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Increased Length of DNA Made by Virions of Murine Leukemia Virus at Limiting Magnesium Ion Concentration

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Conditions have been developed for reverse transcription by detergent-disrupted virions of Moloney murine leukemia virus which permit synthesis of molecules that appear to be complete transcripts of the 35S RNA subunits. At limiting Mg^{2+} concentration, DNA is synthesized in good yield, up to a maximum size of about 2.4×10^6 daltons. DNA larger than 2×10^6 daltons, taken from alkaline sucrose gradients, has no detectable self-complementarity and was protected from digestion by S_1 nuclease to an extent of 90% by annealing to 70S RNA. All size classes of DNA made in these reactions are primed with RNA, because all are initiated with a pApdA junction. To produce such long molecules, it is necessary to keep the concentration of Mg^{2+} in the reaction mixture below the total concentration of deoxyribonucleoside triphosphates. Under these conditions, degradation of the RNA template is minimized. The rate of DNA synthesis is also slowed by 30 to 50%, but products longer than 5,000 nucleotides, which are not found otherwise, are completed between 3 and 6 h of reaction.

An increasing body of evidence indicates that the RNA-dependent DNA polymerases of RNA tumor viruses (18) are competent to synthesize high-molecular-weight DNA transcripts of the viral RNA genome without the involvement of cellular factors (2, 10, 14). These large molecules are made by detergent-treated virions of murine leukemia virus (MuLV) when the concentration of each deoxynucleoside triphosphate in the reaction is at least 10-fold higher than the reported K_m of purified reverse transcriptase (6, 12). Even at very high substrate concentrations, however, we were previously unable to demonstrate synthesis of full-length transcripts of the MuLV genome (14), in contrast to results of Junghans et al. with Rous sarcoma virus (10).

In this paper, we report that DNA molecules of increased length can be synthesized by virions of Moloney MuLV in the presence of rate-limiting concentrations of Mg^{2+} ion. The products are made in significant yield up to a defined limit-length, which is large enough to represent transcription of an entire 35S subunit of the 70S RNA genome. All size-classes of DNA synthesized in these reactions are initiated with a pApdA bond. Thus, a common mechanism of initiation appears to exist for the limit-length DNA transcripts as well as the smaller DNA products previously shown to initiate at an unique site (W. A. Haseltine, D. Kleid, A. Panet, E. Rothenberg, and D. Baltimore, J. Mol. Biol., in press). At least part of the effect of restricting the Mg^{2+} concentration is to stabilize the RNA template during the course of the incubation.

MATERIALS AND METHODS

Virus. The virus used in these studies was the clone 1 strain of Moloney MuLV (5). Both virus harvested at 3- to 4-h intervals and virus harvested after 12 h were used, with interchangeable results. Virus and viral RNA were purified as described previously (14).

Endogenous reverse transcriptase reaction. The endogenous polymerase reaction conditions were essentially as described previously (14). Detergent-disrupted virions of Moloney MuLV were incubated at 37°C in the presence of 100 μg of actinomycin D per ml, 50 mM Tris-hydrochloride, 10 to 20 mM dithiothreitol, 60 mM NaCl, and 0.01% Nonidet P-40 (Shell). Deoxyribonucleoside triphosphates (dNTP's) and magnesium acetate were added at the concentrations indicated in the text and figure legends. All unlabeled dNTP stock solutions were neutralized, treated with Chelex 100 resin, and quantitated by absorbance at λ_{max}. [^{3}H]dCTP and [α-^{32}P]dATP were obtained from New England Nuclear Corp. The reaction products were purified as described (14), except that the Sephadex G-50 chromatography step was omitted for all but preparative samples.

Alkaline sucrose gradients. Alkaline sucrose gradients and samples were prepared as described previously (14). Conditions of centrifugation were as described in the figure legends. For direct soluble

counting, fractions were diluted, neutralized, and counted in Handifluor as described (14); for preparative gradients or for acid-precipitated samples, 0.36-ml fractions were neutralized with 0.5 ml of 0.1 M Tris-0.2 M CH_3COOH.

SDS-sucrose gradients. RNA samples were centrifuged through 15 to 30% sucrose gradients containing 0.5% sodium dodecyl sulfate (SDS) as described previously (4). Viral 70S RNA was denatured by heating to 78°C for 2 min in 10 mM Tris, pH 7.5, 2 mM EDTA, and 0.5% SDS, and quick-chilling.

Polyacrylamide gel electrophoresis. Preparation of samples and conditions for electrophoresis were as described (Haseltine et al., in press).

Agarose gel electrophoresis. Samples to be subjected to electrophoresis through agarose gels were first ethanol-precipitated in plastic Brinkmann microtest tubes. The pellets were dried in a lyophilizer, dissolved in 15 μl of 0.1 N NaOH, and incubated for 10 to 30 min at 37°C to denature double-stranded nucleic acids and to partially degrade residual template RNA. They were then chilled on ice and diluted with 30 μl of ice-cold 2 mM EDTA. Five to ten microliters of bromophenol blue tracking dye in 60% sucrose or 50% glycerol was added to each sample immediately before loading onto a 3-mm-thick slab gel of 1.4% agarose in 40 mM Tris-acetate, 50 mM Na acetate, 1 mM EDTA (pH 8.3) (E′ buffer). The upper buffer chamber of the gel apparatus was filled just before loading with E′ buffer that had been chilled to 0°C. The samples were initially run into the gel for 5 min at 100 V while supplemental ice was added as needed to the upper buffer chamber to keep it cold. Then electrophoresis was carried out for 10 to 12 h at 20 V (about 2 V/cm) with recirculation of buffer. Under these conditions, single-strand DNA migrated as a linear function of the logarithm of its molecular weight (Fig. 5; unpublished data of D. Donoghue and E. Rothenberg; also reference 8). The denatured double-strand DNAs used as markers appeared as doublets in some cases, but the error contributed was not more than 5 to 10% in most cases. Uniformity of salt concentration among the samples was, of course, crucial for accurate comparisons of their mobilities.

Tritium fluorography was carried out as described (11). Unincorporated dNTP was eluted from the gel in the course of the fluorography procedure. For the preparative gel shown in Fig. 9A, a 1-mm-thick agarose slab was cast on a plug of 15% acrylamide–0.75% *N,N*-methylene bisacrylamide in E′ buffer. Glycerol was added to 10% concentration in the thin slab to improve handling. The DNA of appropriate size was located by autoradiography, and the slices containing the DNA were excised with a razor blade. DNA was eluted from the gel as follows. The gel slices were placed in Brinkmann microtest tubes and weighed, and 1.25 ml of 5 M $NaClO_4$ was added per g of gel, in this case 0.2 to 0.3 g. The tubes were then incubated 7 min at 67°C, which was sufficient to dissolve the agarose. Sodium phosphate buffer at neutral pH was then added to a final concentration of 10 mM, and approximately 30 μg of hydroxylapatite (Bio-Gel HTP, Bio-Rad) was mixed with each sample and incubated for 30 min at 37°C with occasional agitation. After a brief low-speed centrifugation, the hydroxylapatite pellet was washed twice with 1 ml of 10 mM phosphate buffer, and nucleic acid was finally eluted from the pellet with two 0.5-ml washes of 0.48 M phosphate buffer at 67°C. The DNA was desalted by passage over a Sephadex G-50 column in water and concentrated by lyophilization. The yield was 50 to 70% of the radioactivity in the excised gel band.

