**Discontinuous DNA Synthesis by Purified Mammalian Proteins**

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Five proteins purified from mouse cells acting together efficiently convert a single-stranded circular DNA template to covalently closed duplex circle by a discontinuous mechanism. DNA polymerase \( \alpha / \)primase, and with the assistance of alpha accessory factor covers the single-stranded circle with RNA-primed DNA fragments. Primers are removed by a combination of RNase H-1 and a 5'-exonuclease that was identified by its ability to complete this *in vitro* system. The 5'-exonuclease is required to remove residual one or two ribonucleotides at the primer/DNA junction that are resistant to RNase H-1. Gap filling is by the DNA polymerase \( \alpha / \)primase, and the enzyme and its responses to N-ethylmaleimide, salt, and divalent metals will be published elsewhere.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—The cells used as starting material were the mouse lymphoblast line (L1210) grown in suspension culture (2). Published procedures were used to purify DNA polymerase \( \alpha / \)primase (2), AAF (Fraction VI) (3), and bacteriophage T4 3'-nucleotidase (4).

To prepare DNA ligase I (5) and RNase H-1 (6, 7), extract from 25 g of mouse cells was fractionated on phosphocellulose (50-ml bed) by gradient elution (0-0.4 M KCl in 500 ml). Both DNA ligase I and RNase H-1 were eluted together between -70 and 140 mM KCl (by conductivity) with only slight overlap with DNA polymerase \( \alpha / \)primase, AAF, circle closing activity (see below) and RNase H-2, all of which overlapped extensively with each other and were eluted between -115 and 225 mM KCl. The fraction containing both ligase I and RNase H-1 was fractionated on hydroxyapatite (6-ml bed) by gradient elution (0.2 M KCl, 0-0.4 M KPO₄, pH 7.6, in 60 ml) separating the lagging strand and the replication fork.

**Experimental Procedures**

**DNA polymerase \( \alpha / \)primase** acting on a single-stranded DNA template (with rNTPs and dNTPs) synthesizes oligoribonucleotide primers that are extended to form short DNA fragments. In the presence of alpha accessory factor (AAF) the polymerase-primase complex remains associated with the template DNA while synthesizing fragments covering thousands of nucleotides (1). The mechanism is productive to the extent required for synthesis of Okazaki-sized fragments; and the enzyme complex can traverse double-stranded regions without detaching (1). These features are consistent with a role in discontinuous synthesis of the lagging strand of the replication fork; subsequent steps would include removal of primer RNA, filling gaps, and ligation. We report here the development of a system of five proteins that includes these additional steps and carries out the facile conversion of a single-stranded circular DNA to a covalently closed duplex circle by a discontinuous mechanism. Four of the five components are purified proteins of defined activities that would be expected to participate in the overall reaction. The fifth protein was required to complete the system allowing DNA fragments to join together into high molecular weight continuous strand. An exonuclease activity associated with the fifth protein facilitated its purification and characterization, and additional studies provided an explanation for the relationship between the exonuclease activity and function in the *in vitro* discontinuous DNA synthesis system.

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exonuclease activity on various polymers and DNA were carried out in the same mixture except Tris(Cl) was pH 0.5, magnesium acetate was 2 mM, and the amount of labeled substrate was 25 pmol/20 μl.

The circle closing activity (cca) assay was carried out in two stages. The first stage was in 10 μl containing 50 mM Hepes(K.), pH 7.6, 20 μM potassium acetate, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mg/ml bovine plasma albumin, 0.01% Nonidet P-40, 10% glycerol, 2 mM rATP, 0.2 μM each of rGTP, rCTP, and rUTP, 0.1 mM each of dGTP, dCTP, and dTTP, 20 μM [α-32P]dATP (5 μCi/μmol), 5 ng fd single-stranded circular DNA, 0.5-2 units of ligase I, 0.5-1 unit of RNase H, and 1-0.2 units of a bacterial exonuclease γ/primease. The mixture was incubated 30 min (37°C) for the first stage, after which 2 μl containing ca was added, and the mixture was reincubated 1 h (37°C) for the second stage of the assay reaction. This was followed by addition of 3 μl containing 0.5 mM NaOH, 0.1 mM EDTA, 0.2% bromphenol blue, 20% glycerol, and the mixture was electrophoresed in a 1% alkaline gel, then neutralized, dried, and autoradiographed. For some purposes requiring many assays, e.g. identifying active fractions in a column effluent, the volumes of the assay were halved to conserve enzymes. When the composition of the first stage was the same in all of the assays, it was assembled and incubated in a single large mixture, following which it was distributed into separate tubes for the second stage reaction. The two-stage protocol for the assay was necessary when crude fractions (i.e. containing endonucleases) were the source of cca. Purified fractions of cca could be included along with all of the other components in a single reaction mixture, which was incubated only once (90 min), with the same result as the two-stage reaction.

Endonuclease assay was carried out in 20 μl containing 50 mM Tris(Cl) pH 7.6, 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 0.2 mg/ml bovine plasma albumin, 0.01% Nonidet P-40, 10% glycerol, and 100 fmol dATP. The product was purified as described elsewhere (2).

Substrates—[3H]Poly(dA-dT) used as substrate for the ligase assay (and for the exonuclease assay, below) was synthesized in a 600-μl mixture containing 50 mM Tris(Cl), pH 7.6, 50 mM potassium acetate, 10 mM magnesium acetate, 5 mM dATP, 2 mM [3H]dTTTP (0.125 μCi/μmol), 0.1 mM EDTA, 100 μg/ml bovine plasma albumin, 0.01% Nonidet P-40, 10% glycerol, 50 μg/ml of poly(dA-dT), and 15 units of Klenow fragment of E. coli DNA polymerase I. After incubation for 16 h (37°C), EDTA, Sarkosyl, and Proteinase K were added to 20 mM, 0.2% and 0.2 mg/ml, respectively, and the mixture was held at 50°C for at least 1 h. It was then applied to a spin column containing Sephacryl S-200 in 10 mM Tris(Cl), pH 7.6, 1 mM EDTA. Between 80 and 98% of the radioactivity was recovered as acid-insoluble form. Prior to use in either the ligase or exonuclease assays, [3H]poly(dA-dT) (0.25 μm) was incubated 30 min (37°C) in a mixture containing 25 mM Tris(Cl), pH 7.6, 1 mM MgCl₂, and 2.5 mg/ml pancreatic DNase I. EDTA (to 2 mM) and NaOH (to 0.2 μl) were added and the mixture was incubated at 50°C for 1 h, following which it was neutralized with 10 M acetic acid and used without further treatment. Average fragment size was ~1300 nucleotides (by alkaline gel electrophoresis).

