# TRANSCRIPTIONAL ROLE IN DNA REPLICATION: DEGRADATION OF RNA PRIMER DURING DNA SYNTHESIS+

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Summary- DNA synthesis catalyzed in vitro by E.coli DNA polymeraseI in the presence of single stranded fd DNA or poly (dT) as template is stimulated by RNA primers. When poly(dT) fully or partially saturated with polyriboadenylic acid strands is used as template - primer, DNA synthesis proceeds with concomitant degradation of the ribostrands to 5'-adenosine monophosphate. The fragment of DNA polymerase lacking the 5'→3' exonuclease shows comparable RNA primer dependency but reduced efficiency for the degradation of the RNA primer from the 5'-end.

Involvement of RNA polymerase in DNA replication has recently been demonstrated by several groups (1-6) and the possibility of a primer function of RNA in DNA synthesis has been further underlined by the finding, that newly synthesized DNA is covalently linked to the RNA primer (2,5,6). Very little, however, is known about the fate of the RNA primer during and after DNA synthesis.

In this communication we wish to present evidence that homogeneous DNA polymeraseI from E.coli is capable of degrading the ribo-strand of an RNA-DNA hybrid during the course of DNA synthesis.

Materials- Unlabeled ribo- and deoxyribonucleoside triphosphates were obtained from Schwarz Bioresearch and Boehringer. [$^{3}$H]rATP, [$^{14}$C]rGTP, [$^{14}$C]dCTP and [$^{14}$C]dATP were obtained from Radiochemical Center,Amersham. [$\alpha$-$^{32}$P]rATP was a gift from Mr. S. Kühn of this Department.fd DNA was kindly supplied by Mr. D. Fischer of this Department. The preparation had about 80% closed circles and 20% linear molecules of chain length equal to those of circles(7). $d(T)_{\overline{1000}}$ was prepared using $d(T)_6$ and dTTP in the presence of terminal transferase(8). DNA polymeraseI,isolated according to Jovin et al.followed by an additional phosphocellulose chromatography step(9), was a gift of Dr.L.Loeb, Institute for Cancer Research, Fox Chase, Philadelphia,U.S.A. The enzyme, homogeneous as analyzed by isoelectric focussing had a specific activity of 20,000 units/mg with poly[d(A-T)] as template-primer and 240,000 units/mg with $d(A)_{\overline{1000}}.d(T)_{\overline{32}}$ assay, with units

+The abbreviation and symbols follow recommendation of IUPAC system published in Eur.J.Biochem. 15, 203 (1970).

defined according to Richardson et al.(10). The large fragment of DNA polymeraseI was obtained from Boehringer. E.coli RNA polymerase,isolated according to Burgess(11) was supplied by Mr. Kühn. The specific activity was 160 units/mg (11) with $d(T)_{1000}$ as template under conditions specified below. Rifampicin was obtained from CIBA.

Methods- All incubations were carried out at 37° and contained 50mM Glycyl-glycine-KOH buffer (pH 7·5), 10mM $MgCl_2$, 75 mM KCl, 100 µM EDTA, 100 µM dithiothreitol and 5% glycerol. Other components are listed under the legends. Acid insoluble radioactivity in aliquots (10 or 20 µl) of the reaction mixtures was determined according to Bollum (12) as modified by Mans and Novelli (13). When $^3H$-labeled substrates were used, measurements were made after acid precipitation (14) and washing on Whatman GF/C glass fiber filters (10). A Beckman model LS-233 counter was used for discriminate counting. Molarity of nucleotides and polynucleotides was determined spectrophotometrically and refers to the concentration of nucleotide residues.

## RESULTS

Discontinuously coupled synthesis- Similar to work previously reported (5) we noticed that in a simultaneously coupled synthesis (rNTPs, dNTPs, E.coli RNA polymerase and DNA polymeraseI, all added together) an RNA primer formation is necessary for in vitro replication of single stranded circular fd DNA or $d(T)_{1000}$ (Roychoudhury and Kössel, manuscript in preparation). When the possibility was envisaged, that DNA polymerase itself may cause the primer degradation by means of the exonucleases known to be associated with this enzyme molecule (16,17), the question arose, whether the large fragment (molecular weight 75,000) of DNA polymeraseI catalyzes similar reaction. Furthermore, it seemed important to clarify whether DNA polymeraseI or its fragment can cause chain extension from the ribo-3' terminus, if the deoxy-template strand is already saturated with newly synthesized ribo-strands, because in this case degradation and/or displacement of the primer would be necessary.

