Inman, R. B., & Baldwin, R. L. (1962) *J. Mol. Biol. 5*, 172.
Jensen, D. E., & von Hippel, P. H. (1977) *Anal. Biochem. 80*, 267.
Johnsrud, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5314.
Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980a) *J. Mol. Biol. 136*, 65.
Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980b) *J. Mol. Biol. 136*, 79.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lohman, T. M., Wensley, C. G., Cina, J., Burgess, R. R., & Record, M. T., Jr. (1980) *Biochemistry 19*, 3516.
Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469.
Nüsslein, C., & Heyden, B. (1972) *Biochem. Biophys. Res. Commun. 47*, 282.
Pettijohn, D., & Kamiya, T. (1967) *J. Mol. Biol. 29*, 275.
Pilz, I., Kratky, O., & Rabussay, D. (1972) *Eur. J. Biochem. 28*, 205.
Record, M. T., Jr., Lohman, T. M., & deHaseth, P. (1976) *J. Mol. Biol. 107*, 145.
Reiner, A. M. (1969) *J. Bacteriol. 97*, 1437.
Reisbig, R. R., Woody, A.-Y. M., & Woody, R. W. (1979) *J. Biol. Chem. 254*, 11208.
Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4769.
Richardson, J. P. (1966) *J. Mol. Biol. 21*, 83.
Rünzi, W., & Matzura, H. (1976) *J. Bacteriol. 125*, 1237.
Saxe, S. A., & Revzin, A. (1979) *Biochemistry 18*, 255.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Siebenlist, U. (1979) *Nature (London) 279*, 651.
Studier, F. W. (1965) *J. Mol. Biol. 11*, 373.
Wells, R. D., Larsen, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465.
Williams, R. C., & Chamberlin, M. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3740.

Mechanism of Action of Moloney Murine Leukemia Virus RNA-Directed DNA Polymerase Associated RNase H (RNase H I)†

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ABSTRACT: The mechanism of action of the ribonuclease H (RNase H) activity associated with Moloney murine leukemia virus RNA-directed DNA polymerase (RNase H I) and the two-subunit ($\alpha\beta$) form of avian myeloblastosis virus DNA polymerase were compared by utilizing the model substrate $(A)_n \cdot (dT)_n$ and polyacrylamide gel electrophoresis in 7 M urea to analyze digestion products. Examination on 25% polyacrylamide gels revealed that a larger proportion of the RNase H I oligonucleotide products generated by limited digestion of $[^3H](A)_{\langle 1100 \rangle} \cdot (dT)_n$ were acid insoluble (15–26 nucleotides long) than acid soluble (less than 15 nucleotides long), while the opposite was true for products generated by $\alpha\beta$ RNase H. RNase H I was capable of attacking RNA in RNA·DNA in the 5′ to 3′ and 3′ to 5′ directions, as demonstrated by the use of $[^3H,3'\text{- or } 5'\text{-}^{32}P](A)_{\langle 380 \rangle} \cdot (dT)_n$ and cellulose–$[^3H](A)_n \cdot (dT)_n$. Both RNase H I and $\alpha\beta$ RNase H degraded $[^3H](A)_n \cdot (dT)_n$ with a partially processive mechanism, based upon classical substrate competition experiments and analyses of the kinetics of degradation of $[^3H,3'\text{- or } 5'\text{-}^{32}P](A)_{\langle 380 \rangle} \cdot (dT)_n$. That is, both enzymes remain bound to a RNA·DNA substrate through a finite number of hydrolytic events but dissociate before the RNA is completely degraded. Both RNase H I and $\alpha\beta$ RNase H were capable of degrading $[^{14}C](A)_n$ in $[^3H](C)_n$–$[^{14}C](A)_n$–$[^{32}P](dA)_n \cdot (dT)_n$, suggesting that retroviral RNase H is capable of removing the tRNA primer at the 5′ terminus of minus strand DNA at the appropriate time during retroviral DNA synthesis in vitro.

Retrovirus RNA-directed DNA polymerase (reverse transcriptase) has been studied extensively in recent years (Temin & Baltimore, 1972; Green & Gerard, 1974; Wu & Gallo, 1977; Verma, 1977; Gerard & Grandgenett, 1980). Such effort is warranted since the reverse transcriptase molecule is extremely complex. The prototype for the avian enzyme, avian myeloblastosis virus (AMV)[1] $\alpha\beta$ reverse transcriptase, has multiple enzymatic activities, i.e., DNA polymerase (Baltimore, 1970; Mizutani & Temin, 1970), RNase H (Moelling et al., 1971), and DNA endonuclease (Golomb & Grandgenett, 1979; Grandgenett et al., 1980), as well as distinct nucleic acid binding activities, i.e., ability to selectively bind to $tRNA^{Trp}$ (Panet et al., 1975) and to unwind RNA·DNA and duplex DNA (Collett et al., 1978). The single-subunit α form of AMV reverse transcriptase lacks at least two of these activities, DNA endonuclease (Golomb & Grandgenett, 1979) and ability to selectively bind to $tRNA^{Trp}$ (Grandgenett et al., 1976). Murine retrovirus reverse transcriptase resembles AMV α in being a single-subunit enzyme (Moelling, 1976; Verma, 1975) that lacks DNA endonuclease activity (Moelling, 1974; Verma, 1975) and ability to selectively bind to its putative primer, $tRNA^{Pro}$ (Haseltine et al., 1977).

The RNase H activity of the avian enzymes has been thoroughly characterized. Both AMV α and $\alpha\beta$ RNase H are exonucleases that attack RNA·DNA in either the 5′ to 3′ or

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[1] Abbreviations used: AMV, avian myeloblastosis virus; M-MLV, Moloney murine leukemia virus; the symbols ⟨⟩ indicate average values.

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3′ to 5′ direction (Keller & Crouch, 1972; Leis et al., 1973; Grandgenett & Green, 1974). The enzymes differ in that α has been characterized as random (Grandgenett & Green, 1974) and $\alpha\beta$ as processive in mode of action (Leis et al., 1973; Grandgenett & Green, 1974). The question as to which avian enzyme the murine reverse transcriptase most closely resembles in its mode of RNase H action is a matter of controversy (Verma, 1975; Moelling, 1976). In addition, the ability of the murine enzyme to degrade a substrate molecule in both the 5′ to 3′ and 3′ to 5′ directions has not been documented.

Utilizing the same classical method, substrate competition, Verma (1975) and Moelling (1976) obtained conflicting results regarding the mode of action of murine retrovirus RNase H I. In assaying RNase H activity these workers, as well as others in this area, have relied on acid precipitation of substrate–product mixtures to separate undigested substrate from digestion products. Size analysis by gel electrophoresis reveals that many of the digestion products generated by reverse transcriptase associated RNase H are large enough to be acid insoluble (Gerard & Grandgenett, 1975; Gerard, 1978). Utilizing gel electrophoresis to separate reaction products from substrate and applying the classical methods of substrate competition and substrate end labeling, I demonstrate in this report that M-MLV RNase H I and AMV $\alpha\beta$ RNase H are similar in their mode of action and differ principally in the size distribution of their digestion products. Both enzymes appear to be partially processive in mode of action when assayed by these methods. Experiments with a synthetic model substrate that suggest a function for retroviral RNase H in proviral DNA synthesis were also performed.

Experimental Procedures

Reagents. Some biochemical reagents used were obtained from sources identified previously (Gerard & Grandgenett, 1975; Gerard, 1978). The following is a list of additional reagents and their sources: [α-^{32}P]dATP, [γ-^{32}P]ATP, [^{3}-H]CDP, [^{14}C]ADP, and [α-^{32}P]ATP, New England Nuclear Corp.; *E. coli* alkaline phosphatase, T_4 polynucleotide kinase, *E. coli* RNase H, and calf thymus deoxyribonucleotidyl transferase, Enzo Biochemicals; snake venom phosphodiesterase and bovine pancreatic DNase I, Worthington Biochemicals; *E. coli* RNA polymerase and $(C)_n$ ($s_{20,w}$ = 3.1 S), Miles Laboratories, Inc.; *E. coli* DNA polymerase I, Boehringer Mannheim; *M. luteus* primer-independent and primer-dependent polynucleotide phosphorylase, P-L Biochemicals; Sephacryl S-200 superfine and Sepharose 4B, Pharmacia Fine Chemicals. *E. coli* RNase II was purified from a mutant of *E. coli* K-12 lacking RNase I (Enzo Biochemicals) by the method of Gupta et al. (1977). AMV $\alpha\beta$ DNA polymerase purified by phosphocellulose and heparin–Sepharose chromatography (Golomb et al., 1980) was a gift from D. Grandgenett of this Institute.

Preparation of Heparin–Sepharose. One gram of heparin (Sigma, Grade I) was coupled to 50 mL of cyanogen bromide activated Sepharose 4B (Iverius, 1971) in 0.1 M $NaHCO_3$.

Virus. M-MLV was grown in Swiss high-passage mouse embryo cells (Riggin et al., 1974). Virus was purified by sequential discontinuous and continuous isopycnic centrifugation after precipitation with poly(ethylene glycol) or pelleting by centrifugation at 10000*g* for 16 h (Gerard, 1978).

