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DNA Polymerase Iota-Like Activity in Crude Cell Extracts of Different Mouse Organs

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Abstract—The recently discovered DNA polymerase iota differs greatly from the numerous eukaryotic and prokaryotic DNA polymerases known previously in its ability to catalyze error-prone DNA synthesis. Using homogeneous preparations of the enzyme, it was shown previously that DNA polymerase iota incorporated preferentially dGMP opposite the thymidine of the template in the growing DNA chain. To elucidate the role of this enzyme in the mammals, its activity was assayed in crude cell extracts of different mouse organs. It is shown that the extracts of the brain and testis cells exhibit the highest activity of DNA polymerase iota, which is not in agreement with the results of other authors. The data suggest that the tissue specific expression of DNA polymerase iota is regulated to a significant degree at the posttranscriptional and posttranslational levels.

Key words: iota DNA polymerase, activity, tissue specificity, genome stability

In recent years, about twenty DNA polymerases have been found in eukaryotic cells [1]. Each has its own functions and is characterized by certain properties. One of the representatives of this group of polymerases is the recently discovered DNA polymerase iota [2]. This protein possesses a number of unusual properties. On one hand, it exhibits some activities directed to maintenance of genome stability, such as removal of the phosphate of the deoxyribose that is necessary for the correct reconstruction of a damaged site of the DNA molecule [3]. On the other hand, *in vitro* it inserts dGMP into the growing DNA chain opposite thymidine of the template, contrary to the Watson–Crick rule, more effectively than dAMP corresponding to this position [4]. It was also shown that the ability of DNA polymerase iota to incorporate guanine opposite uracil by the same mechanism of that opposite thymidine can be used by the cell to decrease the mutagenic potential of DNA damage caused by deamination of cytosine [5].

However, it is clear that the complete manifestation of such a function of the enzyme in the cell would seriously change its genome. Nevertheless, the experiments on detection of RNA encoding DNA polymerase iota demonstrated that the corresponding gene is transcribed in virtually all studied mouse tissues. The most intensive transcription of the enzyme was observed in testes, heart, and pancreas [6].

Thus, presumably DNA polymerase iota (or at least one of its activities) is necessary for the normal metabolism in cells of various mammalian tissues. This might be connected with the fact that some cells under certain conditions require some changes in the nucleotide sequence at a certain site of DNA. For example, this takes place in the genes of immunoglobulins, and DNA polymerase iota was shown to take part in their rearrangement [7, 8]. But it is evident that the cell needs a mechanism or a number of mechanisms directed to the neutralization of the mutagenic action of DNA polymerase iota on the whole genome.

Theoretically, factors of such neutralization can act on different stages of cell functioning, for example, synthesis of the full-length protein, post-translational modification, inclusion of DNA polymerase iota into the replisome and, finally, removal of the incorrectly attached nucleotides from the DNA molecule by autonomic exonucleases. The use of the exonuclease activity of other DNA polymerases is also possible, so DNA polymerase iota does not exhibit such activity [4, 9-11]. It is rather complex to reveal all these factors and the contribution of each of them into the total neutralizing activity. Clearly, certain biochemical tests are required to estimate the efficiency of the action of separate factors.

Therefore, the goal of the present study was to investigate crude tissue extracts in terms of their ability to exhibit DNA polymerase iota-like activity, i.e., to incorporate dGMP opposite the thymidine of the template,

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under conditions providing simultaneous action of other DNA polymerases together with other enzymes existing in extracts. In the extracts of most mouse tissues, this activity was insignificant, but it was easily detected in extracts of brain and testes.

MATERIALS AND METHODS

Substrate for assaying the DNA polymerase activity. To assay the activity of DNA polymerases, we used two complementary oligodeoxyribonucleotides (Ge17 and Ge30) [4] that hybridize yielding a duplex with a free 5′-end (Fig. 1).

The 5′-end of the Ge17 oligonucleotide was labeled using phage T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP. The reaction was performed in 70 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 5 mM dithiothreitol (DTT) (BioRad, USA) at 37°C for 30 min. Originally, after this procedure, the Ge17 oligonucleotide was separated from the free [γ-^{32}P]ATP using a Sephadex column. However, further experiments showed that such a separation was not necessary because the results obtained with the purified Ge17 did not differ from those obtained with the unpurified Ge17.

