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MINI-REVIEW

Properties of Autonomous 3′→5′ Exonucleases

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Abstract—Autonomous 3′→5′ exonucleases (AE) are not bound covalently to DNA polymerases, but they are often included into the replicative complexes. Intracellular AE overproduction in bacteria results in sharp suppression of mutagenesis, whereas inactivation of these enzymes in bacteria and fungi leads to an increase in mutagenesis frequency by 2-3 orders of magnitude. Correction of DNA polymerase errors *in vitro* occurs after addition of AE to the incubation medium. This correction is clearly manifested under conditions of mutational stress (during induced but not spontaneous mutagenesis), for instance, with an imbalance of dNTPs – error-prone conditions. At equimolar dNTP (error-free conditions), the correction is relatively weak. The gene knockout of both alleles of the major AE gene in mice does not influence spontaneous mutagenesis though a substantial increase could be expected. The frequency of induced mutagenesis has not been yet measured, though the inactivation of AE could increase the frequency of mutagenesis. Complete inactivation of the major AE leads to inflammatory myocarditis and a 5-fold reduction of life span of mice. Dominant heterozygous mutations were found in various loci of the AE gene, which caused the development of Aicardi–Goutieres (autosomal recessive encephalopathy) syndrome, familial chilblain lupus, systemic lupus erythematosus, retinal vasculopathy, and cerebral leukodystrophy. In the nucleus, AE have a corrective function, but after transition into cytoplasm these enzymes destroy aberrant DNA that appears during replication and thereby save the cells from autoimmune diseases. Depending on their intracellular localization, AE carry out various biological functions but employ the same mechanism of the catalyzed reactions.

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Autonomous (individual) 3′→5′ exonucleases (AE) are not covalently bound to DNA polymerases, but they are often included in their complexes with other proteins. The epsilon subunit of *Escherichia coli* DNA polymerase III may be an AE. The gene of the ε subunit has been cloned, and therefore it is possible to carry out its overproduction. Intracellular overproduction of the ε subunit is accompanied by a 10-100-fold reduction in the level of spontaneous and induced mutagenesis [1, 2]. In contrast, inactivation of the exonuclease function of the ε subunit due to substitution of two conservative amino acid residues at the enzyme active site results in "error catastrophe" and therefore to total mutagenesis incompatible with survival of *E. coli* cells [3]. Simultaneous inactivation of AE I and VII in *E. coli* cells causes a 10-30-fold increase in the probability of spontaneous mutagenesis [4]. Different modes of inactivation of an AE gene in the basidiomycete fungus *Ustilago maydis* caused a 10-100-fold increase in the frequency of spontaneous mutagenesis [5, 6]. Addition of AE to DNA polymerases *in vitro* has clearly demonstrated an increase in the fidelity of DNA synthesis under dNTP imbalance (e.g. 1000-fold decrease in TTP concentration compared with other dNTP, which models conditions of inaccurate DNA synthesis) (figure). In living cells the concentration of pyrimidine dNTP is 5-10-fold higher that that of purine dNTP [7], whereas concentrations of individual dNTP may exhibit a 38-fold difference [8]. Consequently, statistically significant results require experimentation at equimolar and non-equimolar dNTP concentrations. Qualitative estimation of the effect of AE on the fidelity of DNA synthesis (figure) is supported by quantitative estimation (table) during determination of the frequency of forward and reverse mutations due to DNA polymerase synthesis *in vitro*.

Briefly, the methods are based on the following [9]:

– a synthetic primer is hybridized with the plus strand of bacteriophage φX174 amber 3 DNA and its 3′-end is positioned at a distance of three nucleotides from the point amber mutation. Then DNA synthesis is performed by means of DNA polymerase using the primer template. If an error associated with the amber codon does not appear, subsequent DNA transfection into spheroplasts of *E. coli* cells would not yield phage production (which is observed using suppressor cells). In the case

Abbreviations: AE, autonomous 3′→5′ exonucleases.

Removal of autonomous 3′→5′-exonucleases from the membrane complex of rat hepatocyte DNA polymerase α decreases its fidelity during inaccurate DNA synthesis

Preparations of DNA polymerase α	Equimolar concentrations (5 μM) of dNTP – conditions of accurate DNA synthesis	Equimolar concentrations (5 μM) of dNTP + 2.5 mM dGTP (dGTP excess) – conditions of inaccurate DNA synthesis	Addition of Dna Q (1000 U) with the excess of dGTP
Membrane complex of DNA polymerase α with 3′-exonuclease and other proteins	3.2 ± 0.6	9 ± 1	—
Autonomous exonucleases removed from the complex by ultracentrifugation	4.1 ± 1	100 ± 9	8.3 ± 1.5
Autonomous exonucleases removed from the complex by chromatography	2.0 ± 0.9	84 ± 6	—

Note: The mutation frequency ($\times 10^{-6}$) of DNA copied by the membrane complex and its remaining forms in the reversion experiment with φX174 amber 3.

