Role of Calf RTH-1 Nuclease in Removal of 5'-Ribonucleotides during Okazaki Fragment Processing †

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Role of Calf RTH-1 Nuclease in Removal of 5′-Ribonucleotides during Okazaki Fragment Processing†

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ABSTRACT: The role of the exonucleolytic activity of the calf 5′ to 3′ exo/endonuclease, a RAD2 homolog 1 (RTH-1) class nuclease, in lagging-strand DNA replication has been examined using model Okazaki fragment substrates. These substrates exemplify the situation in Okazaki fragment processing which occurs after the initiator RNA primer is cleaved off, and released intact, by calf RNase HI, leaving a single ribonucleotide at the 5′ end of the RNA−DNA junction. This final RNA is then removed by the calf RTH-1 nuclease [Turchi et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9803−9807]. The cleavage specificity of calf RTH-1 nuclease for different junction ribonucleotides was compared. These were removed without the usual requirement of calf RTH-1 for an immediately adjacent upstream primer. In most cases, the presence of an upstream DNA or RNA primer, separated from the monoribonucleotide−DNA segment by either a nick or a gap, reduced the efficiency of removal of the monoribonucleotide compared to the removal seen with no upstream primer. Substrates in which the monoribonucleotide−DNA segment had been replaced by an oligomer of the same sequence but consisting entirely of DNA also exhibited upstream primer inhibition. Results with various sequences indicated that the upstream primer is generally inhibitory for ribonucleotide removal but is sometimes neutral. For deoxynucleotide removal it could be stimulatory, neutral, or inhibitory. Possible reasons for the unexpected lack of upstream primer dependence have been explored. The ratio of RNase HI to RTH-1 was also shown to be critical for both enzymes to work together efficiently. These results suggest that regions of upstream primer inhibition within the genome may play a role in determining the mechanism by which mammalian Okazaki fragments are processed.

During Okazaki fragment processing, initiator RNA cleavage is the first and a necessary step to complete lagging-strand DNA replication (Kornberg & Baker, 1992). In *Escherichia coli*, the removal of initiator RNA requires the intrinsic 5′ to 3′ exonuclease activity of DNA polymerase I, with the assistance of RNase H (Funnel et al., 1986). In eukaryotes, none of the known DNA polymerases have an intrinsic 5′ to 3′ exonuclease activity (Bambara & Huang, 1995). Reconstitution of simian virus 40 replication *in vitro* suggested that initiator RNAs were completely removed by a combination of a 44-kDa 5′ to 3′ exonuclease and the RNase HI (Ishimi et al., 1988). A similar observation was made using enzymes purified from mouse cells (Goulian et al., 1990). However, definition of the specific reactions performed by the two nucleases in processing the initiator RNA was only very recently reported from our laboratory, using purified calf enzymes (Turchi et al., 1994). We found that calf RNase HI makes a structure-specific cleavage, removing the RNA primer intact but leaving one ribonucleotide at the RNA−DNA junction, which is then removed by a calf 5′ to 3′ exonuclease, now designated the RTH-1[1] class nuclease. DNA polymerization then creates the substrate for ligation (Turchi et al., 1994).

Given a DNA substrate having two primers annealed to a template and separated by a nick, the calf RTH-1 nuclease will remove a nucleotide from the downstream primer (Murante et al., 1994). Further cleavage was found to be inefficient, suggesting that the gapped structure is a poor substrate for the exonuclease. When the downstream primer had a 5′ unannealed tail, with an immediately adjacent upstream primer, the exonuclease displayed endonuclease activity, cleaving away the tail as an intact segment (Murante et al., 1994). Surprisingly, the enzyme also has a strict 5′ end requirement for its endonucleolytic activity (Murante et al., 1995). It must recognize a 5′ end of an unannealed tail, before sliding along the single strand to the point of cleavage, which leaves a nick or a one nucleotide gap between upstream and downstream primers. The enzyme was also found to slide past adducts on the tail and cut behind them, suggesting a mechanism of DNA repair (Murante et al., 1995).

A counterpart of the mammalian nuclease was found in *Saccharomyces cerevisiae* and named the RAD2 homolog

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[1] Abbreviations: RTH-1, RAD2 homolog 1; FEN-1, flap endonuclease 1; pol, polymerase; SV40, simian virus 40; NP-40, Nonidet P40; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taq, *Thermus aquaticus*; Tth, *Thermus thermophilus*; Tfl, *Thermus flavus*; exo, exonuclease; endo, endonuclease, nt, nucleotide.

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or RTH-1 protein (Sommers et al., 1995). Yeast RTH-1 is highly homologous to the yeast RAD2 family of proteins, noted for their role in DNA repair (Prakash et al., 1993). Null mutants of yeast RTH-1 are temperature-sensitive for DNA replication and have a hyperrecombination phenotype (Sommers et al., 1995). This is consistent with a defect in joining of Okazaki fragments, since an abundance of nicks and gaps in the chromosomal DNA promotes recombination. Such mutants are also sensitive to methylmethanesulfonate-derived base adducts, indicating a role for the enzyme in DNA repair (Sommers et al., 1995). Yeast RTH-1 was also necessary to maintain stability of DNA repeats, suggesting that mutation of the human counterpart could increase risk for colorectal cancer through impairment of the protective mismatch repair pathway (Johnson et al., 1995).

Harrington and Lieber (1994a) identified an enzyme from mouse cells with similar substrate specificity, which they named flap endonuclease 1 (FEN-1). Activity and gel mobility shift results indicated that an upstream DNA primer adjacent to the unannealed, or flap, strand is required for efficient FEN-1 substrate recognition, binding, and cleavage activity (Harrington & Lieber, 1995). The gene encoding mouse FEN-1 has been cloned and sequenced (Harrington & Lieber, 1994b). The human FEN-1 has also been studied in several laboratories and given a variety of different names, including maturation factor 1, MF-1 (Waga et al., 1994), DNase IV (Robins et al., 1994), and the human homolog of the *Schizosaccharomyces pombe* rad2 gene product (Murray et al., 1994). DNase IV has structural and functional homology with the 5′-nuclease domain of *E. coli* DNA polymerase I (Robins et al., 1994). Like FEN-1, the intrinsic 5′ to 3′ exonucleases of *E. coli* DNA pol I and of Taq polymerase can also act as structure-specific endonucleases (Lyamichev et al., 1993). The human FEN-1 gene has been independently cloned by Murray et al. (1994) and Hiraoka et al. (1995) and localized at human chromosome 11q12 (Hiraoka et al., 1995).

Exonucleolytic activity of the calf RTH-1 nuclease was shown to be stimulated by synthesis from an upstream primer, presumably due to continuous generation of the preferred nicked substrate (Siegal et al., 1992). RTH-1 can work with DNA polymerases α, δ, or ϵ to carry out nick translation (Turchi & Bambara, 1993). The presence of calf RNase HI, RTH-1 nuclease, DNA polymerase ϵ, and DNA ligase I results in initiator RNA primer removal, gap closure, and ligation on a model Okazaki substrate (Turchi et al., 1994). We found that in the reconstituted reaction, the RNase HI structure-specific cleavage is RNA sequence- and length-independent and that the reaction is solely dependent on Mg^{2+} (Huang et al., 1994). In the current report, we further define the substrate specificity of the RTH-1 nuclease for removal of the 5′ junction ribonucleotide left after RNase HI action. Surprisingly, results show that the exonuclease does not have an upstream primer requirement for removing the remaining ribonucleotide. In fact, presence of an upstream primer is often inhibitory. Interestingly, similar results were seen for substrates with downstream primers consisting entirely of DNA. These observations have caused us to reevaluate the upstream primer requirement in general. The likelihood of inhibition by upstream primers has helped us to formulate additional details of the mechanism by which mammalian Okazaki fragments are processed.

EXPERIMENTAL PROCEDURES

Materials

Unlabeled nucleotides were purchased from Pharmacia Biotech Inc. (Piscataway, NJ), and radiolabeled nucleotides (3000 mCi/mmol) were from DuPont New England Nuclear (Boston, MA). Oligonucleotides were synthesized by Genosys Inc. (The Woodlands, TX). T3 RNA polymerase, T4 polynucleotide kinase, and Sequenase (version 2.0) were obtained from U.S. Biochemical Corp. (Cleveland, OH). All the restriction enzymes were purchased from New England Biolabs (Beverly, MA). pBS+ plasmid was from Stratagene Cloning Systems (La Jolla, CA). RNase inhibitor and snake venom phosphodiesterase were from Boehringer Mannheim (Indianapolis, IN). For calf RNase HI purification, the ([^{3}H]-rA)−poly(dT) substrate was made by annealing a ([^{3}H]rA) primer, purchased from Amersham Life Science Inc. (Arlington Heights, IL) to a poly(dT) template obtained from Midland Scientific (Midland, TX). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Methods

Enzyme Purification. Calf RTH-1 was purified through hydroxyapatite chromatography as described previously (Murante et al., 1994). To remove contaminating RNase H activity, this preparation was further purified. First, over an 8-mL heparin−Sepharose column developed with an 80-mL gradient from 100 to 750 mM KCl, the nuclease was purified 2-fold. This pool was then subjected to chromatography on a 1-mL CM-Sepharose column using a 10-mL gradient from 100 to 750 mM KCl. Final specific activity was 194 000 units/mg, with 1 unit defined as the amount of nuclease required to release 5 pmol of [^{32}P]TMP from 5′-[^{32}P]dT_{16}−dA_{2000} in 15 min at 37 °C.