The substrate for the enzymatic reaction was obtained after annealing (73°C, 3 min with subsequent gradual cooling to room temperature) of 300 pmol of labeled Ge17 with 350 pmol of cold Ge30 in 50 μl of the buffer for the PNK reaction containing 100 mM NaCl.

Preparation of tissue extracts. To prepare tissue extracts, the corresponding mouse organ was washed in 10 ml of 0.14 M ice-cooled sodium phosphate buffer, pH 7.4, and then ground in a glass homogenizer in the same buffer. The buffer volume for homogenization was 1 μl per 1 mg of the tissue. The resulting homogenate was centrifuged at 4°C (3 min, 14,000*g*). The supernatant was used as the enzyme preparation. Protein concentration was determined using the Protein Assay kit (BioRad). Protein content in the extract varied in different experiments from 15 to 30 mg/ml. The mixture of the tissue extract with the substrate without addition of dNTPs was used as the negative control.

Assay of the activity of DNA polymerases. The reaction was performed at 37°C in 20 μl of mixture containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT, 60 pmol of the substrate, 5 μl of the investigated extract, and dNTP. The concentration of dNTP was 1 μM in all experiments. The reaction was stopped by ice-cooling with the subsequent addition of an equal volume of mixture containing 95% formamide, 50 mM EDTA, and 0.05% bromphenol blue.

Electrophoresis of the reaction products was performed in 20% polyacrylamide gel (acrylamide to bisacrylamide ratio was 30 : 1) in the presence of 7 M urea in Tris-borate buffer at 30 mA. Electrophoresis was run until the bromphenol blue left the gel. After the electrophoresis, the gel was placed onto a polypropylene film and autoradiographed for 1 h.

RESULTS

To test the DNA polymerase activity in crude cell extracts without purification of the enzyme, we used the oligonucleotide duplex (Fig. 1). This substrate was used previously by the other authors for investigation of the homogeneous preparations of DNA polymerase iota [4]. Thus, if the ^{32}P-label is attached to the 5′-end of the 17-bp oligonucleotide Ge17 that is a component part of the substrate, the change in the mobility of this oligonucleotide revealed on the autoradiograph allows determination of the enzymes acting on this oligonucleotide. For example, a decrease in the mobility indicates an increase in its weight caused by the action of DNA polymerases, while an increase in the mobility is a result of the action of nucleases.

To determine if the extracts of different mouse organs possess the DNA polymerase iota activity (namely, if they are capable of catalyzing the incorporation of dGTP opposite thymidine of the template), the oligonucleotide substrate was incubated with the extract of the corresponding organ in the presence of dGTP for 15 min (the substrate contained the primer labeled at the 5′-end, its 3′-end being located opposite the thymidine of the template) (Fig. 1). As seen from Fig. 2, under these conditions the extracts of brain and testes increase the molecular weight of the primer by one nucleotide in the presence of dGTP alone in the reaction mixture. The appearance of the second band indicates the formation of not only the 18-bp, but also the 19-bp oligonucleotide. This can be explained by the fact that the template has C in the 19th position from the 5′-end. A guanine can be inserted opposite this C by both DNA polymerase iota and any other DNA polymerase. However, it can be assumed that the formation of the 19-bp product could

Fig. 1. Oligonucleotide duplex used as the substrate. The upper oligonucleotide is Ge17, the lower is Ge30.

be rather a result of the activity of iota DNA polymerase because it is the enzyme for which the T–G pair is the best of all possible incorrectly paired bases to continue the reaction [4]. Besides, as seen in Fig. 2, the characteristic feature of the extracts of brain and testes is the minimal degradation of the substrate by nucleases compared to the extracts of other tissues. In the latter case, under the conditions employed only traces of the polymerizing activity can be revealed, while the degrading activity obviously predominates (as seen from the presence of fragments of smaller size than the substrate). In the spleen extracts, complete degradation of the substrate is observed.