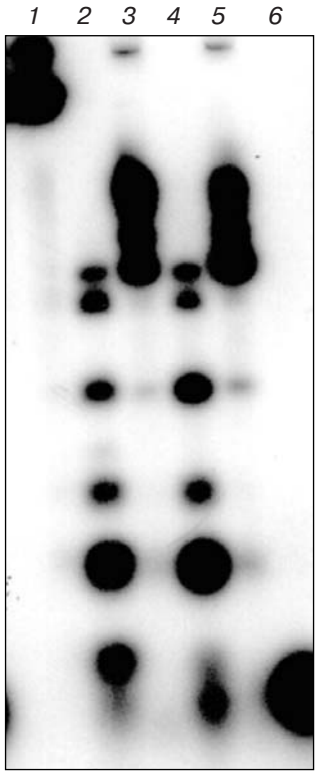
of an error, the DNA polymerase moves along the nonsense amber codon (where subsequent translation of a corresponding protein required for formation of the negative colony, i.e. for lysis of bacterial host cells, normally terminated) and this nonsense codon is thus "converted" into a sense codon (i.e. genotypic or phenotypic reversion occurs) in a newly synthesized strand. In this case, DNA transfection into *E. coli* cells results into production of this phage due to its reversion into a wild type;

– measurement of the frequency of direct mutations in the M13mp2 phage [10]. This phage contains *lac*-operon with a 250-nucleotide single-stranded gap within the β-galactosidase gene. This gap is formed as follows. Initially the restriction endonucleases PvuI and PvuII remove a DNA fragment of 250 nucleotides. The remaining part of the DNA is subjected to denaturation followed by annealing with a single-stranded circular DNA of phage M13. Subsequent gap filling by DNA polymerase may cause errors. Transfection of such DNA into the spheroplasts of *E. coli* cells lacking *lac*-operon the mutant phage forms colorless or light blue colonies on a corresponding medium, whereas the wild type phage colonies are blue.

Originally the correcting AE preferentially removing unpaired nucleotides of the 3′-end of DNA have been described in [11]. Later the correcting capacities of AE have been demonstrated in many studies [12-18]. In complexes with DNA polymerases α, β, and δ, AE caused 10-, 30-, and 50-fold increase in the fidelity of DNA synthesis evaluated by determination of the forward and reverse mutation frequencies under conditions of inaccurate DNA synthesis [14, 17, 19, 20]. However, under conditions of accurate synthesis (i.e. under equimolar concentrations of dNTP) AE removal from a large membrane complex of DNA polymerase α with 3′-exonuclease and some other proteins had a minor influence on the fidelity of DNA synthesis (table). In the case of a 500-fold excess of dGTP over the other dNTP, the fidelity of DNA synthesis sharply decreased; addition of AE concentration corresponding to its cellular level restored this parameter (table). This was demonstrated by both qualitative (figure) and quantitative (table) modes of estimation of the correcting function of AE [20]. It appears that under conditions of accurate synthesis satisfactory result of DNA polymerase functioning can be achieved due to correct selection of complementary nucleotide pairs by active sites of these polymerases and also by the correcting effect of exonucleases covalently bound to DNA polymerase I, δ, and ε. Significant increase in errors (associated with mutational stress, e.g. nucleotide imbalance) requires involvement of AE for correction of these errors.

In *E. coli*, the ε subunit (or Dna Q) of DNA polymerase III exhibits AE function [3], whereas in mammals the major AE are known as Trex1 and Trex2 (molecular masses of 30 and 24 kDa, respectively) [21]. These exonucleases cleave one nucleotide at a time from the 3′-end of DNA. The AE Trex1 and Trex2 have been cloned and sequenced [21]; in solution, they exist as homodimers, as shown by X-ray analysis [22]. Their amino acid sequences share 44% homology [21].

Recently it has been shown that knockout of both alleles of the Trex1 gene (encoding the major mouse AE) caused a 5-fold decrease in life span of the knockout mice [23]. Surprisingly, this inactivation of AE did not influence the frequency of spontaneous mutagenesis. Unfortunately, the frequency of induced mutagenesis under these conditions (when major AE inactivation would increase this parameter) was not determined. The number of malignant tumor in the mutant mice was comparable with that of normal mice exhibiting the 5-fold longer life span. It was also found that Trex1 destroys aberrant DNA that appears during replication and penetrates into cytoplasm [24]. Accumulation of such DNA



Effect of autonomous 3′→5′ exonucleases (Dna Q and TREX 2) on primer elongation catalyzed by DNA polymerase I (Klenow fragment, exo$^+$). The elongated primers were separated from the DNA template by heating at 100°C and analyzed by electrophoresis in 16% polyacrylamide gel in the presence of 7 M urea and 90 mM Tris-borate buffer, pH 8.3, followed by autoradiography. Primers 15 nucleotides in length labeled with ^{32}P at the 5′-end were hybridized with circular single-stranded phage φX174 amber 3 DNA. The reaction was performed for 20 min at 37°C using 0.1 U of DNA polymerase I (Klenow fragment, exo$^+$) in 5 μl of 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 fmol primer/template, four dNTPs 1 mM each (lane *1*) or 1 μM TTP and 1 mM other dNTP (lanes *2-6*). Lanes: *6*) reaction was immediately stopped; *3*, *5*) homogeneous autonomous exonucleases Dna Q (0.9 μg) and TREX 2 (1.2 μg), respectively, were added to the reaction mixture; *2*, *4*) the autonomous exonucleases were not added

under condition of Trex1 inactivation results in the development of inflammatory myocarditis [23]. It remains unclear why Trex2 and other nucleases cannot cleave this DNA. It was also found that dominant heterozygous mutations in various loci of the Trex1 exonuclease gene cause the development of Aicardi–Goutieres syndrome (autosomal recessive encephalopathy), familial chilblain lupus, systemic lupus erythematosus, retinal vasculopathy, and cerebral leukodystrophy [25, 26]. Such diseases are characterized by appearance of antibodies against the organism's own double-stranded DNA [27]. Other reasons responsible for the development of these autoimmune diseases remain unknown.

Thus, in the nucleus AE provide a corrective function, but after transition into cytoplasm these enzymes destroy aberrant DNA and thereby protect the cells from (auto)immune diseases. Depending on their intracellular localization, AE carry out various biological functions but employ the same mechanism of the catalyzed reactions.

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