Purification of M-MLV RNase H I (RNA-Directed DNA Polymerase Associated RNase H). M-MLV RNase H I was purified by sequential chromatography on DEAE-Sephadex and phosphocellulose (Verma and Baltimore, 1974) followed by heparin–Sepharose chromatography. Briefly, 30 mg of M-MLV was lysed at 37 °C for 2 min in 20 mM Tris-HCl

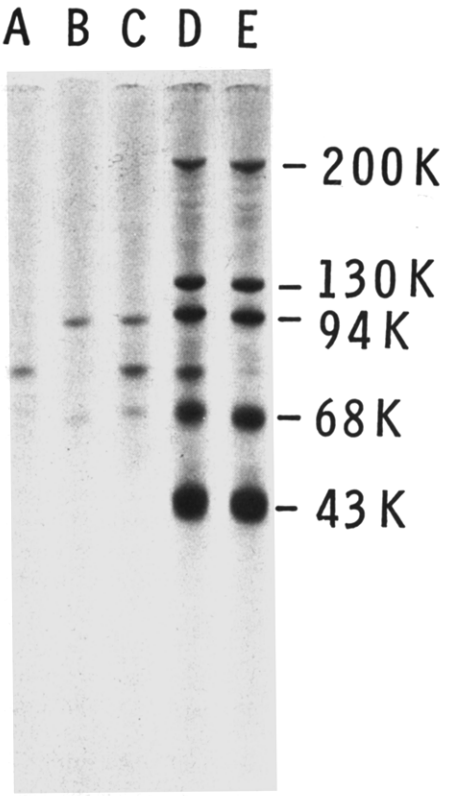

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis of (A) 1 μg of M-MLV RNA directed DNA polymerase–RNase H (RNase H I), (B) 0.8 μg of AMV $\alpha\beta$ DNA polymerase, (C) M-MLV DNA polymerase plus AMV $\alpha\beta$ DNA polymerase, (D) a mixture of protein standards containing 5 μg each of myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, and M-MLV DNA polymerase, and (E) protein standards alone. Five percent polyacrylamide gels were run for 4 h at 10 mA/gel (Experimental Procedures). Gels were stained with Coomassie brilliant blue.

(pH 8.0), 10 mM dithiothreitol, 20% glycerol, 0.1 mM EDTA, 0.2% NP-40, and 1 mM phenylmethanesulfonyl fluoride. DEAE-Sephadex and phosphocellulose chromatography were performed essentially as described (Verma & Blatimore, 1973) except that the DEAE-Sephadex column was equilibrated and washed after being loaded with buffer containing 1 mM phenylmethylsulfonyl fluoride. The RNase H I peak fractions from phosphocellulose were pooled, concentrated by dialysis against 30% poly(ethylene glycol) (Gerard, 1978), and then applied to a 2-mL Heparin–Sepharose column equilibrated in 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.01% NP-40, and 10% glycerol (buffer A). After the column was washed with 20 mL of buffer A–0.25 M NaCl, RNase H I was either batch eluted with 1 M NaCl or eluted with a linear gradient of 0.25 M to 1 M NaCl in buffer A. Peak activity fractions were concentrated by dialysis against 30% poly(ethylene glycol) (Gerard, 1978). M-MLV RNase H I purified by this procedure appeared as a single band on sodium dodecyl sulfate polyacrylamide gels (see Figure 1).

Sodium Dodecyl Sulfate–Polyacrylamide Disc Gel Electrophoresis. Protein samples were precipitated with 15% trichloroacetic acid in the presence of 50% ethanol and 30 μg/mL *E. coli* tRNA at −20 °C for 12 h. After centrifugation (12000*g*, 20 min), the pellet was washed with acetone at −20 °C. Samples were subjected to sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis as described (Grandgenett et al., 1973).

Protein Determination. The method of Lowry et al. (1951) was used with BSA as standard to determine protein con-

centrations except when dilute solutions were involved; then the method of McKnight (1977) was employed.

RNase H Assays. Assays for RNase H activity were carried out at 37 °C for various times with $[^3H](A)_n \cdot (dT)_n$ and monitored the degradation of $[^3H](A)_n$ by various means (Gerard & Grandgenett, 1975; Gerard, 1978). Standard reaction mixtures (100 μL) contained 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, $(dT)_n$ and radioactively labeled $(A)_n$ at various concentrations, and KCl and $MnCl_2$ or $MgCl_2$ as indicated below. The $(dT)_n$ used in these studies was greater than 1000 nucleotides long. Assays contained 40 mM KCl and 2 mM $MnCl_2$ with M-MLV RNase H I (Gerard, 1978), 20 mM KCl and 10 mM $MgCl_2$ with AMV αβ RNase H (Grandgenett & Green, 1974) and *E. coli* DNA polymerase I, and 20 mM KCl and 5 mM $MgCl_2$ with *E. coli* RNase H (Leis et al., 1973). The reaction conditions used for M-MLV RNase H I, AMV αβ RNase H, and *E. coli* RNase H have been established to be optimal for these enzymes with $[^3H](A)_n \cdot (dT)_n$ as substrate. One unit of RNase H activity is defined as the amount of enzyme required to solubilize 100 pmol of $[^3H](A)_n$ in $[^3H](A)_n \cdot (dT)_n$ in 20 min at 37 °C.

$(A)_n$. Oligomers of $(A)_n$ used as standards for electrophoresis on 7 M urea–polyacrylamide gels were obtained from various sources. $A(A)_9$ was purchased from Collaborative Research Inc. $A_{(44)}$ (2.4 S), $A_{(54)}$ (2.6 S), $A_{(90)}$ (3.3 S), $A_{(120)}$ (3.7 S), and $A_{(220)}$ (5.0 S) were obtained from Miles Laboratories, Inc. $[^3H](A)_{(33)}$ (2.1 S), $[^3H](A)_{(24)}$ (1.8 S), $[^3H](A)_{(17)}$ (1.5 S), and $[^3H](A)_{(10)}$ (1.2 S) were prepared as follows. A single lot of $[^3H](A)_n$ (50 μCi and 60 cpm/pmol; Miles Laboratories, Inc.) containing substantial quantities of acid-soluble $[^3H](A)_n$ was fractionated into four pools containing $[^3H](A)_n$ of increasing size by chromatography on a 0.9 × 90 Sephacryl S-200 superfine column in buffer containing 0.02 M Tris-HCl (pH 8.0), 0.1 M NaCl, and 6 M urea. After dialysis against the same buffer without urea the $[^3H](A)_n$ pools were concentrated by ethanol precipitation in the presence of 0.3 M NaCl. The sedimentation coefficient of the $[^3H](A)_n$ in each pool was determined in 1 M NaCl and 0.02 M potassium phosphate (pH 7.5) at 20 °C by utilizing either $A_{(16)}$ or $A_{(54)}$ (see above) as markers (Eisenberg & Felsenfeld, 1967). The average chain length of $[^3H](A)_n$ in each pool was then calculated from the empirical formula (Eisenberg & Felsenfeld, 1967) $s = 0.405Z^{0.467}$ where s and Z are the sedimentation coefficient and the chain length, respectively.

Preparation of $[^3H](A)_n$ of High Specific Radioactivity. $[^3H](A)_n$ of high specific radioactivity was synthesized in a reaction mixture (2 mL) containing 100 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 100 mM NaCl, 2 mM $MnCl_2$, 0.2 mM [^{3}H]ATP (2,100 cpm/pmol), 0.3 mM $(dT)_n$, and 24 units of *E. coli* RNA polymerase. After 60 min at 37 °C, 100 μL each of 0.2 M EDTA and 10% (w/v) sodium dodecyl sulfate were added. After phenol and ether extraction, the product was ethanol precipitated at −20 °C. Poly(dT) was removed by DNase I digestion (40 μg) containing 20 mM Tris-HCl (pH 7.4) and 6 mM $MgCl_2$. The reaction mixture was heated at 80 °C for 3 min after addition of 20 μL of 0.2 M EDTA and 120 mg of urea and was then fractionated on a 10-mL Sephacryl S-200 column run in 20 mM Tris-HCl (pH 8.0) and 6 M urea. The gel-excluded $[^3H](A)_n$ was dialyzed against 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 1 mM EDTA, and ethanol precipitated. Approximately 220 nmol of $[^3H](A)_n$ were recovered.

One half of the $[^3H](A)_n$ product was fractionated according to size as already described (Eisenberg & Felsenfeld, 1967). $[^3H](A)_n$ with an $s_{20} = 10.7$ S (average number of nucleotides = 1100; Eisenberg & Felsenfeld, 1967) was recovered by ethanol precipitation. The other half of the $[^3H](A)_n$ was dephosphorylated with *E. coli* alkaline phosphatase (Grandgenett & Green, 1974) and fractionated by sedimentation. $[^3H](A)_n$ with an $s_{20} = 6.5$ S (average length = 380 nucleotides) was recovered by ethanol precipitation.

Preparation of $[^3H,5'\text{-}^{32}P](A)_{(380)}$. The 5′ end of dephosphorylated $[^3H](A)_{(380)}$ (2100 cpm/pmol) was phosphorylated with [γ-^{32}P]ATP (1.9×10^5 cpm/pmol) and polynucleotide kinase as already described (Grandgenett & Green, 1974). $[^3H,5'\text{-}^{32}P](A)_{(380)}$ with a specific activity in ^{32}P of 400 cpm/pmol of total phosphate was obtained.