Under the conditions employed, endonucleases are not inhibited [12]. Nevertheless, the fact that even these conditions allow determination of a reliable incorporation of dGMP opposite T of the template indicates the enhanced ability of the extracts (at least those of brain and testes) to catalyze this reaction.

Since among the tissues investigated the extracts of brain and testes possess the highest ability to incorporate G opposite T of the oligonucleotide duplex, we assayed for the control the enzymatic activity of these extracts in the absence of dNTPs, as well as in the presence of three other dNTPs. Figure 3 shows that no polymerase activity was revealed when no dNTP was added in the reaction mixture (lanes *6* and *12*). On the addition of different dNTPs, dAMP was incorporated with the most efficiency. The same was observed in the presence of the mixture of dATP and dGTP. As in the previous experiment (Fig. 2), the incorporation of dGMP was observed; however, it was more efficient in the case of the testis extract (Fig. 3, lane *8*). In the latter case the reaction was performed for 4 min, since, as shown below (Fig. 4), this time is optimal. On the addition to the reaction mixture of dTTP or dCTP (Fig. 3), an insignificant amount of the 18-bp product was revealed, the 19-bp product being not detected. Presumably, this can be a result of the action of some other unknown polymerases.

It should be noted that the formation of the 18- and 19-bp products in the presence of dGTP is not likely to be connected with the activity of terminal transferases. Experiments on the incubation of the labeled Ge17 oligonucleotide without its annealing under the standard conditions with the extracts of brain and testes in the presence of dGTP support this assumption (data not shown). In the latter case, no DNA polymerase activity was observed, and the substrate degraded more intensively than the oligonucleotide duplex in the absence of dNTPs (Fig. 3, lanes *6* and *12*).

We investigated further the dependence of the dGMP incorporation on time of incubation of the oligonucleotide duplex with the extracts of brain and testes. The results of the experiment are presented in Fig. 4. It is seen that the maximal incorporation of dGMP in the presence of the testis extract is observed after 4 min of

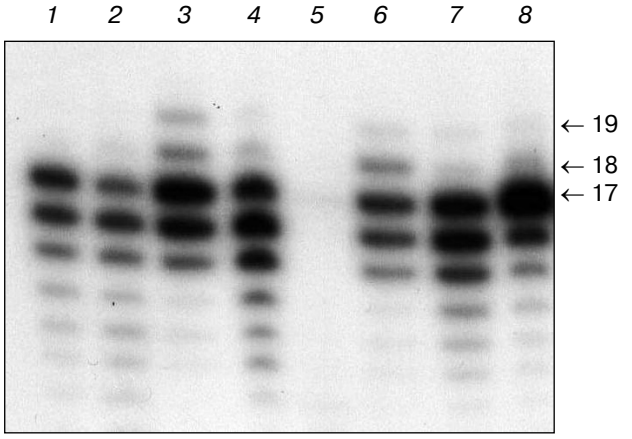

Fig. 2. Effect of extracts of different mouse organs on the oligonucleotide duplex while incubating in the presence of dGTP for 15 min: *1*) heart; *2*) kidney; *3*) brain; *4*) liver; *5*) spleen; *6*) testes; *7*) lungs; *8*) pancreas.

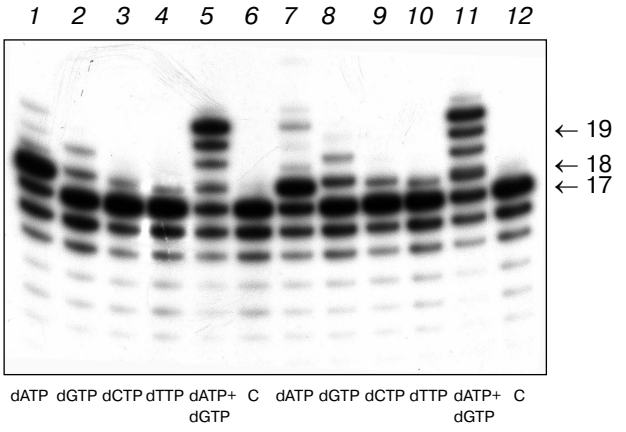

Fig. 3. Effect of extracts of brain and testes on oligonucleotide duplex in the absence and in the presence of different dNTPs: *1-6*) the substrate was incubated with the brain extract for 15 min; *7-12*) incubation with the testis extract for 4 min. dNTPs added into the reaction mixture are indicated at the bottom. C is negative control (without dNTPs).