Calf thymus RNase HI was purified by the procedure of Eder and Walder (1991). Details of the purification have been described in Turchi et al. (1994) except that the heparin−Sepharose column chromatography was carried out directly following phenyl-Sepharose chromatography, and only Mono S was employed after blue Sepharose chromatography. The resultant active fractions were dialyzed in final dialysis buffer which contained 50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 2 mM EDTA/EGTA, and 50% glycerol. The enzyme remains stable when stored at −80 °C for several months. The final pool of RNase HI had a specific activity of 150 000 units/mg as measured on a poly[([^{3}H]rA)−(dT)] substrate according to Eder and Walder (1991).

Substrate Construction. The sequences of the primers and structures of the substrates used in this study are described in Table 1 and in the figures. The nomenclature of the substrates is devised as follows: the letter A, G, C, U, or T in a substrate name indicates the base of the 5′ nucleotide to be removed by the RTH-1 nuclease. When the substrate is named only by this single letter, there is no upstream primer and the 5′ residue of the primer is a ribonucleotide. When this letter is followed by an N, the substrate has an upstream primer forming a nick with the downstream primer. When this letter is followed by a G, the substrate has an upstream primer which leaves a gap. A 2 following the letter indicates that the substrate is the second substrate to have the particular base at the 5′ end of a primer, now located in a different

Table 1

	length (nt)	sequence
		(A) Primer Sequences (5′ to 3′)[a]
1	25	CTCACTAAAGGGAACAAAAGCTTGC
2	25	CACTAAAGGGAACAAAAGCTTGCAT
3n	20	GGGAACAAAAGCTTGCATGC
3g	24	AGCTCGAAATTAACCCTCACTAAA
4	20	TCACTAAAGGGAACAAAAGC
5	12	**GGGAACAAAAGC**
6	25	CGCCAGGGTTTTCCCAGTCACGACC
7	35	**A**TGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTA
8	33	**G**CCTGCAGGTCGACTCTAGAGGATCCCCGGGTA
9	31	**C**TGCAGGTCGACTCTAGAGGATCCCCGGGTA
10	39	**U**TGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTA
11	36	**C**ATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTA
12[b]	34	**U**GCCTGCAGGTCGACTCTAGAGGATCCCCGGGTA
17	51	**GGGAACAAAAGCU**TGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTA
18	51	**GGGAACAAAAGCTTGCATGCC**TGCAGGTCGACTCTAGAGGATCCCCGGGTA
		(B) Templates (3′ to 5′)
T1	75	TCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTCGAACGTACGGACGTCCAGCTGAGATCTCCTAGGGGCCCAT
T2	64	GCGGTCCCAAAAGGGTCAGTGCTGGAACGTACGGACGTCCAGCTGAGATCTCCTAGGGGCCCAT

(C) Structures[c]

substrate	template	UP	DP	substrate	template	UP	DP	substrate	template	UP	DP
AN	T1	1	7	UN2	T1	1+	12	uC	T1	3n+	9
A	T1		7	U2	T1		12	uG	T1	2+	8
CN	T1	3n	9	dAN	T1	1	13	uU	T1	4+	10
C	T1		9	dA	T1		13	SWN	T2	6	10
CG	T1	3g	9	dCN	T1	3n	15	SW	T2		10
CN2	T1	4+	11	dC	T1		15	dSWN	T2	6	14
C2	T1		11	dGN	T1	2	14	dSW	T2		14
GN	T1	2	8	dG	T1		14	P	T1		18
G	T1		8	dTN	T1	4	16	H	T1		17
UN	T1	4	10	dT	T1		16	R	T1	5	
U	T1		10	uA	T1	1+	7	UR	T1	5	10

[a] Nucleotides shown in boldface type are RNA; others are DNA. Primers 1−6 are upstream primers [UP]; primers 7−18 are downstream primers [DP]. [b] Primers 13−16 are the same oligomers as 7−10, respectively, but consist entirely of DNA, including the 5′-most nt, i.e., r**U** → dT, etc. [c] + indicates that nucleotides have been added to the given primer as described in the text.

position on the template. A d preceding the letter indicates the 5′ residue is a deoxynucleotide. A u preceding the letter indicates that the 5′ nucleotide has been unannealed due to extension of the upstream primer by a single nucleotide. SW indicates a substrate in which a second template was used in order to "swap" the upstream primer regions. P is a substrate made using restriction enzyme *Pst*I; construction of H used *Hin*dIII. R indicates use of a primer made completely of RNA, with or without another downstream primer.

T4 polynucleotide kinase was used to 5′-phosphorylate the downstream primers as specified using [^{32}P]ATP according to the manufacturer's protocol. Substrates designed to assay 5′ to 3′ exonuclease junction ribonucleotide cleavage activity were constructed by annealing the appropriate 5′ end-labeled synthetic monoribonucleotide−DNA segment, with or without the appropriate upstream primer, to the template and then isolated via 12% native gel electrophoresis (Sambrook et al., 1989). The substrates were eluted from the gel using elution buffer (0.5 M ammonium acetate, 0.1% sodium dodecyl sulfate, and 0.1 mM EDTA), ethanol-precipitated, and resuspended in 1× annealing buffer (50 mM Tris, pH 8, 10 mM magnesium acetate, 50 mM NaCl, and 1 mM DTT). Substrates with downstream primers consisting entirely of DNA were made similarly. For the endonucleolytic cleavage substrates, the upstream primer was first annealed and extended with 10 units of Sequenase by a single deoxynucleotide. Thus, when the downstream primer was annealed, the 5′ nucleotide would be in rapid equilibrium with the 3′ nucleotide of the upstream primer and would presumably be accessible for endonucleolytic cleavage at least 50% of the time. The upstream primers for substrates UN2 and CN2 were made similarly by first extending the upstream primers for substrates AN and UN by 1 and 3 nucleotides, respectively, so as to produce a nick with the downstream primer. The 13mer RNA primer for substrate R was made by runoff transcription from a T3 promoter using the restriction enzyme *Hin*dIII to linearize pBS+ plasmid as template. The reaction conditions were the same as used in Huang *et al*. (1994).

Enzyme Assays. The calf RNase HI assay was performed in a buffer containing 20 mM HEPES (pH 7.0), 50 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, and 0.001% Nonidet P-40 in a volume of 10 μL. Reactions were stopped by the addition of 98% formamide and 10 mM EDTA (pH 8.0) with 0.01% (w/v) each xylene cyanol and bromophenol blue. Reaction mixtures were heated at 90 °C for 5 min and then separated by 12% polyacrylamide/7 M urea gel electrophoresis (Sambrook et al., 1989). Products were visualized by autoradiography using a double Dupont Cronex Lightning Plus intensifying screen at −80 °C.

The calf RTH-1 exo/endonuclease assay was performed in a solution containing 60 mM BisTris (pH 7.0), 5% glycerol, 0.1 mg/mL BSA, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 70 fmol of substrate in a volume of 168 μL. Reactions were initiated by the addition of 1.2 units of enzyme and incubated at 37 °C. Reactions were stopped at the appropriate times by adding 25 μL of sample to an equal