To answer these questions, a discontinuously coupled synthesis (without removing RNA polymerase and rNTPs) was employed in order to test the capacity of an 80% saturated hybrid to support DNA synthesis. As shown in fig. 1B, both the whole enzyme and the large fragment are able to replicate such a hybrid structure. The extent of replication of about 70% for the whole enzyme and 50% for the large fragment indicates that considerable displacement synthesis was operative together with

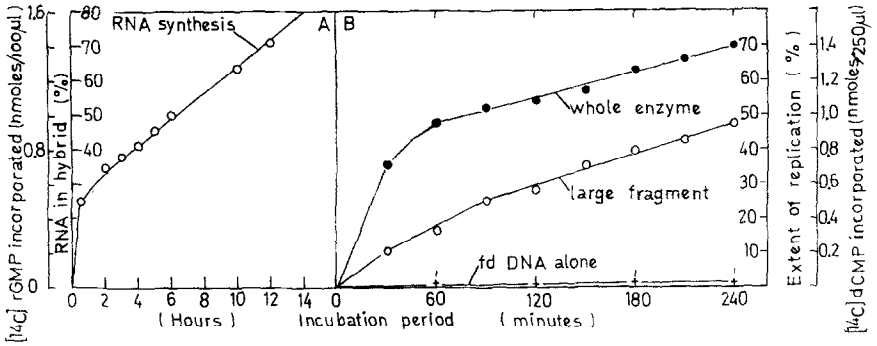


Fig. 1. DNA synthesis with RNA-DNA hybrid as primer-template

Reaction mixtures contained (A) 10 nmoles of fd DNA, 25 nmoles each of rATP, rCTP, rUTP, [$^{14}$C]rGTP (6600 cpm/nmole) and 12.5 µg of RNA polymerase in 100 µl. The total nucleotide incorporation was calculated from [$^{14}$C]rGMP incorporation, i.e. 1.6 nmoles of rGMP incorporation = 8 nmoles of total nucleotides according to the base composition of fd DNA (15).

(B) From a parallel incubation (400 µl) with components in the same proportion as above but all 4 rNTPs unlabeled, aliquots (100 µl) were transferred after 14 hours to tubes containing 25 nmoles each of dATP, dGTP, dTTP and 12.5 nmoles of [$^{14}$C]dCTP (19,400 cpm/nmole). Whole DNA polymeraseI (0.5 unit) and its large fragment (0.3 unit) were then added and the volume made upto 250 µl with water. The curve 'fd DNA alone' refers to an assay without rNTPs during the preincubation.

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repair synthesis, since with repair synthesis only, a maximum of only 20% replication would be expected.

Degradation of the ribo-strands- Random chain initiation with DNA dependent RNA polymerase on a synthetic template, such as poly(dT), is expected to produce after saturation, a duplex structure schematically represented by:  When such fully transcribed duplex is used as template-primer, DNA synthesis takes place with a concomitant degradation of the ribo-strands (fig. 2). That this degradation is not due to a contaminating RNase activity (RNase H) in the RNA polymerase preparation or a bacterial contamination of the reaction mixture is evident from the fact that no degradation takes place in the absence of DNA polymerase. As the DNA polymerase preparation consisted of an electrophoretically homogeneous protein, it thus appears, that DNA polymerase itself has the degradative activity.



It, therefore, seemed of interest to examine, whether this degradation is due to a 5→3' exonuclease or due to a 3'→5' exonuclease activity or to both. Accordingly, a partial hybrid [d(T)$_{1000}$ 60 n moles, poly(rA) 12·5 n moles] in which the ribo-strand had $^{32}$P-activity at the 5'end

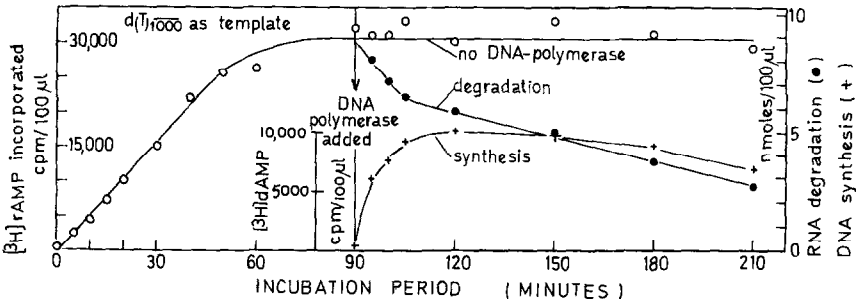


Fig.2. Degradation of ribo- strands by DNA polymeraseI.

