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In Vitro Transcription of DNA from the 70S RNA of Rous Sarcoma Virus: Identification and Characterization of Various Size Classes of DNA Transcripts

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DNA transcripts, ranging from 1,500 to 4,500 nucleotides in length, can be identified in the DNA product synthesized in vitro by the Rous sarcoma virus-associated RNA-directed DNA polymerase. Although these DNA transcripts are considerably larger than the size classes of the DNA product previously reported, they only represent a minor proportion (<5%) of the total DNA synthesized in standard reaction mixtures containing rate-limiting concentration of one of the four deoxynucleoside triphosphates. However, the proportion of these larger transcripts relative to the total DNA product increases substantially when the enzymatic synthesis of DNA is performed in the presence of equimolar concentrations of deoxynucleoside triphosphate precursors. Both rate-zonal sedimentation in alkaline sucrose and nucleic acid hybridization techniques have confirmed the length and genetic complexity of these larger DNA transcripts. The identification of large DNA chains in the DNA product synthesized in vitro by the avian oncornavirus RNA-directed DNA polymerase provides an explanation for the paradox that exists between the limited number of primer sites per 70S RNA genome, the small size of the bulk of the DNA product, and the extent of the Rous sarcoma virus genome represented by the DNA product.

The RNA-directed DNA polymerase associated with virions of RNA tumor viruses uses RNA as a template to synthesize single- and double-stranded DNA (7, 10, 12, 19, 21, 22, 31, 32). The DNA product transcribed from the oncornavirus 70S RNA genome in vitro is small in size, ranging from 75 to 300 nucleotides in length (19, 32). Similar-sized DNA transcripts are synthesized by both detergent-activated virions that may or may not contain DNase activity (23, 27) and purified preparations of RNA-directed DNA polymerase that can be rendered free from detectable DNase activity (13, 21). The selective inhibition of RNase H activity, which appears to be an integral part of the avian RNA-directed DNA polymerase (18, 34, 35), also fails to exhibit any major effect on the size distribution of the DNA product (3; M. Collett and A. Faras, manuscript in preparation). Virion-associated ribonucleases, other than RNase H, appear not to be responsible for the small size of the DNA transcripts since no effect on the size of the DNA product was observed when purified preparations of RNA-directed DNA polymerase, that were apparently relatively free from detectable RNase activity, were employed in the synthesis of DNA (13, 20, 21). Furthermore, neither denaturation of the 70S RNA template nor addition of exogenous primer molecules (9, 30; A. Faras and N. Dibble, unpublished data) affects the size distribution of the resultant DNA product. It is, therefore, generally thought that the limitation of the in vitro transcription of the RNA tumor virus 70S genome into DNA is either a result of extensive secondary structure of the RNA template or of the inability of the enzyme to transcribe large regions of heteropolymeric RNA without the additional requirement of some host cell factor(s).

In this communication, we present data indicating that the RNA-directed DNA polymerase of Rous sarcoma virus (RSV) is capable of transcribing large regions of RSV 70S RNA into DNA. Although these larger DNA transcripts represent only a minor proportion of the total DNA product synthesized in vitro, they nevertheless can be identified and isolated by electrophoresis in polyacrylamide gels. Rate-zonal sedimentation analysis in alkaline sucrose indicates that some of the DNA transcripts eluted from these gels range between 1,500 and 4,500

nucleotides in length. Furthermore, the larger DNA transcripts contain most of the sequences of the RSV genome and appear to be relatively uniform with respect to their nucleotide sequence representation. The identification of DNA chains that were an order of magnitude larger than originally thought to be transcribed by the RSV RNA-directed DNA polymerase in vitro provides at least one explanation of how most of the nucleotide sequences of the RSV genome are present in the DNA product when there appears to be a limited number of primer sites per 70S RNA genome (4; J. Taylor et al., manuscript submitted for publication, and A. Faras and N. Dibble, manuscript in preparation).

MATERIALS AND METHODS

Reagents. The sources and preparation of most of the pertinent materials have been previously described (8, 11, 13). [^{3}H]TTP (15 to 20 Ci/mmol or 53 Ci/mmol) was from Schwarz Bio Research Inc. Unlabeled deoxynucleoside triphosphates and Takadiastase (Sanzyme) were obtained from Calbiochem. Carrier-free [^{32}P]P_i was from New England Nuclear Corp.