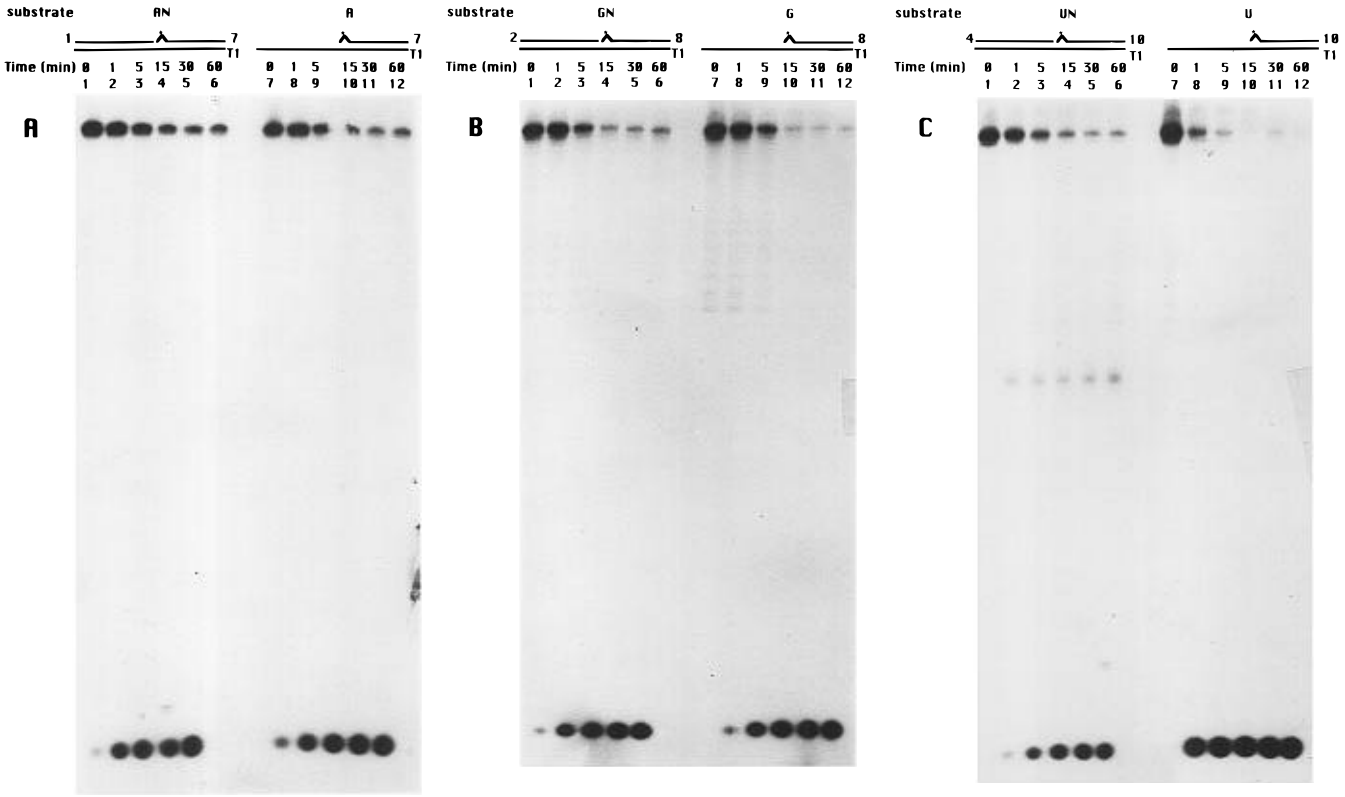

FIGURE 1: Removal of 5′ monoribonucleotides by calf RTH-1 nuclease with or without an upstream DNA primer. Assays measuring exonucleolytic cleavage of the 5′ end of a downstream primer are depicted. For this and subsequent figures, percent of labeled terminal mononucleotides released from starting material by calf RTH-1 nuclease after a 30-min incubation was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software and is given in parentheses as a mean of 3−6 experiments ± standard error. (A) Lanes 1−6 show a time course for removal of monoribonucleotide A from a substrate with an upstream primer forming a nick with the downstream primer (65.00 ± 1.04); substrate lacking an upstream primer is shown in lanes 7−12 (73.60 ± 1.84). (B) Lanes 1−6 show a time course for removal of monoribonucleotide G from a substrate with an upstream primer forming a nick with the downstream primer (51.10 ± 10.45); substrate lacking an upstream primer is shown in lanes 7−12 (70.51 ± 2.84). (C) Lanes 1−6 show a time course for removal of monoribonucleotide U from a substrate with an upstream primer forming a nick with the downstream primer (48.39 ± 2.18); substrate lacking an upstream primer is shown in lanes 7−12 (81.36 ± 2.72). Substrate structures and oligonucleotide compositions are shown above the appropriate lanes. In this and subsequent figures, the numbers next to the primers refer to the key to oligonucleotide sequences found in Table 1. Downstream primers were 5′ radiolabeled, and reaction conditions were as described under Methods. The upper band represents starting substrate and the lower band represents the removed monoribonucleotide.

volume of 98% formamide and 10 mM EDTA (pH 8.0) with 0.01% (w/v) each xylene cyanol and bromophenol blue and heating at 90 °C for 5 min. Zero time controls were removed from the reaction mixture before addition of enzyme. Products were separated by 18% polyacrylamide/7 M urea gel electrophoresis (Sambrook et al., 1989), visualized by autoradiography using a double Dupont Cronex Lightning Plus intensifying screen at −80 °C, and analyzed via Phosphorimager.

Calculation of RNase HI to RTH-1 Molar Ratio. Protein concentrations were determined by the method of Bradford (1976) using protein assay dye from Bio-Rad. Molecular weights of proteins were determined by running against known molecular weight standards on a protein gel. Multiplying the amounts of protein used by the molecular weights allowed determination of the ratio of molar amounts used.

RESULTS

Removal of 5′ Terminal RNA−DNA Junction Monoribonucleotides by Calf RTH-1 Nuclease Is Inhibited by the Presence of an Upstream DNA Primer. The cleavage specificity of calf RTH-1 nuclease for different junction ribonucleotides was compared, using substrates AN, GN, UN, and CN. These substrates have structures which should appear during Okazaki fragment processing. RNase H1 should remove most of the initiator RNA, leaving a single ribonucleotide at the 5′ terminus. This ribonucleotide must be removed by the RTH-1 nuclease prior to completion of elongation of the upstream Okazaki fragment, forming a nick flanked by deoxynucleotides that can be ligated. Removal of ribonucleotides with any of the four bases from the 5′ end of the downstream primer showed varying degrees of inhibition in the presence of the upstream primer. Figure 1A shows that the efficiency of removal of a monoribonucleotide, in this case A, is reduced compared to the removal seen with no upstream primer. Similar results were obtained for bases G (Figure 1B), U (Figure 1C), and C (Figure 2). Though in some cases the upstream primer inhibition is slight, it is statistically significant and the extent of inhibition in each case is evident from PhosphorImager analysis presented in the figure legends.

Upstream Primer Inhibition Occurs Even If the Upstream Primer Is Separated from the Downstream Primer by a Gap Rather than a Nick. Figure 2 shows that efficiency of exonucleolytic cleavage of monoribonucleotide C is significantly reduced not only by generation of a nicked substrate (lanes 5−8) but also by annealing of a 24mer DNA to leave a 20-nucleotide-long gap (lanes 9−12). Reduced cleavage

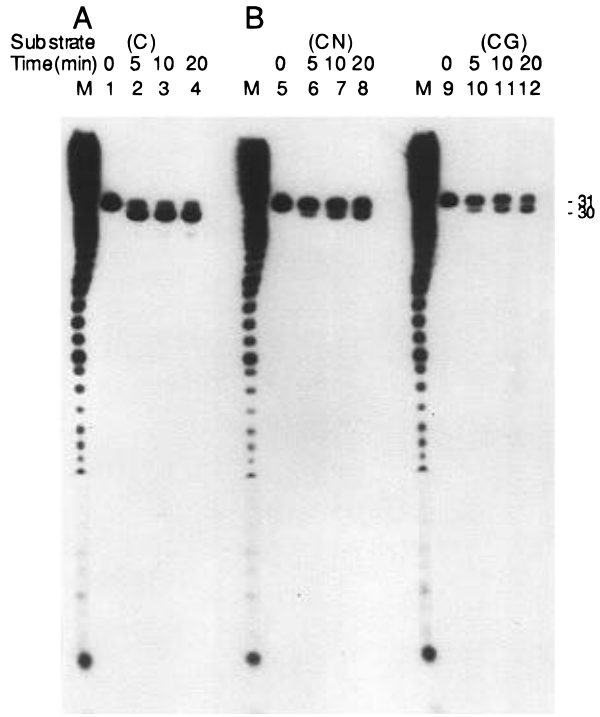

FIGURE 2: Time course of calf RTH-1 nuclease cleavage of the 5′ monoribonucleotide C remaining after RNase HI degradation of an initiator RNA, without an upstream DNA primer versus with a nicked or gapped upstream DNA primer. Quantitation of mononucleotide release is given in parentheses as described in Figure 1. (A) Time course of RTH-1 nuclease cleavage in the absence of an upstream primer (83.70 ± 1.48). (B) Time course of RTH-1 nuclease cleavage in the presence of an upstream primer, separated by either a nick (lanes 5−8) (60.78 ± 6.50) or a gap (lanes 9−12). Substrates are shown above the appropriate lanes. See Table 1 for oligonucleotide compositions. Downstream primers were labeled internally with ^{32}P. Therefore, the visible cleavage product is one nucleotide shorter than the starting primer. The amount of calf RTH-1 nuclease used was 0.3 unit/lane. Otherwise, reaction conditions were as described under Methods. Lane M shows a 5′ ^{32}P-labeled 39mer of unrelated DNA after limited snake venom phosphodiesterase digestion. Size markers at the right indicate product length in nucleotides.

was also observed for monoribonucleotide U with both nick- and gap-containing substrates (data not shown).

In vivo, monoribonucleotide-terminated primers are expected to be made by RNase HI-directed cleavage of the initiator RNA segments of Okazaki fragments (Turchi et al., 1994). Thus, to verify that a monoribonucleotide−DNA produced by this enzymatic reaction behaved equivalently to the chemically produced substrates that we had used in Figure 1, we constructed substrate C for this experiment. We began with substrate P, which has an RNA primer made exclusively by runoff transcription from a T3 promoter using the restriction enzyme *Pst*I to linearize pBS+ plasmid as template. The RNA primer was elongated on T1 with labeled deoxynucleotides using Sequenase. RNase HI was then used to cleave the RNA, leaving a 5′ monoribonucleotide-terminated DNA 31 nucleotides long. The 31-nucleotide fragment was then purified by gel electrophoresis. The presence of a 5′ monoribonucleotide in a sample of the 31mer was confirmed by alkaline digestion (data not shown). The intact 31mer was then reannealed to template T1, with or without upstream primer. Figure 2 shows that, in the presence of RTH-1, a significant proportion of the monoribonucleotide was removed after only 5 min, especially for the substrate which lacked an upstream primer. The same observation was also made using substrate U, constructed by RNase HI cleavage of substrate H (data not shown).

