

Enzymatic completion of mammalian lagging-strand DNA replication

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ABSTRACT Using purified proteins from calf and a synthetic substrate, we have reconstituted the enzymatic reactions required for mammalian Okazaki fragment processing *in vitro*. The required reactions are removal of initiator RNA, synthesis from an upstream fragment to generate a nick, and then ligation. With our substrate, RNase H type I (RNase HI) makes a single cut in the initiator RNA, one nucleotide 5' of the RNA-DNA junction. The double strand specific 5' to 3' exonuclease removes the remaining monoribonucleotide. After dissociation of cleaved RNA, synthesis by DNA polymerase generates a nick, which is then sealed by DNA ligase I. The unique specificities of the two nucleases for primers with initiator RNA strongly suggest that they perform the same reactions *in vivo*.

Considerable progress has been made in the last decade toward identifying and characterizing the proteins involved in mammalian DNA replication (reviewed in ref. 1). A substantial proportion of this information is derived from reconstitution of simian virus 40 DNA replication *in vitro*. The results support a two-polymerase system for replication of nuclear DNA, with polymerase δ and polymerase α /primase responsible for leading- and lagging-strand synthesis (2, 3). The recent demonstration that these enzymes, plus DNA polymerase ϵ , are all required for viability in yeast (4–6) has prompted several proposed roles for the latter enzyme in DNA replication (7–10). While the viral model has clarified priming and elongation steps in the replication fork, the exact steps involved in completion of lagging-strand synthesis are not yet clear. Simian virus 40 reconstitution reactions (11–13) indicated roles for the mammalian 5'-to-3' exonuclease, a nuclear DNA polymerase, DNA ligase, and possibly RNase H type I (RNase HI) in joining of nascent segments of the lagging strand. DNA ligase I, rather than III, was shown to be required (12, 13). The specific function of RNase HI and the cleavage mechanism involved remained to be determined.

In *Escherichia coli*, initiator RNA of Okazaki fragments is thought to be removed by the 5'-to-3' exonuclease of DNA polymerase I, although a role for RNase H cannot be totally excluded (14). Double strand-specific 5'-to-3' exonucleases, which appear to be functional homologs of each other, have been isolated from human cells, mice, and calf (11–13, 15, 16). The calf nuclease displayed an endonuclease function that cleaves the unannealed 5' tail of a primer on a template, in the presence of a directly adjacent upstream primer (17). This very specific function is also exhibited by *E. coli* DNA polymerase I (18), which strongly suggests that the bacterial and mammalian enzymes are also functional homologs.

Mammalian RNase H enzymes have been identified and characterized in detail. RNase H enzymes can be placed into one of two classes (I and II) based on subunit structure, peptide molecular mass, and the ability to use manganese as a cofactor (19, 20). They have been postulated to be involved

in DNA replication and transcription (21), but no specific roles have been established for either class.

We had shown (22) that the calf 5'-to-3' exonuclease could work with calf DNA polymerases α , δ , or ϵ and DNA ligase I to join two DNA primers annealed to a template with a four-base gap. In addition, we have demonstrated that 5'-to-3' exonuclease activity is stimulated by synthesis from an upstream primer and that the stimulation is the result of the formation of a nick between the two primers, which is a preferred substrate for the exonuclease (17). These results suggest that a nick translation process occurs prior to the joining reaction catalyzed by DNA ligase I. Goulian *et al.* (15) previously showed that a combination of RNase HI and 5'-to-3' exonuclease can remove initiator RNA from primer-templates *in vitro*. Here we define the specific reactions by which these two nucleases can remove initiator RNA in a model Okazaki fragment system before polymerization can make the substrate for ligation.