Alkaline hydrolysis and high-voltage electrophoresis. The RNA moieties of reverse transcriptase products were hydrolyzed to mononucleotides by incubation in 10% (vol/vol) piperidine for 24 h at 55°C. The samples were then lyophilized to dryness, redissolved in 20 to 25 μl of 0.1 mM EDTA with the four 2′,3′-ribonucleotides as markers, and applied to a sheet of Whatman 3MM paper. The samples were subjected to electrophoresis for 1.5 h at 2,500 V in pyridine-acetic acid buffer (pH 3.5) (22). The markers were located by visualization under short-wave ultraviolet light.

Preparation of DNA markers. Conditions for cleavage with *Eco*RI restriction endonuclease (13) were 10 mM Tris-hydrochloride, pH 7.6, and 10 mM $MgCl_2$ and with or without 100 mM KCl at 37°C for 20 to 60 min. Supercoiled simian virus 40 (SV40) DNA labeled with [^{14}C]thymidine was isolated by cesium chloride-ethidium bromide banding. The SV40 marker shown in Fig. 4 was the product of an incomplete digestion; single-strand circles are visible as a band of lesser intensity above the doublet of the linear strands. In other cases digestion was complete. DNAs from the bacteriophages λgt-λC (19; molecular weights as revised in 20) and P22 were generous gifts of Daniel Donoghue, who performed some of the endonuclease digestions. A fragment of molecular weight 4.5×10^6 (9), obtained by *Eco*RI digestion of P22 DNA, was found to contain a single-strand breakage such that it produced fragments of 2.3×10^6, 1.6×10^6, and 0.7×10^6 daltons upon denaturation (D. Donoghue and E. Rothenberg, unpublished data). These molecular weights were verified by electron microscopy with single-strand φX DNA as a standard.

Nucleic acid hybridization. Conditions were essentially as described previously (14). Samples were boiled for 3 min to denature before addition of LiCl to a final concentration of 0.9 M. The extent of hybridization was determined by resistance to S_1 nuclease (4).

RESULTS

Effect of Mg^{2+} concentration on DNA synthesis. The kinetics of reverse transcription by detergent-disrupted virions of Moloney MuLV were measured in the presence of different concentrations of Mg acetate, 0.2 mM [^{3}H]dCTP, and 5 mM each dATP, dGTP, and dTTP. The maximal rate of DNA synthesis occurred when the concentration of Mg acetate equaled or slightly exceeded the total nucleotide concentration of 15.2 mM (Fig. 1). With Mg^{2+} present at 12 mM, the incorporation of [^{3}H]dCMP was

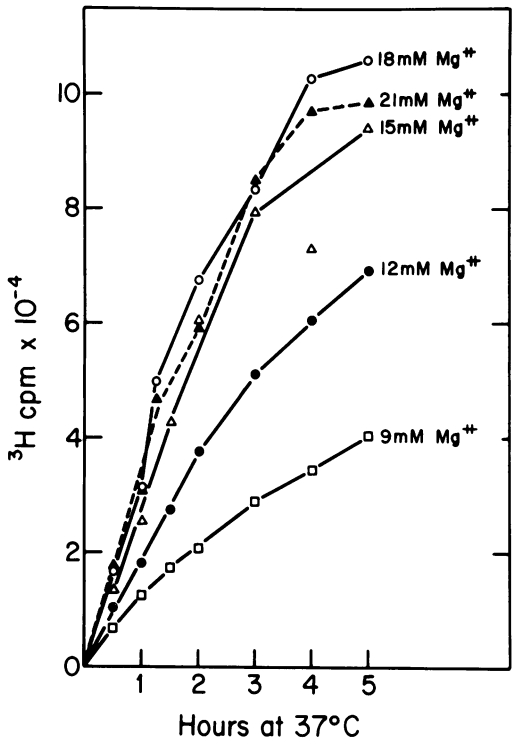


FIG. 1. *Effect of magnesium acetate concentration on the kinetics of DNA synthesis by virions of MuLV. Virions were incubated in 200 μl of endogenous reverse transcriptase reaction mixtures containing 5 mM each of dATP, dGTP, and dTTP and 0.2 mM [³H]dCTP. Magnesium acetate was present at 9, 12, 15, 18, and 21 mM in the different mixtures. At the indicated times, portions of 20 μl were taken and diluted into 0.5 ml of 0.1 M sodium pyrophosphate containing 20 mM EDTA and carrier RNA. Trichloroacetic acid-precipitable material was collected on filters and the radioactivity was determined by scintillation counting.*

reduced to about 70% of maximum; at 9 mM Mg^{2+}, the rate of DNA synthesis was reduced by more than 50%.

When the sizes of the DNA products made at different Mg^{2+} concentrations were analyzed by electrophoresis through 1.4% agarose gels, it was found that DNA made in the presence of lower concentrations of Mg^{2+} was significantly longer than DNA made in conditions of Mg^{2+} excess (Fig. 2). The DNA made with limiting Mg^{2+} after 5 h not only had a greater maximum length, but also showed a reduced proportion of molecules smaller than about 300,000 daltons (arrow on Fig. 2). Net elongation was so slow, however, that the longest molecules only appeared reproducibly after 3 to 6 h of synthesis. Because the total amount of DNA synthesis with limiting Mg^{2+} was less than that made in Mg^{2+} excess, it appears that an initiation event at low Mg^{2+} concentration was more likely to yield a long DNA molecule than an initiation event in Mg^{2+} excess.

In previous work (14), extremely high concentrations of dNTP had been found to promote the synthesis of long, but still incomplete, DNA transcripts. To compare the effects of varying both the dNTP concentration and the amount of free Mg^{2+}, virions were incubated at different Mg^{2+} concentrations with dATP, dGTP, and dTTP each at 1 mM or at 5 mM and [³H]dCTP at 0.01, 0.2, or 5 mM. DNA was purified from the reaction mixtures after 6 h of synthesis and analyzed by electrophoresis through agarose slab gels. The Mg^{2+} concentrations tested spanned the incorporation optima for the 3.01 and 3.2 mM dNTP reactions, but were all below the nucleotide concentrations and incorporation optima for the 15.2 and 20 mM dNTP reactions (Fig. 3). The sizes of the products shown in Fig. 4 thus reflected three effects: the enhancement of the rate of DNA synthesis by high substrate levels, the slowing of DNA synthesis in limiting Mg^{2+}, and the increased length of DNA molecules made at limiting Mg^{2+} concentration.