To prepare double (block) labeled [5'-3H, 3'-32P]poly(dA-dT), [3H]poly(dA-dT) was first synthesized as described (above) except that the primer for the incubation mixture (60 μl) consisted of 6 nmoles of DNase-treated [3H]poly(dA-dT) (0.125 ng of pancreatic DNase/6 mM of [3H]poly(dA-dT)). The product was purified as described above for [3H]poly(dA-dT) (EDTA/Sarkosyl/Proteinase K; Sephacryl S-200 spin column). It was then reincubated in the same mixture (100 μl) as previously except [32P]dATP was now 2.5 μM (auxiliary labeling), dATP was 1 mM and labeled with [α-32P]dCTP (33 Ci/mmol), and Klenow enzyme was 25 units. The product was again purified as before. Recovery was ~40%, and based on incorporation of radioactive label the 3'-5'-labeled segment of each strand (on average) was approximately three times the length of the 5'-3'-labeled segment.

H-Labeled E. coli DNA was prepared from a T' strain of E. coli (D13a) grown in 85 μCi/ml [3H]dThd (total dThd, 2 μg/ml). The cells were lysed and the DNA was extracted by successive treatment with lysozyme/EDTA, RNAse A and T, SDS/Pronase, phenol, and chloroform, followed by precipitation with ethanol, and this sequence, beginning with the treatment with RNAse, was repeated twice. The purified DNA had a specific activity of 1320 cpm/μmol.

32P-Labeled M13 DNA was made by extending a 15-nucleotide primer (New England Biolabs) annealed to M13mp8 to ~84% of the length of M13mp8 using the Klenow enzyme, limiting dGTP, and excess dCTP, dTTP, and [α-32P]dATP (60 μCi/nmol).

Homopolymerases of dAMP, dCMP, and dTMP were prepared in a 20-μl volume containing 0.1 mM sodium cacodylate, pH 7.0, 0.2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine plasma albumin, 0.01% Nonidet P-40, 10% glycerol, 1 mM [α-32P]dATP (4 μCi/nmol), 0.5 μg of d(dN)₈, and 5 units of terminal deoxynucleotidyl transferase (P-L. Biochemicals, American Type Culture Collection, Rockville, Md.). These were distributed into separate tubes for the second stage reaction. The product was again purified as described for the labeled poly(dA-dT), above.

RESULTS

Formation of Continuous DNA Strands via Short Fragment Intermediates

In a previous report it was shown that DNA polymerase α/primease in the presence of AAF efficiently converts single-stranded circular DNA to duplex circles (1). The product strands in the latter consisted of many RNA-prime1 Okazaki-like fragments ranging in size from 150 to 900 nucleotides, with the maximum at ~250 nucleotides (1). This would be equivalent to approximately 20-30 fragments/circle of bacteriophage fd DNA. The products of two reactions are shown...
in the neutral gel of Fig. 1, 1-a and 2-a. When a crude cell extract was added and the mixture was reincubated a large portion of the DNA (~1/2 in 1-b and somewhat less than half in 2-b) was converted to covalently closed circles. The mixture of purified DNA polymerase α/primase and AAF plus crude extract evidently was able to remove the RNA primers at the 5' end of each fragment, fill the gaps by extending the 3' ends of fragments, and ligate the fragments together.

Although the crude extract also contained DNA polymerase α/primase and AAF it was incapable of accomplishing the conversion to covalently closed circular product if incubated directly with single-stranded circular DNA (not shown). The nuclease in the extract partially or completely degraded the template DNA before it could be covered with fragments. However, when the single strand was first covered with fragments it left few or no single-stranded gaps as a target for endonucleases, and nicks in a double-stranded region could be repaired by the ligase present in the extract. A moderate amount of duplex linear molecules appeared (Fig. 1, b, c, d), and relatively more with the higher amount of template DNA, presumably because of the lower ratio of DNA to ligase and/or DNA polymerase in the reaction with the lesser amount of template DNA. With longer incubations the proportion of DNA in the form of covalently closed circles diminished, probably due to partial inactivation of one or more of the previously referred to enzymes that filled gaps and ligated nicks, and possibly also in part a fall in the concentration of ATP as phosphoenolpyruvate was exhausted.

Several unsuccessful attempts were made to purify directly from crude extracts the components that brought about the conversion from RNA-primed fragments to continuous strand. The most important reason for the failure was the "unmasking" of nuclease activity in fractions. To reconstitute the activity of the crude extract, fractions were prepared and recombined; however, the usual result was degradation of the DNA fragment-covered template (first incubation product). It appeared that nuclease activity, inhibited in the crude extract, became much more active after fractionation.

An alternative approach was taken of supplementing DNA polymerase α/primase and AAF with the additional activities that were assumed to be required. DNA ligase I (5) and RNase H-1 (6, 7) were purified from cultured mouse cell extracts ("Experimental Procedures"). (The reasons behind the choice of these two enzymes are given under "Discussion.") When both ligase I and RNase H-1 were included in the incubation with DNA polymerase α/primase and AAF no covalently closed duplex circles resulted. However, if a small amount of a crude extract was added and the mixture was reincubated, the result was complete joining in a large proportion of the DNA molecules (not shown), similar to what was seen with a large amount of crude extract in the absence of purified RNase H and ligase (Fig. 1). It appeared that removal of the RNA primer by RNase H-1 and the presence of DNA ligase I were insufficient to join the fragments; however, the crude extract supplied one or more additional activities that were needed.

An Assay for Circle Closing Activity

The reaction that converted single-stranded circles to duplex closed circles was simplified to facilitate its use as an assay for the activity that was still required when RNase H-1 and ligase I were supplied, referred to as circle closing activity. All of the components except for cca, i.e. DNA polymerase α/primase, AAF, RNase H-1, ligase I, and single-stranded circular DNA, together with rNTPs and dNTPs, were incubated together for 30 min. A sample containing cca was then added and the mixture was reincubated for 1 h ("Experimental Procedures"). Instead of using neutral gel electrophoresis, which requires complete joining of all fragments on a circular template to score as a positive event (Fig. 1), analysis of reaction products was carried out in an alkaline gel. The latter provided additional information, i.e. intermediate products of joining, indicated by increase in size of the fragments (Fig. 2). Attempts to convert to a quantitative result, i.e. by cutting up and counting gel fragments, proved too cumbersome for most purposes and not very useful because of substantial day-to-day fluctuations. Accordingly, the assay was used to identify active fractions but provided only a semiquantitative estimate of amount.