Preparation of $[^3H,3'\text{-}^{32}P](A)_{(380)}$. Elongation of $[^3H](A)_{(380)}$ (2100 cpm/pmol) at the 3′ terminus with a limiting amount of [α-^{32}P]ATP with *E. coli* RNA polymerase was performed as described (Baltimore & Smoler, 1972; Grandgenett & Green, 1974). The reaction mixture (100 μL) contained 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 10 mM $MgCl_2$, 150 mM KCl, 7 nmol $[^3H](A)_{(380)}$ (18 pmol of 3′ ends), 0.1 mM $(dT)_n$, 100 pmol of [α-^{32}P]ATP (1.3×10^5 cpm/pmol), and 13 μg (15 units) of *E. coli* RNA polymerase. After a 5-min incubation at 37 °C, 10 μL each of 0.2 M EDTA and 10% sodium dodecyl sulfate were added. The reaction mixture was phenol and ether extracted and subjected to Sephadex G-75 gel filtration, and the gel-excluded product was ethanol precipitated. Approximately 2.5 nmol of $[^3H,3'\text{-}^{32}P](A)_{(380)}$ were recovered containing 15 pmol of 3′-^{32}P label. Since *E. coli* RNA polymerase is primer dependent under these conditions (Niyogi, 1972) and the number of enzyme molecules exceeded the number of $(A)_{(380)}$ 3′ ends, each $[^3H](A)_{(380)}$ molecule should have been extended by approximately two [^{32}P]AMP residues.

Synthesis of $[5'\text{-}^{32}P](C)_{(100)}$–$[^3H](A)_{(100)}$. A commercial preparation of $(C)_n$ was fractionated on the basis of size by centrifugation in a 5–20% sucrose gradient in 20 mM potassium phosphate (pH 7.5) and 1 M NaCl at 20 °C for 17.5 h at 66000*g*. Gradient fractions containing $(C)_n$ with an average sedimentation coefficient of 3.5 S (average chain length = 100; Eisenberg & Felsenfeld, 1967) relative to $A_{(220)}$ ($s_{20,w} = 5$ S) as a marker were pooled and ethanol precipitated. $(C)_{(100)}$ (1500 nmol) was dephosphorylated with alkaline phosphatase and was phosphorylated with 200 nmol of [γ-^{32}P]ATP (6000 cpm/pmol) and polynucleotide kinase as already described (Grandgenett & Green, 1974). $[5'\text{-}^{32}P](C)_{(100)}$–$[^3H](A)_{(100)}$ was synthesized from $[5'\text{-}^{32}P](C)_{(100)}$ with primer-dependent polynucleotide phosphorylase and [^{3}H]ADP (200 cpm/pmol) essentially as described (Leis et al., 1973). With the preparation of primer-dependent polynucleotide phosphorylase used in this and subsequent experiments, the amount of $(A)_n$ synthesized without $(C)_n$ as primer represented only 3% of the total product synthesized. Approximately 820 nmol of $[^3H](A)_n$ and 8.2 nmol of [^{32}P] were recovered in $[5'\text{-}^{32}P](C)_{(100)}$–$[^3H](A)_n$. On the assumption that 100% of the 5′ ends of $[5'\text{-}^{32}P](C)_{(100)}$ were labeled, the average chain length of $[^3H](A)_n$ was 100.

Preparation of $[^3H](C)_n$–$[^{14}C](A)_n$–$[^{32}P](dA)_n$. A uniformly labeled preparation of $[^3H](C)_n$ was synthesized in a reaction mixture (250 μL) containing 100 mM Tris-HCl (pH 8.0), 60 μg/mL BSA, 10 mM $MgCl_2$, 23 mM [^{3}H]CDP (30 cpm/pmol), and 21 units of primer-independent polynucleotide phosphorylase. After a 1-min incubation at 37 °C, 30 μL of 0.2 M EDTA was added and the mixture was heated at 80 °C for 2 min and clarified by sedimentation at 10000*g* for 10 min. After ethanol precipitation, the product was sedimented

on a 5–20% sucrose gradient as already described for $(C)_n$. The fractions from the gradient containing the peak of $[^3H](C)_n$ were pooled, the $[^3H](C)_n$ was ethanol precipitated, and the preparation of $[^3H](C)_n$ was subjected to Sephadex G-75 gel filtration. When $[^3H](C)_n$ was utilized as primer, $[^3H](C)_n$–$[^{14}C](A)_n$ was synthesized in a reaction mixture (60 μL) containing 100 mM Tris-HCl (pH 8.0), 60 μg/mL BSA, 8 mM $MgCl_2$, 20 mM [^{14}C]ADP (50 cpm/pmol), 150 nmol of $[^3H](C)_n$, and two units of primer-dependent polynucleotide phosphorylase. After incubation at 37 °C for 80 min, the mixture was heated at 80 °C for 2 min and subjected to Sephadex G-75 gel filtration to remove unreacted [^{14}C]ADP. The product was recovered by ethanol precipitation. $[^3H](C)_n$ with no or short oligomers of $[^{14}C](A)_n$ attached (less than 20 nucleotides) were removed from the $[^3H](C)_n$–$[^{14}C](A)_n$ product by chromatography on a 1-mL oligo(dT)-cellulose column (Collaborative Research, Inc., type T1). The sample was bound and the column was washed in 0.5 M NaCl–0.01 M Tris-HCl (pH 7.5) and $[^3H](C)_n$–$[^{14}C](A)_n$ was eluted with 0.01 M Tris-HCl (pH 7.5).

$[^3H](C)_n$–$[^{14}C](A)_n$ will not serve as a primer for calf thymus terminal transferase since the enzyme requires a DNA primer (Bollum, 1974). However, I have observed that by addition of one or a few dA residues to $[^3H](C)_n$–$[^{14}C](A)_n$, the homopolymeric RNA becomes a primer for terminal transferase. One or few residues of dA were added to $[^3H](C)_n$–$[^{14}C](A)_n$ by incubation with dADP and polynucleotide phosphorylase (Chou & Singer, 1971). A reaction mixture (100 μL) containing 100 mM Tris-HCl (pH 8.0), 60 μg/mL BSA, 5 mM $MnCl_2$, 20 mM dADP, 0.6 unit of primer-dependent polynucleotide phosphorylase, and $[^3H](C)_n$–$[^{14}C](A)_n$ (10 nmol of $[^3H](C)_n$ and 15 nmol of $[^{14}C](A)_n$) were incubated at 37 °C for 80 min. After being heated at 80 °C for 2 min, the product was subjected to Sephadex G-75 gel filtration and was ethanol precipitated. $[^3H](C)_n$–$[^{14}C](A)_n$–$[^{32}P](dA)_n$ was synthesized from $[^3H](C)_n$–$[^{14}C](A)_n$–dA in a reaction mixture (100 μL) containing 40 mM potassium cocadylate (pH 7.2), 8 mM $MgCl_2$, 60 μM [^{32}P]dATP (375 cpm/pmol), 1 mM 2-mercaptoethanol, 120 units of terminal transferase, and $[^3H](C)_n$–$[^{14}C](A)_n$–dA (5.5 nmol of $[^3H](C)_n$ and 8.5 nmol of $[^{14}C](A)_n$). After incubation at 37 °C for 1 h, the reaction was terminated by the addition of 10 μL of 0.2 M EDTA. The final $[^3H](C)_n$–$[^{14}C](A)_n$–$[^{32}P](dA)_n$ product was freed of reaction mixture components by chromatography on a 0.5-mL oligo(dT)–cellulose column as already described. Finally, the product was subjected to oligo(dG)–cellulose chromatography. Polymer was bound to a 2-mL column in 0.01 M Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.5 M LiCl, and 0.5% $NaDodSO_4$ and eluted with the same buffer minus salt plus 90% formamide. Only polymer bound by the column was recovered by ethanol precipitation, ensuring the presence of $[^3H](C)_n$ at the 5′ end of the polymer. The final product contained 200 pmol of $[^3H](C)_n$, 900 pmol of $[^{14}C](A)_n$, and 350 pmol of $[^{32}P](dA)_n$. Ninety percent of the ^{14}C in an aliquot of the final product was resistant to degradation by a 20-fold excess of *E. coli* RNase II, indicating that most of the molecules were protected at their 3′ termini by $(dA)_n$. *E. coli* RNase II is a 3′-exonuclease that will not degrade DNA, but will degrade single-stranded RNA effectively (Gupta et al., 1977).

Preparation of Cellulose–$[^3H](A)_n$. $[^3H](A)_n$ (5 μmol; 40 cpm/pmol) as supplied by the manufacturer was covalently linked at its 5′ end to cellulose with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodimide *p*-toluenesulfonate (Aldrich Chemical Co.) as described (Leis et al., 1973; Gilham, 1971). Care was taken after completion of the reaction to remove unreacted $[^3H](A)_n$ with extensive washing as described (Leis et al., 1973). Digestion with micrococcal nuclease (20 μg) released 50% of the $[^3H](A)_n$ in a 0.1-mL suspension of the cellulose–$[^3H](A)_n$ in 60 min.

7 M Urea–Polyacrylamide Disc Gel Electrophoresis. Electrophoresis on 5% and 25% polyacrylamide gels containing 7 M urea for examination of the size distribution of labeled polymers after digestion with RNase H was carried out as described previously (Grandgenett & Green; 1974; Gerard, 1978). The position of migration of unlabeled $(A)_n$ standards was determined by staining gels with Stains-All (Bio-Rad Laboratories) (Dahlberg et al., 1969). Gels containing radioactively labeled $(A)_n$ were fractionated by cutting into 2-mm slices (Grandgenett & Green, 1974; Gerard, 1978). Radioactivity was eluted from gel slices by incubation at 37 °C in a toluene base scintillation fluid containing 3% (v/v) Protosol (New England Nuclear); 60–80% of the radioactivity applied to gels was recovered during this procedure.