The highest proportion of very large DNA, as well as the largest absolute amount, was made in the reaction mixture containing the highest nucleotide concentration (20 mM) and the least severely restricted Mg^{2+} concentration (15 mM; Fig. 4, lane 15). Further reductions in Mg^{2+} concentration appeared to decrease the proportion of very long DNA products, probably because elongation was so sluggish (Fig. 4, lanes 13, 14; also cf. lanes 10 to 12). On the other hand, high nucleotide levels were not absolutely necessary for the synthesis of very large DNA: as long as the Mg^{2+} concentration was slightly below that of the substrates, long DNA was made even at moderate dNTP concentrations (lanes 1 and 5, Fig. 4). In the reactions analyzed in lanes 1 through 4, Fig. 4, although dCTP was sharply limiting at 0.01 M and overall DNA synthesis was very inefficient (Fig. 3), molecules as long as 1.0×10^6 to 1.2×10^6 daltons could be found when the Mg^{2+} concentration was kept below the nucleotide concentration (Fig. 4, lane 1). This was in contrast with previous results in Mg^{2+} excess (14), when products longer than 0.4×10^6 to 0.5×10^6 daltons were not detectable in low-substrate reactions.

Limit-length product. The longest DNA molecules synthesized at 12 mM Mg^{2+} and 15.2 mM nucleotides were examined by alkaline sucrose gradient sedimentation and electrophoresis in

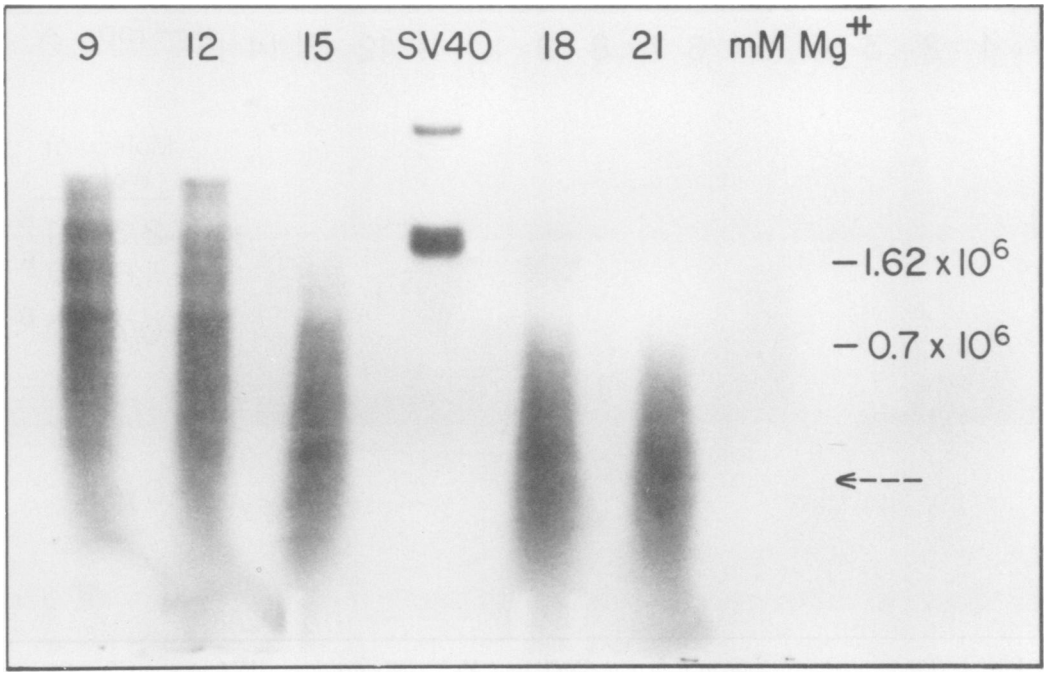


FIG. 2. *Effect of magnesium acetate concentration on the size of DNA made by virions of MuLV. Samples from the reactions described in the legend to Fig. 1 were taken after 5 h of synthesis. Portions of each containing 20,000 to 24,000 ^3H cpm were denatured and subjected to electrophoresis through a 1.4% agarose slab gel for 10 h at 20 V. From left to right, the samples are the products of reactions containing 9 mM, 12 mM, and 15 mM Mg^{2+}, respectively; EcoRI-cleaved SV40 DNA; and products of reactions containing 18 and 21 mM Mg^{2+}. Some reassociated SV40 double-stranded DNA is visible above the major doublet. A nonradioactive fragment of P22 phage DNA cleaved with EcoRI of single-strand molecular weight 0.7 × 10^6 (9) was run as an additional marker. For fluorography, the film was exposed at −70°C for 3 days.*

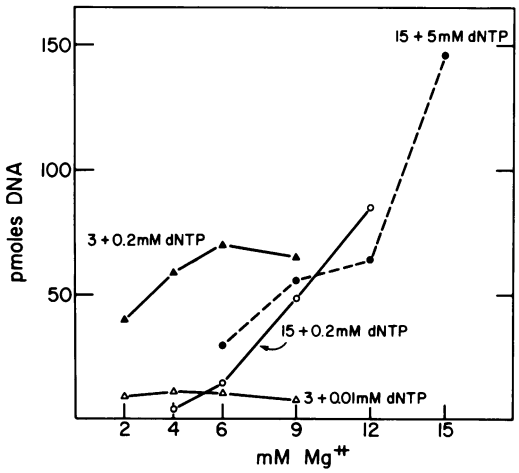


FIG. 3. *Effect on DNA synthesis of varying both Mg^{2+} and dNTP concentrations. Virions were incubated in endogenous reactions containing Mg^{2+} at the indicated concentrations and the following concentrations of nucleotides: 1 mM each dATP, dGTP, and dTTP with [^3H]dCTP at 0.01 mM (3 + 0.01 mM dNTP) or 0.2 mM (3 + 0.2 mM dNTP); or 5 mM each dATP, dGTP, and dTTP with [^3H]dCTP at 0.2 mM (15 + 0.2 mM dNTP) or 5 mM (15 + 5 mM dNTP). Five-microliter portions of the reaction mixtures were acid-precipitated after 6 h of incubation. Incorporated radioactivity was converted to picomoles of DNA synthesized using the known specific activity of the precursors and assuming equimolar incorporation of all four dNTP's.*

1.4% agarose gels with markers of known size. Agarose gels were used because they could resolve single-strand DNA molecules larger than the RNA tumor virus genome and provide accurate sizes for them (8). The pattern of bands of reverse transcriptase products was, however, less sharply defined than on polyacrylamide gels (Haseltine et al., in press). Under the electrophoresis conditions used, the mobility of single-strand DNA was linearly related to the logarithm of its molecular weight (Fig. 5). As shown in Fig. 2 and 4, the largest DNA products made by the MuLV polymerase migrated at a defined limit size. This band contained 4% of the total radioactivity of the sample shown in Fig. 4, lane 15, as calculated from a scan of the fluorograph. The molecular weight of the larg-

