FIFTEENTH SYMPOSIUM ON NUCLEIC ACIDS CHEMISTRY

HELD AT
SAPPORO, JAPAN
SEPTEMBER 19th – 21st, 1988

NUCLEIC ACIDS SYMPOSIUM SERIES

No.20

IRL PRESS
OXFORD · WASHINGTON DC

FIFTEENTH SYMPOSIUM ON NUCLEIC ACIDS CHEMISTRY

HELD AT
SAPPORO, JAPAN
SEPTEMBER 19th – 21st, 1988

Symposium Organizer:

Professor Eiko Ohtsuka
Faculty of Pharmaceutical Sciences
Hokkaido University
Sapporo 060
Japan

NUCLEIC ACIDS SYMPOSIUM SERIES
No.20

IRL PRESS
OXFORD · WASHINGTON DC

IRL Press
Eynsham
Oxford
England

First published 1988

British Library Cataloguing in Publication Data

Symposium on Nucleic Acid Chemistry (*15th : 1988 : Sapparo, Japan*)
Fifteenth Symposium on Nucleic Acid Chemistry
1. Nucleic acids. Components. Synthesis
I. Title II. Ohtsuka, Eiko. III. Series
547.7'90459

ISBN 1 85221 106 7

Printed by Information Printing Ltd, Oxford, England.

Contents

Nucleic Acids Symposium Series No. 20 1988

Contents continued overleaf

Contents continued overleaf

Analysis of mutants induced by *in vitro* incorporation of N^4-aminodeoxycytidine 5′-triphosphate

Keiko Matsumoto, Tadayoshi Bessho, Kazuo Negishi and Hikoya Hayatsu

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

N^4-Aminocytidine is a potent mutagen of a nucleoside(base)-analog type. We have reported that N^4-aminocytidine is mutagenic to prokaryotes and eukaryotes. This nucleoside analog can be incorporated into cellular DNA as N^4-aminodeoxycytidine (dC^{am}).

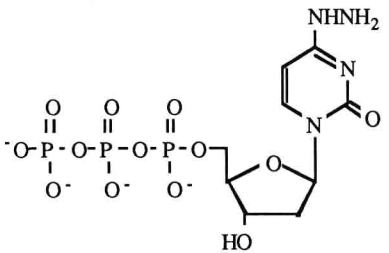

Fig. 1. N^4-Aminodeoxycytidine 5′-triphosphate

The incorporation of N^4-aminodeoxycytidine 5′-triphosphate ($dC^{am}TP$) (Fig. 1), a putative metabolite of N^4-aminocytidine, into replicative form DNA of phage φX174am3 by nick-translation resulted in the production of revertant phages (1). This shows clearly that the N^4-aminocytosine-containing DNA is mutagenic.

Both *E. coli* DNA polymerase I (2) and mammalian DNA polymerase α (3) catalyze incorporation of $dC^{am}TP$ into DNA efficiently in place of dCTP opposite guanine, and less efficiently, but to a significant extent, in place of dTTP opposite adenine. This dual incorporation property can be attributed to an ambiguous base pairing of N^4-aminocytosine to either G or A: the easy tautomerization of N^4-aminocytosine between the amino and the imino forms will allow its pairing with guanine or adenine. Consistent with these findings, N^4-aminocytidine induces both GC to AT and AT to GC transitions in bacteriophages φX174 and M13mp2 *in vivo*, but no transversion is inducible .

Here we studied the induction of forward mutations in *lacZ*α region of M13mp2. The mutations should result in the abolishment of α-complementation for restoration of the β-galactosidase activity. This system is widely used for studying the mutational specificities: with this system, all types of mutations can be detected, and DNA sequences of many mutants can be easily obtained .

Single-stranded M13mp2 DNA samples were annealed to complementary oligodeoxynucleotides. The DNA was incubated with $dC^{am}TP$ in the presence of two of normal dNTPs and DNA polymerase I large fragment from *E. coli* (Klenow enzyme), and further incubated after the addition of saturated amounts of four normal dNTPs to obtain the DNA with dC^{am} in a defined region . A portion of the products was transfected into the Ca^{2+}-treated *E. coli* NR9099, carrying chromosomal deletion of the *lac* operon and containing F′ plasmid having a defective β-galactosidase gene. The transfected bacteria were plated with growing *E. coli*. White and light blue plaques, which mutant progeny phages produce, and wild-type blue plaques were scored to measure mutation frequencies.