MATERIALS AND METHODS

Protein Purification. DNA polymerase ϵ , DNA ligase I, and calf 5'-to-3' exonuclease were purified from calf thymus as described (22). Calf thymus RNase HI was purified by adapting the procedure reported by Eder and Walder (23) for use with calf thymus. Frozen fetal thymus (400 g) was homogenized in 1.2 liter of buffer A (10 mM Tris, pH 8.4/0.5 mM EDTA/0.5 mM EGTA/1 mM dithiothreitol) containing 50 mM KCl. The homogenate was centrifuged at low speed (9000 $\times g$) for 30 min, and then the supernatant was centrifuged at 100,000 $\times g$ for 60 min. The supernatant from high-speed centrifugation was filtered through four layers of cheese cloth and loaded onto a DEAE-Sepharose column (500 ml) equilibrated with buffer A containing 50 mM KCl. The protein that flowed through the column was applied directly to a CM-Sepharose column (500 ml) equilibrated with buffer A containing 50 mM KCl. The column was developed with a 2.5-liter gradient from 50 to 400 mM KCl. Fractions containing RNase H activity were pooled and precipitated with 60% ammonium sulfate. The protein pellet was collected by centrifugation and dissolved in buffer A containing 1 M ammonium sulfate. The protein was loaded on a 40-ml phenyl-Sepharose column and developed with a decreasing salt gradient from 1 M to 0.4 M ammonium sulfate in buffer A. Active fractions were pooled and dialyzed versus 10 volumes of buffer A containing 10% (vol/vol) glycerol and 100 mM KCl. The RNase H activity flowed through both Mono Q and Mono S columns. The flow-through protein from these columns was then applied to a 1-ml heparin-Sepharose column equilibrated in buffer A containing 10% glycerol and 100 mM KCl. The activity was eluted with a 15-ml gradient

Abbreviation: RNase HI, RNase H type I.

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from 100 to 600 mM KCl in buffer A containing 10% glycerol. Active fractions were pooled, diluted to a conductivity equal to buffer A containing 100 mM KCl and applied to a 1-ml blue-Sepharose column equilibrated in the same buffer. RNase H activity was eluted from the column with buffer A containing 500 mM KCl and 2 M MgCl₂. Active fractions were dialyzed versus buffer A containing 50% glycerol and frozen at -70°C. The final pool of RNase HI had a specific activity of 30,000 units/mg as measured on poly([³H]rA)·oligo(dT) according to Eder and Walder (23).

Lagging-Strand Substrate Construction. The lagging-strand substrate was prepared by PCR amplification of a 130-bp region of the plasmid pBS(+) (Stratagene) encompassing the multicloning site and T3 RNA polymerase promoter. The oligonucleotides used for PCR of the pBS(+) plasmid were DNA primer 1 (5'-ATGTTAGGCGCCGGGCGAATTC-GAGCTCGG-3') and DNA primer 2 (5'-AACAGCTATGAC-CATGATTA-3'). The oligonucleotides were synthesized on an Applied Biosystems 360 oligonucleotide synthesizer and purified by 15% polyacrylamide/7 M urea gel electrophoresis. PCR reactions were performed for 30 cycles, and the 130-bp product was purified by electrophoresis through 4% NuSieve GTG agarose (Seakem). The purified double-stranded 130-mer was then subjected to single-primer amplification using only primer 1. The single-strand 130-mer template DNA product was purified by the same method. *In vitro* transcription from the T3 RNA polymerase promoter was performed after *Hind*III or *Pst* I digestion to yield RNA primers of 13 and 21 nucleotides, respectively. RNA primers were purified by 15% polyacrylamide/7 M urea gel electrophoresis. RNA primers were hybridized to the single-strand 130-mer template and were extended by using Sequenase™ (version 2.0) and dNTPs. After extension, the substrate was digested with *Nar*I, producing a 3' recessed terminus, which was labeled by extension with dCTP and [α -³²P]dGTP (3000 Ci/mmol; 1 Ci = 37 GBq). A 20-nucleotide-long DNA primer was hybridized to the 130-mer DNA so that its 3' terminus was 29 nucleotides upstream of the RNA-DNA fragment. This substrate is depicted in Fig. 1.