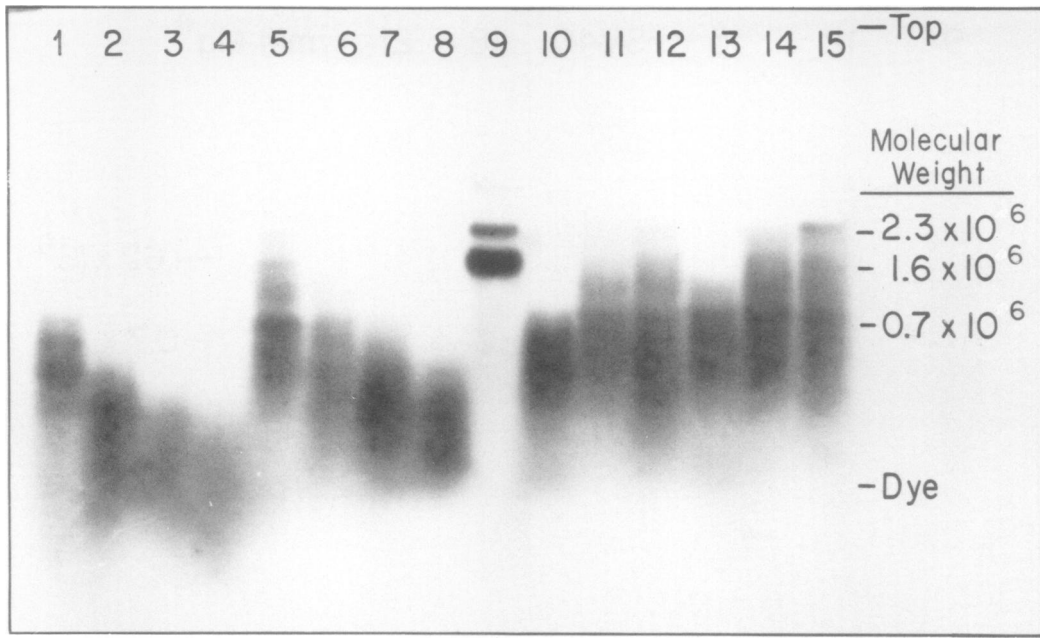


FIG. 4. *Size of products made at different dNTP and Mg^{2+} concentrations. DNA was purified from the reaction mixtures described in the legend to Fig. 3. Portions of each sample containing 10,000 to 13,000 ^3H cpm were denatured and subjected to electrophoresis through a 1.4% agarose slab gel. Electrophoresis was for 10 h at 15 V. Lanes 1–4: DNA made at (3 + 0.01) mM dNTP with 2 mM, 4 mM, 6 mM, and 9 mM Mg^{2+}, respectively. Lanes 5–8: DNA made at (3 + 0.2) mM dNTP with 2 mM, 4 mM, 6 mM, and 9 mM Mg^{2+}. Lane 9: EcoRI endonuclease-cleaved SV40 DNA, including some circular molecules, due to incomplete digestion, migrating slower than the doublet of linear strands. Lanes 10–12: DNA made at (15 + 0.2) mM dNTP with 6 mM, 9 mM, and 12 mM Mg^{2+}. Lanes 13–15: DNA made at (15 + 5) mM dNTP with 9 mM, 12 mM, and 15 mM Mg^{2+}. Unlabeled markers of denatured P22 DNA of 2.3 × 10^6, 1.6 × 10^6, and 0.7 × 10^6 daltons were located by ethidium bromide staining. Fluorography was at −70°C for 7 days.*

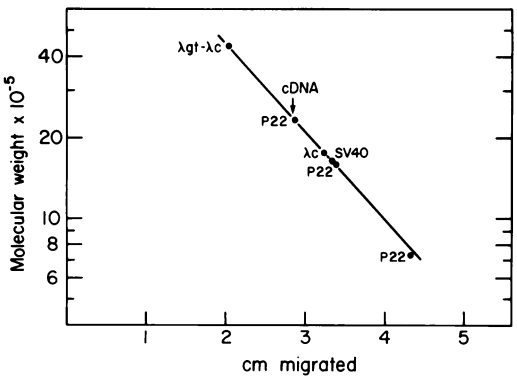


FIG. 5. *Sizing of limit-length polymerase product on agarose gels. The product of a 3-h reaction as described in the text was freed of unincorporated substrate by Sephadex G-50 chromatography, denatured, and subjected to electrophoresis through an agarose slab gel. Identically treated markers of SV40 [^{14}C]DNA digested with EcoRI, nonradioactive bacteriophage λgt-λC cleaved with EcoRI, and a nonradioactive EcoRI fragment of bacteriophage P22 DNA, as described in Materials and Methods, were also analyzed on the gel. Nonradioactive DNA and SV40 DNA were visualized by ethidium bromide staining. Radioactive DNAs were located by fluorography. Distance migrated was plotted against molecular weight for the marker DNAs. The position of the limit-length polymerase product is indicated by an arrow.*

est material was estimated to be 2.4 × 10^6 daltons (arrow in Fig. 5). Thus, the molecules appeared to have achieved a limit-length, which may be long enough to represent complete transcription of a 35S RNA subunit of the viral RNA.

On alkaline sucrose gradients, the product of a 3-h endogenous reaction contained some DNA sedimenting faster than linear SV40 DNA (Fig. 6A); this represented 4% of the total. Resedimentation of this DNA through alkaline sucrose (Fig. 6B) showed that it was truly larger than the SV40 DNA, with a mean calculated molecular weight of 2.3 × 10^6 to 2.5 × 10^6 (16).

The longest DNA molecules were shown to be faithful transcripts of the RNA template by annealing them to 70S Moloney MuLV virion RNA. Portions of the pooled limit-length

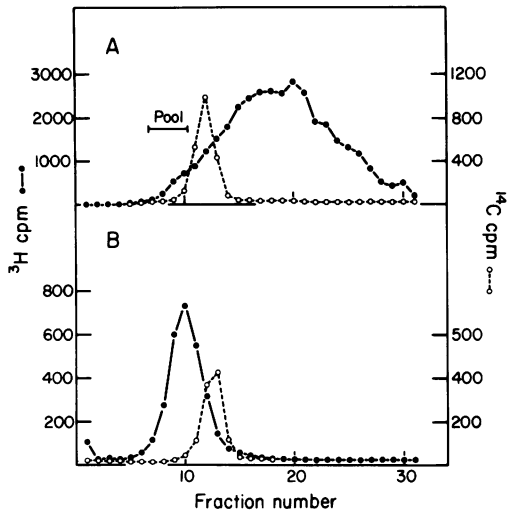


FIG. 6. *Maximum reverse transcriptase product size on alkaline sucrose gradients. (A) A portion of the endogenous reaction product described in the legend to Fig. 5 was centrifuged through a 5 to 20% alkaline sucrose gradient for 17 h at 35,500 rpm in the SW41 rotor at 4°C. Fractions were neutralized, and 1/6 portions of each were counted in 10 ml of Handifluor with 1 ml of water. The ^{14}C-labeled DNA marker of SV40 cleaved with EcoRI was centrifuged in a parallel gradient. The direction of sedimentation was from right to left. (B) The fractions indicated in (A) were pooled and ethanol-precipitated. One-half of the pooled DNA was subjected to centrifugation on a 15 to 30% alkaline sucrose gradient, for 18 h at 35,000 in the SW41 rotor at 22°C. SV40 [^{14}C]DNA was run in a parallel gradient.*

[^{3}H]DNA from the alkaline sucrose gradient shown in Fig. 6A were denatured at 100°C and then incubated at 67°C with and without viral RNA. More than 90% of the [^{3}H]DNA was resistant to digestion by S_1 nuclease after annealing with viral RNA, whereas less than 1% was resistant when annealed without RNA (Table 1). Thus, at least 90% of the limit-length DNA was complementary to the virion RNA, and no self-complementarity was detectable.