Cells and virus. The Schmidt-Ruppin and B77 strains of RSV were propagated in chicken and duck embryo fibroblasts and purified as previously described (9).

Enzymatic synthesis of virus-specific DNA. The synthesis of DNA by detergent-disrupted virions of RSV was performed as follows. Standard reaction mixtures contained 300 μg of virus protein per ml; 0.1 M Tris-hydrochloride, pH 8.1; 0.003 M $MgCl_2$; 6×10^{-5} M unlabeled deoxynucleoside triphosphates (dATP, dCTP, and dGTP); 1.4% (vol/vol) β-mercaptoethanol; and 0.018% (vol/vol) Nonidet P-40. Reactions in which DNA was synthesized in the presence of rate-limiting concentrations of deoxynucleoside triphosphate precursors contained [^{3}H]TTP at 2.4×10^{-6} M (specific activity of the DNA product was approximately 8,900 counts/min per ng). Reactions in which DNA was synthesized in the presence of equimolar concentrations of deoxynucleoside triphosphates contained [^{3}H]TTP at 1×10^{-6} M and unlabeled TTP at 5.9×10^{-5} M (specific activity of the DNA product was approximately 540 counts/min per ng). All reaction mixtures contained freshly purified virus and were incubated for 6 h at 37 C. Termination of enzymatic synthesis was accomplished by adjusting the reaction mixtures to 0.01 M EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, and 500 μg of predigested Pronase per ml. After incubation for 30 min at 37 C, the nucleic acids were extracted twice with STE (0.1 M NaCl; 0.02 M Tris-hydrochloride, pH 7.4; 0.01 M EDTA)-saturated phenol and subjected to ethanol precipitation. Viral RNA was removed from the DNA product by hydrolysis with 0.6 M NaOH for 4 h at 37 C. The ^{3}H-labeled DNA was then neutralized, precipitated with ethanol, centrifuged, and suspended in TE buffer (0.02 M Tris-hydrochloride, pH 7.4; 0.01 M EDTA). These conditions of alkaline hydrolysis are sufficient to reduce RNA to ribonucleotide 3-monophosphates but do not affect DNA, as evidenced by the stability of simian virus 40 DNA when subjected to a similar treatment. Total DNA product was also analyzed after removal of RNA by RNase A treatment (50 μg/ml, 30 min, 37 C) in a solution of 0.02 M Tris-hydrochloride (pH 7.4) and 0.001 M EDTA. No difference in sedimentation was observed between alkali- or RNase A-treated DNA product (Collet and Faras, unpublished data). The yield of DNA product per microgram of virus protein remained relatively constant from reaction to reaction. Approximately 50 to 100 and 200 to 300 ng of DNA product (Collett and Faras, unpublished data). mixtures containing rate-limiting or equimolar concentrations of TTP, respectively.

Electrophoresis in polyacrylamide gels. The procedures for electrophoresis in 2.25% polyacrylamide gels (0.4 by 10 cm) have been previously described (12, 31). The conditions of electrophoresis are given in the legend to Fig. 2. Subsequent to electrophoresis, each gel was sliced into 1-mm segments and either analyzed for radioactivity after solubilization of the individual gel slices with 0.2 ml of 30% H_2O_2 at 60 C for 18 h or incubated in TE buffer containing 0.2% sodium dodecyl sulfate for 36 to 48 h at 4 C to elute the DNA. After elution, the DNA product was precipitated with ethanol, centrifuged, and stored in TE buffer.

Alkaline sucrose sedimentation analysis. Rate-zonal sedimentation in alkaline sucrose gradients was performed essentially as previously described (27). Conditions of sedimentation are described in the legends to Fig. 1 and 3.

Preparation of ^{32}P-labeled RSV 70S RNA. The conditions for labeling RSV RNA with [^{32}P]P_i and its subsequent purification have been detailed elsewhere (4, 21). The specific activity of the ^{32}P-labeled 70S RNA was approximately 3×10^6 to 4×10^6 counts/min per μg.