Enzymatic Reactions. Lagging-strand reactions were performed in 20 mM Hepes, pH 7.0/1 mM dithiothreitol/10% glycerol/1 mM ATP/10 mM MgCl₂/0.1 mM dNTPs in a volume of 20 μ l. Reaction mixtures were incubated at 37°C

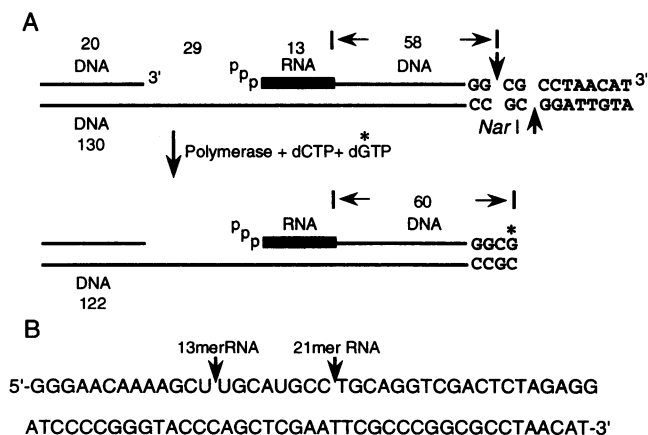


FIG. 1. Model lagging-strand substrate. The lagging strand substrate was constructed as described in text. (A) The lengths of segments in this and subsequent figures are given in nucleotides. The staggered cut by *Nar*I is shown. DNA synthesis adds two nucleotides, with the label on the 3' nucleotide. The final substrate has a 13-nucleotide RNA attached covalently to a 60-nucleotide DNA. (B) The sequence of the RNA-initiated DNA segment is shown. The covalent RNA-DNA junctions for the 13-nucleotide RNA used in most experiments and the 21-nucleotide RNA used in the experiment in Fig. 4 are indicated.

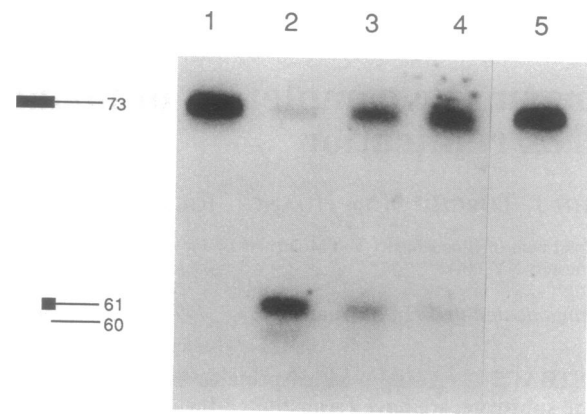


FIG. 2. An activity with the ability to degrade initiator RNA primers can be purified away from calf 5'-to-3' exonuclease. The 3' labeled lagging-strand substrate was incubated in 20- μ l reaction mixtures with various preparations of 5'-to-3' exonuclease purified from calf thymus. Reaction products were separated by 10% polyacrylamide/7 M urea DNA sequencing gels, dried, and detected by autoradiography. In each lane the amount and specific activity of the 5'-to-3' exonuclease preparations in units and units/mg of protein are as follows: no enzyme (lane 1), 0.8 and 29,000 (lane 2), 0.4 and 20,000 (lane 3), 0.8 and 85,000 (lane 4), and 0.8 and 180,000 (lane 5). The lengths of the RNA-initiated DNA strands are given at the left. The wide and narrow black lines represent RNA and DNA respectively, in this and subsequent figures. Sizes are shown in nucleotides.

for the time indicated in the figure legends, and reactions were stopped by the addition of 90% formamide/10 mM EDTA. Reaction products were heated at 95°C for 5 min and separated by denaturing polyacrylamide gel electrophoresis. Products were visualized by autoradiography by using DuPont Cronex Lightning Plus intensifying screens at -70°C.