Initiation of DNA synthesis in limiting Mg^{2+}. It is known that the initial sequence of DNA copied from the Moloney MuLV template under conventional polymerase conditions is the sequence d-AATGAAAGA, which is covalently linked to the 3'-terminal riboadenylic acid of proline tRNA (W. A. Haseltine and D. Baltimore, *in* A. S. Huang, D. Baltimore, and C. F. Fox, ed., *Animal Virology*, in press). Therefore, the mechanism of initiation of the synthesis of long DNA molecules made at low Mg^{2+} concentration could be studied by examining the fate of ^{32}P from [α-^{32}P]dATP in a pulse-chase experiment. Virions were incubated in

TABLE 1. *Hybridization of limit-length DNA*[a]

Sample	S_1 nuclease	cpm (acid-precipitable)	Fraction (%) S_1 resistant
[^{3}H]DNA, incubated	−	368	(100)
[^{3}H]DNA, unincubated	+	18	1.3
[^{3}H]DNA, incubated	+	14	0.2
[^{3}H]DNA, incubated with RNA	+	336	91
Filter background		15, 12	

[a] [^{3}H]DNA (approximately 0.3 ng) from the pooled alkaline sucrose gradient fractions shown in Fig. 6A was incubated for 3 h in 0.9 M LiCl at 68°C in the presence or absence of 4 μg of 70S Moloney MuLV virion RNA per ml. Equivalent C_0t value attained with RNA was 0.75. The hybrids were analyzed by resistance to S_1 nuclease.

appropriate salts and detergent at 37°C for 15 min with only 4 to 5 μM [α-^{32}P]dATP present. The label was then chased by diluting the sample into a fivefold larger reaction volume containing a 5,000-fold excess of unlabeled dATP as well as the other dNTP's, actinomycin D, and Mg^{2+}. During the chase, incorporation of further ^{32}P was drastically reduced (Fig. 7).

All of the label incorporated during the pulse migrated with the tRNA fraction during electrophoresis through a 10% polyacrylamide gel (Fig. 8). For unknown reasons, the label appeared as a diffuse doublet. During the chase, all the ^{32}P originally attached to tRNA was moved into larger molecules. The first and major species of DNA into which label was chased was the 135-nucleotide "strong stop" DNA linked to its tRNA primer, which was previously characterized by Haseltine et al. (in press). As the chase period was extended, increasing amounts of radioactivity were found in DNA that could not penetrate the 10% gel, but even after 90 min, one-half of the labeled molecules migrated as "strong stop" DNA.

To further characterize the longest DNA molecules labeled by this pulse-chase protocol, molecules labeled after 25 and 200 min of chase at limiting Mg^{2+} concentration were fractionated by electrophoresis through agarose gels after a brief alkali treatment to degrade the template (Fig. 9). ^{32}P label appeared in all size-classes of DNA including limit-length DNA. Molecules of different sizes were eluted from the gel, and the RNA moieties were completely hydrolyzed with piperidine. The hydrolysate was then analyzed by paper electrophoresis to detect any ^{32}P that had been transferred to ribonucleotides. As shown in Fig. 9B, transfer of the ^{32}P initially in [α-^{32}P]dATP to rAMP was demonstrable in all size-classes of reverse-transcribed DNA.

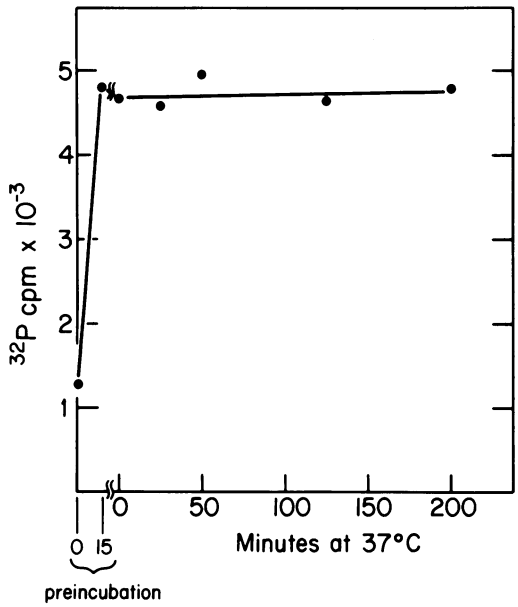


FIG. 7. *Incorporation of [α-^{32}P]dATP in a pulse-chase experiment. To pulse-label the viral polymerase product, virions were incubated in an endogenous reaction at 37°C with only one substrate, 5 μM [α-^{32}P]dATP, and 6 mM Mg^{2+}. No actinomycin D was present initially, and the reaction was supplemented by 10 mM creatine phosphate and 5 μg of creatine phosphokinase per ml in a final volume of 0.5 ml. After 15 min, the label was chased by increasing the reaction volume fivefold to 2.5 ml and adjusting it to 5 mM each dATP, dGTP, and dTTP, 0.2 mM dCTP, 100 μg of actinomycin D per ml, and 12 mM Mg^{2+}. Incubation was then continued for 200 min. At the indicated times, 5-μl portions were taken from the "pulse" mixture and 25-μl portions were taken from the "chase" mixture, and acid-precipitable radioactivity was determined.*

It may be noted in Fig. 9B that the ratio of ^{32}P in rAMP to ^{32}P in DNA at the origin decreased with increasing length of the DNA. This was not unexpected, since in such an experiment longer DNA molecules are more likely to contain in internal positions any ^{32}P that was incorporated after the chase. Furthermore, a certain amount of material at the origin may have been trapped there nonspecifically. These effects make precise quantitation difficult, but it is clear that every size-class of DNA synthesized in the low Mg^{2+} polymerase reaction contained molecules with an initial pApdA linkage, consistent with the $tRNA^{Pro}$ initiation mechanism demonstrated in more limited reactions.

Effect of Mg^{2+} on nucleic acid stability. Because the long DNA molecules synthesized under limiting Mg^{2+} conditions appeared to be initiated in the same way as shorter molecules made in Mg^{2+} excess, it appeared that excess Mg^{2+} somehow inhibits complete transcription of the 35S RNA subunit. This might be due to destruction of the product or template in excess Mg^{2+} or to some other mechanism, such as alteration of the template conformation or of the binding of the polymerase to the template.

To study possible activation of DNase inside the virion by free Mg^{2+}, excess Mg^{2+} was added during the course of an endogenous reaction and the size of the resulting DNA was analyzed by sedimentation through alkaline sucrose gradients (Fig. 10). After 90 min in limiting Mg^{2+}, the largest DNA was about 5,000 nucleotides long, or 1.6×10^6 daltons (Fig. 10A, closed symbols). If incubation was continued, somewhat more large DNA was formed (Fig. 6A). In a sample incubated 90 min in limiting Mg^{2+} and then 90 min more with excess Mg^{2+}, the longest molecules made before the shift remained intact; the additional radioactivity incorporated was almost all in molecules shorter than 1,000 nucleotides long (Fig. 10A, open symbols). Incubation with excess Mg^{2+} for the entire 180 min yielded only molecules shorter than SV40 DNA (Fig. 10B). Therefore, the effect of the high Mg^{2+} concentration was not to destroy DNA that was already made, but to stimulate synthesis of molecules that could only be elongated to a limited extent.