The reaction mixture(400 µl) per 100 µl contained 10 nmoles of $d(T)_{1000}$, 25 nmoles of [$^3$H]rATP(34,000 cpm/nmole), 10 nmoles of unlabeled dATP and 12.5 µg of RNA polymerase. After 90 min. the reaction was stopped temporarily by plunging the tube in an ice bath. 100 µl were withdrawn and transferred to a new tube to which 1 unit(5 µl) of DNA polymeraseI was added. The incubations were resumed with both the tubes at 37°and the radioactivity in 10 µl aliquots determined at the time intervals indicated. For monitoring DNA synthesis, a parallel incubation (200 µl) containing unlabeled rATP and [$^3$H]dATP(2000 cpm/nmole) was carried out in the same way.

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and $^3$H-activity at the 3'-end was prepared. Chain initiation and partial RNA synthesis was carried out with [$\alpha$-$^{32}$P]rATP, followed by addition of rifampicin to prevent further chain initiation. An excess of [$^3$H]rATP was then added in order to complete the chains already started before the addition of rifampicin. When this hybrid was employed as template-primer, both the whole enzyme and the large fragment catalyzed incorporation of [$^{14}$C]dAMP (fig. 3). Simultaneously, $^{32}$P-activity was released immediately in the presence of the whole enzyme. In contrast, no $^{32}$P-activity was released with the large fragment which lacks the 5'→3' exonuclease activity (fig. 3). Degradation of $^3$H-activity, however, took place with both the enzyme preparations only after a lag period of 10 minutes. Whether this degradation is caused by 3'→5' exonuclease during repair synthesis and/or after strand displacement remains to be clarified.

Product of nucleolytic degradation- An isolated hybrid, in which the ribo-strand was labeled with [$^3$H]rAMP residues was first allowed to be degraded in the presence of DNA polymeraseI and unlabeled dATP. A paper chromatographic analysis of the products of degradation revealed almost all the radioactivity to be located in the mononucleotide region. A 5 fold higher amount of material incubated without enzyme, failed to show any radioactivity in the AMP area of the chromatogram and all the material remained confined to the origin, indicating the absence of

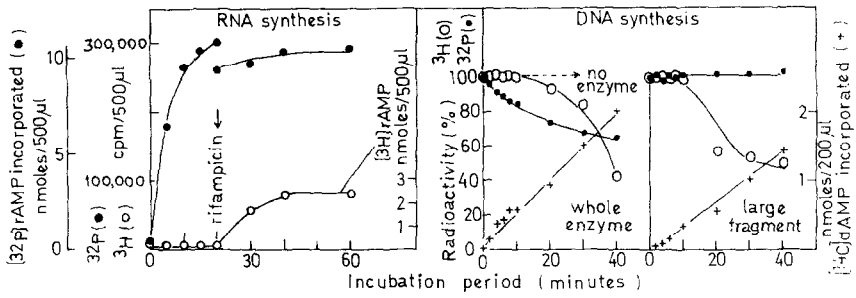


Fig.3. Degradation of newly synthesized RNA by DNA polymeraseI and its large fragment.

The reaction mixture(500 µl) for RNA synthesis contained 60 nmoles of $d(T)_{1\overline{000}}$, 100 nmoles of [α-$^{32}$P]rATP(28,000 cpm/nmole) and 62.5 µg of RNA polymerase. 10 µl aliquots were removed for monitoring acid insoluble radioactivity at the time intervals indicated. At 20 min. the tube was brought to ice bath temperature and 5 µg of rifampicin(dissolved in 10 µl of water) was added. An excess of [$^{3}$H]rATP(2500 nmoles in 50 ul) was then added and the incubation resumed at 37°. After 40 min. 100 µl aliquots containing now 12 nmoles of $d(T)_{1\overline{000}}$ and 2.5 nmoles of newly synthesized RNA were directly used for DNA synthesis with 10 nmoles of [$^{14}$C]dATP(4000 cpm/nmole) and 1 unit each of DNA polymeraseI or its large fragment supplemented with water to final volumes of 200 µl. The $^{32}$P-incorporation kinetics at 20 min. point indicates dilution of $^{32}$P-activity(450 µl reaction mixture plus 60 µl of rifampicin + rATP). [$^{14}$C]dAMP incorporation was measured after cross-over corrections.

bacterial contamination during degradation (fig. 4A). Elution of the radioactive mononucleotide followed by rechromatography in a solvent system, which separates 5'-rAMP from 2'(3')rAMP, shows the radioactivity to be located in 5'-rAMP (fig. 4B). With shorter incubations times also, mainly mononucleotides were found to be the products of degradation (not shown) indicating the exonucleolytic mode of degradation during DNA synthesis.