Upstream Primer Inhibition Also Occurs If the 5′-Terminal Nucleotide Is a Deoxynucleotide Rather than a Ribonucleotide. Our previous studies of the specificity of the calf RTH-1 nuclease demonstrated that the cleavage was greatly stimulated by annealing an upstream DNA primer directly adjacent to the cleavage point; *i.e.*, the enzyme favors a nicked substrate (Murante et al., 1994). On the basis of these observations, the inhibition by an upstream DNA primer shown above is quite surprising. It was surmised that the inhibition occurs because the nucleotide being removed is a ribonucleotide rather than a deoxynucleotide. If true, these observations suggest that steps in initiator RNA removal are coordinated with respect to timing, so that ribonucleotide removal is complete before extension of the upstream Okazaki fragment approaches the junction point, in preparation for ligation. To test this hypothesis, substrates were created in which the downstream monoribonucleotide−DNA segment was replaced by an oligomer of the same sequence but consisting entirely of DNA, including the 5′-terminal nucleotide (substrates dAN, dGN, dCN, and dTN). Unexpectedly, removal of three of the four DNA nucleotides showed upstream primer inhibition similar to that of all four RNA nucleotides. Figure 3A shows that the efficiency of removal of a 5′ dT is reduced compared to the removal seen with no upstream primer. Similar results were obtained for bases dC and dA. Figure 3B shows that removal of 5′ dG exhibited the expected upstream primer stimulation. Thus, at least in one case, the presence of a 5′ ribonucleotide allows inhibition by an upstream primer, whereas the corresponding deoxynucleotide does not. The PhosphorImager quantitation presented in the legends of Figures 1, 2, and 3 summarizes the data for all eight deoxy- and ribonucleotides, with or without an upstream primer. These results indicate that different junction nucleotides are removed by calf RTH-1 nuclease with different efficiencies. Significantly, they also show that upstream primers often can be inhibitory, even on fully DNA substrates.

Shifting the Downstream Primer on the Template Allows for Upstream Primer Stimulation of Removal of a 5′ Monoribonucleotide. The above results suggested that upstream primer-directed inhibition of exonucleolytic cleavage did not occur solely because the 5′ nucleotide was RNA rather than DNA. We then considered that the sequence around a potential cleavage site, or its particular location on the template, influences its requirement for an upstream primer. To test this hypothesis, substrates were created in which the downstream monoribonucleotide−DNA segment was shifted to a new position on the same template. Thus, it was possible to observe the removal of the 5′-terminal U and C when they were located in a different environment. Interestingly, removal of C at the new annealing position was very sensitive to upstream primer inhibition (Figure 4A), much more than at the previous location (data not shown), while removal of U actually showed slight upstream primer-directed stimulation (Figure 4B), contrary to all the previous observations of 5′ monoribonucleotide removal.

Changing the Upstream Primer−Template Region Allows for Upstream-Primer-Directed Stimulation of Cleavage of a 5′ Monodeoxynucleotide but Not a 5′ Monoribonucleotide. We surmised that the upstream primer region sequence itself could influence the inhibitory *versus* stimulatory action of

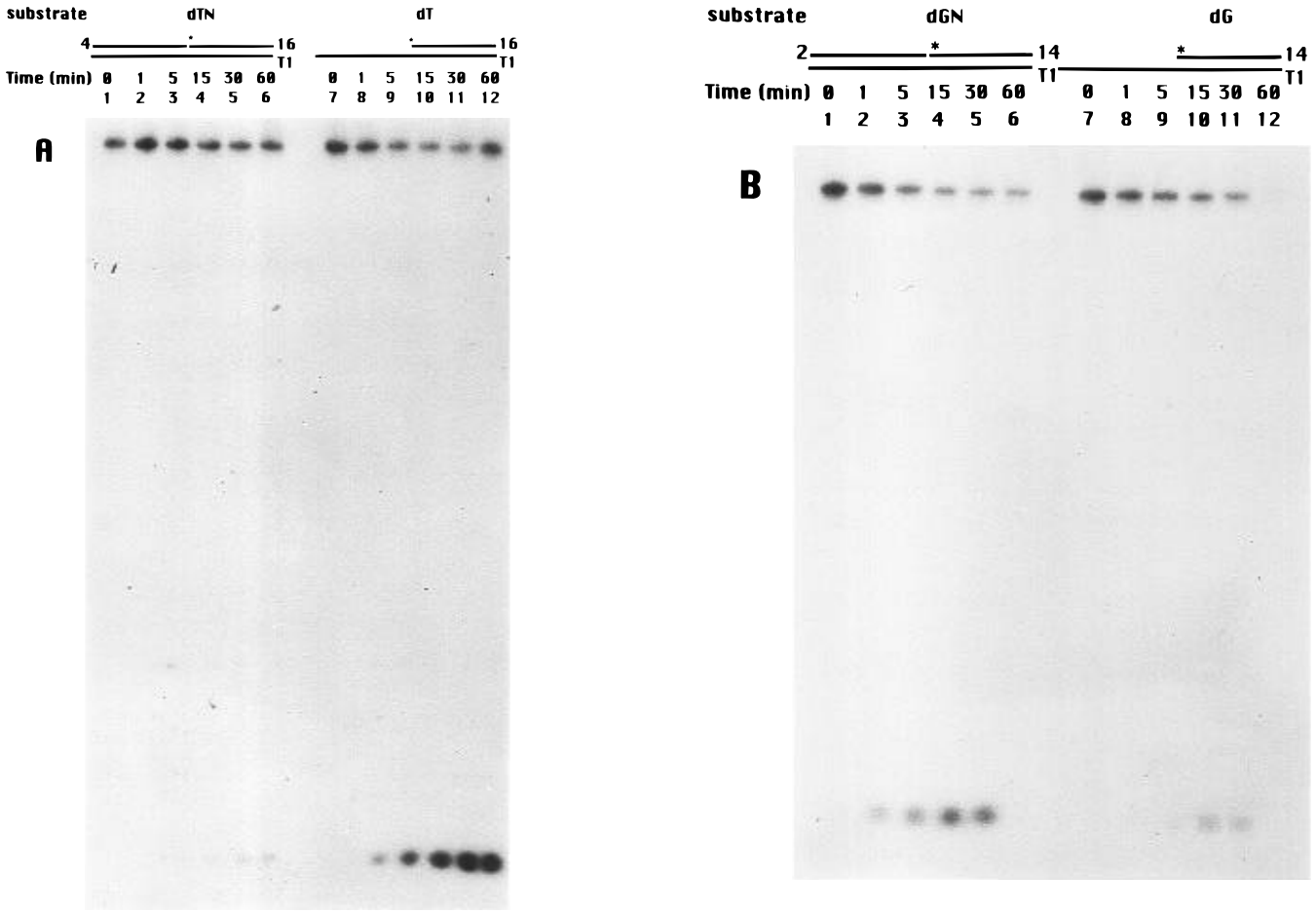

FIGURE 3: Removal of 5′ deoxynucleotides by calf RTH-1 nuclease with or without an upstream DNA primer. Assays measuring exonucleolytic cleavage of the 5′ end of a downstream primer are depicted. (A) Lanes 1−6 show a time course for removal of dT from a substrate with an upstream primer forming a nick with the downstream primer (12.01 ± 3.37); substrate lacking an upstream primer is shown in lanes 7−12 (70.55 ± 2.18). (B) Lanes 1−6 show a time course for removal of dG from a substrate with an upstream primer forming a nick with the downstream primer (50.09 ± 4.66); substrate lacking an upstream primer is shown in lanes 7−12 (25.32 ± 3.41). Substrate structures and oligonucleotide compositions are shown above the appropriate lanes. Downstream primers were 5′ radiolabeled, and reaction conditions were as described under Methods. The upper band represents starting substrate and the lower band represents the removed monodeoxyribonucleotide. Not shown: dA + upstream primer (13.10 ± 4.21), dA − UP (53.80 ± 6.82), dC + UP (0.09 ± 2.12), dC − UP (42.35 ± 6.08).

the upstream primer. Thus, template T2 was created, for which the downstream primer was the same as in substrates UN and dTN, while the upstream primer region was altered (substrates SW, SWN, dSW, and dSWN). We used an upstream sequence that was present in a fully DNA substrate having upstream primer-directed stimulation of cleavage (Murante et al., 1994). Intriguingly, in this situation, removal of T was stimulated by the upstream primer and removal of U was inhibited (data not shown), whereas these cleavages had both been inhibited in the presence of the upstream primer on template T1. A reasonable overall conclusion is that the upstream primer is generally inhibitory for ribonucleotide removal but is sometimes neutral. For deoxynucleotide removal it could be stimulatory, neutral, or inhibitory.