In an attempt to explain the formation of short DNA in excess Mg^{2+}, the stability of the template RNA in the presence of different concentrations of Mg^{2+} was examined. Virions labeled with [^{3}H]uridine were incubated with 5 mM each dATP, dGTP, and dTTP and 12 or 18 mM Mg acetate. The fourth nucleotide, dCTP, was omitted so that DNA could not be made and the template RNA would not be degraded by the RNase H activity of the polymerase (21). After 0 or 4 h of incubation, RNA was extracted and subjected to neutral sucrose gradient centrifugation. The sedimentation profiles of the native RNA samples were identical (data not shown). RNA pooled from the 70S region of each gradient was then denatured by a brief heat treatment and then centrifuged through a second sucrose gradient (Fig. 11). Both incubated samples (Fig. 11A) were more degraded than the control (Fig. 11B), but the extent of the degradation was clearly greater in the sample incubated in excess Mg^{2+}. In limiting Mg^{2+} some full-length 35S RNA was still present at 4 h, but in excess Mg^{2+} virtually no full-length molecules remained intact. Therefore, it appears that at least part of the effect of excess Mg^{2+} was to promote the degradation of the template. Mg^{2+}-activated degradation of the template may, however, not be a complete

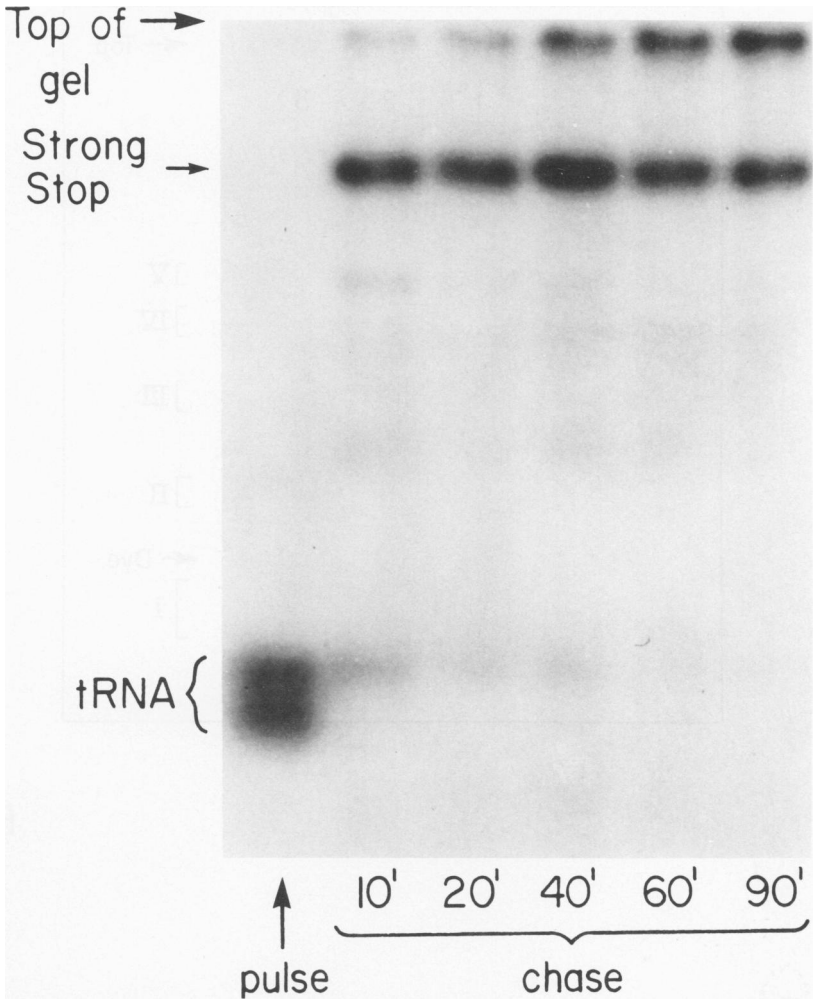
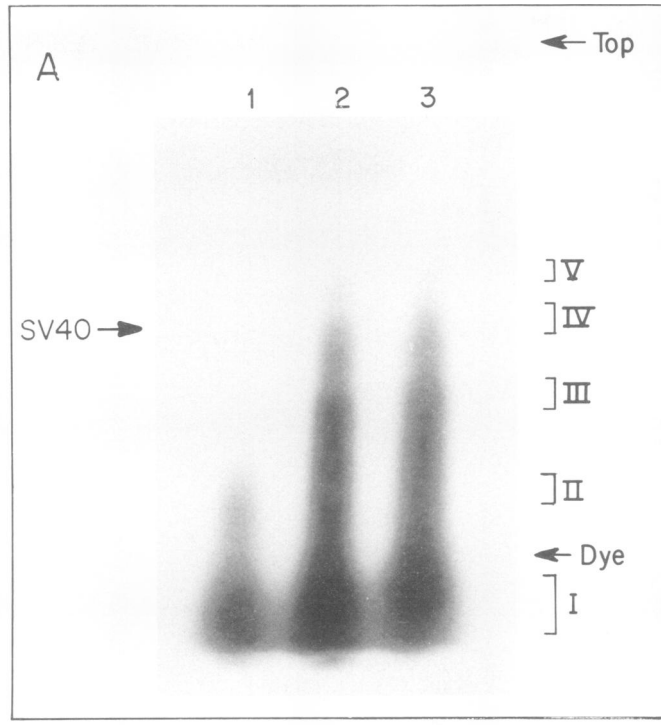


FIG. 8. *Size of pulse-labeled material on 10% acrylamide gels. Pulse-labeled DNA was made as in the legend to Fig. 7, with the following differences. Initially, 3.7 μM [α-^{32}P]dATP was present in a reaction volume of 0.25 ml; after the chase, the reaction contained 5 mM each dATP, dCTP, and dTTP, 1 mM dGTP, and 26 mM Mg^{2+} (excess Mg^{2+}) in a final volume of 1.25 ml. Portions of 0.2 ml were taken from the reaction mixture at 0, 10, 20, 40, 60, and 90 min after addition of the chase mixture. DNA was extracted, freed of unincorporated nucleotides by Sephadex G-50 chromatography, and subjected to electrophoresis on a slab gel of 9.5% polyacrylamide-0.5% bisacrylamide as described by Haseltine et al. (in press). Electrophoresis was for 3 h at 400 V in a water-cooled EC-140 apparatus.*

explanation of the effect of Mg^{2+} on DNA size, because increased Mg^{2+} seemed to limit DNA size more severely (Fig. 10A and B) than would be predicted from its effect on RNA (Fig. 11).