Preparation of SV40 DNA fragments by restriction endonuclease treatment. Specific DNA fragments of known length and molecular weight for use as reference markers in alkaline sucrose gradients or polyacrylamide gels were obtained by the digestion of simian virus 40 DNA with restriction endonucleases. The Hind II restriction endonuclease was purified from *Haemophilus influenzae* Rd by W. Folk and D. Anderson of the University of Michigan, Ann Arbor, according to previously published methods (29). The Hind II enzyme was resolved from Hind III by chromatography on DEAE-cellulose (24). Enzymatic digestion was performed in a solution of 0.01 M Tris-hydrochloride (pH 7.4), 0.01 M $MgCl_2$, 10^{-4} M dithiothreitol, 0.006 M KCl, and 0.005 M NaCl for 6 h at 37 C. The resultant fragments were separated from one another by electrophoresis in 4% polyacrylamide slab gels at 100 V, 23 mA, for 18 h (14). The five double-stranded DNA fragments resolved by this method include Hind II-A (molecular weight, 1.23×10^6), Hind II-B (molecular weight, 9.6×10^5), Hind II-C (molecular weight, 6.4×10^5), Hind II-D (molecular weight, 2.2×10^5) and Hind II-E (molecular weight,

1.44×10^5). (The five DNA fragments generated by the Hind II enzyme [fragments A to E] are named in accordance with the suggested nomenclature for fragments of DNA generated by restriction endonucleases [28].) The fragments were eluted from the gels by three separate incubations at 37 C for 24 h in TE buffer containing 0.2% sodium dodecyl sulfate. The fragments were ethanol precipitated, centrifuged, and dissolved in TE buffer. The double-stranded fragments were denatured to single-stranded DNA before use as reference markers in alkaline sucrose gradients and polyacrylamide gels.

Preparation of DNA product enriched for the "minus" strand. ^{3}H-labeled DNA transcripts (see legend to Fig. 5) were heat-denatured and hybridized to an excess (270-fold) of RSV 70*S* RNA in a solution of 0.3 M NaCl, 0.02 M Tris-hydrochloride (pH 7.2), and 0.001 M EDTA at 68 C to a C_rt (product of the concentration of moles of ribonucleotide and time) value of 3.0. Samples were quenched in ice, diluted with 0.01 M phosphate buffer (pH 6.8), and adsorbed to hydroxyapatite to separate duplex from single-stranded nucleic acid by previously described methods (7). The 0.4 M phosphate buffer eluate that contained minus-strand-enriched DNA was desalted by Sephadex G-50 chromatography and ethanol precipitated. The RNA:DNA hybrids were treated with 0.6 M NaOH at 37 C for 4 h to remove the RNA. After neutralization and ethanol precipitation, the minus-strand-enriched DNA fractions were suspended in TE buffer.

Hybridization of nucleic acids. Conditions of hybridization of ^{3}H-labeled DNA with ^{32}P-labeled 70*S* RNA are described in the legend to Fig. 4. Extent of hybridization was determined by treatment of the samples with 50 μg of pancreatic RNase per ml in 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) at 37 C for 30 min.

RESULTS

Effect of deoxyribonucleoside triphosphate concentration on the size distribution of the DNA synthesized by detergent-disrupted RSV. The DNA product synthesized by either detergent-disrupted virions of RSV or the purified DNA polymerase consists predominantly of small pieces of DNA ranging between 100 and 300 nucleotides in length. We have attempted previously to overcome this apparent restriction of transcription by varying several of the reaction conditions, including pH, buffer, divalent cation, detergent concentration, and concentration of deoxynucleoside triphosphates (13, 16; Collett and Faras, unpublished data). To date, manipulation of only the latter parameter appears to affect the synthesis of DNA by the virion-associated RSV RNA-directed DNA polymerase (31). Both the efficiency of transcription and the extent and uniformity of nucleotide sequence representation of the RSV RNA genome in the DNA product appear to be affected when all of the deoxynucleoside triphosphate precursors are present in the reaction mixtures in equal concentrations (17, 31; see Fig. 4 and 5). A comparison of the size distribution of the total DNA product synthesized in the presence of deoxynucleoside triphosphates containing an equimolar concentration of all four precursors and DNA product synthesized in the presence of deoxynucleoside triphosphates containing rate-limiting concentrations of TTP is presented in Fig. 1. DNA synthesized in the presence of equimolar concentrations of all deoxynucleoside triphosphates exhibits a slight, but reproducible, skewing toward the single-strand components (18*S* and 16*S*) of simian virus 40 form II DNA when analyzed under denaturing conditions in alkaline sucrose (Fig. 1b). DNA product synthesized under reaction conditions containing rate-limiting concentrations of one (TTP) of the four deoxynucleoside triphosphates exhibits less skewing in these gradients. A more dramatic difference between the DNA transcripts