The Ratio of RNase HI to RTH-1 Nuclease Is Critical for Both Enzymes To Work Together Efficiently. Since RNase HI and RTH-1 were hypothesized to collaborate in removing initiator RNA of Okazaki fragments (Turchi et al., 1994), we examined the interaction of the two nucleases in carrying out the reaction. Substrate H, having a 13-nucleotide RNA primer, was employed because the average length of natural mammalian Okazaki fragment initiator RNA ranges around 10 nucleotides (Tseng & Goulian, 1977; Tseng et al., 1979). The cleavage products of the two nucleases are shown in Figure 5. The RNase HI-specific 12mer cleavage product, and the subsequently generated junction ribomononucleotide cleavage product, made by exonuclease action, have been indicated by arrows (Figure 5). Lane 3 has a control experiment showing the action of calf RTH-1 nuclease alone. Interestingly, a trace amount of RNase HI contamination in the RTH-1 preparation allowed for a significant amount of RTH-1 nuclease junction nucleotide cleavage activity (lane 3). The fact that a trace amount of RNase HI and an excess of RTH-1 could produce a considerable, though small, amount of monoribonucleotide suggests that the two enzymes work well together at a low ratio of RNase HI to RTH-1. Lanes 4−6 show the results of predigesting substrate H with RNase HI for 15 min and then adding the indicated amount of RTH-1 for an additional 15 min. Results in lanes 7−9 were obtained by adding RNase HI and RTH-1 nuclease simultaneously and then incubating the samples for 15 min. Clearly, predigestion of the substrate with RNase HI helps RTH-1 to remove the junction ribonucleotide. Both calf RTH-1 mononucleotide digestion products and RNase HI-specific 12mer products were reduced when both enzymes were added to the reaction mixture simultaneously (lanes 7−9 vs lanes 4−6). Lanes 10 and 11 show the same

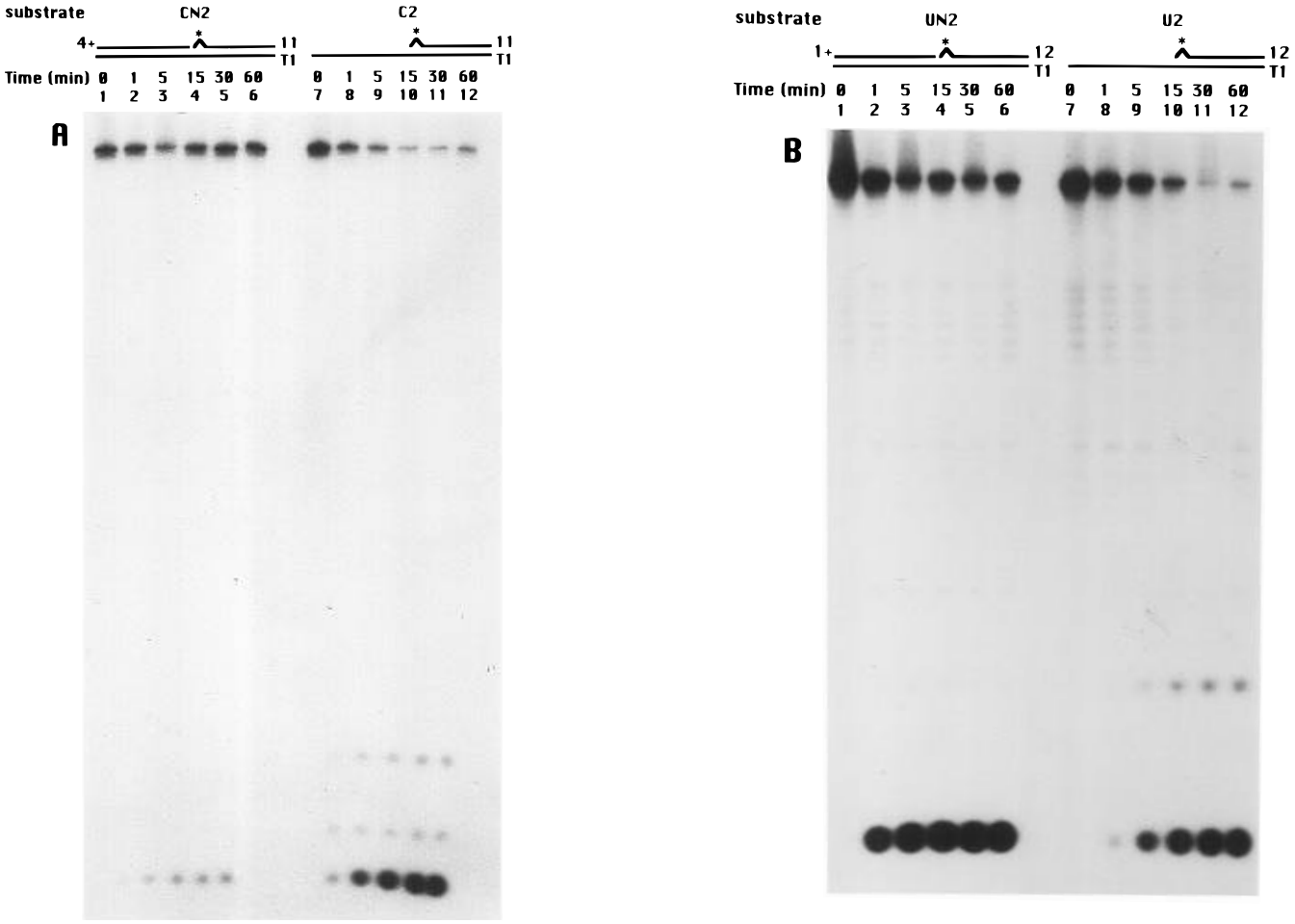

FIGURE 4: Removal of 5′ monoribonucleotides, C and U, at a different template location, by calf RTH-1 nuclease with or without an upstream DNA primer. Assays measuring exonucleolytic cleavage of the 5′ end of a downstream primer are depicted. (A) Lanes 1−6 show a time course for removal of monoribonucleotide C from a substrate with an upstream primer forming a nick with the downstream primer; substrate lacking an upstream primer is shown in lanes 7−12. (B) Lanes 1−6 show a time course for removal of monoribonucleotide U from a substrate with an upstream primer forming a nick with the downstream primer; substrate lacking an upstream primer is shown in lanes 7−12. Substrate structures and oligonucleotide compositions are shown above the appropriate lanes. In this and subsequent figures, primers which have been extended by one or more nucleotides, as detailed in the text, have been indicated by a "+" next to the oligonucleotide sequence number. Downstream primers were 5′ radiolabeled, and reaction conditions were as described under Methods. The upper band represents starting substrate and the lower band represents the removed monoribonucleotide.

reactions as in lanes 7 and 8, except additional units of calf RTH-1 were added as indicated, followed by an additional 15-min incubation. Compared to lane 7, a significant increase in the amount of monoribonucleotide release can be observed in lane 10. These results indicate that RTH-1 can only work after RNase HI structure-specific cleavage, and the function of RNase HI is to prepare the substrate for RTH-1. Furthermore, an excess of either enzyme over an ideal amount interferes with cleavage efficiency of the other enzyme. This implies either that the two enzymes compete for the same substrate or that there is protein−protein interaction, with a resultant disturbance of catalysis.

In an attempt to find out the most effective ratio of RNase HI and calf RTH-1 nuclease for complete RNA removal from the Okazaki fragment substrate, an enzyme titration experiment was performed (Figure 6). Optimal RNA primer processing occurs in lane 8. In this lane, 1.2 fmol of RNase HI and 1.4 pmol of RTH-1 were used, indicating that the best molar ratio of RNase HI:RTH-1 nuclease is approximately 1:1000. Figure 6 clearly shows that less exonuclease, i.e., a higher ratio, reduces processing. The true optimal ratio could actually be lower than 1:1000, but this could not be tested because further increasing the RTH-1 concentration increases the RNase H contamination to a level approaching that of the RNase H intentionally added. Regardless of the exact ratio, a ratio as low or lower than 1:1000 is unexpected and will be considered in the Discussion section. These results demonstrate that the two enzymes must functionally coordinate to process initiator RNA during Okazaki fragment maturation.

Upstream RNA Products of the RNase HI Cleavage Might Remain Bound to the Template. Since natural initiator RNA segments range in length from approximately 6 to 14 nucleotides (Tseng & Goulian, 1977; Tseng et al., 1979), it is likely that after RNase HI cleavage, some of the RNA products remain bound to the template during RTH-1 cleavage of the junction ribonucleotide. We wanted to know whether such segments influence the reaction by stimulation or inhibition. We first determined whether the RNase HI 12mer RNA cleavage product from substrate H remained bound to the template using native polyacrylamide gel electrophoresis. Figure 7 shows that it remained bound during a 1-h incubation (lane 5 *vs* 10), suggesting that it may remain bound long enough to inhibit the RTH-1 cleavage. However, the 7mer RNase HI nonspecific cleavage product dissociated from template after a 30−60-min

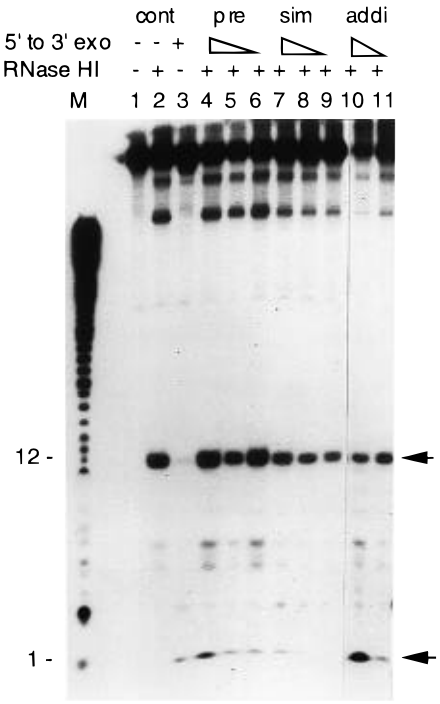

FIGURE 5: Effect of coincubation with calf RTH-1 nuclease on RNase HI activity. Substrate H (see Table 1) was incubated with or without enzyme as described below. Top arrow, RNase HI product; bottom arrow, RTH-1 exonuclease product. Lane 1, no enzyme; lane 2, 0.3 unit of RNase HI; lane 3, 0.3 unit of RTH-1 nuclease. Lanes 4−6, 0.3 unit of RNase HI was preincubated with substrate H for 15 min, followed by digestion for 15 min with calf RTH-1 nuclease (0.5, 0.3, and 0.1 unit, respectively). Lanes 7−9, substrate was incubated with 0.3 unit of RNase HI and 0.5 unit (lane 7), 0.3 unit (lane 8), or 0.1 unit (lane 9) of exonuclease simultaneously for 15 min; lanes 10 and 11 are like lanes 7 and 8 except an additional 0.5 and 0.3 unit of exonuclease were added for another 15-min incubation, respectively. Lane M shows size markers as in Figure 2. Reaction conditions were as described under Methods and in the text. Size markers at the left indicate product length in nucleotides.