Results

Size of M-MLV DNA Polymerase–RNase H (RNase H I). Murine DNA polymerase–RNase H (RNase H I) used in mechanistic studies should be established to be structurally intact. Moelling (1976) has demonstrated that murine retrovirus preparations contain protease(s) which will attack reverse transcriptase, particularly during initial enzyme purification steps. Consequently, during purification of M-MLV RNase H I, the protease inhibitor phenylmethanesulfonyl fluoride was added during virion lysis and initial DEAE-Sephadex chromatography, and these steps were performed as rapidly as possible. A Coomassie blue stained sodium dodecyl sulfate gel representative of the M-MLV RNase H I used in these studies is shown in Figure 1A. On the basis of the rate of migration of M-MLV RNase H I relative to a series of protein standards (Figure 1D), its molecular weight can be calculated to be 80 000 daltons (data not shown). This value is in agreement with the estimate made by Verma (1975) for the molecular weight of M-MLV RNase H I. Moelling (1976) has reported the molecular weight of Friend leukemia virus RNase H I to be 84 000 relative to the α and β subunits of AMV reverse transcriptase. When α and β are assigned molecular weights of 70 000 and 110 000, respectively (Moelling, 1976), the molecular weight of M-MLV RNase H I relative to α and β as markers is also 84 000 (Figure 1C, and data not shown). On the basis of more recent determinations, however, the molecular weights of AMV α and β are 62 000 to 65 000 and 92 000 to 94 000, respectively (Gerard & Grandgenett, 1980). The best estimate of the molecular weight of M-MLV RNase H I is therefore 80 000.

Comparison of the Digestion Products of $[^3H](A)_{\langle 1100\rangle}$·$(dT)_n$ *Generated by M-MLV RNase H I and AMV αβ RNase H.* Previous estimates of the size of oligomers generated by the action of M-MLV RNase H I and AMV αβ RNase H on $[^3H](A)_n$·$(dT)_n$ indicated that these enzymes produce oligomers approximately 10–40 nucleotides long (Grandgenett & Green, 1974; Gerard & Grandgenett, 1975; Gerard, 1978). The gels used in these studies were not adequately standardized with reference markers, however, so that these product sizes were only rough estimates. For more accurate determinations of product sizes, 7 M urea polyacrylamide gels were standardized with various species of $(A)_n$ where $8 \leq n \leq 220$ (see Figure 2 and Figure 6). The products generated from $[^3H](A)_{\langle 1100\rangle}$·$(dT)_n$ by M-MLV RNase H I and AMV αβ RNase H after various periods of incubation were then analyzed on 25% polyacrylamide gels (Figure 2). $[^3H](A)_n$ of

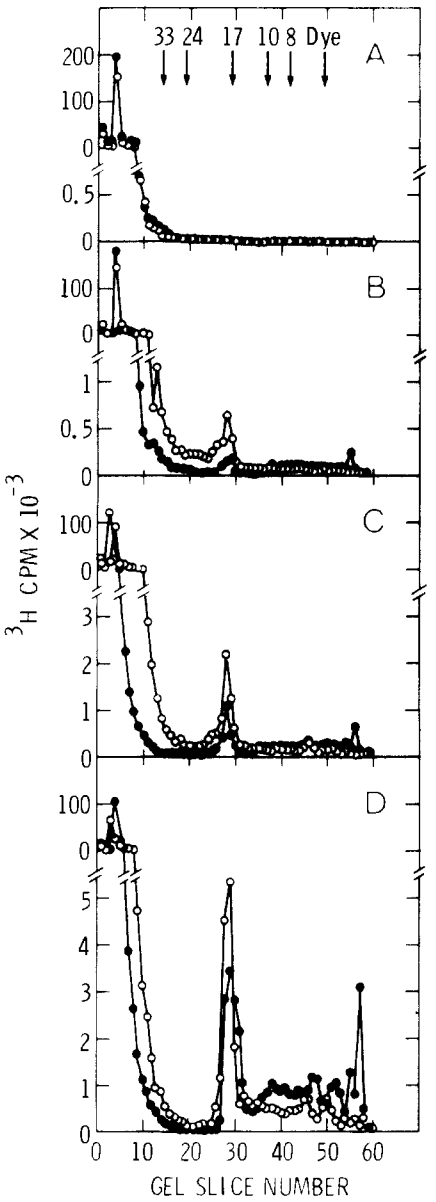

FIGURE 2: Polyacrylamide gel electrophoresis in 7 M urea of digestion products of $[^3H](A)_{(1100)}\cdot(dT)_n$ generated by M-MLV RNase H I (○) and AMV $\alpha\beta$ RNase H (●). Standard RNase H reaction mixtures (Experimental Procedures) containing 240 pmol of $[^3H](A)_{(1100)}$ (0.22 pmol of $A_{(1100)}$ molecules) and 290 pmol of $(dT)_n$ were incubated for 0 (A), 0.75 (B), 5 (C), and 20 min (D) in the presence of either 0.1 unit (0.1 pmol of enzyme) of M-MLV RNase H I or 0.1 unit (0.08 pmol of enzyme) of AMV $\alpha\beta$ RNase H. The digestion products were analyzed on 25% polyacrylamide gels. The position of migration of $(A)_n$ standards of known chain lengths are shown by the arrows.

high specific radioactivity was used in these and subsequent experiments so that small amounts of product could be detected subsequent to polyacrylamide gel fractionation of substrate–product mixtures. Very early in a reaction catalyzed by either M-MLV RNase H I or AMV $\alpha\beta$ RNase H (Figure 2B), the predominant single species of digestion product generated is 18 nucleotides long (fractions 26–30). Both enzymes also generated smaller products with a cross section of lengths (fractions 32–60) ranging from 18 down to a minimum of 2 (Keller & Crouch, 1973; G. F. Gerard, unpublished data). Two other features should be noted concerning the profiles in Figure 2B. First, both enzymes generated small amounts of products 20–26 nucleotides long (fractions 15–24), and the murine enzyme produced more than the avian. The level of these products did not increase substantially during the course

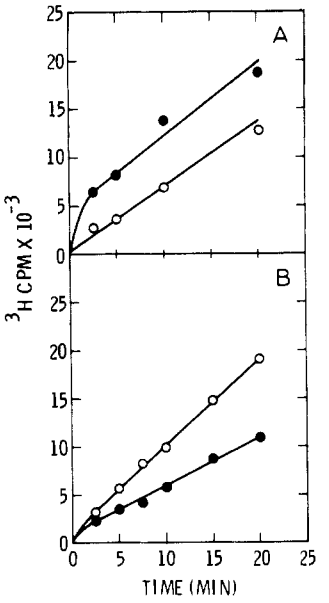

FIGURE 3: Time courses of the degradation of $[^3H](A)_{(1100)}\cdot(dT)_n$ catalyzed by M-MLV RNase H I and AMV $\alpha\beta$ RNase H. Standard RNase H reaction mixtures (Experimental Procedures) containing (A) 240 pmol of $[^3H](A)_{(1100)}$ (0.22 pmol of $A_{(1100)}$ molecules), 290 pmol $(dT)_n$, and 0.1 unit (0.1 pmol enzyme) of M-MLV RNase H I or (B) 240 pmol of $[^3H](A)_{(1100)}$, 290 pmol of $(dT)_n$, and 0.1 unit (0.08 pmol of enzyme) of AMV $\alpha\beta$ RNase H were incubated for the times indicated. The digestion products from each time point were analyzed on 25% polyacrylamide gels as shown in Figure 2. For each gel, total radioactivity in $[^3H](A)_n$ of the following two size classes is plotted against the time of incubation: $15 \leq n \leq 26$ (●) and $n \leq 14$ (○).

of the reaction (Figure 2C,D). Second, the avian enzyme produced larger quantities, relative to the murine enzyme, of small products that were shorter than 10 nucleotides (Figure 2C,D). As the reaction proceeded, the products of various size classes of both enzymes were produced in quantities roughly proportional to the levels present early (Figure 2C,D), with the exception of the products 20–26 nucleotides long mentioned above. From these results it is apparent that at early times in a reaction, as well as late, retrovirus RNase H produces oligonucleotide products ranging in length from 19 to less than 10, so that the larger oligonucleotides are produced simultaneously with the smaller.

To place the results from Figure 2 and subsequent experiments on a more quantitative basis and to facilitate comparisons with data from other laboratories, which have relied on acid precipitation rather than gel analysis to study reaction kinetics, I have taken the following approach. Oligonucleotide products 14 nucleotides or smaller are assumed to be 90% soluble in trichloroacetic acid, while oligonucleotide products in the 15–26 nucleotide range are assumed to be totally acid insoluble (Cleaver & Boyer, 1972). The radioactivity in these two size regions from each gel can then be totaled, yielding a quantitative estimate of the acid-soluble and -insoluble products generated by each enzyme. Application of such an analysis to data similar to that in Figure 2 produced the time courses shown in Figure 3A,B. With substrate in excess over enzyme, M-MLV RNase H I catalyzed an initial rapid rate of formation of acid-insoluble product. After 2.5 min, the rate of formation of acid-insoluble product decreased rapidly to a rate that paralleled that of acid-soluble product, which was linear throughout the course of a 20-min reaction (Figure 3A). In contrast, at a similar molar ratio of substrate to enzyme, AMV $\alpha\beta$ RNase H catalyzed a more rapid rate of formation of acid-soluble than acid-insoluble product throughout the

reaction course (Figure 3B). These results illustrate that when acid-precipitation techniques are utilized to assay retrovirus RNase H, the activity of both the avian and murine enzyme is underestimated, and the murine enzyme more so than the avian.