RESULTS AND DISCUSSION

Our previous results have shown that calf 5'-to-3' exonuclease can degrade a downstream DNA primer and, in conjunction with polymerization by DNA polymerase α , δ , or ϵ , could generate a product that is capable of being joined by DNA ligase I (22). Therefore, we assessed the ability of the exonuclease to degrade an initiator RNA primer using the substrate shown in Fig. 1. This substrate contains a triphosphorylated 5' terminus similar to initiator RNA primers synthesized by DNA polymerase α /primase (24). The 3' end of the RNA-initiated DNA strand was labeled, so that the fate of the 3' portion of the segment could be evaluated. Fig. 2

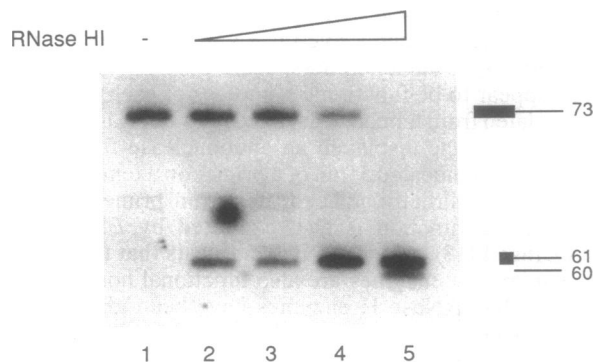


FIG. 3. Purified calf thymus RNase HI degradation of initiator RNA primers. Calf thymus RNase HI was purified to a specific activity of 30,000 units/mg of protein as described in text. The reactions were incubated for 15 min at 37°C and processed as described in Fig. 2. Lanes: 1, no enzyme; 2, 0.08 units; 3, 0.15 units; 4, 0.3 units; 5, 0.6 units. Sizes are shown in nucleotides.

shows the results obtained when various preparations of 5'-to-3' exonuclease purified from calf with specific activities ranging from 20,000 to 180,000 units/mg of protein were assayed (Fig. 2). The results demonstrate that the lower specific activity preparations of calf 5'-to-3' exonuclease were capable of degrading the RNA (Fig. 2, lanes 2-4). However, this property was lost as the enzyme was further purified to higher specific activities (Fig. 2, lane 5). The 5'-to-3' exonuclease preparations used were from independent purifications; therefore, specific activity measurements do not correlate absolutely to the level of the contaminating activity degrading the RNA portion of the substrate.

We reasoned that the most likely protein contaminating the 5'-to-3' exonuclease preparation that was capable of degrading an RNA primer hybridized to DNA, was ribonuclease H (RNase H). RNase H activity is present at high levels in mammalian cells and is a common contaminant of purified proteins. To test this hypothesis, we purified calf RNase HI and assessed its ability to degrade the RNA portion of the substrate. In a time (data not shown)- and concentration-dependent manner (Fig. 3), calf thymus RNase HI could degrade the RNA segment of the substrate generating a

primary product, approximately the length of the DNA portion of the substrate with a single remaining ribonucleotide at the 5' end. At the highest concentration of the RNase HI, loss of the last ribonucleotide was evident. This is presumably the result of a low level contamination of the 5'-to-3' exonuclease in the preparation of RNase HI.

Curiously, we did not observe any intermediates with various lengths of RNA left attached to the DNA segment. RNase H enzymes from diverse organisms generally release short RNA oligomers (25, 26). If the calf RNase HI acted in this fashion, individual enzymes would have had to move in a highly processive fashion, completely degrading one RNA segment before moving on to another. Alternatively, the calf RNase HI might have made a single specific cleavage at a site just upstream of the RNA-DNA junction.

To distinguish between these two possibilities, we modified the substrate to contain an internally ³²P-labeled RNA primer 21 nucleotides long, extended by unlabeled DNA. This substrate allowed us to monitor the RNA degradation products generated by incubation with RNase HI (Fig. 4). With increasing concentrations of RNase HI, the major product released was an RNA molecule 20 nucleotides long. This length is one nucleotide shorter than the RNA segment that was hybridized and extended to generate the full-length RNA-DNA substrate. Although obtained with one sequence, these results suggest that calf RNase HI makes a specific cleavage at the phosphodiester bond one nucleotide 5' of the RNA-DNA junction. Additional preliminary results indicate that the same cleavage specificity occurs at other RNA-DNA junction sequences (data not shown). We did observe a very low level of shorter oligoribonucleotides when high RNase HI concentrations were used to degrade the substrate. These products may be the result of multiple enzymes binding to a single substrate or degradation of the released RNA after the initial digestion. Observation of the high specificity of cleavage suggests that RNase HI is designed to recognize the initiator RNA of Okazaki fragments.