incubation. Further increase in incubation time resulted in a decrease in the intensity of the corresponding bands. The minimal intensity of the bands is observed after 30 min of incubation. In the case of the assay of the activity in the brain extract, the decrease in the intensity of the bands with time is less pronounced. Besides, on comparing the activity of the extracts of brain and testes on the 2nd and 4th min of incubation, it is seen that in the extract of testes the incorporation of dGMP is more intensive, this concerning particularly the band corresponding to the 18-bp product. However, upon longer incubation, the picture virtually equalizes, and the sam-

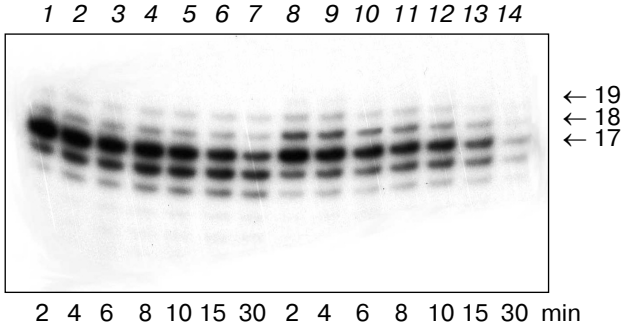

Fig. 4. Time-dependent incorporation of dGMP into the oligonucleotide duplex catalyzed by DNA polymerases of the extracts of mouse brain and testes: *1-7*) incubation in the presence of the brain extract; *8-14*) incubation with the extract of testes.

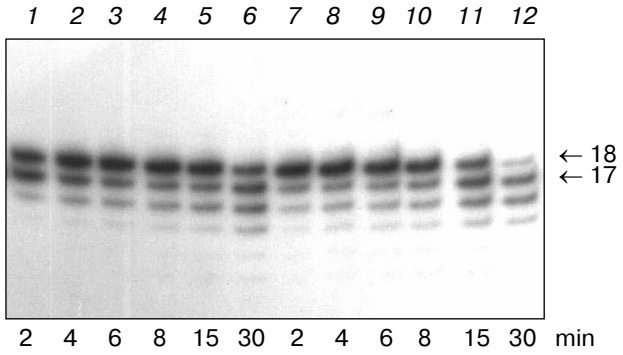

Fig. 5. Time-dependence of dAMP incorporation into the oligonucleotide duplex catalyzed by DNA polymerases of the extracts of mouse brain and testes: *1-6*) incubation in the presence of the brain extract; *7-12*) incubation with the extract of testes.

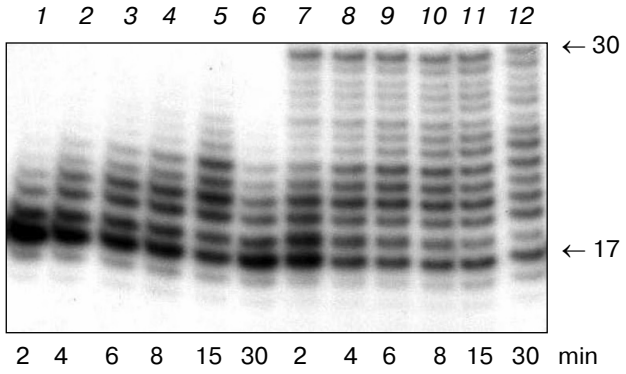

Fig. 6. Time-dependence of the completion of the oligonucleotide duplex by DNA polymerases of the extracts of brain and testes in the presence of the mixture of dATP, dGTP, and dTTP: *1-6*) incubation in the presence of brain extract; *7-12*) in the presence of extract from testes.

ples that were incubated for 10 min with the extracts of testes and brain are similar in terms of the label incorporation into the 18- and 19-bp products. Also, it should be noted that a higher activity of nucleases was observed in the brain extracts.