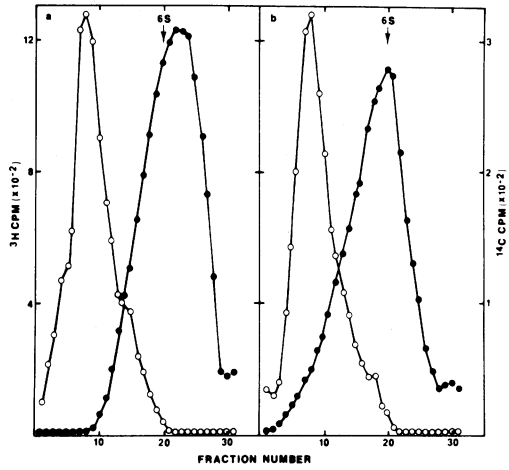

FIG. 1. *Alkaline sucrose sedimentation of total ^{3}H-labeled DNA synthesized by detergent-disrupted preparations of RSV. ^{3}H-labeled DNA was synthesized in the presence of either rate-limiting or equimolar concentrations of deoxynucleoside triphosphates and then purified. Samples of the total ^{3}H-labeled DNA product (12,000 counts/min) and ^{14}C-labeled SV40 form III DNA (form III was obtained by treatment of form I with the Escherichia coli RI restriction endonuclease) (2000 counts/min), which was employed as a sedimentation reference, were adjusted to 0.6 M NaOH. After 15 min of incubation at 37 C, the samples were layered onto 5 to 20% (wt/wt) gradients of alkaline sucrose (0.9 M NaCl, 0.6 M NaOH, 0.01 M EDTA) and centrifuged in an SW50.1 rotor at 50,000 rpm for 6 h at 20 C. (a) Total DNA product synthesized in the presence of rate-limiting concentrations of TTP. (b) Total DNA product synthesized in the presence of equimolar concentrations of deoxynucleoside triphosphates. Symbols: ●, ^{3}H-labeled DNA product; ○, ^{14}C-labeled SV40 DNA.*

synthesized under these two concentrations of deoxynucleoside triphosphates was obtained by electrophoresis in gels of 2.25% polyacrylamide, which affords greater resolution of nucleic acids than rate-zonal sedimentation analysis. Electropherograms of alkali-denatured DNA product synthesized in reactions containing either equimolar or rate-limiting concentrations of TTP are presented in Fig. 2. Although the bulk (75%) of the DNA synthesized under rate-limiting concentrations of precursor migrates in the range of 4-7S RNA markers, a portion of the DNA migrates between the 7S and 18S RNA markers. This observation is consistent with our previous analyses of RSV-specific DNA synthesized under similar reaction conditions (12, 31). The DNA product synthesized in the presence of equimolar concentrations of deoxynucleoside triphosphates and analyzed under identical conditions of electrophoresis exhibits a shift in electrophoretic mobility toward the high-molecular-weight RNA markers (Fig. 2b). Less than 40% of the DNA transcripts synthesized under these conditions are found in the regions of the gel containing the 4S and 7S RNA markers.

Size and structure of virus-specific DNA transcripts resolved by 2.25% polyacrylamide gels. The differences in electrophoretic mobility exhibited by the DNA transcripts in 2.25% polyacrylamide gels could be the result of differences not only in the length of the DNA product, but also in secondary structure. To determine whether one or both of these parameters are responsible for the wide range of electrophoretic mobilities observed, the DNA transcripts were eluted from arbitrarily assigned regions of the polyacrylamide gels and analyzed for chain length under denaturing conditions in alkaline sucrose gradients with appropriate DNA markers and content of secondary structure with S1 nuclease. Although the DNA transcripts from regions I, II, III, IV, and V contain very little secondary structure (<10%), significant size differences can be detected between the various classes of DNA eluted from the gels (Fig. 3). DNA transcripts obtained from regions II, III, IV, and V of the gels exhibit sedimentation coefficients of 10-11S, 9S, 6S, and 5S, respectively, when compared with the single-strand components of simian virus 40 form II DNA included as sedimentation marker. However, by far the largest DNA transcripts eluted from the gel are those in region I. The DNA obtained from this region of the gel contains DNA transcripts that sediment in the range of the 16-18S DNA markers (Fig. 3a).