When fractionated on a DNA cellulose column (Fig. 2) or by other fractionation procedures the cca behaved as if it were a single component. However, because excessive amounts of cca were inhibitory in the reaction (see below), if too large an aliquot were taken for the assays a spurious double peak would appear due to inhibition of joining at the true maximum and apparent peaks on either side of the true maximum at the points in the up- and downslope where maximum joining occurred.

By alkaline gel electrophoresis the completed product from these reactions consisted of a mixture of linear single strands, close to full-length, and (denatured) covalently closed circles. This was usually evident as a double band (Fig. 2) and in some gels was very distinct (10). The most likely source for the linear single strands was the product of synthesis on linear single-stranded template DNA, present already as a contaminant in the stocks of single-stranded circular DNA and probably also to some extent produced during the incubations due to traces of nuclease(s). Linear single strands of DNA served as templates and were covered with DNA fragments by DNA polymerase α/primase and AAF, entirely similar to the reaction with single-stranded DNA circles (data not shown). Of the other possible sources of linear single strand product, incomplete joining, and cutting (nuclease)
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### Purification of Circle Closing Activity/Exonuclease

#### Initial Steps in Purification

In the current procedure the cca/exo is purified along with six other proteins, DNA polymerase α/primase (2), AAF (3), DNA polymerase δ (11), DNA ligase I, RNase H-1 (2). "Experimental Procedures"). The initial steps in the procedure have been described in detail for the purification of DNA polymerase α/primase (2) and DNA ligase I.

An early objective was to remove contaminating activities and, if possible, identify a particular activity with the cca that would account for its function in the circle closing reaction. Most of the endonuclease activity was removed with the first one or two purification steps; however, exonuclease activity persisted, and appeared coincident with cca activity through trials of phosphocellulose, DNA cellulose, Bio-Rex 70, hydroxyapatite and glycerol gradient, forcing the tentative conclusion that cca and exonuclease (exo) were associated (cca/exo).

The exonuclease assay made subsequent purification efforts considerably easier.

### Purification of Circle Closing Activity/Exonuclease

**FIG. 2. Circle closing activity assay on DNA cellulose column fractions.** The active fractions from a phosphocellulose column (to which a crude extract had been applied) were applied to a DEAE-cellulose column in 50 mM Hepes(K+), pH 7.6, 50 mM KCl. A portion of the non-binding fractions, which contained the activity from the DEAE-cellulose column, was applied to a 1-ml bed column of DNA cellulose (2.5 mg DNA/ml bed) equilibrated with 50 mM KPO4, pH 7.6, 0.2 mM PO4 fraction; Fraction III (2)). Table I summarizes the procedure beginning with the next step.

**DEAE-Cellulose Chromatography**—The hydroxyapatite, 0.3 mM PO4 fraction from a preparation that began with 40 g of cells was dialyzed against 50 mM Tris(Cl-), pH 7.6 and applied to a column of DEAE-cellulose (Whatman DE52) (1-ml bed volume/5 mg of protein) that had been equilibrated with 50 mM Tris(Cl-), pH 7.6 (buffer A), and this was followed by 4 column volumes of buffer A. The effluent from sample application and subsequent wash with buffer A contained the cca/exo, as measured by the exonuclease assay (as well as RNase H-1) (DEAE-cellulose fraction, Fraction IV). Following the wash with buffer A the column was eluted with a salt gradient to recover DNA polymerase α/primase (2) and DNA ligase I.

**Q-Sepharose Chromatography**—Fraction IV was adjusted to pH 9.0 with 2 mM Tris(OH-), diluted with 2 volumes of 5 mM Tris(Cl-), pH 9.0, and applied to a column of Q-Sepharose, Fast Flow (Pharmacia LKB Biotechnology, Inc.) (1-ml bed volume/5 mg of protein) that had been equilibrated with 20 mM Tris(Cl-), pH 9.0 (buffer B). The column was washed with 5 column volumes of buffer B followed by a 20-column volume gradient from 0 to 0.5 mM KCl in buffer B. The effluent from the gradient was collected in 40 fractions of which those containing most of the exonuclease activity (fractions 14–19) were pooled and adjusted to pH 7.6 with 2 mM acetic acid. (Q-Sepharose fraction, Fraction V).

**DNA-Cellulose Chromatography**—Fraction V was diluted with 3 volumes of 5 mM Tris(Cl-), pH 7.6, and applied to a 1-ml bed column of DNA-cellulose (2.5 mg DNA/ml bed) equilibrated with buffer A. The column was washed with 10 ml of buffer A followed by a 20-ml gradient of 0–0.4 mM KCl in buffer A, collected in 21 fractions. The peak exonuclease fractions (13 and 14) were pooled (DNA-cellulose fraction, Fraction VI) (Fig. 3).

**Glycerol Gradient**—Seventy μl of Fraction VI was dialyzed against 50 mM Hepes(K+), pH 7.6, 0.2 mM KCl, 5% glycerol and applied to a 3.9-ml glycerol gradient (10–30%) in the same buffer. It was centrifuged in the Beckman SW60 Ti rotor at 55,000 rpm for 42 h, collected from below, and the fractions with maximum exonuclease activity pooled (glycerol gradient fraction, Fraction VII) (Fig. 4).

### Stability

The cca/exo was quite stable; there was little or no loss of activity after several months at −20 °C in 50% glycerol nor after several cycles of freeze (liquid nitrogen)/thaw in either 30 or 50% glycerol. No loss was seen after 1 year at −70 °C in 30 or 50% glycerol after freezing in liquid

### Properties of Circle Closing Activity/Exonuclease

**TABLE I**

| Fraction          | Protein | Exonuclease | Specific activity |
|-------------------|---------|-------------|------------------|
| IV DEAE-cellulose | 19.9    | 68,000      | 3,400            |
| V Q-Sepharose     | 7.6     | 44,000      | 5,900            |
| VI DNA-cellulose  | 1.50    | 24,000      | 16,500           |
| VII Glycerol gradient | 0.095 | 12,400      | 130,000          |

*The procedures for preparing fractions I (crude extract/S-100), II (phosphocellulose), and III (hydroxyapatite) are part of a procedure reported previously ("Results" and Ref. 2).

Glycerol gradient was carried out on a portion of Fraction VI; the values for this step were corrected to apply to the total fraction.

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1. In this procedure RNase H-1 is present along with cca/exo in the non-binding fraction from DEAE-cellulose and is separated from it in the next few steps. DNA ligase separates from (precedes) DNA polymerase α/primase in the DEAE-cellulose gradient. Details of their purification by this procedure and their characterization will be published elsewhere.