To compare the efficiency of the enzyme catalyzing the incorporation of dGMP opposite T of the substrate with the efficiency of other polymerases that are contained in the extracts of brain and testes, we incubated these extracts in the presence of dATP that can be incorporated into the substrate by other DNA polymerases according to the Watson–Crick rule. The reaction was also performed in the presence of the mixture of dNTPs that were necessary for the completion of the template. As expected, the incorporation of dAMP into the 18-bp product (Fig. 5) was much more intensive than the incorporation of dGMP into the 18- and 19-bp products (Fig. 4), this being virtually the same for the extracts of brain and testes. As for the action of nucleases, more active removal of the attached dAMP was observed in the testis extracts than in the brain extracts. At least after 30 min of incubation, the dAMP attached to the substrate was virtually completely removed. Total degradation of the substrate that is revealed by the appearance of the fractions containing the 16-bp and smaller products was more pronounced in the case of the brain extract. The same tendency was observed while incubation of these extracts in the presence of dGTP (Fig. 4).

As seen from the nucleotide sequence of the oligonucleotide duplex, three deoxynucleoside triphosphates (dATP, dGTP, and dTTP) are sufficient for its completion. Extracts of brain and testes differ significantly in their ability to complete the second strand of the substrate (Fig. 6). In the testis extract, both partial and full completion of the duplex is observed. In the case of the brain extract, only the partial completion took place, the maximal incorporation being achieved after 15 min. After 30 min, a partial degradation of the products was observed. It should be also noted that on the incubation of the substrate with the extracts of brain and testes in the presence of a mixture of three dNTPs the nuclease activity was significantly lower than that in the case of the incubation in the presence of dGTP or dATP. This suggests that in the investigated organs the activity of DNA polymerases is significantly higher than the nuclease activity.

DISCUSSION

The maintenance of genome stability is an important function of the living cell. This can be proved by the numerous data on the detection of caretaker genes, whose products take part in the processes of the maintenance of the correct nucleotide DNA sequences in the cell [12-15]. These systems repair the disturbances arising in the

nucleotide sequence of DNA during its synthesis or as a result of the influence of numerous intra- and extracellular factors.

Besides random factors capable of disturbing genome structure, there are a number of enzymes, mostly DNA polymerases, that under certain conditions can directionally change the nucleotide sequence in DNA [10, 11]. Detailed investigation of the biochemical properties of the homogeneous preparations of human DNA polymerase iota expressed in yeast cells demonstrated that this enzyme very probably could be such a factor [4]. To clarify this, it is necessary to know in what mammalian organs and tissues DNA polymerase iota functions and how it interacts with the enzymes responsible for the maintenance of the genome structure.

In the present work, we suggest a simple and easily replicable method for the assay of the activity specific for DNA polymerase iota in crude extracts of different organs. Since the ability to incorporate G opposite T against the Watson–Crick rule is unique, it can be considered that it was the activity of this DNA polymerase that was observed in our experiments. This is confirmed by the fact that the investigated extracts of brain and testes continued the synthesis after the incorrect incorporation of G. The electrophoretic separation of the products of the DNA polymerase reaction was analogous to that obtained by the authors that investigated homogeneous preparations of DNA polymerase iota using the same oligonucleotide duplex [4].

The results obtained on the DNA polymerase iota-like mutagenic activity can be interpreted based on some assumptions. This is connected with the fact that there are a number of different enzymes besides DNA polymerases in the extracts, and these enzymes can affect the used substrate in different ways, affecting the final results of the experiment. Nevertheless, it can be noted that the total effect of the extract from a particular mouse organ on the substrate under standard conditions yields a specific distribution of bands on the electrophoregram that can be used for identification of the organ. To a certain extent, the electrophoregram can serve as a kind of passport of an organ.

Among the enzymes that are present in the cell extracts, both DNA polymerases and different exo- and endonucleases are capable of affecting directly the results of experiments. The results presented in Figs. 2-6 indicate that in our system the action of nucleases does not prevent the detection of the products resulting from the activity of DNA polymerases.

Indirectly, the results of the experiments can be affected by other enzymes, for example proteases. It can be assumed that DNA polymerase iota is affected strongly by the action of proteases in the extract of testes, since after 4 min of incubation of the reaction mixture, the accumulation of the product ceases and the intensity of the bands decreases (Fig. 4).