Is M-MLV RNase H I a Random or Processive Exonuclease? M-MLV RNase H I is an exonuclease that requires free ends in an RNA·DNA substrate, on the basis of its inability to cleave RNA inserted in one strand of the double-stranded superhelical *E. coli* plasmid DNA, $ColE_1$ (Moelling, 1974; Verma, 1975). In recent years, there has been some disagreement over whether murine retrovirus RNase H I is a processive or random exonuclease (Verma 1975; Moelling, 1976). A processive nuclease is an enzyme that, once bound to a polynucleotide chain substrate, does not dissociate until the substrate chain is completely degraded. A random exonuclease dissociates from the substrate molecule after each hydrolytic event. An exonuclease may also be partially processive and stay bound to a chain through a finite, average number of catalytic events, but dissociate before the substrate chain is completely degraded.

Analysis of Processivity by Substrate Competition. Both Verma (1975) and Moelling (1976) used the same experimental approach to study the mode of action of RNase H I. A large excess of cold $(A)_n$·$(dT)_n$ was added to an ongoing reaction that contained $[^3H](A)_n$·$(dT)_n$ and RNase H I. Verma (1975) found that the cold $(A)_n$·$(dT)_n$ inhibited the release of acid-soluble radioactivity while Moelling (1976) did not, leading to opposite conclusions. In the reports of Verma (1975) and Moelling (1976), it is not clear from the data whether the enzyme or the substrate was in molar excess, and to obtain interpretable results, the substrate must be in excess over the enzyme. A number of laboratories have performed similar types of experiments with AMV $\alpha\beta$ RNase H and $[^3H](A)_n$·$(dT)_n$ as substrate (Leis et al., 1973; Grandgenett & Green, 1974; Verma, 1975; Moelling, 1976). The results have consistently indicated a processive mode of action for this enzyme. In all of these studies, only the formation of acid-soluble product was monitored.

Figure 4 shows the results of similar experiments in which an excess of $[^3H](A)_{(1100)}$·$(dT)_n$ served as substrate for M-MLV RNase H I (Figure 4A) and AMV $\alpha\beta$ RNase H (Figure 4B) and in which the total amount of all products generated was determined by gel electrophoresis. Neither enzyme was inhibited during the first 5 min of reaction by the addition of a 10-fold molar excess of cold $(A)_n$·$(dT)_n$ 1 min after the start of the reaction. An exonuclease functioning randomly would have been inhibited immediately after addition of excess $(A)_n$·$(dT)_n$. As the reaction proceeded beyond 5 min, however, the activity of both enzymes was inhibited by a progressingly greater amount. The progressive inhibition observed after 5 min was probably created by trapping by excess cold $(A)_n$·$(dT)_n$ of enzyme molecules which were dissociating from partially degraded substrate molecules. On the basis of the initial rate of formation of all products catalyzed by M-MLV RNase H I and AMV $\alpha\beta$ RNase H under the reaction conditions described in Figure 2, 3A,B, and 4 and on the assumption of an average product length of 16 and 10 nucleotides, respectively, the murine enzyme would require approximately 40 min and the avian enzyme 70 min to degrade a molecule of $[^3H](A)_{(1100)}$·$(dT)_n$. These results, therefore, support a partially processive mode of action for both retroviral enzymes, as does the evidence presented below.

Analysis of Processivity by the Use of End-Labeled Substrate. A second more quantitative experimental approach

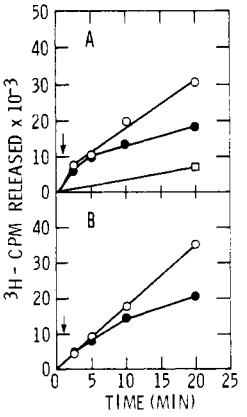

FIGURE 4: Effect of addition of excess $(A)_n$·$(dT)_n$ on the rate of degradation of $[^3H](A)_{(1100)}$·$(dT)_n$ by M-MLV RNase H I and AMV $\alpha\beta$ RNase H. (A) $[^3H](A)_{(1100)}$ (2400 pmol), $(dT)_n$ (2900 pmol), and 1 unit of M-MLV RNase H (1 pmol) were added to three standard scaled-up (10-fold) reaction mixtures. Unlabeled $(A)_n$ (26 nmol) and $(dT)_n$ (31 nmol) were either not added (○), added before start of the reaction (□), or added one min after the start of the reaction (●). (B) $[^3H](A)_{(1100)}$ (2400 pmol), $(dT)_n$ (2900 pmol), and 1 unit of AMV $\alpha\beta$ RNase H (0.9 pmol) were added to two standard scaled-up (10-fold) reaction mixtures. Unlabeled $(A)_n$ (26 nmol) and $(dT)_n$ (31 nmol) were either not added (○) or added 1 min after the start of the reaction (●). Samples (100 μL) were withdrawn at various times and analyzed by electrophoresis on 25% polyacrylamide gels. The total radioactivity from each gel present in $[^3H](A)_n$ smaller than 27 nucleotides is plotted.

that yields information regarding the processivity of an exohybridase relies on monitoring the relative rates of degradation of $(A)_n$·$(dT)_n$ where $(A)_n$ is labeled uniformly with one isotope and at one end with a different isotope. A random exohybridase attacking from a particular end of a substrate molecule should catalyze preferential release of label from that end, while a processive exohybridase or an endohybridase should hydrolyze internal label and end label at approximately equal rates. As in the previous experiment, care was taken to ensure that the substrate was in molar excess over the enzyme used, and only initial reaction rates were monitored. The relative rates were determined at which M-MLV RNase H I and AMV $\alpha\beta$ RNase H catalyzed the formation of ^3H- and ^{32}P-labeled $(A)_n$ products from $[^3H,5'\text{-}^{32}P](A)_{(380)}$·$(dT)_n$. Total product populations were analyzed by electrophoresis on 25% polyacrylamide gels. In the absence of $(dT)_n$, neither enzyme catalyzed any significant release of ^3H or ^{32}P label from the polymer. The kinetics of total ^{32}P- and ^3H-labeled product formation catalyzed by M-MLV RNase H I and AMV $\alpha\beta$ RNase H were essentially identical and are plotted in Figure 5A as the total percentage of ^{32}P vs. ^3H released at various time points. A processive exonuclease catalyzing the formation of ^3H- and ^{32}P-labeled products at equal rates will generate a line with a slope of 45° as illustrated by the broken line. In general, as processivity decreases, the slope of this line should deviate increasingly from the 45° line (Thomas & Olivera, 1978). The plots of the data generated by RNase H I and $\alpha\beta$ RNase H are almost identical in slope and deviate somewhat from 45° toward preferential release of ^{32}P. This deviation is much smaller than that observed with *E. coli* DNA polymerase I, which has random 5′ to 3′ exonuclease activity (Baltimore & Smoler, 1972; Leis et al., 1973). M-MLV RNase H I and AMV $\alpha\beta$ RNase H therefore attack the RNA in RNA·DNA in a 5′ to 3′ direction and function with equal degrees of partial processivity, and more processively than DNA polymerase I.

Similar experiments were performed with $[^3H,3'\text{-}^{32}P]$-$(A)_{(380)}$·$(dT)_n$ as substrate for M-MLV RNase H I and AMV

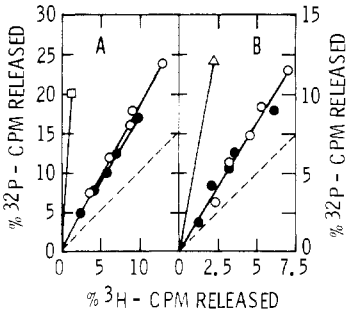

FIGURE 5: Comparison of the degree of processivity of M-MLV RNase H I and AMV $\alpha\beta$ RNase H. (A) The kinetics of digestion of [^{3}H,5′-^{32}P](A)$_{(380)}$·(dT)$_n$ by M-MLV RNase H I (○) and AMV $\alpha\beta$ RNase H (●) were plotted as percent ^{32}P vs. ^{3}H released. The percent ^{32}P vs. ^{3}H solubilized by 50 pg of *E. coli* DNA polymerase I (□) during a 2.5-min digestion in a standard reaction mixture containing 24 pmol of [^{3}H,5′-^{32}P](A)$_{(380)}$ and 29 pmol of (dT)$_n$ is also shown. (B) The kinetics of digestion of [^{3}H,3′-^{32}P](A)$_{(380)}$·(dT)$_n$ by M-MLV RNase H I (○) and AMV $\alpha\beta$ RNase H (●) were plotted as percent ^{32}P vs. ^{3}H released. The percent ^{32}P vs. ^{3}H solubilized by snake venom phosphodiesterase (△) during a 2.5-min incubation is shown as a control. Standard RNase H reaction mixtures contained 100 pmol of [^{3}H,3′-^{32}P](A)$_{(380)}$ (0.26 pmol of (A)$_{(380)}$ molecules) and 120 pmol of (dT)$_n$ and one of the following: 0.07 unit of M-MLV RNase H I (0.07 pmol of enzyme molecules), 0.045 units of AMV $\alpha\beta$ RNase H (0.04 pmol of enzyme molecules), or 0.2 μg of snake venom phosphodiesterase. The broken line represents equal rates of release of ^{32}P and ^{3}H.