2. These were followed by the elution of DNA polymerase α/primase (along with cca/exo, RNase H-1, and DNA ligase I) with 0.3 mM KPO4, pH 7.6, 0.2 mM KCl (hydroxyapatite, 0.3 mM PO4 fraction; Fraction III).
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FIG. 3. DNA-cellulose chromatography on cca/exo. Fraction V (Q-Sepharose fraction) was applied to a DNA-cellulose column and eluted with a salt gradient (“Results”). Two µl of 1/50 dilution of each fraction was assayed for exonuclease (above); aliquots of each sample were also analyzed in SDS-polyacrylamide gel which was stained with silver (below). (Exonuclease assays were carried out on pools of fractions 1-4 and 18-21). Polypeptide size markers for the gel are given in Fig. 5 legend with the exception that fumarase and ovalbumin were omitted here.

FIG. 4. Correlation of exonuclease activity, circle closing activity, and polypeptide size in glycerol gradient fractions. DNA-cellulose fractions 13 and 14 (Fig. 3) were pooled and centrifuged in a glycerol gradient (“Results”). Each of the fractions were assayed for exonuclease (2 µl of 1/50 dilution) (“Experimental Procedures”) (top) and circle closing activity (1/500 dilution) (“Experimental Procedures”) (bottom). SDS-polyacrylamide gel electrophoresis was also carried out on each fraction (middle). Polypeptide and DNA gel markers are given in the legends to Figs. 5 and 2, respectively.

nitrogen. The glycerol gradient fraction represented an 18% recovery compared to the DEAE-cellulose fraction. Since assays for exonuclease were not done prior to DEAE-cellulose overall recovery is not known. A notable exception to generally good recoveries with the enzyme was with gel filtration; multiple attempts (that included fractions less pure than the current glycerol gradient fraction), with Sephadex and Sephacryl (Pharmacia), Bio-Gel P (Bio-Rad), and Ultrogel AcA (LKB), all resulted in very large losses. With purified fractions (e.g. DNA-cellulose fraction), if salt concentration was allowed to fall too low (e.g. by dialysis against 10 or 20 mM Tris buffer) a precipitate would appear and the activity would largely disappear. We were unable to dissolve the precipitate and/or recover activity by increasing salt concentration; nor was it established whether the precipitate was primarily the enzyme itself or some other component(s) in that fraction that aggregated and occluded the cca/exo in the process.

Structure and Purity—The intensity of a ~50-kDa polypeptide in silver-stained SDS-polyacrylamide gels of DNA cellulose fractions 13 and 14 correlated with the peak of exonuclease activity (Fig. 3). In the glycerol gradient step that followed, the maxima of exonuclease and cca remained congruent and were closely correlated with the ~50-kDa polypeptide. In addition, essentially all of the other polypeptides were widely separated from the ~50-kDa polypeptide and in several
fractons the latter was the only polypeptide detected (Fig. 4). The data from denaturing gel electrophoresis gave a size of 49 kDa for the polypeptide (Fig. 5A). Calibration of the glycerol gradient provided a sedimentation coefficient of 3.2 S (Fig. 5B). Previously mentioned problems with recovery of activity from gel filtration interfered with determination of Stokes radius; however, from the sedimentation coefficient of 3.2 S we assume that the native protein is a monomer.

In the procedure described here, RNase H-1 appeared in the nonbinding fraction from DEAE-cellulose, along with cca/exo (above). A large portion of the RNase H-1 remained in the cca/exo fraction from Q-Sepharose; however, all but a minute amount (~0.7%) was removed by the DNA-cellulose. On the glycerol gradient (Fig. 4) the peak of the RNase H-1 was in fractions 4 and 5 while the exonuclease peaked in fractions 8 and 9. About half of the RNase H-1 applied to the gradient was recovered and, of that, ~1% could be detected in fraction 8 (and none in fractions 9 and 10) under assay conditions modified to increase sensitivity ("Experimental Procedures"). There was also a trace contamination with an endonuclease, the peak of which was between fractions 7 and 8 of the glycerol gradient (Fig. 4); this also was detected only under maximally sensitive assay conditions ("Experimental Procedures"). Thus far we have not been able to carry out an additional fractionation step to remove residual RNase H-1 and/or endonuclease in the highly purified enzyme without drastic losses. Even the highly purified cca/exo is inhibitory if used in amounts far in excess of what is required for optimum joining (Fig. 6). However, it is unlikely that this inhibition results from the minute contamination with endonuclease since much larger amounts of nuclease in crude fractions did not have such an effect (above). No other activities have been detected in the highly purified fractions of cca/exo including DNA polymerase, DNA ligase, topoisomerase, or DNA-dependent ATPase.

Characterization of Exonuclease Activity

On poly(dA-dT) optimal activity was at 2 mM Mg2+; activity was ~90% of maximum at 1 mM Mg2+, 85% at 5 mM Mg2+, and 75% at 10 mM Mg2+. Mn2+ at 1–2 mM gave ~55% of the activity with Mg2+. The pH response was also fairly flat; with Tris(Cl-) the optimum pH was 8.5–9.0, with 90% of optimal activity at pH 8.0 and 9.5, and ~85% at pH 7.5. With glycine(K+) the result was about the same as with optimum Tris(Cl-) and there was little or no difference in activity from pH 8.0 to 9.5. Even relatively low concentrations of salt were inhibitory; KCl at 10, 20, 50, 75, and 100 mM resulted in 88, 77, 54, and 35% (respectively) of the activity in absence of added salt. In the presence of N-ethylmaleimide <5% activity remained.

A dual labeled poly(dA-dT) substrate showed that the cca/exo attacks solely at the 5' terminus (Fig. 7). Controls with the 3'-exonuclease of bacteriophage T4 DNA polymerase, and the 5'-specific bacteriophage T7 gene 6 exonuclease confirmed the specificity of the test (Fig. 7).