A specific feature of the testis extract is that if it was incubated in the presence of three dNTPs sufficient for the completion of the template, no decrease in the intensity of the bands corresponding to the products of 18-30 bp was observed. Presumably, in this case the polymerases are protected in some way from the protease action, and the substrate is protected from the action of nucleases and phosphatases.

A disagreement of our data with the results of other authors [6] also needs explanation. The other authors determined the expression of DNA polymerase iota gene in different mouse organs by the content in these organs of the corresponding mRNA. They demonstrated a rather high level of the transcription of the gene of DNA polymerase iota in the cells of heart and pancreas and its low level in brain cells. We did not reveal any DNA polymerase iota-like activity in the extracts of either heart or pancreas cells, while the activity was significant in the brain and testes (Fig. 2). In the experiments on organs of mice of different sex and age (11 individuals were analyzed), the extracts of brain and testes exhibited steadily the DNA polymerase iota activity except for the organs of one male. In this case, we revealed virtually no DNA polymerase iota activity in the brain extract, while in the testis extract the activity exceeded the level that was characteristic for other individuals of the same sex and age.

The contradiction of the results can be explained by the tissue specific posttranscriptional or posttranslational regulations of the DNA polymerase iota activity in cells. Particularly, it was demonstrated that iota DNA polymerase is involved in the mechanism of DNA replication due to its interaction with PCNA (Proliferating Cell Nuclear Antigen) [16]. The presence or absence of the corresponding product can significantly affect the DNA polymerase activity.

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REFERENCES

1. Hubscher, U., Maga, G., and Spadari, S. (2002) *Annu. Rev. Biochem.*, **71**, 133-163.
2. Tissier, A., McDonald, J. P., Frank, E. G., and Woodgate, R. (2000) *Genes and Development*, **14**, 1642-1650.
3. Bebenek, K., Tissier, A. K., Frank, E. G., McDonald, J. P., Prasad, S., Wilson, S. H., Woodgate, R., and Kunkel, T. A. (2001) *Science*, **291**, 2156-2159.
4. Zhang, Y., Yuan, F., Wu, X., and Wang, Z. (2000) *Mol. Cell. Biol.*, **200**, 7099-7108.
5. Valisman, A., and Woodgate, R. (2001) *EMBO J.*, **20**, 6520-6529.

6. Frank, E. G., Tissier, A., McDonald, J. P., Rapic-Otrin, V., Zeng, X., Geahart, P. J., and Woodgate, R. (2001) *EMBO J.*, **20**, 2914-2922.
7. Faili, A., Aoufouchi, S., Flatter, E., Gueranger, Q., Reynaud, C.-A., and Well, J.-C. (2002) *Nature*, **419**, 945-946.
8. Poltoratsky, V., Woo, C. J., Tippin, B., Martin, A., Goodman, M. F., and Scharff, M. D. (2001) *Proc. Natl. Acad. Sci.*, **98**, 7976-7981.
9. Shcherbakova, P. V., Bebenek, K., and Kunkel, Th. A. (2003) Sageke.sciencemag.org/cgi/content/full/sageke; 2003/8/re3.
10. Goodman, M. F. (2002) *Annu. Rev. Biochem.*, **71**, 17-50.
11. Friedberg, E. C., and Gerlach, V. L. (1999) *Cell*, **98**, 413-416.
12. Shevelev, I. V., Legina, O. K., Tutaev, K. Yu., and Krutyakov, V. M. (1998) *Mol. Biol. (Moscow)*, **32**, 741-744.
13. Difilippantonio, M. J., Zhu, J., Chen, H. T., Meffre, E., Nussenzweig, M. C., Max, E. E., Ried, T., and Nussenzweig, A. (2000) *Nature*, **404**, 510-514.
14. Levitt, N. C., and Hickson, I. D. (2002) *Trends Mol. Med.*, **8**, 179-186.
15. Hickson, I. D. (2003) *Nat. Rev. Cancer*, **3**, 169-178.
16. Haraska, L., Johnson, R. E., Unk, I., Phillips, B. B., Hurwitz, J., Prakash, L., and Prakash, S. (2001) *Proc. Natl. Acad. Sci.*, **98**, 14256-14261.