, 1824-1838.
2. Tambets K., Kivisild T., Metspalu E. et al. (1999) The Topology of the Maternal lineages of the Anatolian and Trans-Caucasus Populations and the Peopling of Europe: Some Preliminary Considerations. In Renfrew C., Boyle K. (eds), McDonald Institute monographs. Chapter. 25, 219-235.
3. Torroni A., Wallace D.C. (1994) Mitochondrial DNA variation in human populations and implications for detection of mitochondrial DNA mutations of pathological significance. J. Bioenerg Biomembr. 26, 261-271.
4. Wallace D.C., Shoffner J.M., Trounce I. et al. (1995) Mitochondrial DNA mutations in human degenerative diseases and aging. Biochim Biophis Acta. 1271, 141-151.