The poly(dT) strand of poly(dA)-oligo(dT) was digested at ~4% of the rate with poly(dA-dT), whereas the rate on the complementary strand, poly(dA), was ~1/20 the rate with poly(dT) (Table II). In each case, when the homopolymer was not paired with the complementary strand the rate of digestion was ~2% of the rate when paired. In contrast, the rates of digestion of native and denatured DNA were approximately the same, and actually slightly greater for the single strands.
The activity of a poly(dA-dT) substrate was reduced to ~2% removal of 5'-PO4 with calf intestinal alkaline phosphatase poly(dA-dT) (-0.05-0.3%). Poly(dC) was an even poorer substrate for T4 3'-nucleotidase (4). Treatment with bacterial alkaline phosphodiesterase converted the labeled product to dAMP, which was verified as 5'dAMP by its susceptibility to 5'-nucleotidase and insusceptibility to bacterial phage T4 3'-nucleotidase (4). Snake venom phosphodiesterase converted the labeled product into a single spot that was almost, but not completely, separated from (slower than) dAMP in 0.09 M LiCl, but was clearly separated from (slower than) DNP in 0.09 M KPO4, pH 3.5. When 32P-labeled poly(dA-dT) synthesized with [α-32P]dATP was digested with cca/exo and analyzed by polyethyleneimine-cellulose thin layer chromatography ~90% of the labeled product was in a single spot that was almost, but not quite, congruent with dAMP in 0.5 M LiCl, but was clearly separated from (slower than) DNP in 0.09 M KPO4, pH 3.5. Snake venom phosphodiesterase converted the labeled product to DNP, which was verified as 5'-DNP by its susceptibility to 5'-nucleotidase and insusceptibility to bacterial phage T4 3'-nucleotidase (4). Treatment with bacterial alkaline phosphodiesterase transformed ~65% of the DNP of the unknown product into 32P, and the remaining ~35% into a slightly less charged than (slightly faster than) DNP. It was concluded that the product of digestion of poly(dA-dT) by cca/exo was a mixture of dinucleotides, ~7% 5'-pdAdT and ~93% 5'-pdTdT. Smaller amounts of the digestion products, usually ~15%, consisted of larger oligonucleotides that were multiples of the dinucleotides (presumably p(dAdT), and p(dTdT)), which was inferred by comparison with pancreatic DNase digest of poly(dA-dT), which consists of p(dTdT), (12). In addition there was a small amount of [32P]dAMP (2-3%).

In contrast to the predominantly dinucleotide product from digestion of poly(dA-dT), the principal product of digestion by cca/exo for all other substrates tested was the 5'-mononucleotide (80-95% of the soluble product). This included the homopolymers poly(dT), poly(dA), and poly(dC) both when digested alone as well as when the complementary strand was present, and both native and denatured DNA. The remaining 0-20% in each case consisted of oligonucleotides, decreasing in amount with increasing chain length. Neither the presence/absence of complementary strand, nor extent of digestion (~2-30%), had a significant effect on the proportions of mononucleotide and oligonucleotide products.

A 5'-OH terminus strongly inhibited the exonuclease. After removal of 5'-PO4 with calf intestinal alkaline phosphatase the activity of a poly(dA-dT) substrate was reduced to ~2% of its previous level.

**Mechanism of Circle Closing Activity**

The determination that circle closing activity and an exonuclease activity were properties of the same protein (Fig. 4) did not immediately resolve the relationship although it did suggest a mechanism. Some possibilities were easily eliminated, i.e. the protein did not have DNA polymerase activity (for gap closing), nor did it have RNA ligase activity. It also did not have intrinsic RNase H activity, as discussed above. The possibility that the cca/exo may facilitate the circle closing reaction by stimulating one or more of the other components was tested by including it in the standard assays with purified DNA polymerase α/primase, DNA ligase I, and RNase H-1. In addition, cca/exo was added in the self-primed reactions of DNA polymerase α/primase, with single-stranded circular DNA and with unprimed poly(dT), in the presence and absence of AAF (1, 3). In no instance there was there stimulation of any of these activities.

The identification of the exonuclease activity as 5' to 3' suggested that it may aid in the removal of primer ribonucleotides. RNase H-1 is an endonuclease (6) and, although it has been stated that, at high concentrations, the enzyme is able to completely remove a RNA primer (13), there are no published data on this capability. The possibility was considered that, at least under the conditions of our experiments, RNase H-1 does not remove the last one or two ribonucleotides at the DNA-DNA junction, preventing ligation. The function considered for cca/exo was to remove the residual one or a few ribonucleotides, allowing ligation to take place. The ability of the cca/exo to perform this function was tested by determining the susceptibility of the primer, or a portion of it, to the cca/exo. The experiments were carried out in two ways; in one, labeled RNA primers (along with their unlabelled DNA extensions) were synthesized by DNA polymerase α/primase and AAF and, in some of the reactions, RNase H-1 and cca/exo were included, singly and together (Table III). The cca/exo by itself had a relatively small effect on the amount of primer that remained. RNase H-1 alone removed ~80%; but with both RNase H-1 and cca/exo present most of the remaining primer was eliminated (Table III).

In the second type of experiment, the reaction was carried out in two stages; in the first stage the RNA primer-labeled product of DNA polymerase α/primase and AAF was synthesized and purified. In the second reaction the purified product suggested by a purification. Some possibilities were easily eliminated, i.e. the protein did not have DNA polymerase activity (for gap closing), nor did it have RNA ligase activity. It also did not have intrinsic RNase H activity, as discussed above. The possibility that the cca/exo may facilitate the circle closing reaction by stimulating one or more of the other components was tested by including it in the standard assays with purified DNA polymerase α/primase, DNA ligase I, and RNase H-1. In addition, cca/exo was added in the self-primed reactions of DNA polymerase α/primase, with single-stranded circular DNA and with unprimed poly(dT), in the presence and absence of AAF (1, 3). In no instance there was there stimulation of any of these activities.

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### Table II

**Polynucleotide Activity**

| Polynucleotide | Activity |
|---------------|---------|
| Poly(dA-dT)   | (100)   |
| Poly(dA)      | 0.001   |
| Poly(dA) + unlabeled poly(dT) | 0.056 |
| Poly(dT)      | 0.066   |
| Poly(dT) + unlabeled poly(dA) | 3.89   |
| Poly(dC)      | 0.009   |
| Poly(dC) + unlabeled poly(dG) | 0.011 |
| DNA, E. coli, denatured | 0.285 |
| DNA, E. coli, native | 0.066 |
| DNA, M13, denatured | 0.074 |
| DNA, M13, native | 0.045 |

(Table II). The rates for DNA were very low compared with poly(dA-dT) (~0.05-0.3%). Poly(dC) was an even poorer substrate for cca/exo and was essentially unaffected by the presence or absence of poly(dG).