The induced mutation frequencies increased from < 0.04 % in the control samples to 0.2-1.0 % of surviving phages by the incorporation of N^4-aminocytosine into phage DNA. Among 42 mutants sequenced, 35 mutants were found to have sequence alterations in the regions proximate to 3′-terminal of annealed oligonucleotides (41 altered bases in total). As expected, all the mutations were transitions, and A to G and G to A in viral strands were the predominant changes (36 mutations). However, 5 mutations were identified as T to C in viral strands. One possible explanation for this puzzling observation is that the erroneous incorporation of dGTP opposite to thymine in the template is enhanced by the incorporated N^4-aminocytosine.

In *in vitro* site-directed mutagenesis, usually one defined nucleotide in a gene is targeted. However, it seems to be also useful to be able to induce by a simple technique many mutations in a given region of a gene. For example, production of many mutant proteins with respect to a region corresponding to a specific domain would facilitate studies on biochemistry of that protein. A candidate for it is a mutagenesis by incorporation of a mutagenic nucleotide, because such nucleoside-triphosphates can be incorporated randomly in gapped DNA. We expect that $dC^{am}TP$ is suitable for this purpose, because it can be incorporated efficiently and phage DNAs containing N^4-aminocytosine are as infective as normal DNA.

REFERENCES

1. Takahashi, M., Negishi, K., and Hayatsu, H. (1985) Biochem. Biophys. Res. Comm., 131, 104-109.
2. Negishi, K. Takahashi, M., Yamashita, Y., Nishizawa, M., and Hayatsu, H. (1985) Biochemistry, 24, 7273-7278.
3. Takahashi, M., Nishizawa, M., Negishi, K., Hanaoka, F., Yamada, M., and Hayatsu, H. (1988) Mol. Cell. Biol., 8, 347-352.

Photoaffinity labelling reagent for eukaryotic DNA-dependent DNA polymerase α; photoreactive 1-β-D-arabinofuranosyl-5-(E)-(4-azidostyryl)-uracil 5′-triphosphate

Shunji Izuta and Mineo Saneyoshi

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

ABSTRACT: We had synthesized 5-(E)-(4-azidostyryl) araUTP as photoaffinity labelling reagent for eukaryotic DNA polymerase α. This nucleotide showed a strong and selective inhibitory effect on DNA polymerase α purified from cherry salmon (Oncorhynchus masou)testes, with Ki value of 0.7 uM and Ki/Km value of 0.28. The inhibition mode has been shown as mixed type when the enzyme reaction carried out under room light, and was competitive to dTTP in the dark during incubation. From the results of experiments using a [γ-^{32}P]-nucleotide, it was appeared that this compound needed poly(dA)-oligo(dT) as a temprate-primer for binding to DNA polymerase α, and that this analogue could bind to dTTP recognition site of this enzyme.

INTRODUCTION

Among three kinds of eukaryotic DNA-dependent DNA polymerases (α, β and γ), DNA polymerase α is thought to have essential role for nuclear DNA replication (1). For clarifying the mechanism of eukaryotic DNA replication, it is very important to study this enzyme in detail using unique approach. In this context, we have attempted to develope an photoaffinity labelling reagent for DNA polymerase α. We reported previously that araUTP analogues bearing strong hydrophobic styryl group at 5-position showed the strong and selective inhibitory effects on DNA polymerase α purified from cherry salmon (Oncorhynchus masou) testes (2). Based on these results, we have designed 5-(E)-(4-azidostyryl) ara UTP which has photoreactive aryl azido group. In this paper, we describe the synthesis and utilization of this nucleotide analogue as an affinity labeling reagent for DNA polymerase α from cherry salmon (Oncorhynchus masou).

MATERIALS & METHODS

Synthesis: 5-(E)-(4-azidostyryl) araUTP was synthesized from 5-(E)-(4-aminostyryl) araUTP (2) via diazonium intermediate followed by replacement with azido group. [γ-^{32}P]-5-(E)-(4-azidostyryl)-araUTP was prepared from [γ-^{32}P]-GTP to corresponding araUDP derivative by phosphate transfer reaction

which catalyzed by nucleoside diphosphate kinase.