To obtain a more precise size estimate of these DNA transcripts, a similar analysis was performed with shorter DNA molecules employed as sedimentation markers. DNA fragments of known lengths and molecular weights

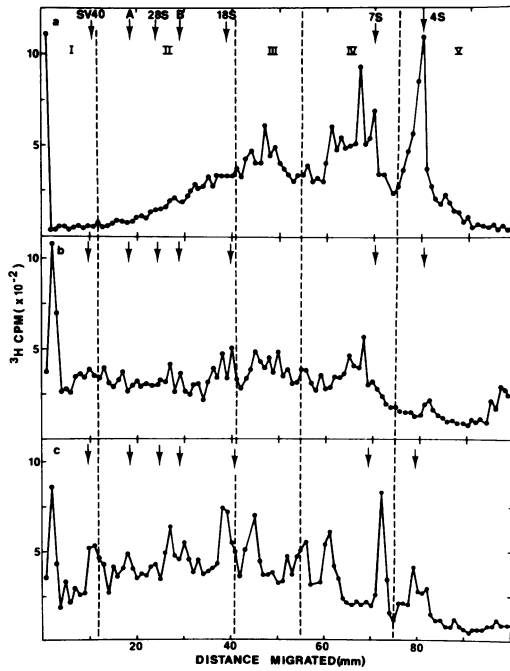

FIG. 2. *Electrophoresis of total DNA product in 2.25% polyacrylamide gels. ^{3}H-labeled DNA was prepared with detergent-disrupted preparations of RSV under rate-limiting or equimolar concentrations of deoxynucleoside triphosphate precursors and then purified. The DNA product was treated with 0.6 M NaOH for 4 h at 37 C, neutralized, ethanol precipitated, and suspended in TE buffer. Samples of the ^{3}H-labeled DNA (50,000 counts/min) containing 3,000 counts/min each of ^{32}P-labeled 28S, 18S, 7S, and 4S cellular RNA as references were subjected to electrophoresis in 2.25% polyacrylamide gels at 5 mA/gel for 3 h. In addition to RNA markers, specific single-stranded DNA fragments obtained by H. influenzae Hind II digestion of SV40 DNA were included in an effort to further calibrate the gels relative to DNA markers of known length and molecular weight. (a) Total DNA product synthesized in the presence of rate-limiting concentrations of TTP. (b) Total DNA product synthesized in the presence of equimolar concentrations of deoxynucleoside triphosphates. (c) Total DNA product synthesized in the presence of equimolar concentrations of precursors by purified RSV harvested at 4-h intervals from infected duck embryo fibroblasts. The arrows represent either 28S, 18S, 7S, or 4S cell RNA markers, 16S SV40 linear DNA, or the Hind II-A and Hind II-B fragments of SV40 DNA. The vertical broken lines represent the regions (denoted by the roman numerals) of corresponding preparative gels of the same DNA products from which the ^{3}H-labeled DNA was eluted.*