When 32P-labeled poly(dA-dT) synthesized with [α-32P]dATP was digested with cca/exo and analyzed by polyethyleneimine-cellulose thin layer chromatography ~90% of the labeled product was in a single spot that was almost, but not quite, congruent with dAMP in 0.5 M LiCl, but was clearly separated from (slower than) DNP in 0.09 M KPO4, pH 3.5. Snake venom phosphodiesterase converted the labeled product to DNP, which was verified as 5'-DNP by its susceptibility to 5'-nucleotidase and insusceptibility to bacterial phage T4 3'-nucleotidase (4). Treatment with bacterial alkaline phosphodiesterase transformed ~65% of the DNP of the unknown product into 32P, and the remaining ~35% into a slightly less charged than (slightly faster than) DNP. It was concluded that the product of digestion of poly(dA-dT) by cca/exo was a mixture of dinucleotides, ~7% 5'-pdAdT and ~93% 5'-pdTdT. Smaller amounts of the digestion products, usually ~15%, consisted of larger oligonucleotides that were multiples of the dinucleotides (presumably p(dAdT), and p(dTdT)), which was inferred by comparison with pancreatic DNase digest of poly(dA-dT), which consists of p(dTdT), (12). In addition there was a small amount of [32P]dAMP (2-3%).

In contrast to the predominantly dinucleotide product from digestion of poly(dA-dT), the principal product of digestion by cca/exo for all other substrates tested was the 5'-mononucleotide (80-95% of the soluble product). This included the homopolymers poly(dT), poly(dA), and poly(dC) both when digested alone as well as when the complementary strand was present, and both native and denatured DNA. The remaining 0-20% in each case consisted of oligonucleotides, decreasing in amount with increasing chain length. Neither the presence/absence of complementary strand, nor extent of digestion (~2-30%), had a significant effect on the proportions of mononucleotide and oligonucleotide products.

A 5'-OH terminus strongly inhibited the exonuclease. After removal of 5'-PO4 with calf intestinal alkaline phosphatase the activity of a poly(dA-dT) substrate was reduced to ~2% of its previous level.

### Table III

**Effect of cca/exo and RNase H-1 on RNA primers**

| RNase H-1 | cca/exo | RNMPF |
|-----------|---------|-------|
| Experiment A | 1 - - 407 | 2 - + 224 | 3 + - 91 |
| Experiment B | 4 + + 7 | 1 - - 61 | 2 - + 39 |
| 3 + - 5.9 | 4 + + 1.2 | |
was incubated with RNase H-1 or cca/exo, or both (Table III). The result was very similar to the previous. With cca/exo alone there was only a small reduction in the amount of primer. RNase H-1 alone removed 90%. Again, most of the remainder disappeared when both RNase H-1 and cca/exo were present. The results of both experiments strongly support the proposed mechanism. If most of the primers were intact in the absence of both RNase H-1 and cca/exo, the proportions removed by RNase H-1 alone would be consistent with one or two ribonucleotides remaining, since the usual size is nine or 10 nucleotides (14, 15). The small amount of removal by cca/exo could have been a result of the traces of contaminating RNase H-1 that have, thus far, eluded efforts to remove them completely (see above). Other possibilities are that some of the RNA primers were only a few nucleotides in length (and, therefore, susceptible to removal as an oligonucleotide by cca/exo) either because smaller sizes were formed under the conditions used and/or resulted from undetected traces of RNase H in the preparations of DNA polymerase α/primase and AAF.

**Requirements for Discontinuous DNA Synthesis**

An excess of RNase H-1 did not compensate for the absence of cca/exo in the circle closing reaction (not shown). Although RNase H-1 stimulated the reaction with the most highly purified fractions of cca/exo (Fig. 8), the stimulatory effect could be eliminated by using an excess of cca/exo. It will not be possible to know whether this means that the cca/exo, alone, is capable of removing intact nona- and decaribonucleotide primers until trace contamination by RNase H-1 is reduced even further.

Other 5′-exonucleases were tested to see if they would replace the cca/exo in the circle closing reaction. Bacteriophage λ exonuclease had no effect, consistent with the fact that it excises only mononucleotides and has no detectable RNase H activity (16). E. coli DNA polymerase I did replace cca/exo as would be expected from the fact that it normally functions in primer removal (as well as gap filling) (17–19). Bacteriophage T7 gene 6 exonuclease also has RNase H activity and is thought to serve in primer excision (20); however, interestingly, it did not replace cca/exo in the circle closing reaction (not shown).

The amounts of DNA polymerase α/primase required for the circle closing reaction seem large when compared with the effects of similar amounts of the enzyme, in the presence of AAF, with much larger amounts of template DNA (1). AAF had a stimulatory effect, but if additional DNA polymerase α/primase was used (e.g. two to four times) there was little or no stimulation with AAF, and it could be omitted without significant effect (not shown).

**DISCUSSION**

The facility with which a single-stranded circular DNA is covered with Okazaki-like DNA fragments by DNA polymerase α/primase plus AAF encouraged the effort to use a single-stranded circle as a model template for the lagging strand. A similar approach has been used in the past for studies on lagging strand synthesis in bacteriophage T7 DNA replication (20). A crude mouse cell extract readily completed the steps from the fragment-covered circle to covalently closed circular duplex. When direct attempts to fractionate the crude extract were not successful, two candidate enzymes, RNase H-1 and DNA ligase I, were purified and used to supplement the fragment-synthesizing system, initially with the view that these activities might be all that is required to join the fragments. When RNase H-1 and ligase I were added no joining ensued; however, with those enzymes present addition of a very small amount of crude extract provided a “circle closing activity,” resulting in efficient joining.

An exonuclease activity was identified with circle closing activity and, guided by both assays, it was purified to apparent homogeneity and several of its features characterized. The cca/exo is a 3.2 S single polypeptide of 49 kDa. It is relatively stable, abundant in cell extracts, and fairly easily purified with a procedure by which six other DNA-related enzymes are also obtained. The exonuclease attacks at the 5′ terminus and requires the presence of the 5′-PO₂⁻ for activity. It is slightly more active on denatured DNA than on native DNA, whereas it is much less active on the unpaired homopolymers of dA and dT, compared with each annealed to its complementary strand. Poly(dA-dT) is, by far, the most active substrate; with the exception of a modest activity on poly(dT) (paired with poly(dA)), the activities with DNA and the other homopolymers are three to five orders of magnitude less than the activity with poly(dA-dT). The principal product is mononucleotides except when the substrate is poly(dA-dT), from which it is dinucleotides. The product includes, in addition, a variable proportion (5–20%) of oligonucleotides.