Photoaffinity labelling of DNA polymerase α: DNA polymerase α was labelled under following condition. The mixture containing 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml poly(dA)-oligo(dT), 4 μCi [γ-^{32}P]-5-(E)-(4-azidostyryl)araUTP and 30 units of cherry salmon DNA polymerase α, was incubated for 30 min at 37°C without light, then irradiated light above 300 nm using high pressure Hg lamp with Pyrex filter for 5 min at 5 cm distance. The radiolabelled product was separated from an unreacted analogue by SDS-10% polyacrylamide gel electrophoresis and detected by autoradiography with X-ray film.

RESULTS & DISCUSSION

Inhibotor properties of photoreactive analogue : From kinetic analysis, in the case of DNA polymerase α, two different kind of inhibition mode were demonstrated. One of these cases, was mixed type when the reaction was performed under room light, and other was competitive to dTTP when there was dark during incubation. On the other hand, in the case of DNA polymerase β, the inhibition mode was competitive to dTTP either under light or dark during incubation, indicate that this analogue would fail to bind to this enzyme if light condition.

Photoaffinity labelling of DNA polymerase α : We next examined whether this analogue really binds to enzyme by photolysis or not, using radioactive derivative. The radioactive product was yielded when poly(dA)-oligo(dT) was used as template-primer, and poly(dA) itself or activated DNA was not effecti ve to produce photoadduct. This indicates that this nucleotide would need the condition to recognize only dTTP for binding to DNA polymerase α. Effect of dTTP in the reaction was remarkable in this photoreaction. The radiolabelled enzyme was decreased according to an increasing concentration of dTTP, suggesting that this analogue could bind to dTTP binding site of the enzyme. Thus, our newly developed 5-(E)-(4-azidostyryl) araUTP should be very useful probe for analysis of the nucleotide binding site of DNA polymerases. We also found that this analogue was inhibited herpesvirus-induced DNA polymerases (3), then this compound should also be applicable to affinity labelling of herpes DNA polymerases.

REFERENCES

1. Fry, M. and Loeb,L.A. (1986) Animal Cell DNA Polymerases, CRC Press, Boca Raton FL.
2. Izuta,S. and Saneyoshi, M. (1987) Chem. Pharm. Bull., 35, 4829-4838.
3. Suzuki,S., Izuta,S., Nakayama, C. and Saneyoshi, M. (1987) J. Biochem. (Tokyo), 102, 853-857.

Inhibitory effects of various derivatives of azidothymidine triphosphate on reverse transcriptase and DNA polymerases

Katsuhiko Ono, Hideo Nakane, Piet Herdewijn[1], Jan Balzarini[1] and Erik De Clercq[1]

Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan and [1]Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

ABSTRACT

Inhibitory effects of various derivatives (sugar or triphosphate moiety was modified) of 3'-azido-2',3'-dideoxythymidine 5'-triphosphate (AZTTP) on the activities of reverse transcriptase and cellular DNA polymerases were examined and compared with each other. The mother compound, AZTTP, was found to be the most potent and selective inhibitor of reverse transcriptase. On the other hand, 2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate was strong but nonspecific inhibitor for all the polymerases tested except for DNA polymerase α.

INTRODUCTION

Since the causative agent of Acquired Immune Deficiency Syndrome (AIDS) was identified as a retrovirus, now designated as human immunodeficiency virus (HIV), several compounds have been found to be effective as anti-HIV agents.[1,2] Some of them are inhibitors for the viral reverse transcriptase (RT), which is prerequisite for provirus synthesis and its integration into host cell DNA. The most potent and selective inhibitors so far reported belong to a family of 2',3'-dideoxynucleosides. Among them, 3'-azido-2',3'-dideoxythymidine (AZT) is the strongest inhibitor and is currently used as a chemotherapeutic agent against AIDS.

AZT is phosphorylated in the cell to be the active triphosphate form (AZTTP) to inhibit RT. In search of efficient and selective inhibitors for RT, we examined the inhibitory effects of several AZTTP derivatives on RT in comparison with those on cellular DNA polymerases, the latter being indices of the drugs for cellular toxicity.