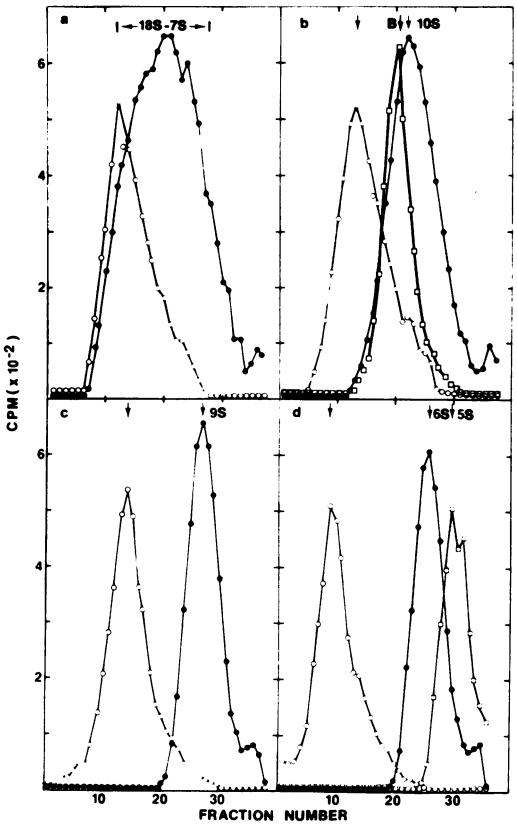

FIG. 3. *Alkaline sucrose sedimentation of ^{3}H-labeled DNA products eluted from 2.25% polyacrylamide gels. ^{3}H-labeled DNA product synthesized under equimolar concentrations of deoxynucleoside triphosphates was subjected to preparative electrophoresis in 2.25% polyacrylamide gels as described in the legend to Fig. 2. The preparative gels were sliced (1 mm), and the slices within the regions indicated in Fig. 2 were pooled for elution of DNA. Greater than 75% of the DNA was eluted, ethanol precipitated, and suspended in TE buffer. Samples of the DNA (5,000 counts/min) were adjusted to 0.6 M NaOH, and ^{32}P-labeled SV40 form II DNA (4,000 counts/min) was added as a sedimentation marker. In some instances, the SV40 DNA fragment Hind II-B was also included as a sedimentation marker for more precise measurements of size. Sedimentation in 5 to 20% alkaline sucrose gradients was performed in an SW50.1 rotor at 50,000 rpm for 6 h at 20 C. (a) Region I DNA, polyacrylamide gel fractions 1 to 12. (b) Region II DNA, polyacrylamide gel fractions 13 to 41. (c) Region III DNA, polyacrylamide gel fractions 42 to 55. (d) Region IV DNA (●), polyacrylamide gel fractions 56 to 75; and region V DNA (□), polyacrylamide gel fractions 76 to 100. Symbols: ●, ^{3}H-labeled DNA product, ○, ^{32}P-labeled SV40 form II DNA; □ (panel b), ^{32}P-labeled Hind II B fragment of SV40 DNA. The Hind II B fragment was run in a parallel gradient with ^{3}H-labeled RSV-specific DNA, but its position was superimposed in panel b for comparative purposes.*

were obtained from simian virus 40 form I DNA treated with the restriction endonuclease Hind II from *H. influenzae*. The five resultant DNA fragments were purified by polyacrylamide gel electrophoresis as described above and were denatured prior to their use as reference markers. The single-stranded DNA fragments exhibited chain lengths of 1,880 nucleotides (molecular weight, 620,000), 1,450 nucleotides (molecular weight, 480,000) 970 nucleotides (molecular weight, 320,000), 333 nucleotides (molecular weight, 110,000), and 218 nucleotides (molecular weight, 72,000). The position of the second-largest DNA fragment (fragment B) relative to the RSV-specific DNA transcripts in alkaline sucrose gradients is included in the sedimentation profiles in Fig. 3. We have also subjected several of these fragments to electrophoresis in 2.25% polyacrylamide gels in an effort to further calibrate the gel system with respect to DNA. The relative electrophoretic mobilities of two of these fragments have been included in the electropherograms presented in Fig. 2. DNA fragments with sedimentation coefficients similar to those of RSV-specific DNA transcripts in alkaline sucrose also migrate with similar electrophoretic mobilities in polyacrylamide gels. These results substantiate our alkaline sucrose analyses of the RSV-specific DNA transcripts eluted from various regions of the gel and further document the fact that resolution of the DNA product in our polyacrylamide gel system is predominantly a function of the chain length of the transcripts.

We must also emphasize the differences in electrophoretic mobilities of the simian virus 40 DNA fragments and the various RNA species employed as markers in these gels. Although the larger single-strand DNA fragments (1,880 nucleotides, molecular weight, 620,000; 1,450 nucleotides, molecular weight, 480,000) exhibit electrophoretic mobilities in the range of 28*S* rRNA (4,500 nucleotides, molecular weight, 1.6×10^6), they differ considerably with respect to chain length and molecular weight. The 18*S* rRNA marker (2,000 nucleotides, molecular weight, 0.7×10^6), which exhibits a chain length similar to the largest simian virus 40 DNA fragment, migrates much more rapidly under these conditions of electrophoresis. We conclude that significant differences can be detected between similarly sized DNA and RNA molecules and that RNA is not a valid marker for DNA chains in this polyacrylamide gel system.