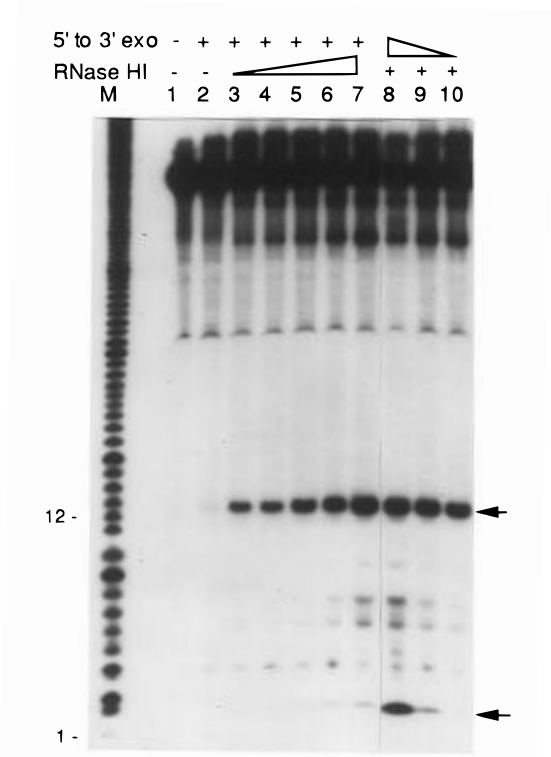

FIGURE 6: Enzyme titrations of RNase HI and calf RTH-1 nuclease in coincubation. Substrate H was incubated for 20 min as follows: lane 1, no enzyme; lane 2, incubation with 0.3 unit of calf RTH-1 nuclease; lanes 3−7: 0.4 unit of calf RTH-1 nuclease in addition to RNase HI at 0.1, 0.2, 0.3, 0.4, and 0.5 unit, respectively; Lanes 8−10, 0.3 unit of RNase HI in addition to exonuclease at 1, 0.5, and 0.3 unit, respectively. Top arrow, RNase HI product; bottom arrow, RTH-1 exonuclease product. Lane M shows a 5′ ^{32}P-labeled 140mer of unrelated RNA following limited alkaline hydrolysis. Reaction conditions were as described under Methods and in text. Size markers at the left indicate product length in nucleotides.

incubation time (lanes 4 and 5). This band is very faint because it is a minor, nonspecific, RNA product. Nevertheless, its presence suggests that shorter RNA primers can spontaneously dissociate and are, therefore, less likely to cause upstream inhibition of the RTH-1 nuclease. Such a conclusion was further supported when we tested the stability of another 10-nucleotide-long RNA primer. Forty percent dissociated after a 15-min incubation (data not shown).

The Presence of an Adjacent RNA Upstream Primer Reduces Junction Ribonucleotide Cleavage Efficiency. The likely presence of some bound upstream RNA left after RNase HI cleavage prompted us to investigate whether that RNA would have a stimulatory or inhibitory effect on junction ribonucleotide removal. Substrate UR was constructed by isolating the 12mer incomplete in vitro transcription product from *Hin*dIII-digested plasmid and then annealing it to template T1 along with the downstream monoribonucleotide−DNA segment, so that the effect of an RNA upstream primer could be seen on a previously tested downstream primer. RTH-1 nuclease cleavage efficiency for this substrate was compared to that for substrate U which has no upstream primer. Judging from the mononucleotide product in Figure 8B, calf RTH-1 nuclease cleavage efficiency is decreased in the presence of an adjacent upstream RNA primer (lanes 1−3 *vs* 4−6). Furthermore, the upstream inhibition is of approximately the same amount seen with an upstream DNA primer (data not shown). Thus, it seems that whether an upstream primer is RNA or DNA may make little difference. In addition, adding RNase HI to the reaction does not have a significant effect (lanes 7 and 8 *vs* 5 and 6). Substrate R has only the upstream RNA primer without a downstream primer. Thus, Figure 8, panel A and lanes 10−12 of panel B, are control experiments showing that the calf RTH-1 nuclease itself has no affinity for a simple RNA/DNA hybrid.

The Endonucleolytic Activity of Calf RTH-1 Nuclease Can Cleave a Downstream Primer Even in the Presence of an Inhibitory Upstream Primer. With an inhibitory upstream primer, the RTH-1 nuclease might be able to proceed with junction ribonucleotide removal by an alternative mechanism. We created substrates to model cases in which extension of the upstream primer displaces the junction ribonucleotide. Substrates uA, uG, uC, and uU were prepared as described under Methods. Figure 9A shows that an exonucleolytically resistant monoribonucleotide, in this case G, is readily removed endonucleolytically. As we have previously shown, endonucleolytic cleavage occurs both at the point of annealing and one nucleotide into the annealed portion of the downstream primer (Murante et al., 1994). Figure 9A shows the expected monomer and dimer resulting from endonucleolytic removal of the unannealed monoribonucleotide. Similar results were obtained for removal of rA (data not shown). Interestingly, the exonucleolytically resistant rU and rC had a different endonucleolytic cleavage specificity, so that only

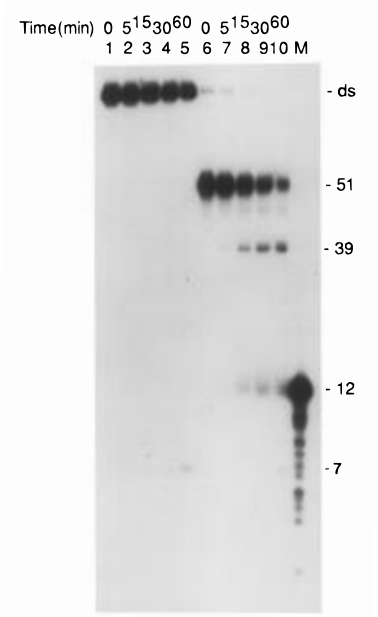

FIGURE 7: Native gel electrophoresis of RNase HI cleavage products. Substrate H was subjected to RNase HI digestion (0.3 unit) for the indicated time periods. Lanes 1−5, no heat denaturation; lanes 6−10, following heat denaturation. Lane M is a 13mer RNA made by transcription in vitro and subjected to limited alkaline hydrolysis. Reaction conditions were as described under Methods and in the text. Size markers at the right indicate product length in nucleotides.

the monomer was obtained. Figure 9B shows that endonucleolytic removal occurs readily for the monoribonucleotide U and results in only the monomeric product (data for C are similar but not shown). To assure that the upstream primer was appropriately extended and that endonucleolytic cleavage was responsible for the monomer seen with uC and uU, despite no observed dimer, the upstream primers of UN, CN, uC, and uU were 5′ end-labeled with [^{32}P]ATP by polynucleotide kinase. The substrates were then fractionated on an 18% denaturing gel to verify that the upstream primers of uU and uC were one nucleotide longer than those of UN and CN (data not shown).

DISCUSSION

We proposed a mechanism for maturation of mammalian Okazaki fragments in which the RNA primers were removed by the action of two nucleases (Turchi et al., 1994). RNase HI makes a single cleavage one nucleotide upstream of the RNA−DNA junction, releasing the RNA primer. The remaining junction ribonucleotide is removed by the action of calf RTH-1 nuclease, a functional homolog of the yeast RTH-1 and human FEN-1 nucleases. In this report, the specificity with which the two nucleases from calf act on the RNA primer of Okazaki fragment model substrates was further examined. We have previously shown that, on substrates with two adjacent DNA primers annealed to a DNA template, a nick structure between the primers greatly stimulates cleavage of the downstream primer, relative to substrates with either no upstream primer or a gap between the primers (Murante et al., 1994). We have shown similar requirements for endonucleolytic activity (Murante et al., 1994), and similar structure specificity has also been reported from studies of the nuclease activities of *E. coli* DNA polymerase I, Taq, Tth, and Tfl DNA polymerases (Lyamichev et al., 1993), mouse FEN-1 (Harrington & Lieber, 1994a), and the human FEN-1 nuclease, DNase IV (Robins et al., 1994). However, on the Okazaki fragment substrate, the calf RTH-1 nuclease is capable of cleaving off the 5′ end RNA−DNA junction monoribonucleotide efficiently in the absence of an upstream primer (Figure 1). This result is consistent with observations presented here suggesting that RTH-1 also does not exhibit upstream primer dependency on some DNA substrates. Dahlberg and colleagues have also demonstrated that, for endonucleolytic cleavage of an RNA/DNA hybrid substrate, Taq and Tth polymerase do not require an upstream primer (Lyamichev et al., 1993). The mouse FEN-1 displayed no cleavage activity on tested RNA/DNA hybrids (Harrington & Lieber, 1994a) but did cleave some substrates without an upstream primer dependence or with changed substrate cleavage specificity (Harrington & Lieber, 1994a).