MATERIALS AND METHODS

AZTTP derivatives tested were as follows; 3'-azido-2',3'-dideoxythymidine 5'-triphosphate (AZTTP), 2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate (ddeTTP), 3'-azido-2',3'-dideoxythymidine 5'-diphosphate

(AZTDP), 3'-azido-2',3'-dideoxythymidine 5'-triphosphate ($\alpha \rightarrow \beta$), 3'-azido-2',3'-dideoxythymidine 5'-triphosphate ($\beta \rightarrow \gamma$), and 2',3'-didehydro-2',3'-dideoxycytidine 5'-triphosphate (ddeCTP).

RT, DNA polymerases α, β and γ, and terminal deoxynucleotidyltransferase (TdT) were purified from Rauscher murine leukemia virus, cultured human KBIII cells, and calf thymus, respectively.[3] DNA polymerase I was obtained from commercial source. Assay for these DNA polymrase activities were carried out under the reaction conditions described previously.[3] Inhibitory potentials of the compounds were expressed as inhibition constants (Ki's) obtained by the kinetic analyses.

RESULTS AND DISCUSSION

1) None of the derivatives tested were inhibitory to DNA polymerase α at drug concentrations up to 50 μM.
2) Only ddeTTP was inhibitory to DNA polymerase β.
3) All the compounds, particularly AZTTP and ddeTTP, were inhibitory to DNA polymerase γ.
4) TdT was moderately sensitive to inhibition by any of the compounds.
5) RT was sensitive to all the compounds, especially to AZTTP (Ki; 42 nM).
6) DNA polymerase I was inhibited by either of AZTTP, ddeTTP and ddeCTP, but was insensitive to either of AZTDT, AZTTP ($\alpha \rightarrow \beta$) and AZTTP ($\beta \rightarrow \gamma$).

All the results obtained in the present study indicate that the original compound, AZTTP, is still the most potent and selective inhibitor for the reverse transcriptase and that ddeTTP is inhibitory over a wide variety of DNA polymerases, particularly to DNA polymerase γ (Ki; 3.5 nM).

REFERENCES

1. De Clercq, E. (1986) J. med. Chem., 29, 1561-1569.
2. Mitsuya, H. and Broder, S. (1987) Nature, 325, 773-778.
3. Ono, K., Nakane, H. and Fukushima, M. (1988) Eur. J. Biochem., 172, 349-353.

The gene for PCNA (DNA polymerase-delta auxiliary protein) is present in higher plant genomes

Tetsuya Moriuchi, Iwao Suzuka[1], Koh-ichi Kadowaki[1], Hideyuki Daidoji[2], Yoshinari Takasaki[2] and Paul K.Nakane

Department of Cell Biology, Tokai University School of Medicine, Isehara 259-11, [1]Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba Science City 305 and [2]Department of Internal Medicine, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT

The homologue of PCNA (DNA polymerase-delta auxiliary protein) was searched in higher plants. In DNA blot analysis rat PCNA cDNA probe hybridized with homologous sequences in genomic DNAs from rice, soybean, tobacco and red pepper. The PCNA-related molecular clone (pCJ-1) was isolated from rice DNA and used as a probe for RNA blot analysis. The pCJ-1 probe hybridized with a 1.2 kb transcript in RNA from rice root tips and shoots. Immunoblot analysis of the soluble extract of soybean root tips using monospecific anti-PCNA antibodies identified a 34 kd protein. Immunohistochemical analysis revealed the presence of immuno-reactive PCNA protein in the nuclei of cells in the meristem of soybean root tips. The highly homologous nature of PCNA gene and PCNA protein throughout animal and plant kingdoms suggests that PCNA play an essential role in DNA replication in eukaryotes.

INTRODUCTION

PCNA (cyclin) was originally described in proliferating mammalian cells as a 33-36 kd nuclear protein and was recently found to be a DNA polymerase-delta auxiliary protein (1,2). Whether PCNA is an universal protein necessary for proliferation of eukaryotes, PCNA homologue was searched in higher plants.

MATERIALS AND METHODS

DNA and RNA blot analysis: DNAs were extracted from leaves of rice (*Oriza sativa* L.), soybean (*Glycine max* Merril), tobacco (*Nicotiana tobacum* L.) and red pepper (*Capsicum frutescence* L.). RNA was extracted from shoots and root tips of the rice 5 days after seeding. Blot analyses were performed as described (3).

Isolation of PCNA-related genomic clones: The recombinant phage library from rice DNA was screened with rat PCNA cDNA (pCR-1)(3).