Table I: Test of the Ability of RNase H I to Degrade [^{3}H](A)$_n$ Blocked at the 5′ Terminus[a]

enzyme	% [^{3}H](A)$_n$ solubilized with: [5′-^{32}P](C)$_{(100)}$-[^{3}H](A)$_{(100)}$ −(dT)$_n$	[5′-^{32}P](C)$_{(100)}$-[^{3}H](A)$_{(100)}$ +(dT)$_n$	cellulose-[^{3}H](A)$_n$ −(dT)$_n$	cellulose-[^{3}H](A)$_n$ +(dT)$_n$
M-MLV RNase H I	<1	62	6	31
AMV $\alpha\beta$ RNase H	0	38	0	21
E. coli RNase H	13	70	1	23

[a] Substrates were synthesized and [^{3}H](A)$_n$ was covalently attached at its 5′ end to cellulose as described under Experimental Procedures. Standard reaction mixtures (100 μL) contained [5′-^{32}P](C)$_{(100)}$-[^{3}H](A)$_{(100)}$ (256 pmol of [^{3}H](A)$_{(100)}$) and (dT)$_n$ as indicated at 1050 pmol, and one of the following: 1.5 units of RNase H I, 1.1 units of AMV $\alpha\beta$ RNase H, or 2.8 units of *E. coli* RNase H. Incubation was for 20 min. A suspension (0.1 mL) of cellulose-[^{3}H](A)$_n$ (9200 cpm of ^{3}H, 40 cpm/pmol) was centrifuged for 4 min in a Microfuge B (Beckman), and the supernatant was discarded. The cellulose was suspended in standard reaction mixtures (100 μL) containing 1050 pmol (dT)$_n$ where indicated and one of the following: 1.3 units of RNase H I, 1.8 units of AMV $\alpha\beta$ RNase H, or 10 units of *E. coli* RNase H. During a 20-min incubation, tubes were mixed every 5 min. The reaction was stopped by the addition of 200 μL of cold water and centrifuged as above. The supernatant was counted in aqueous scintillation fluid. The amount of [^{3}H](A)$_n$ released by micrococcal nuclease (50%) was assumed to be all the [^{3}H](A)$_n$ acessible to attack by the enzymes.

$\alpha\beta$ RNase H, and the results are illustrated in Figure 5B. Again, the plots of the data generated by RNase H I and $\alpha\beta$ RNase H have identical slopes, and the slopes deviate from 45° by almost the same extent as in Figure 5A. This deviation is smaller than that observed with snake venom phosphodiesterase, a random 3′ to 5′ exonuclease. These results substantiate the conclusion that both retroviral enzymes function as partially processive exonucleases. The fact that M-MLV RNase H I preferentially releases ^{32}P over ^{3}H with [^{3}H,3′-^{32}P](A)$_{(380)}$·(dT)$_n$ as substrate, coupled with the observation that M-MLV RNase H I can also degrade [^{3}H](A)·(dT)$_n$ when the (A)$_n$ is blocked at the 5′ end with cellulose (Table I), demonstrates that M-MLV RNase H I attacks a substrate in the 3′ to 5′ direction as well as in the 5′ to 3′ direction. It should, therefore, be emphasized that the data in Figure 5 probably overestimate the processivities of the retroviral enzymes, since while ^{32}P and ^{3}H are released as the result of enzymatic attack in one direction, ^{3}H is released by enzyme molecules attacking in the other direction.

Assessment of the Ability of Retroviral RNase H to Degrade (A)$_n$ Blocked at the 5′ End with RNA and at the 3′ End with DNA. On the basis of the inability of retroviral DNA polymerase associated RNase H to degrade the RNA inserted in *E. coli ColE*$_1$ superhelical DNA (Keller & Crouch, 1972; Grandgenett & Green, 1974; Moelling, 1974; Verma, 1975) or circular poly(A) hybridized to (dT)$_n$ (Leis et al., 1973), it was concluded that the viral enzyme requires free ends in its RNA substrate for activity. What this concept means in terms of the ability of retroviral hybridase to degrade RNA in RNA·DNA in which the RNA termini overlap the ends of the DNA has yet to be established. I have tested the ability of M-MLV RNase H I and AMV $\alpha\beta$ RNase H to degrade [^{3}H](A)$_n$ with (C)$_{(100)}$ attached at the 5′ end (Table I). In the presence (dT)$_n$, the [^{3}H](A)$_n$ in (C)$_{(100)}$-[^{3}H](A)$_{(100)}$ was degraded by RNase H I and $\alpha\beta$ (Leis et al., 1973). However, this experiment suffers from a lack of independent confirmation that the presence of (C)$_{(100)}$ at the 5′ end of [^{3}H]-(A)$_{(100)}$ actually clocks the 5′ to 3′ exonuclease activity of retroviral RNase H, since the 3′ end of the substrate was not blocked. For such confirmation, (C)$_n$-(A)$_n$ was synthesized which contained (dA)$_n$ attached at the 3′ terminus. DNA was selected to block the 3′ end of (A)$_n$ since a positive demonstration of the ability of retroviral RNase H to degrade a substrate such as (C)$_n$-(A)$_n$-(dA)$_n$·(dT)$_n$ would have important implications for the potential function of the enzyme in vivo (see Discussion).

[^{3}H](C)$_n$-[^{14}C](A)$_n$-[^{32}P](dA)$_n$ was synthesized in several successive reactions catalyzed by polynucleotide phosphorylase and calf thymus terminal transferase as described in Experimental Procedures. Examination on 7 M urea–5% polyacrylamide gels (Figure 6A) showed that most of the polymer was 120–320 nucleotides long. Removal of the [^{3}H](C)$_n$ and [^{14}C](A)$_n$ in the polymer preparation by digestion with pancreatic RNase A demonstrated that the [^{32}P](dA)$_n$ present was fairly uniform in size and had an average length of 60 nucleotides (Figure 6B). Therefore, the sum of the lengths of [^{3}H](C)$_n$ and [^{14}C](A)$_n$ in the polymer probably was in the range of 60–260 nucleotides, and on the average, the lengths of [^{3}H](C)$_n$ and [^{14}C](A)$_n$ were in the range of 15–60 and 45–200 nucleotides, respectively, based on the molar ratios of ^{3}H and ^{14}C in the polymer.

In the absence of (dT)$_n$, neither preparation of RNase H solubilized significant amounts of any of the labels contained in [^{3}H](C)$_n$-[^{14}C](A)$_n$-[^{32}P](dA)$_n$. In addition, the gel profile of the substrate in the absence of (dT)$_n$ was not significantly altered in the presence of AMV $\alpha\beta$ RNase H (compare Figure 6A,C) or M-MLV RNase H I (data not shown). In the presence of (dT)$_n$, both enzymes attacked polymer of all size classes (Figure 6D,E), releasing [^{32}P](dA)$_{(60)}$, [^{3}H](C)$_n$ that migrated at the same position or ahead of (dA)$_{(60)}$, and smaller oligonucleotides of [^{14}C](A)$_n$. Product formation was proportional to enzyme concentration. In the presence of 0.5 unit of murine enzyme, the amount of ^{14}C and ^{32}P in polynucleotides less than 95 nucleotides long increased 22% and 12%, respectively; in the presence of 1 unit of avian enzyme, the increases were 37% and 27%, respectively. With each enzyme, the proportion of ^{14}C released comes into line with the ^{32}P released assuming the [^{14}C](A)$_n$ in all substrate