Digestion by RNase HI results in a molecule containing a single 5' ribonucleotide followed by DNA. This ribonucleotide must be removed to prepare the segments for DNA-to-DNA ligation. Removal of the last ribonucleotide can be accomplished by the 5'-to-3' exonuclease. Fig. 5 shows that the combined action of the RNase HI and the 5'-to-3' exonuclease completely removed the initiator RNA segment. Lanes 2 and 6 of Fig. 5 show the removal of the first 12 of the 13 ribonucleotides by RNase HI alone, and lanes 3 and 7 confirm the inability of the 5'-to-3' exonuclease to demonstrate any nucleolytic activity against the intact substrate. One possible explanation for this result is that the 5'-to-3'

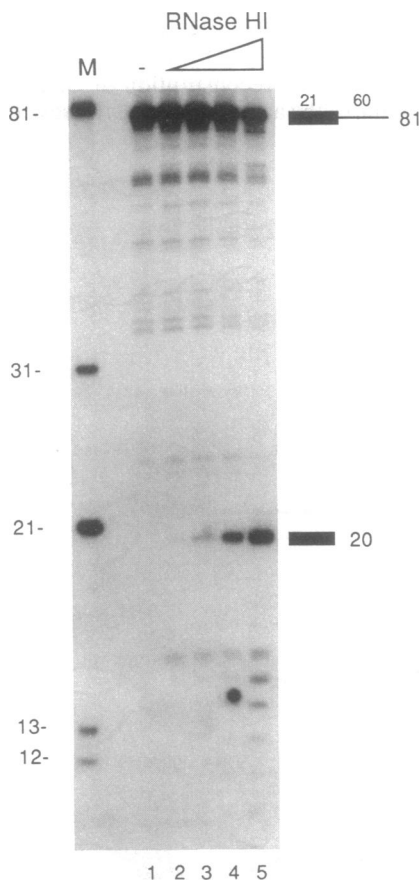


FIG. 4. RNase HI makes a site-specific cut one nucleotide 5' of the RNA-DNA junction. The substrate shown in Fig. 1 was modified to contain a 21-nucleotide RNA transcript internally labeled with [α -³²P]UMP. The transcript was extended to the full length of a template that had not been cut by *Nar*I. This substrate was incubated with the same concentrations of RNase HI listed in Fig. 3. The reaction mixtures were incubated and processed as described in Fig. 2 except that the products were separated on a 15% polyacrylamide/7 M urea gel. The RNA molecular size markers in lane M were generated by transcription *in vitro* from the T3 RNA polymerase promoter of the plasmid pBS(+) digested with *Hind*III to generate a 13-nucleotide (nt) RNA, with *Pst*I for a 21-nt RNA, with *Sal*I for a 31-nt RNA, or with the 130-nt PCR fragment to generate an 81-nt RNA.

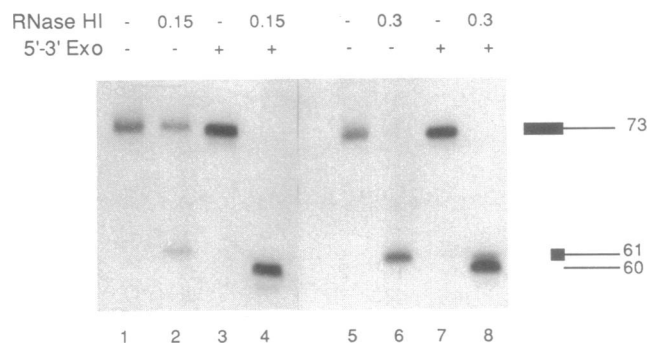


FIG. 5. The combined action of RNase HI and 5'-to-3' exonuclease is sufficient for complete removal of RNA primers. The 3'-labeled substrate was incubated with 0.3 units of 5'-to-3' exonuclease and with the units of RNase HI designated in the figure. Reactions were incubated and processed as described in Fig. 2. Sizes are shown in nucleotides.