With optimal amounts of each of the five purified proteins the in vitro discontinuous DNA synthesis reaction was very efficient, resulting in replication of all of the input template and yielding a full-length continuous strand product. Based on several kinds of evidence (21–24), including the appropriate dual functions (primase and polymerase) of the enzyme complex, it is assumed that DNA polymerase α/primase performs similar functions in lagging strand synthesis in vivo. The results here show that DNA polymerase α/primase is also capable of completely closing gaps, a point on which some previous reports had not agreed (25, 26). A recent report also shows that DNA polymerase α/primase is able to fill gaps completely but, in addition, indicates that the final nucleotide is inserted with difficulty (27). Relevant to this difficulty is the rather large amount of DNA polymerase α/primase required by our in vitro system for discontinuous DNA synthesis. It is possible that this reflects the absence of additional accessory factors, or that another DNA polymerase normally serves in filling gaps. DNA polymerase I is thought to serve this function in E. coli but in its absence it is possible that

**Fig. 8. Titration of RNase H-1 in the circle closing reaction.**

Fraction 10 of the glycerol gradient (Fig. 4) was used as the source of cca/exo in the cca assay. The amount of RNase H-1 in a reaction was 0.05 units multiplied by the numeral above each lane (0, none; 1, 0.05 units; 2, 0.1 units etc.). See Fig. 2 for abbreviations.
DNA polymerase III fills gaps, albeit more slowly than DNA polymerase I (17–19).

The specificity of AAF (for DNA polymerase α/primase) and its uniquely suited functions suggest a role in assisting DNA polymerase α/primase in lagging strand synthesis (1). In the in vitro model system described here AAF was stimulatory if polymerase α/primase was limiting. It is possible that the large excess of DNA polymerase α/primase required for gap filling obscured a more prominent effect of AAF.

There was an absolute requirement for DNA ligase I; no joining was seen in the purified system in its absence. Support preparation with cca/exo.

that the cca/exo, itself, can cut between ribonucleotides sug-

would probably be as the intact 9- or 10-mer, or larger.

mined. It is also not yet known whether cca/exo can cut the

in the past; one of these, DNase IV, partially purified from

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for gap filling obscured a more prominent effect of AAF.

The results here indicate that under our conditions RNase H-l (and of endonuclease) that remained. From our own

 Interestingly, the 5'-OH terminus of a polydeoxynucleotide

uncovering a short region of the template strand 3' terminus

exonuclease purified by Hurwitz and associates (33) had sim-

same enzyme. Similar to the results reported here, the 5'-

DNA ligase II, accounts for most of the DNA ligase activity

of ligase I in this role in vivo is indirect. DNA ligase I

DNA and homopolymer, whereas cca/exo and DNase IV (29,

in dividing cells, and there is a large increase in ligase I

activity when cells undergo the change from nondividing to

dividing state, e.g. with mitogen treatment of lymphocytes,

amylic mass -200 kDa) is the predominant ligase activity

adult liver compared with regenerating liver (5). In contrast,

surprising since it is an endonuclease (6). Probably only one

activities on homopolymer model substrates it appears unlikely

of its DNA ligase activity in nondividing cells and does not change significantly as the

latter become dividing cells (5).

There was also no joining in the absence of cca/exo. The

later functions in primer removal along with RNase H-1. The

results here indicate that under our conditions RNase H-

removes most, but not all, of the RNA primer, a result not

surprising since it is an endonuclease (6). Probably only one

or two ribonucleotides remain and it is the function of cca/

exo to remove these so that ligation can occur. (Judging from

studies on homopolymer model substrates it appears unlikely

that a 3' terminus of DNA would be joined to a 5'-terminal

ribonucleotide by mammalian ligase I (29) but, even if this

occurred, it would not have been detected in our reactions,

which used alkaline gels to assess degree of joining.) Higher

amounts of RNase H-1 did not replace the requirement for

cca/exo. Very high amounts of RNase H-1 were inhibitory to

the circle closing reaction (carried out with cca/exo present).

We cannot exclude the possibility that the latter may have

been due to a contaminant in the preparation of RNase H-1

and that this might have prevented our seeing the removal of

all ribonucleotides by RNase H-1 without the aid of cca/exo.

However, considerable excess of RNase H-1 was used (e.g.

at least 30-fold), without causing inhibition and without affect-

ing the strict requirement for cca/exo. We believe it is more

likely that RNase H-1 is incapable of removing the last

ribonucleotide(s); observations that indicated otherwise (6)

may have resulted from slight contamination of the RNase H

preparation with cca/exo.

It is not known at this time whether cca/exo can remove

an intact (9- or 10-nucleotide) RNA primer without the aid of

a RNase H. This is because, although the cca/exo appeared

homogeneous on SDS-gels, there were minute traces of RNase

H-1 (and of endonuclease) that remained. From our own

results with DNA/[32P]JRNA substrate, the lack of evidence

that the cca/exo, itself, can cut between ribonucleotides sug-

suggests that, if unaided cca/exo were capable of removing a
decarboxyribonucleotide primer from an Okazaki-like fragment, it

would probably be as the intact 9- or 10-mer, or larger.

Interestingly, the 5'-OH terminus of a polydeoxynucleotide

was apparently not removed by the enzyme; the effect of the

5'-terminal triphosphate of a primer remains to be deter-

mined. It is also not yet known whether cca/exo can cut the

(RNA/DNA) junctional phosphodiester or whether it must

remove even a single 5'-terminal ribonucleotidyl as a dinucleotide or larger.

Two mammalian 5'-exonucleases have been characterized

in the past; one of these, DNase IV, partially purified from

rabbit bone marrow (29) and lung (30), had a sedimentation

coefficient of 3.4 S and a calculated molecular mass of 42 kDa; the other, DNase VIII, was 2.8 S and purified from human

tissue contained DNA polymerase α/primase and DNA ligase I

with poly(dA-dT) and homopolymers paired with the complementary

strand, much less activity with native DNA, and essentially

none with unpaired homopolymers and denatured DNA (29,

30). DNase VIII had similar activities with poly(dA-dT),

unpaired homopolymers, and denatured DNA and very little

activity on native DNA (31). Like DNase IV, cca/exo was

much more active on the homopolymers (poly(dA) and

poly(dT)) when paired with the complementary strand (com-

pared with the unpaired homopolymer); but cca/exo was much

more active with poly(dA-dT) than with either homopolymers

(paired) or DNA, and it was equally active with native and

denatured DNA.

Like cca/exo (and unlike DNase IV) DNase VIII was in-

active on substrates with 5'-OH termini (31). DNase VIII

produced primarily dinucleotides and lesser amounts of larger

oligonucleotides (and virtually no mononucleotides) from

DNA and homopolymer, whereas cca/exo and DNase IV (29,

30) released mostly mononucleotides (with lesser amounts of

oligonucleotides) from DNA and homopolymers. However, like cca/exo, DNase VIII produced dinucleotides and their

multiples from poly(dA-dT) whereas with DNase IV the pro-

duct was mostly mononucleotides. Both DNase IV and VIII

acted at nicks 5' to pyrimidine dimers, liberating pyrimidine

dimers (a property not tested for cca/exo).