DISCUSSION

Detergent-disrupted virions of Moloney MuLV are competent to synthesize transcripts of the 35S subunit of the virion RNA up to a defined limit-length. At least part of the cause of abortive transcription is the degradation of the template RNA in the course of the reaction. This degradation is minimized if the concentration of Mg^{2+} in the reaction mixture is lower than the concentration of nucleotides, so that essentially all the Mg^{2+} is chelated into 1:1 Mg-dNTP complexes (17). The kinetics of DNA synthesis are slower when Mg^{2+} is made limiting, but longer products can ultimately be made. Molecules up to half the maximum length are made even in very low dNTP concentrations (Fig. 4, lane 1). The mechanism of the Mg^{2+}-enhanced RNA degradation is not known; contaminating RNases in the virions may be activated, oxidative breakage, perhaps involving dithiothreitol (1), may be accelerated in the


A
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1
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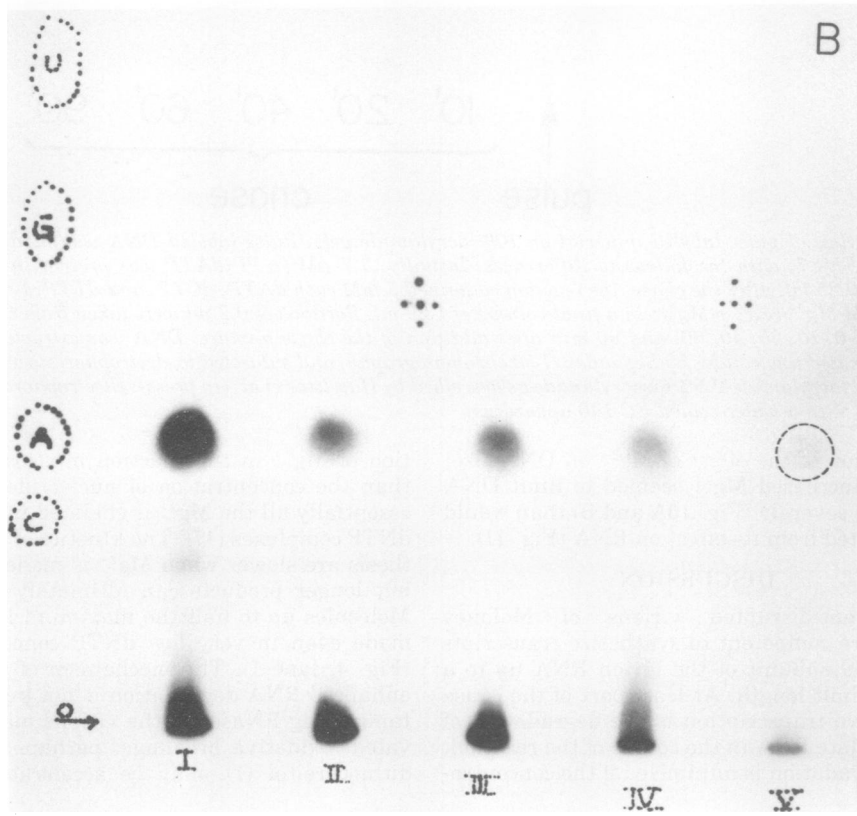
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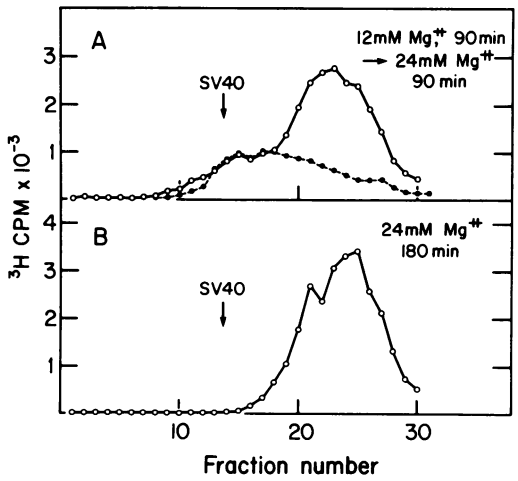

FIG. 10. *Effect of Mg^{2+} concentration on reverse transcriptase product stability. Virions were incubated in endogenous reactions with 15.2 mM nucleotides, [^{3}H]dCTP limiting, at 12 mM Mg^{2+} for 90 min (A, closed symbols), at 12 mM Mg^{2+} for 90 min, and then at 24 mM Mg^{2+} for an additional 90 min (A, open symbols), or at 24 mM Mg^{2+} for 180 min (B). DNA was extracted from each and centrifuged through alkaline sucrose gradients for 17 h at 35,000 rpm, 24°C, in an SW41 rotor. Linear SV40 DNA was centrifuged on a parallel gradient as a marker. Fractions were acid-precipitated for scintillation counting.*

presence of free Mg^{2+}, or a combination of effects may be responsible.

The effect of Mg^{2+} on the rate of DNA synthesis may be partly due to changes in the level of available Mg-dNTP substrate (3), but this cannot be the sole factor. In Fig. 3, less DNA was made with 20 mM dNTP and 6 mM Mg^{2+}, where the concentration of Mg-dNTP was about 6 mM, than in the reaction containing only 3.2 mM dNTP and 6 mM Mg^{2+}, where the concentration of Mg-dNTP was no more than 3.2 mM. It is probable that the enzyme needs a small amount of free Mg^{2+} either to maintain its own conformation or to bind the template efficiently.

In the polymerase reactions with slightly limiting Mg^{2+}, the products have a defined maximum size. The limit-length appears to be 2.4×10^6 daltons, by alkaline sucrose gradient sedimentation and agarose gel electrophoresis. This number is probably accurate to about 10%: in the case of the alkaline sucrose gradient centrifugation, it reflects the weight-average

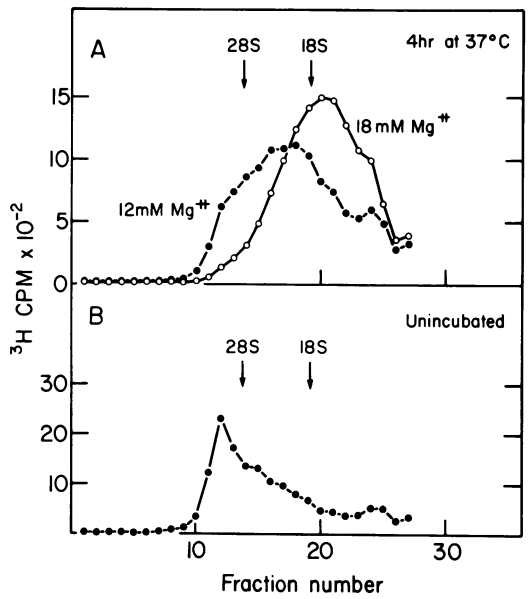

FIG. 11. *Size of template RNA after incubation at excess and limiting Mg^{2+} concentrations. [^{3}H]uridine-labeled virions were incubated in endogenous reactions with 5 mM each dATP, dGTP, and dTTP and 12 or 18 mM Mg^{2+}. After 4 h at 37°C, RNA was extracted from the incubated samples and an unincubated control by the SDS-phenol-chloroform method (4) and centrifuged on 11-ml 0.5% SDS-sucrose gradients for 2 h at 35,000 rpm, 22°C, in an SW41 rotor. The fractions containing 70S RNA were pooled, ethanol-precipitated, denatured at 78°C, and centrifuged on 11-ml 0.5% SDS-sucrose gradients as before for 4 h at 40,000 rpm. The nonradioactive internal markers of HeLa 28S and 18S RNA were located by absorbance at 260 nm. Fractions were counted directly in Handifluor. (A) RNA incubated for 4 h in 12 mM Mg^{2+} (●) or 18 mM Mg^{2+} (○). (B) Unincubated control.*