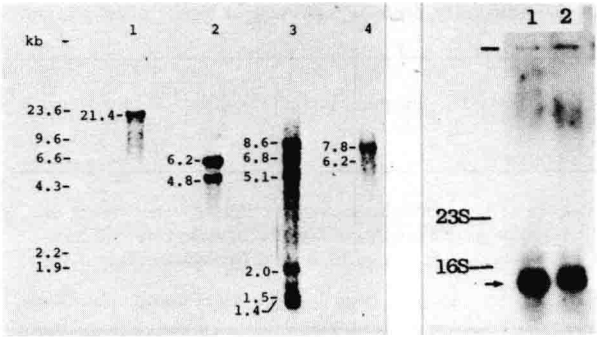

Fig. 1

Lane 1, Bgl II-digest of soybean DNA.
Lane 2, Hind III-digest of soybean DNA.
Lane 3, Eco RI-digest of soybean DNA.
Lane 4, Hind III-digest of tobacco DNA.

Fig. 2

Lane 1, poly(A) RNA from rice shoots.
Lane 2. poly(A) RNA from rice root tips.

Fig. 1. (left) DNA blot hybridization of rat PCNA cDNA with plant DNAs
Fig. 2. (right) RNA blot hybridization of rice PCNA probe (pCJ-1) with rice mRNA.

RESULTS AND DISCUSSION

A 0.75 kb Pst I fragment which contains 88% of the coding region of rat PCNA/cyclin cDNA was radiolabeled and used as a hybridization probe. As shown in Fig. 1. the probe hybridized with sequences in genomic DNAs from soybean and tobacco. A genomic library from rice DNA in Charon 28 was screened with the probe and a 5 kb fragment of the positive clone was subcloned into the Hind III site of pUC 18 (pCJ-1). In RNA blot analysis a 0.6 kb Bal I fragment of pCJ-1 insert hybridized with a single 1.2 kb transcript in RNAs from rice root tips and shoots, the size of which coincides with that of full length cDNA of rat PCNA/cyclin (Fig. 2). These results indicated that the PCNA/cyclin gene in rice is actively transcribed. The extraordinary conservation of the PCNA/cyclin gene throughout eukaryotes implies that the biological function is essential to the maintenance of species.

REFERENCES

1. Prelich, G., Tan, C-K., Kostura, M. et al. (1987) Nature 326, 517-520.
2. Bravo, R., Frank, R., Blundell, P. A. et al. (1987) Nature 326, 515-517.
3. Matsumoto, K., Moriuchi, T., Koji, T. et al. (1987) EMBO J. 6, 637-642.

Studies on the recognition mode of *E.coli* RNase H using modified oligonucleotide probes

Hideo Inoue, Yoji Hayase, Asami Ito, Toshiro Sasaki, Yasuhiro Miura, Makoto Koizumi, Shigenori Iwai and Eiko Ohtsuka

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

ABSTRACT

Nonanucleotide duplexes of RNA and modified DNA which contained 5-phenyl-2'-deoxyuridines or 2'-O-methoxymethylribonucleosides were prepared for the study of the E. coli RNase H recognition mode. The hydrolysis rates and sites of the ribo strands in the enzyme reaction were compared with those for the unmodified RNA-DNA counterparts.

E. coli RNase H is an endoribonuclease which hydrolyzes RNA in RNA-DNA hybrids, producing oligoribonucleotides with 5'-phosphates. We have recently found that short deoxyoligonucleotides linked with 2'-O-methylribooligonucleotides direct site-specific cleavage of the complementary ribooligonucleotides by this enzyme.[1] The present study was initiated in order to examine the enzyme recognition mode in which modified oligonucleotide probes were used.

An RNA-DNA hybrid adopts the A form; the major groove and minor grooves are similar in size, but the former is very deep and the latter is rather shallow. Therefore, we envisioned that RNase H recognizes and binds to the minor groove in addition to phosphate groups; the 2'-hydroxyl groups and the 2'-deoxy parts are aligned to the rim of the minor groove.

We have synthesized nonadeoxynucleotides containing 5-phenyluracils instead of T-residues and 2'-O-methoxymethylribonucleosides at appropriate positions, respectively, by using an automated DNA synthesizer. The complementary ribo strands were prepared by slight modifications[2] of the reported procedures and labeled at the 5'-end. The RNase H reaction was carried out at 15 or 30 ^{o}C, pH 7.7, and the RNA cleavage sites were