exonuclease could not initiate cleavage at the triphosphorylated 5' terminus of the initiator RNA primer. However, as shown in lanes 4 and 8, the single ribonucleotide left after RNase H digestion, having a 5' monophosphate, was effectively removed by the 5'-to-3' exonuclease. The first DNA nucleotide at the 5' end was also removed, as seen faintly in Fig. 5, lanes 4 and 8. Considerably more was made under conditions of high 5'-to-3' exonuclease concentration or extended incubation times (data not shown).

We previously found that the 5'-to-3' exonuclease was greatly stimulated by the presence of a primer hybridized to DNA just upstream of the primer being digested, separated only by a nick (17). It is likely that the RNA segment cut by the RNase HI temporarily remains bound to the template and stimulates exonucleolytic removal of the last ribonucleotide. This interpretation also explains why the removal of the first DNA nucleotide is slower. If synthesis were occurring from the upstream primer, the RNA segment should dissociate, but the advancing DNA terminus could also serve to stimulate the exonuclease.

Since we had identified the enzymes capable of removing the initiator RNA, we could attempt to combine RNA removal with joining of the two remaining DNA segments (Fig. 6). We previously have demonstrated that DNA polymerase α , δ , or ϵ , in conjunction with 5'-to-3' exonuclease, could fill a gap to support ligation (22). We and others have proposed that DNA polymerase ϵ performs lagging-strand synthesis (8-10). Therefore, we employed DNA polymerase ϵ in reactions with RNase HI, 5'-to-3' exonuclease, and DNA ligase I. This combination of purified enzymes resulted in removal of the initiator RNA and joining of the two DNA segments (Fig. 6, lane 3). The polymerase ϵ preparation was contam-

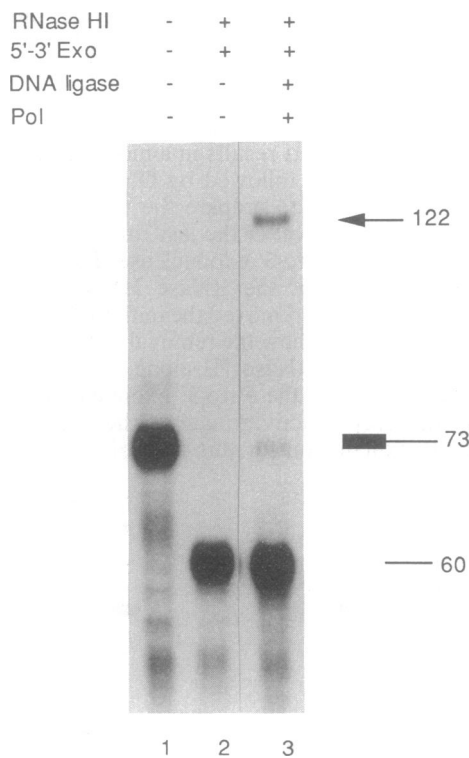


FIG. 6. Complete lagging-strand processing by purified proteins. The lagging-strand substrate was prepared for this experiment by hybridizing the 13-nucleotide RNA to the single-stranded 130-nucleotide template and extending and labeling the 3' terminus. The substrate was incubated with DNA polymerase ϵ (0.1 units), DNA ligase I (0.1 units), RNase HI (0.3 units), and 5'-to-3' exonuclease (0.3 units) as shown in the figure. Reaction mixtures were incubated and processed as described in Fig. 2. The final ligation product, 122 nucleotides in length, is indicated by an arrow.

inated with a small amount of RNase H and exonuclease, but this did not influence the results since the nucleases were added in excess. Also, ligation product observed in our reactions did not contain any RNA nucleotide as determined by resistance to treatment with 1 M KOH following the joining reaction (data not shown). These reactions are a reconstitution of enzymatic activities required for the completion of lagging-strand DNA replication.