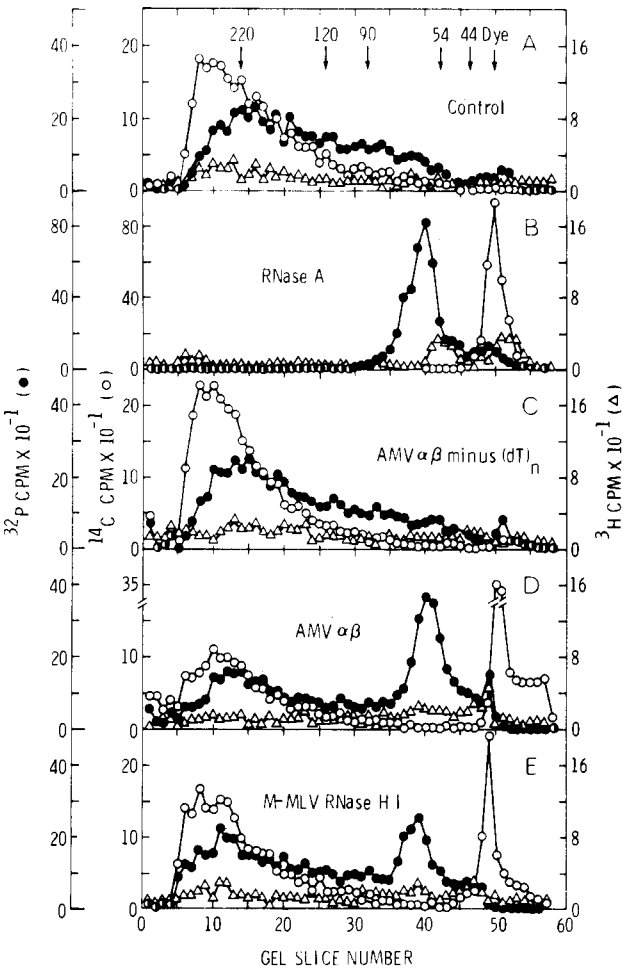

FIGURE 6: Polyacrylamide gel electrophoresis in 7 M urea of digestion products of $[^3H](C)_n$-$[^{14}C](A)_n$-$[^{32}P](dA)_n$ produced by RNase A, M-MLV RNase H I, and AMV $\alpha\beta$ RNase H. Standard reaction mixtures were incubated for 20 min and contained $[^3H]$-$(C)_n$-$[^{14}C](A)_n$-$[^{32}P](dA)_n$ (20 pmol in ^3H; 90 pmol in ^{14}C; and 35 pmol in ^{32}P) and either no added enzyme (A), 2 μg of heated RNase A (B), 1 unit of AMV $\alpha\beta$ RNase H(C), 1 unit of AMV $\alpha\beta$ RNase H and 4.5 μM $(dT)_n$ (D), or 0.5 unit of M-MLV RNase H I, 4.5 μM $(dT)_n$, and 1 μg/mL bentonite (E). Polyacrylamide gels (5%) were analyzed for radioactivity in ^3H (△), ^{14}C (○), and ^{32}P (●). The total radioactivity in ^{14}C was corrected for a 15% overlap from ^{32}P and the total radioactivity in ^3H was corrected for a 30% overlap from ^{14}C. The positions of migration of $(A)_n$ standards of known chain lengths are shown by the arrows.

molecules unprotected by $(dA)_n$ (10%) was digested. I conclude from these results that retroviral RNase H is capable of degrading $[^{14}C](A)_n$ blocked at the 5′ end with $(C)_n$ and at 3′ end with $(dA)_n$ when the $[^{14}C](A)_n$ is annealed to $(dT)_n$.

Discussion

On the basis of the evidence established utilizing classical substrate competition and end-labeling procedures with $[^3H](A)_n$·$(dT)_n$, both M-MLV RNA-directed DNA polymerase associated RNase H (RNase H I) and AMV $\alpha\beta$ RNase H are partially processive exohybridases, i.e., they remain bound to a substrate through a finite number of hydrolytic events, but not until the substrate molecule is completely degraded. These conclusions regarding the mode of action of AMV $\alpha\beta$ RNase H and M-MLV RNase H I differ from the results of earlier work (Leis et al., 1973; Grandgenett & Green, 1974; Verma, 1975; Moelling, 1976). This is explained at least in part by the fact that in the original work, the significant amounts of acid-insoluble oligonucleotide products generated by the enzymes were not included in enzyme kinetic analyses. In the experiments reported here, enzyme activity was assayed by subjecting reaction products to polyacrylamide gel electrophoresis rather than acid precipitation; therefore, all digestion products were recovered for analysis.

The basic difference between the murine and avian enzymes pinpointed by this work was the ratio of acid-insoluble to acid-soluble oligonucleotide products generated during the course of a reaction. Even though both enzymes generated $(A)_n$ with a cross section of lengths $(2 < n < 26)$ all during the course of a reaction, the murine RNase H produced a much higher proportion of $(A)_n$ $(15 \leq n \leq 26)$ than $(A)_n$ $(n \leq 14)$ when the $(A)_{(1100)}$·$(dT)_n$ substrate was in excess over enzyme, while just the opposite was true with AMV $\alpha\beta$ RNase H. The fact that both enzymes produce large cleavage products that probably remain hydrogen bonded to DNA has important implications for the function of retroviral RNase H. Watson et al. (1979) have shown that in a model system containing 3′-$(C)_{(35)}$-$(A)_n$·$(dI)_{(15)}$ as template-primer, AMV $\alpha\beta$ DNA polymerase-RNase H, dGTP, dTTP, and dATP, both $(dT)_n$ and $(dA)_n$ are synthesized, and that the synthesis of $(dA)_n$ is primed by fragments of $(A)_n$ which are generated by the action of $\alpha\beta$ RNase H on the $(A)_n$·$(dT)_n$ hybrid formed initially.

On the basis of results with double-labeled and end-blocked substrates, M-MLV RNase H I can attack the RNA in RNA·DNA in both the 5′ to 3′ and 3′ to 5′ direction. In this respect, M-MLV RNase H I resembles the RNase H activity associated with both the α and $\alpha\beta$ forms of AMV reverse transcriptase (Leis et al., 1973; Grandgenett & Green, 1974).

Keeping in mind this ability to attack RNA·DNA in either direction and the fact that retroviral RNase H seems to generate at random a population of oligonucleotide products with a cross section of lengths (2–26 nucleotides), some suggestions can be made regarding the enzyme's mode of action. In order for an exohybridase to function with any degree of processivity, the enzyme must remain bound to the RNA·DNA chain even after hydrolysis of a phosphodiester bond has occurred and must then be able to move along the chain for a random but limited number of nucleotides before catalyzing another hydrolytic event. Thomas & Olivera (1978) have suggested that processive exonucleases require a second binding site or anchor site in addition to a catalytic site to maintain binding to substrate after hydrolysis, while random exonucleases do not require such a site. They further suggest that this anchor site recognizes some termination signal in the substrate which causes the enzyme to dissociate. Perhaps the termination signal for retrovirus RNase H is a gap in the RNA strand of a RNA·DNA substrate. An enzyme molecule might therefore generate its own termination signal by carrying out processive hydrolysis while moving in both directions on an RNA·DNA molecule and dissociating when it encounters a region in the substrate where the RNA has been degraded sufficiently to dissociate from the DNA and produce a gap. An enzyme functioning in this manner would appear partially processive.

The anchor site postulated in the previous discussion could be located at the DNA polymerase active site of either the murine or avian reverse transcriptase, or perhaps at the DNA endonuclease active site of AMV $\alpha\beta$ DNA polymerase–RNase H (Golomb & Grandgenett, 1979). Substantial evidence exists for the separate identity of the RNase H, DNA polymerase, and DNA endonuclease active sites in these enzymes (Gerard & Grandgenett, 1980). The existence of a random exohybridase (G. F. Gerard, unpublished results) in M-MLV with a molecular weight of 30000 daltons that appears to be derived from M-MLV DNA polymerase–RNase H by proteolytic

cleavage (Gerard, 1978), supports the concept of the DNA polymerase active site serving as an anchor site. In the case of AMV $\alpha\beta$ DNA polymerase–RNase H, either the DNA polymerase or DNA endonuclease (Golomb & Grandgenett, 1979) active site could function an an anchor site for RNase H. Grandgenett & Green (1974) found that the α form of AMV DNA polymerase–RNase H, which lacks the DNA endonuclease active site (Golomb & Grandgenett, 1979), has random exohybridase activity. This has been confirmed in substrate competition experiments similar to those in Figure 4 performed with AMV α RNase H purified to homogeneity as described (Grandgenett, 1976) and $[^3H](A)_{(1100)}\cdot(dT)_n$ (G. F. Gerard, unpublished data). Therefore, loss of the DNA endonuclease site in the conversion of $\alpha\beta$ to α results in a change from partially processive to random in the mode of action of RNase H, consistent with the idea that the DNA endonuclease site serves as an anchor site for AMV RNase H.

Interestingly, I found that $(A)_n$, blocked at the 3′ end by $(dA)_n$ approximately 60 nucleotides long and at the 5′ end by $(C)_n$ an average of 30 nucleotides long, when hybridized to $(dT)_n$, was hydrolyzed by both M-MLV RNase H I and AMV $\alpha\beta$ RNase H. Similar results have been obtained with substrates containing $(A)_n$ protected with $(dA)_n$ 140 nucleotides long and $(C)_n$ an average of 60 nucleotides long (G. F. Gerard, unpublished data). These findings have important implications for the function of retroviral RNase H in vivo. According to recent models of proviral DNA synthesis (Varmus et al., 1978; Shank et al., 1978; Coffin, 1979; Gilboa et al., 1979b; Dina & Benz, 1980) based primarily on data derived from restriction endonuclease mapping of proviral DNA synthesized in vivo (Shank et al., 1978; Hsu et al., 1978; Varmus et al., 1978) and in vitro (Gilboa et al., 1979a,b), two "jumps" must be made by the growing (−) strand DNA. The mechanism of the first "jump" has been discussed in detail (Bishop, 1978) and is not of interest here, except that it illustrates the general mechanism followed in the second "jump", i.e., a short nucleotide sequence is synthesized at the 3′ end of the growing (−) strand DNA which is complementary to a sequence at the 3′ end of the polynucleotide chain to which the growing chain must "jump". Before the second "jump" can be completed, RNA (1) contained in an RNA·DNA hybrid structure approximately 20 nucleotides long, (2) having a section of unhybridized tRNA approximately 60 nucleotides long covalently attached at its 5′ end, and (3) having DNA covalently attached at its 3′ end must be hydrolyzed. The results presented here indicate that retroviral RNase H is capable of removing the unhybridized tRNA as well as the RNA contained in such a RNA·DNA hybrid.