Surprisingly, this report shows not only a lack of upstream primer dependence with many substrates but also actual upstream primer inhibition. On most tested substrates, the presence of an upstream DNA primer separated by either a nick (Figure 1) or a gap (Figure 2) from the downstream monoribonucleotide−DNA segment actually decreased cleavage efficiency significantly. This upstream primer-directed inhibition was seen for the removal of all four RNA nucleotides, suggesting that the removal of the 5′-terminal ribonucleotide *in vivo* is coordinated, so that its removal is complete before extension of the upstream Okazaki fragment can approach and inhibit the calf RTH-1 cleavage. In fact, it may be removed before the next upstream Okazaki fragment is even initiated.

We had suggested that the upstream primer dependence of the calf RTH-1 nuclease allowed alternation of polymerization and exonucleolytic cleavage for nick translation (Siegal et al., 1992). The nuclease would have to wait for the polymerase to close a gap before proceeding and, therefore, could not run ahead. However, a delay in junction ribonucleotide removal until the arrival of the extending upstream primer would not seem necessary. The ribonucleotide could be removed immediately, and then the nuclease could wait for the upstream primer before proceeding to degrade deoxynucleotides. This would allow the option of ligation or further nick translation, after complete RNA removal. The absence of an upstream primer requirement for junction ribonucleotide removal but reestablishment of the requirement during subsequent DNA degradation is consistent with our observations. With no upstream primer, we saw efficient removal of the junction ribonucleotide but no further cleavage (Figure 2). However, if a DNA nucleotide was substituted for the corresponding RNA nucleotide, thus creating a substrate, with two adjacent DNA primers annealed to a DNA template, which had always previously exhibited upstream stimulation, it also showed upstream inhibition in three of the four cases (Figure 3). Together these remarkable results called into question the assumption that calf RTH-1 nuclease always or usually requires an upstream primer and opened up the possibility that many other factors may influence whether or not an upstream primer is inhibitory or stimulatory.

To define these features we first considered that the sequence around a given nucleotide, or its particular location on the template, might influence its requirement for an upstream primer. Thus, we tested the removal of monoribonucleotides C and U from the 5′ end of downstream

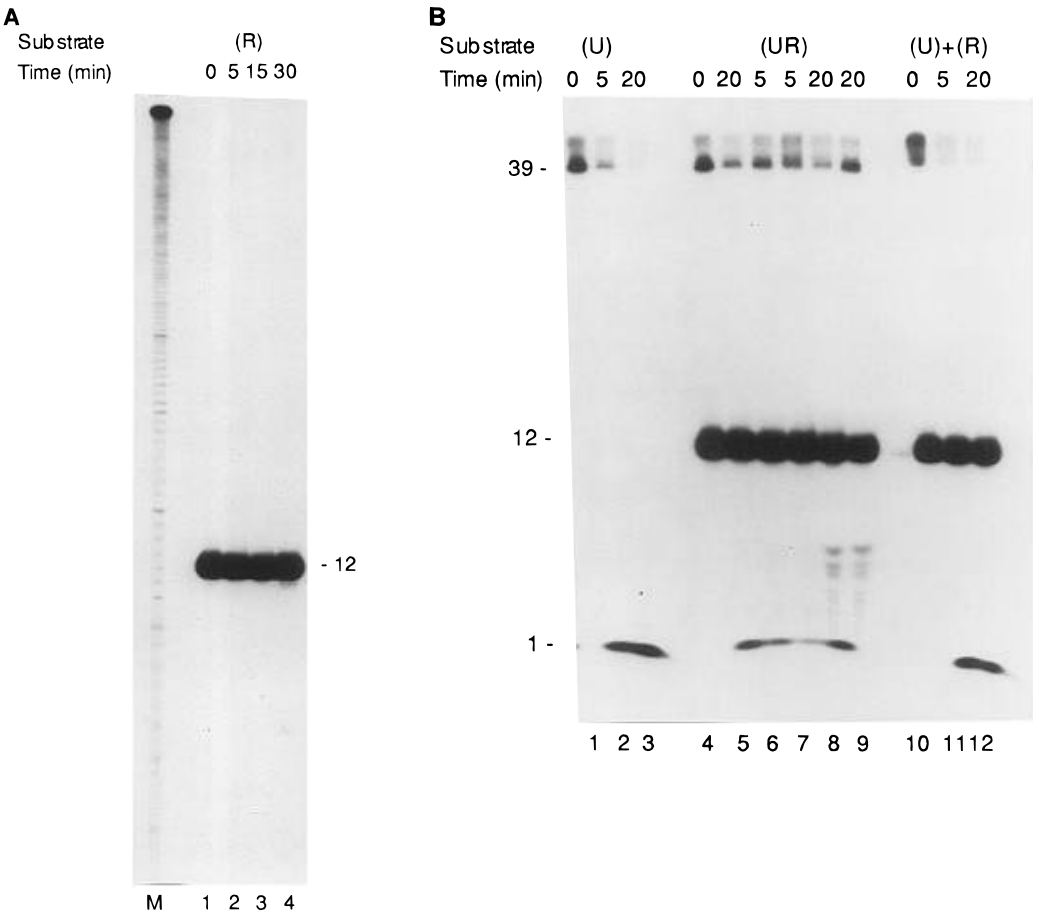

FIGURE 8: Time course of calf RTH-1 nuclease cleavage of the remaining junction ribonucleotide with or without an adjacent upstream RNA primer. In this experiment, both the 12mer cleavage product RNA and the remaining uridine are ^{32}P-labeled. The substrates used and the incubation time are indicated on the top of the figure. (A) Lane 1, no enzyme; lanes 2−4, 0.3 unit of calf RTH-1 nuclease; lane M, a 5′ ^{32}P-labeled 140mer of unrelated RNA following limited alkaline hydrolysis. (B) Lanes 1, 4, and 10, no enzyme; lanes 2−3, 5−8, and 11−12, 0.3 unit of calf RTH-1 nuclease; lanes 7−9: 0.3 unit of RNase HI. Lanes 10−12 are mixed substrate. Ten femtomoles of each substrate was used in each lane. The mixed substrate lanes therefore had a total of 20 fmol of substrate. Otherwise, reaction conditions were as described under Methods and in the text. Size markers indicate product length in nucleotides.

primers annealed to locations on the template differing from that of the original substrates. The C still demonstrated upstream primer inhibition (Figure 4A), even in the new environment, but removal of U showed slight upstream primer stimulation or was essentially neutral (Figure 4B). This latter result indicated for the first time that removal of RNA nucleotides is not always upstream primer-inhibited and that both RNA and DNA cleavage efficiency may vary with the sequence environment. To determine the extent of the influence of sequence environment, we placed a new upstream primer region adjacent to one of the previously tested downstream primer regions and observed its effect. In this situation, removal of T showed slight upstream primer stimulation and removal of U showed slight upstream primer inhibition (data not shown). They were both essentially neutral relative to the considerable inhibition they had both shown in the presence of the previous upstream primer. This result suggests that, along with the local environment of the nucleotide being removed, the sequence of the upstream primer itself plays a role.

Calf RTH-1 cleavage efficiency for removal of the 5′ monoribonucleotide U was reduced in the presence of an upstream RNA primer when compared to that with no upstream primer (Figure 8). In fact, the upstream RNA primer inhibited cleavage to about the same extent as the upstream DNA primer. This suggests that if an upstream primer is inhibitory for removal of a particular nucleotide, it probably makes little difference whether that upstream primer consists of RNA or DNA. This result also suggests that the RNA oligonucleotide cleavage product of RNase HI often inhibits rather than stimulates junction ribonucleotide removal. Since natural initiator RNAs are 6−14 nucleotides long, the RNase HI cleavage products are 5−13 nucleotides long. Most members of this size distribution would be expected to dissociate spontaneously (DeStefano et al., 1994). The longest members may take a long time or may require displacement by cellular helicases, single-stranded DNA binding proteins, or ultimately, synthesis from the upstream primer. It is also possible that the junction ribonucleotides adjacent to the longest natural initiator RNA cleavage products are removed relatively slowly *in vivo*, because the RNA primer acts as an upstream inhibitor.

We considered that cleavage specificity might be influenced by the location of the cleavage site on the helix. If true, upstream primer dependence might be observed for several nucleotides followed by inhibition for several nucleotides, in a periodic manner. Our observations are inconsistent with such a hypothesis. The observed pattern is too random, with upstream primer stimulation or inhibition occurring for one or two nucleotides, then not occurring. To formally rule out this possibility, additional, systematic studies would have to be performed.