We have used DNA polymerase ϵ for the complete reaction shown here. However, we emphasize that polymerase ϵ is not necessarily the enzyme used *in vivo*. The action of RNase HI and 5'-to-3' exonuclease generates two DNA primers separated by a short gap hybridized to a DNA template. We have shown (22) that calf DNA polymerase α , δ /proliferating cell nuclear antigen (PCNA), or ϵ is capable of closure of such a gap to a nick that can be joined by DNA ligase I. Waga and Stillman (13) found that PCNA, replication factor C, and replication protein A blocked ligation in a substrate similar to that used here when DNA polymerase α was the only polymerase present. This indicated a need to switch to polymerase δ or ϵ .

The mammalian 5'-to-3' exonuclease, DNA ligase I (12), and possibly RNase HI were found to be necessary for formation of form I simian virus 40 DNA (11-13). Here we show the reactions by which RNase HI acts with the 5'-to-3' exonuclease to remove the RNA primer of Okazaki fragments.

In summary (Fig. 7), reconstitution of Okazaki-fragment processing requires the action of two nucleases. The reaction

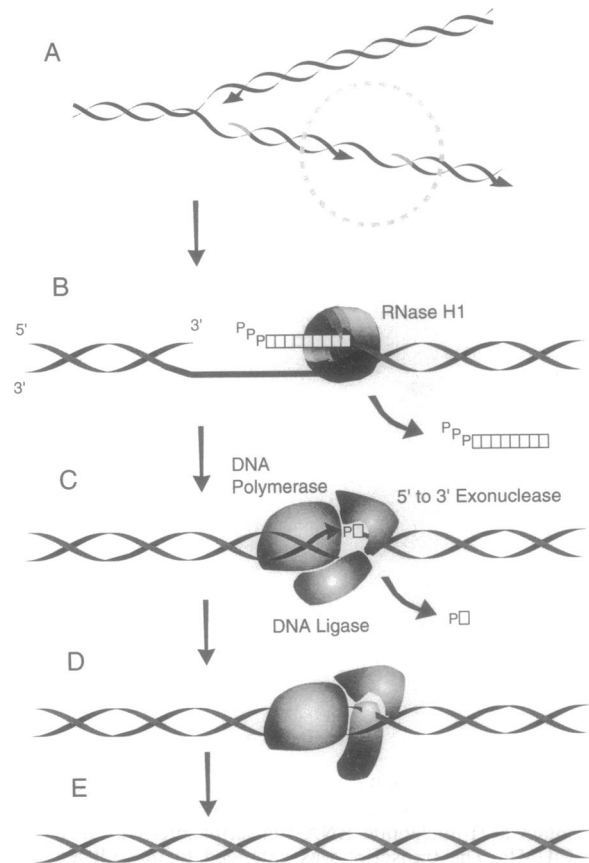


FIG. 7. Model for the completion of mammalian lagging-strand DNA replication. The initial reaction involves a site-specific cleavage by RNase HI at the phosphodiester bond of the nucleotide 5' of the RNA-DNA junction. Removal of the last 5' ribonucleotide is performed by 5'-to-3' exonuclease and results in a 5' phosphorylated DNA terminus. Action of the exonuclease is likely to be stimulated by the cleaved segment of RNA or by elongation of the upstream primer as shown. A nick is then generated between the two DNA termini, which are joined by DNA ligase I.

is very specific in that on our substrate only two cleavages are required. RNase HI makes a specific endonucleolytic cut, leaving a single ribonucleotide that is removed by the 5'-to-3' exonuclease. The nucleases can act in the same reaction with DNA polymerase ϵ and DNA ligase I to complete the reaction of Okazaki-fragment processing. The simian virus 40 *in vitro* DNA replication system has identified and assigned roles for a number of proteins involved in steps of eukaryotic DNA replication (1, 11–13). We have added to this knowledge by identifying steps involved in removal of initiator RNA primers prior to joining of Okazaki fragments, representing the final steps in mammalian DNA replication.

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