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References

Baltimore, D. (1970) *Nature (London) 226*, 1209.
Baltimore, D., & Smoler, D. F. (1972) *J. Biol. Chem. 247*, 7282.
Bishop, J. M. (1978) *Annu. Rev. Biochem. 47*, 35.
Bollum, F. J. (1974) *Enzymes, 3rd Ed. 10*, 145.
Chou, J. Y., & Singer, M. F. (1971) *J. Biol. Chem. 246*, 7486.
Cleaver, J. E., & Boyer, H. W. (1972) *Biochim. Biophys. Acta 262*, 116.
Coffin, J. M. (1979) *J. Gen. Virol. 42*, 1.
Collett, M. S., Leis, J. P., Smith, M. S., & Faras, A. J. (1978) *J. Virol. 26*, 498.
Dahlberg, A. E., Dingman, C. W., & Peacock, A. C. (1969) *J. Mol. Biol. 41*, 139.
Dina, D., & Benz, E. W. (1980) *J. Virol. 33*, 377.
Eisenberg, H., & Felsenfeld, G. (1967) *J. Mol. Biol. 30*, 17.
Felix, F., Potter, J. L., & Laskowski, M. (1960) *J. Biol. Chem. 235*, 1150.
Gerard, G. F. (1978) *J. Virol. 26*, 16.
Gerard, G. F., & Grandgenett, D. P. (1975) *J. Virol. 15*, 785.
Gerard, G. F., & Grandgenett, D. P. (1980) in *The Molecular Biology of RNA Tumor Viruses* (Stephenson, J., Ed.) p 345, Academic Press, New York.
Gilboa, E., Goff, S., Shields, A., Yoshimura, F., Mitra, S., & Baltimore, D. (1979a) *Cell 16*, 863.
Gilboa, E., Mitra, S. W., Goff, S., & Baltimore, D. (1979b) *Cell 18*, 93.
Gilham, P. T. (1971) *Methods Enzymol. 21D*, 191.
Golomb, M., & Grandgenett, D. P. (1979) *J. Biol. Chem. 254*, 1606.
Golomb, M., Vora, A. C., & Grandgenett, D. P. (1980) *J. Virol. Methods 1*, 157.
Grandgenett, D. P. (1976) *J. Virol. 20*, 348.
Grandgenett, D. P., & Green, M. (1974) *J. Biol. Chem. 249*, 5148.
Grandgenett, D. P., Gerard, G. F., & Green, M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 230.
Grandgenett, D. P., Vora, A. C., & Faras, A. J. (1976) *Virology 75*, 26.
Grandgenett, D. P., Golomb, M., & Vora, A. C. (1980) *J. Virol. 33*, 264.
Green, M., & Gerard, G. F. (1974) *Prog. Nucleic Acid Res. Mol. Biol. 17*, 188.
Gupta, R. S., Kasai, T., & Schlessinger, D. (1977) *J. Biol. Chem. 252*, 8945.
Haseltine, W. A., Panet, A., Smoler, D., Baltimore, D., Peters, G., Harada, F., & Dahlberg, J. E. (1977) *Biochemistry 16*, 3635.
Hsu, T. W., Sabran, J. L., Mark, G. E., Guntaka, R. V., & Taylor, J. M. (1978) *J. Virol. 28*, 810.
Iverius, P. H. (1971) *Biochem. J. 124*, 677.
Keller, W., & Crouch, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3360.
Leis, J. P., Berkower, I., & Hurwitz, J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 466.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
McKnight, G. S. (1977) *Anal. Biochem. 78*, 86.
Mizutani, S., & Temin, H. (1970) *Nature (London) 226*, 1211.
Moelling, K. (1974) *Virology 62*, 46.
Moelling, K. (1976) *J. Virol. 18*, 418.
Moelling, K., Bolognesi, D. P., Bauer, H., Busen, W., Plassmann, H. W., & Hausen, P. (1971) *Nature (London), New Biol. 234*, 240.
Niyogi, S. K. (1972) *J. Mol. Biol. 64*, 609.
Panet, A., Haseltine, W. H., Baltimore, D., Peters, G., Harada, F., & Dahlberg, J. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2535.
Riggins, C. H., Bondurant, M. C., & Mitchell, W. M. (1974) *Intervirology 2*, 209.
Shank, P. R., Hughes, S. H., Sung, H. J., Mjors, J. E., Qunitrell, N., Guntaka, R. V., Bishop, J. M., & Varmus, H. E. (1978) *Cell 15*, 1383.

Temin, H., & Baltimore, D. (1972) *Adv. Virus Res. 17*, 129.
Thomas, K. R., & Olivera, B. M. (1978) *J. Biol. Chem. 253*, 424.
Varmus, H. E., Heasley, S., Kung, H. J., Oppermann, H., Smith, V. C., Bishop, J. M., & Shank, P. R. (1978) *J. Mol. Biol. 120*, 55.
Verma, I. (1977) *Biochim. Biophys. Acta 473*, 1.
Verma, I. M. (1975) *J. Virol. 15*, 843.
Verma, I. M., & Baltimore, D. (1973) *Methods Enzymol. 29*, 125.
Watson, K. F., Schendel, P. L., Rosok, H. J., & Ramsey, L. R. (1979) *Biochemistry 18*, 3210.
Wu, A. M., & Gallo, R. C. (1976) *C.R.C. Crit. Rev. Biochem. 3*, 289.

Hydrogen-1 and Phosphorus-31 Nuclear Magnetic Resonance Study of the Solution Structure of *Bacillus licheniformis* 5S Ribonucleic Acid†

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ABSTRACT: The conformation of *Bacillus licheniformis* 5S RNA in solution has been studied by using 360-MHz ^{1}H NMR and 40.5-MHz ^{31}P NMR spectroscopy. The ^{1}H NMR spectra, which are well resolved, have been compared with theoretical spectra derived by ring current shift calculations for various models proposed in the literature for the secondary structure of 5S RNA. The total amount of base pairs is estimated to be around 36. NMR melting experiments indicate that both the molecular stalk and the prokaryotic loop [Fox, G. E., & Woese, C. R. (1975) *Nature (London) 256*, 505] are present in the solution structure. On this basis, some models proposed for the secondary structure of 5S RNA not containing these structural features can be rejected. Several resonances are observed around 10.7 ppm that can be ascribed to protons involved in non-Watson–Crick base pairing most likely present in tertiary interactions in the 5S RNA molecule or to ring N protons of nonpaired bases which as a result of the molecular folding are shielded from the solvent. Under our solution conditions, these structural features disappear at physiological temperature, the process being uncoupled from the collapse of the secondary structure. Using ^{31}P NMR, we demonstrate that the number of phosphate conformations in the sugar phosphate backbone of 5S RNA, deviating from the g^-,g^- conformation normally found in double helices, is far less than in tRNA.

5S RNA is an integral part of all ribosomes with the possible exception of some mitochondrial ribosomes (Borst & Grivell, 1971; Lizardi & Luck, 1971). Reconstitution experiments have clearly demonstrated the importance of prokaryotic 5S RNA for ribosomal function (Erdmann et al., 1971; Dohme & Nierhaus, 1976). It is generally accepted that 5S RNA plays a role in binding, directly or indirectly, some proteins to the large ribosomal subunit. In prokaryotes, 5S RNA is also thought to take part directly in protein synthesis by binding tRNA to the ribosome through base pairing (Erdmann, 1976). Some authors have speculated that cyclic conformational changes in prokaryotic 5S RNA may be the driving force for translocation in protein synthesis (Woese et al., 1975; Weidner et al., 1977).

Knowledge of the three-dimensional structure of 5S RNA derived by X-ray crystallography is, of course, an essential element in understanding its function. So far, studies used for probing the structure of 5S RNA in solution as well as in the ribosome have been limited to other techniques (Erdmann, 1976; Lewis & Doty, 1977; Österberg et al., 1976; Marshall & Smith, 1977; Wrede et al., 1978; Luoma & Marshall, 1978a,b; Chen et al., 1978; Hamill et al., 1978; Noller & Garrett, 1979). This has resulted in an abundance of models for the secondary structure of 5S RNA (Erdmann, 1976). Some studies suggest the existence of tertiary interactions within the 5S RNA molecule (Erdmann, 1976; Douthwaite et al., 1979; Noller & Garrett, 1979), but concrete information on this important aspect of the conformation is not yet available.

One important consideration in judging the feasibility of a proposed base-pairing scheme for prokaryotic 5S RNA ought to be the general applicability of the scheme. There is ample evidence from reconstitution experiments (Erdmann, 1976) that 5S RNA from one prokaryote can be incorporated into the large ribosomal subunit of another prokaryote without loss of biological activity. Therefore, the overall conformation of 5S RNA from various prokaryotes should be closely similar. The first model taking this fact fully into account is the one proposed by Fox & Woese (1975) which includes only those intramolecular double-helical regions that could be formed in all six prokaryotic 5S RNAs known at the time of its publication. Sequences of 5S RNA determined subsequently proved to conform to the model without fail. The model also is in good agreement with studies of the sites in the 5S RNA molecule sensitive to nuclease attack (Vigne & Jordan, 1971; Jordan, 1971; Bellemare et al., 1972a) or modifying agents (Lee & Ingram, 1969; Bellemare et al., 1972b; Gray et al., 1973; Noller & Herr, 1974; Delihas et al., 1975). It is, however, still at odds with other data pertaining to the secondary structure of prokaryotic 5S RNA, notably the oligonucleotide-binding studies of Wrede et al. (1978). More recent models, e.g., the model of Luoma & Marshall (1978b) and the model of Österberg et al. (1976), are also in good agreement with the nuclease-sensitive sites.

Secondary structure interactions between bases in an RNA chain can be studied with the aid of proton magnetic resonance. Although, as yet, it is not possible to deduce the conformation of an RNA molecule directly from the proton spectrum, the

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