## DISCUSSION

The data presented in this communication indicate, that homogeneous DNA polymeraseI from E.coli is able to degrade the RNA primer, while synthesis of DNA strands is still going on. As judged from the preferentially 5'→3' exonucleolytic mode of action, which is not observed, when the DNA polymerase fragment is applied and from the fact, that 5'-ribonucleoside phosphates are the products of reaction, it seems likely that the nuclease activity described here is identical with the known 5'→3' exonuclease of the DNA polymeraseI molecule hitherto considered as DNase (16,17). A second possibility, however, that the RNA degrading

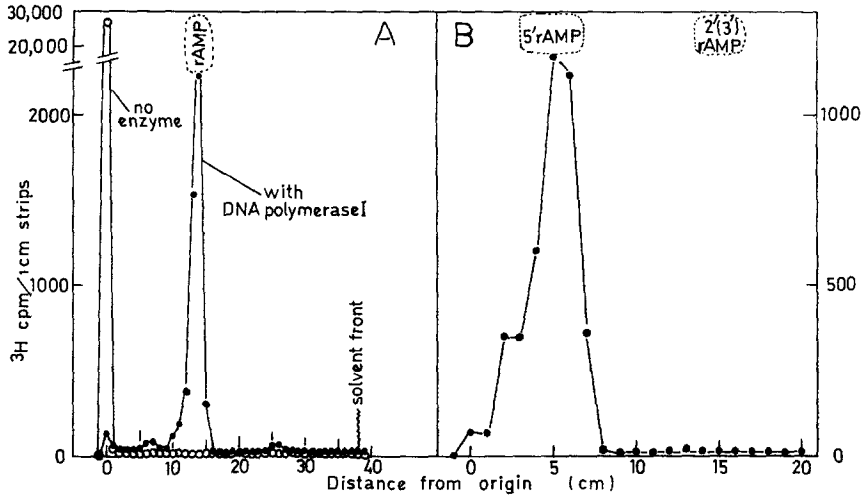


Fig.4. Characterization of the degradation products from DNA polymerase I treatment.
(A) A partial poly[$^3$H](rA).poly(dT) hybrid prepared according to fig.2 and purified by sephadex G100 gel filtration was used for the degradation study. The reaction mixtures(100 µl) contained 2 nmoles of $d(T)_{1000}$ and 0.6 nmole of [$^3$H]poly(rA) in the form of hybrid and 1 unit of DNA polymerase I. Two sealed sterile tubes with or without enzyme were incubated for 24 hours at 37°. All the material from the control tube and 20 µl from the tube containing enzyme, were subjected to paper(2040a Schleicher and Schüll) chromatography(descending) in n-propanol-conc.$NH_4OH$ - $H_2O$(55:10:35 v/v/v) for 8 hours. Paper strips(1 cm) were cut serially and counted.
(B) The material in the peak from (A) corresponding to rAMP was eluted and rechromatographed in Isopropanol -conc.$NH_4OH$- 0.1M Boric acid(7:1:2 v/v/v) and the radioactivity determined as above.

activity is located at a site different from the DNA exonuclease site, can not be excluded. Besides the 5'→3' degradation of RNA, attack from the 3'-end is also observed indicating that altogether two types of RNA degrading activities are effective. Lehman and Richardson noticed (18), that highly purified DNA polymerase from E.coli degrades ribonucleotide residues from double stranded mixed ribo-deoxy-polymers obtained from DNA polymerase reactions in the presence of manganese. In the light of the data presented here it appears, that the substrate range of the nucleolytic activity observed by these authors may now be extended from hybrids with mixed ribo-deoxy strands to hybrids containing all-ribo strands annealed to a large all-deoxy strand.

With respect to the possible in vivo function, it is tempting to speculate that removal of RNA primers may be effected by the nuclease activity discovered here. As an alternative Keller has suggested(5) the possible existence of a special enzyme similar to RNaseH. While we consider that

such a possibility exists, we may add, that in E. coli according to the observations presented here, DNA polymeraseI itself should be capable of performing this function.

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