Some of the properties of cca/exo discussed above are

shared with DNase IV and others with DNase VIII. The

differences could be taken as evidence that all three are
different enzymes. However, from the available information

it appears more likely that at least two, cca/exo and DNase

IV, and possibly all three, are the same enzyme and that the

differences have procedural or technical explanations. Unfor-

tunately, none of the DNase IV or VIII preparations remain

that would allow a direct comparison of the three enzymes to

resolve the discrepancies. 4,5

Although the relationship of cca/exo to DNases IV and

VIII must, for the present, be described with qualifications,

there is no uncertainty about the relationship of cca/exo to
two 5'-exonucleases described more recently, by Hurwitz and

colleagues (32, 33). The first was found as a requirement for

initiation in a defined system for adeno viral DNA replication

under circumstances in which the adeno viral DNA template

lacked the terminal protein (32). Called "factor pL," it was

purified and found to be a 5'-exonuclease, with molecular

mass of 44 kDa on SDS-polyacrylamide gels, and sedimenta-
tion coefficient of 2.8 S. It functioned in that system by
digesting the 5' terminus of the non-template strand, thereby

uncovering a short region of the template strand 3' terminus

and allowing initiation to take place (32). Factor pL was not

required under normal circumstances, when the terminal pro-

tein was present. Hurwitz and colleagues (33) were led to

the 5'-exonuclease a second time in the development of a recon-
nstituted T-antigen-dependent system for replication of SV40

origin-containing DNA. In addition to T-antigen, DNA po-

lymerase α/primase, RNase H, DNA ligase II, and topoisom-

erase II, there was a requirement for a small amount of crude

cell extract. When the active component in the crude extract

was purified it was found to be a 44-kDa 5'-exonuclease.

Factor pL could be substituted and was assumed to be the

same enzyme. Similar to the results reported here, the 5'-
exonuclease purified by Hurwitz and associates (33) had sim-

4 T. Lindahl, personal communication.

5 L. Grossman, personal communication.
ilar activities with single- and double-stranded DNA and strongly preferred paired poly(dA) to the homopolymer alone, and the product of digestion of DNA and homopolymers was primarily 5′-mononucleotides, plus smaller amounts of oligonucleotides. RNase H also gave only modest stimulation (approximately two-fold) in the SV40 origin system (33). The 5′-exonuclease purified from HeLa cells by Hurwitz and associates functioned in the circle closing activity assay in a manner indistinguishable from cca/exo (data not shown), and cca/exo purified from mouse cells by this laboratory replaced the HeLa 5′-exonuclease in the SV40 replication system (33), leaving little doubt that cca/exo is the mouse cell counterpart to the 5′-exonuclease isolated from HeLa cells by Hurwitz and colleagues. Ishimi et al. (33) reached a similar conclusion to the one stated here, regarding the role of the 5′-exonuclease in removing residual ribonucleotides that remained after digestion of the primer by RNase H, and also concerning the likelihood that the 5′-exonuclease and DNase IV are the same protein. In characterizing the 5′-exonuclease, Ishimi et al. (33) made the additional observation that the enzyme released the radioactivity from 5′-[32P]poly(rA)-poly(dT) as the mononucleotide, which we would not have expected from the absence of activity of cca/exo with DNA/[^32P]RNA. Possible explanations include a greater sensitivity of the terminally labeled oligo(rA)-poly(dT) substrate and a qualitative difference between the two substrates in susceptibility to the 5′-exonuclease. The possibility of a trace of RNase H (endonuclease) activity also comes up but, at least with RNA-DNA substrates, mononucleotides are ordinarily a relatively minor product (6, 13).

Two RNases H have been characterized in mammalian cells in the past, RNase H-1 (~60–100 kDa) and the antigenically distinct and smaller, RNase H-2 (~30–40 kDa) (6, 7). RNase H-1 activity was better correlated with DNA synthesis than RNase H-2 in studies with lymphocytes activated by mitogen (34) and in serum-stimulated cells (35); however, the data connecting mammalian RNases H with specific function(s) are limited. In the E. coli oriC system, removal of primer is accomplished by the 5′-exonuclease of DNA polymerase I, with the assistance of RNase H (19), similar to the function of cca/exo and RNase H-1. Also similar to the circle closing reaction and the roles of cca/exo and RNase H-1, the stimulation of the oriC system by E. coli RNase H is a relatively small effect (19). The product of bacteriophage T7 gene 6 is a 5′-exonuclease that, like the 5′-exonuclease of E. coli DNA polymerase I (17–19), has RNase H activity and has been implicated in RNA primer removal (20).

Several 5′-exonucleases have been identified in yeast, one of which, exonuclease IV, has activity on RNA as well as DNA; however, its effect on RNA paired with DNA is not yet known (36). Four RNases H, one each purified from yeast (37), Drosophila embryo (38), calf thymus (39), and mouse F9 cells (40) also have stimulatory activity for their homologous DNA polymerase/primase. The Drosophila embryo, calf thymus, and mouse cell enzymes stimulate the primase itself. Neither the calf thymus nor the mouse enzyme has been clearly identified with RNases H-1 or H-2. The Drosophila embryo enzyme has been shown capable of removing the last ribonucleotide of an RNA primer although this was not an efficient reaction (38); the abilities of the yeast, calf thymus, and mouse enzymes in this regard have not been specifically addressed in the studies reported thus far. The herpes simplex-1 DNA polymerase was recently shown to have a 5′-exonuclease activity that is also a RNase H and is presumed to serve in primer removal (41). The mouse RNase H-1 used in the studies reported here lacked DNA polymerase activity and did not stimulate mouse DNA polymerase α/primase on several different primed and unprimed templates that were tested. It was reported that mammalian RNase H-2 did not have DNA polymerase activity (42), but there is no information on its ability to stimulate DNA polymerase α/primase or to completely remove RNA primers.

Although two independent efforts, the one reported here and that of Ishimi et al. (33), using quite different assay systems, resulted in purification of the same 5′-exonuclease, additional evidence will be needed to establish whether the enzyme serves the same role in replication in vivo as it does in these in vitro systems. In this regard, it would be helpful to know whether the 5′-exonuclease provides this function in a more complex in vitro replication system requiring the participation of additional replication factors not present in the test system used here or the one used by Ishimi et al. (33). A direct search in crude extracts for an activity capable of removing residual primer residues is likely to lead to the same 5′-exonuclease. A different approach may be needed to uncover an alternative mechanism for total removal of primers in mammalian cells, if one exists.

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