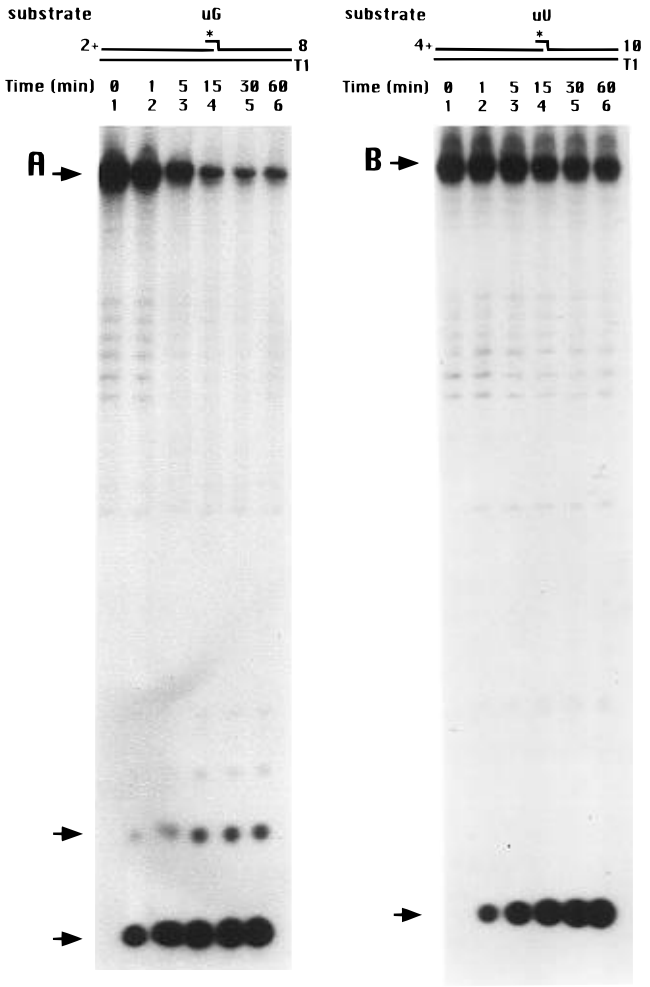

FIGURE 9: Removal of 5′ monoribonucleotides by the endonucleolytic activity of calf RTH-1 nuclease. Assays measuring endonucleolytic cleavage of the 5′ end of a downstream primer are depicted. Panel A shows a time course for removal of monoribonucleotide G from a substrate in which the upstream primer has been extended to form a nick with the first DNA nucleotide of the downstream primer, while the 5′ terminal RNA nucleotide has been unannealed. Panel B shows a time course for removal of monoribonucleotide U from a substrate in which the upstream primer has been extended to form a nick with the first DNA nucleotide of the downstream primer, while the 5′ terminal RNA nucleotide has been unannealed. Top arrow, starting material; middle arrow, dimer product; bottom arrow, monomer product. Substrate structures and oligonucleotide compositions are shown above the appropriate lanes. Downstream primers were 5′ radiolabeled, and reaction conditions were as described under Methods.

We also examined whether RTH-1 homologs displayed the same upstream primer dependence. Since we had not seen the expected upstream stimulation of calf RTH-1 nuclease cleavage on substrate dTN, we tested this same substrate with *E. coli* DNA polymerase I. The exonucleolytic activity of polymerase I was stimulated by the upstream primer (data not shown). This result demonstrates that upstream primer influence is not solely inherent in the substrate. However, upstream primer phenomena are not dependent on any contaminants in the chemically synthesized substrates that we have employed, since enzymatically produced monoribonucleotide−DNA substrates behave similarly.

We wondered why the calf RTH-1 nuclease does not carry out the entire process of primer removal itself. It can, in fact, cleave substrate P exonucleolytically, as indicated by removal of a 5′ end-labeled nucleotide. However, the reaction is very inefficient, requiring a large amount of enzyme and long incubation time (data not shown). The unique substrate specificities of both the calf RNase HI and RTH-1 nuclease with respect to the RNA−DNA junction ribonucleotide may be due to the distinctive helical structure of the RNA−DNA/DNA substrate. Recent determination of the solution structure of a synthetic Okazaki-type molecule revealed that it was a chimeric mixture of hybrid-form (H-form) and B-form structure and that the overall molecule cannot be classified as either an A-form or a B-form duplex (Salazar et al., 1994).

Another unexpected observation was the very low molar ratio of RNase HI to RTH-1 nuclease required for the most efficient complete removal of initiator RNA. Figures 5 and 6 show that the ratio of the nucleases in the solution is critical for both enzymes to work together efficiently. The low ratio is particularly surprising considering that RNase HI is an abundant and active enzyme in the cell (R. Crouch and W. Busen, unpublished information). Our results suggest that only a minute fraction of the total cellular RNase HI is available to participate in processing of initiator RNA. RNase HI is thought to participate in a surveillance process that removes unnecessary RNA primers made by the DNA replication and transcription machinery (R. Crouch, personal communication). It is possible that, within the living cell, nearly all of the RNase HI molecules are engaged in such housekeeping activities, leaving only a small portion to work on DNA replication. If so, the replication system has evolved to utilize the low level of available RNase HI. Alternatively, DNA replication proteins other than those that we have used to reconstitute initiator RNA processing may be responsible for regulating RNase HI *in vivo*. These additional replication factors may direct the RNase HI to release the cleaved Okazaki fragment to the RTH-1 nuclease for junction ribonucleotide removal.

Since it appears that removal of some 5′-terminal monoribonucleotides and deoxynucleotides is very inefficient in the presence of an upstream primer, we wished to explore how Okazaki fragment processing might proceed if RTH-1 is inhibited from performing its necessary function. In such cases, we surmised that the enzyme may still be able to remove the resistant monoribonucleotide *via* its endonucleolytic activity. Figure 9 shows that exonucleolytically resistant monoribonucleotides are readily removed endonucleolytically, and their presence actually alters the endonucleolytic cleavage specificity. The enzyme cleaved directly behind the ribonucleotide at the RNA−DNA junction, with little (A and G) or no (U and C) cleavage observed, as expected for endonucleolytic activity, on the 3′ side of the first deoxynucleotide. Thus, the enzyme achieved endonucleolytically what it could not exonucleolytically—efficient removal of the 5′ monoribonucleotide with an upstream primer present directly adjacent to the first annealed nucleotide of the downstream primer. Perhaps, when the junction ribonucleotide is resistant to exonucleolytic cleavage, the downstream primer is displaced by synthesis on the upstream primer as far as necessary to reach a site of endonucleolytic cleavage. Then, when the tail is removed, the substrate can continue nick translation or the two primers can be ligated.

Figure 10 presents a model of the most favorable sequence of events in the processing of mammalian Okazaki fragments based on the results presented here. RNase HI efficiently

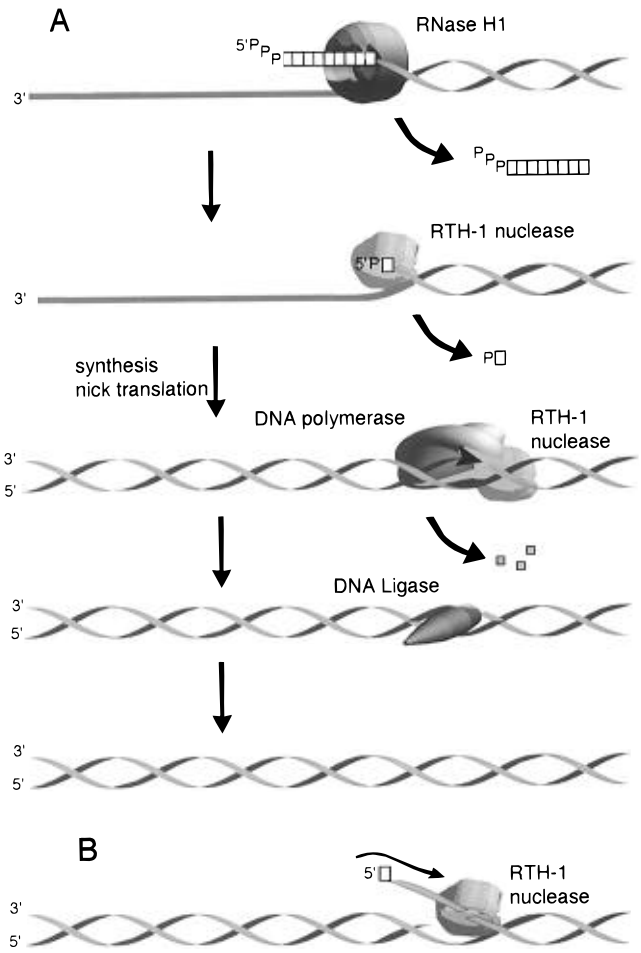

FIGURE 10: Revised model of Okazaki fragment processing. (A) Exonucleolytic model requiring absence of an upstream primer until after RTH-1 cleavage. (B) Endonucleolytic model which may be employed if RTH-1 exonucleolytic cleavage is inhibited by an upstream primer.

cleaves off the initiator RNA, leaving the junction ribonucleotide. The RNA oligomer product of the reaction spontaneously dissociates. The RTH-1 nuclease then cleaves the remaining ribonucleotide. These reactions would occur before the approach of the growing upstream Okazaki fragment. With the arrival of the upstream primer, RTH-1 cleavage-directed nick translation would proceed until a sequence is reached in which there is upstream primer-directed inhibition. Ligation would then take place. If cleavage of the junction ribonucleotide is inefficient at a particular sequence, an endonucleolytic cleavage pathway could come into play. Of course, at nucleotides without upstream inhibition, our previously proposed model in which the upstream primer may approach immediately (Bambara & Huang, 1995) could still be employed. RNA−DNA junctions occur at frequencies which suggest near random distribution on the genome (Kaufmann et al., 1977; Anderson et al., 1977). Thus, it is reasonable to expect that the cell may need to employ various mechanisms of Okazaki fragment processing depending on the requirements of a particular junction.

RTH-1 has recently been demonstrated to be involved in the MSH2−MLH1−PMS1 mismatch repair pathway (Johnson et al., 1995). Mutations in the mammalian mismatch repair pathway are associated with colorectal cancers (Reenan & Kolodner, 1992; Prolla et al., 1994), pointing out the value of investigating the exact mechanisms of the enzyme and the effect mutations may have on its substrate specificity.

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