FIG. 9. *Covalent linkage of pulse-labeled DNA to an RNA primer. (A) Autoradiogram of preparative agarose gel. DNA was prepared from 0.5- and 2.0-ml portions of the reaction mixture described in the legend to Fig. 7 after 25 min and 200 min of chase, respectively, and passed over Sephadex G-50. The samples from the 25- and 200-min incubations were denatured and diluted to 50 and 100 μl, respectively. The 25-min sample was subjected to electrophoresis in lane 1, and the 200-min sample was divided between lanes 2 and 3, with denatured linear SV40 DNA as a marker in another lane. After electrophoresis at 20 V, the marker was located by ethidium bromide staining. (B) High-voltage electrophoresis of pulse-labeled DNA after alkaline hydrolysis. Portions of the agarose gel indicated with brackets in (A) were excised from lanes 2 and 3, except for fraction I, which was taken from lane 1. The approximate molecular weights of the material in the fractions were: I, <2, $\times 10^5$; II, 3–4 $\times 10^5$; III, 8 $\times 10^5$; IV, 1.7 $\times 10^6$; and V, 2.4 $\times 10^6$. The nucleic acids were eluted, treated with piperidine, and subjected to high-voltage electrophoresis on Whatman 3MM paper at pH 3.5. The 2',3'-ribonucleotide markers were located under UV light and their positions were marked in radioactive ink. Autoradiography was for 5 weeks.*

sedimentation rate of the population of all molecules larger than linear SV40 DNA, and in the case of the agarose gel analysis the migration rate is somewhat subject to effects of base composition (20) that have not been taken into account. The expected molecular weight of a complete single-strand transcript would be about 2.7×10^6 daltons, based on electron microscopy and gel electrophoresis of the closed-circular and linear infectious proviral DNAs made in vivo (7, 15).

The limit-length product is more than 90% complementary to virion RNA, by S_1 nuclease resistance after hybridization, and has no detectable hairpin structure that would self-hybridize with zero-order kinetics (Table 1). Furthermore, limit-length as well as shorter molecules can be initiated on a polyribonucleotide primer, presumably $tRNA^{Pro}$ (Haseltine and Baltimore, in press), to give an initial pApdA bond.

Thus, the longest DNA molecules appear to be faithful copies of the template initiated by a specific mechanism. Given the uncertainty in sizing, the limit-length product would appear to be the transcript of a complete 35S RNA subunit. This interpretation is supported by the recent demonstration that polymerase products synthesized under similar in vitro conditions contain infectious DNA molecules (Rothenberg and D. Smotkin, manuscript in preparation).

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LITERATURE CITED

1. **Bode, V. C.** 1967. Single-strand scissions induced in circular and linear λ DNA by the presence of dithiothreitol and other reducing agents. J. Mol. Biol. 26:125–129.
2. **Collett, M. S., and A. J. Faras.** 1975. In vitro transcription of DNA from the 70S RNA of Rous sarcoma virus: identification and characterization of various size classes of DNA transcripts. J. Virol. 16:1220–1228.
3. **Englund, P. T., J. A. Huberman, T. M. Jovin, and A. Kornberg.** 1969. Enzymatic synthesis of deoxyribonucleic acid. XXX. Binding of triphosphates to deoxyribonucleic acid polymerase. J. Biol. Chem. 244:3038–3044.
4. **Fan, H., and D. Baltimore.** 1973. RNA metabolism of murine leukemia virus: detection of virus-specific RNA sequences in infected and uninfected cells and identification of virus-specific messenger RNA. J. Mol. Biol. 80:93–117.
5. **Fan, H., and M. Paskind.** 1974. Measurement of the complexity of cloned Moloney murine leukemia virus 60 to 70S RNA: evidence for a haploid genome. J. Virol. 14:421–429.
6. **Faras, A. J., J. M. Taylor, J. P. McDonnell, W. E. Levinson, and J. M. Bishop.** 1972. Purification and characterization of the deoxyribonucleic acid polymerase associated with Rous sarcoma virus. Biochemistry 11:2334–2342.
7. **Gianni, A. M., J. R. Hutton, D. Smotkin, and R. A. Weinberg.** 1976. Proviral DNA of Moloney leukemia virus: purification and visualization. Science 191:569–571.
8. **Hayward, G. S., and M. C. Smith.** 1972. The chromosome of bacteriophage T5. I. Analysis of the single-stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. 63:383–395.
9. **Helling, R. B., H. M. Goodman, and H. W. Boyer.** 1974. Analysis of endonuclease R·EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis. J. Virol. 14:1235–1244.
10. **Junghans, R. P., P. H. Duesberg, and C. A. Knight.** 1975. In vitro synthesis of full-length DNA transcripts of Rous sarcoma virus RNA by viral DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 72:4895–4899.
11. **Laskey, R. A., A. D. Mills, and J. S. Knowland.** 1975. Revisions and extensions of existing fluorographic procedures. Appendix to R. A. Laskey and A. D. Mills, Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335–341.
12. **Leis, J. P., and J. Hurwitz.** 1974. RNA-dependent DNA polymerase from avian myeloblastosis virus, p. 143–150. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 29E. Academic Press Inc., New York.
13. **Morrow, J. F., and P. Berg.** 1972. Cleavage of Simian virus 40 DNA at a unique site by a bacterial restriction enzyme. Proc. Natl. Acad. Sci. U.S.A. 69:3365–3369.
14. **Rothenberg, E., and D. Baltimore.** 1976. Synthesis of long, representative DNA copies of the murine RNA tumor genome. J. Virol. 17:168–174.
15. **Smotkin, D., A. M. Gianni, S. Rozenblatt, and R. A. Weinberg.** 1975. Infectious viral DNA of murine leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 72:4910–4913.
16. **Studier, W.** 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373–390.
17. **Taqui Rhan, M. M., and A. E. Martell.** 1966. Thermodynamic quantities associated with the interaction of adenosine triphosphate with metal ions. J. Am. Chem. Soc. 88:668–671.
18. **Temin, H., and D. Baltimore.** 1972. RNA-directed DNA synthesis and RNA tumor viruses, p. 129–186. *In* K. M. Smith and M. A. Lauffer (ed.), Advances in virus research, vol. 17. Academic Press Inc., New York.
19. **Thomas, M., J. R. Cameron, and R. W. Davis.** 1974. Viable molecular hybrids of bacteriophage lambda and eukaryotic DNA. Proc. Natl. Acad. Sci. U.S.A. 71:4579–4583.
20. **Thomas, M., and R. W. Davis.** 1975. Studies on the cleavage of bacteriophage lambda DNA with EcoRI restriction endonuclease. J. Mol. Biol. 91:315–328.
21. **Verma, I. M.** 1975. Studies on reverse transcriptase of RNA tumor viruses. III. Properties of purified Moloney murine leukemia virus DNA polymerase and associated RNase H. J. Virol. 15:843–854.
22. **Verma, I. M., N. L. Meuth, and D. Baltimore.** 1972. The covalent linkage between RNA primer and DNA product of the avian myeloblastosis virus DNA polymerase. J. Virol. 10:622–627.