Genetic complexity of the various size classes of RSV-specific DNA transcripts. On the basis of the size estimates of the various

classes of RSV-specific DNA eluted from the 2.25% polyacrylamide gels in Fig. 2 and the current estimates of the genetic complexity of the RSV genome (1, 2, 26), it appears that at least 20% and as much as 50% of the RSV genome is represented by single DNA transcripts contained within regions II and I of the gel, respectively. In an attempt to determine directly the proportion of the RSV genome represented in the different size classes of the RSV-specific DNA transcripts, we have employed nucleic acid hybridization techniques, which enable us to measure the extent to which the RSV-specific DNA sequences protect radiolabeled RSV 70S RNA from hydrolysis by pancreatic RNase (Fig. 4). Although the two largest-sized classes of DNA transcripts protect the RSV 70S RNA genome from RNase hydrolysis to at least some extent, the smaller DNA size classes do not appear to protect the RSV genome (<6%), even at DNA:RNA ratios of 100:1. Analysis of the secondary structure of the DNA transcripts after the hybridization period indicated that regions I, II, III, IV, and V exhibited S_1 nuclease resistance to the extent of 66, 68, 58, 63, and 23%, respectively, indicating that much of the single-stranded DNA transcripts had reassociated to form duplexes during hybridization. These observations are consistent with the computed C_ot values (1.5×10^{-1} mol/s per liter) for the DNA in these hybridization reactions and exceed the C_ot values required to routinely achieve complete reassociation of most, if not all, of the RSV-specific duplex DNA synthesized under our reaction conditions (33). Therefore, the complementary strands of the RSV DNA transcripts reassociate during hybridization and are, thus, not available for duplex formation with RSV 70S RNA. In an effort to obviate this problem and to allow more accurate genetic complexity determinations on all of the size classes of RSV-specific DNA, we attempted to enrich for the minus-strand component of the DNA transcripts by hybridizing the various size classes of the DNA product to a large excess of unlabeled RSV 70S RNA, as described in the legend to Fig. 5. Most of the various size classes of RSV-specific DNA, enriched for the minus strand in this manner, now exhibit the capacity to protect the RSV genome from RNase hydrolysis to various extents (Fig. 5).

From the data presented in Fig. 5, it is apparent that the larger DNA transcripts protect labeled 70S RNA to a greater extent from RNase hydrolysis than do the small-sized transcripts. Furthermore, substantially lower DNA:RNA ratios are required to protect RSV

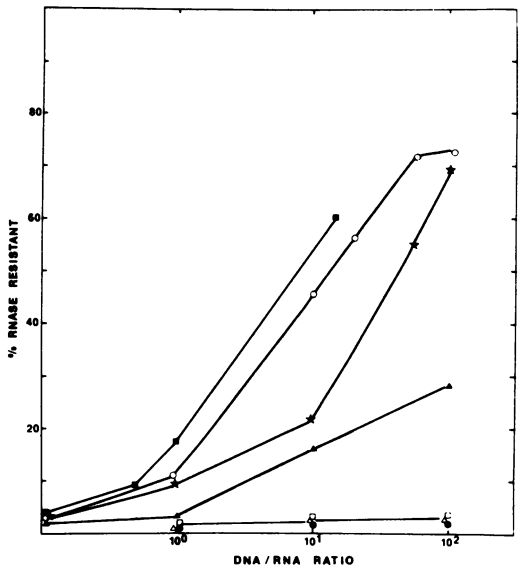

FIG. 4. *Extent of RSV genome represented by different size classes of RSV-specific DNA product. Total ^{3}H-labeled DNA products and ^{3}H-labeled DNA eluted from the various regions of the 2.25% polyacrylamide gel depicted in Fig. 2 (recoveries of DNA were routinely greater than 75%) were hybridized to ^{32}P-labeled 70S RNA of RSV as follows. Approximately 0.5 ng (2,200 counts/min) of ^{32}P-labeled RNA, 40 μg of yeast RNA, and varying amounts of ^{3}H-labeled DNA in 0.1 ml of 3× SSC were overlaid with paraffin oil and incubated for 36 h at 68 C. The extent of hybridization was measured by hydrolysis with pancreatic RNase. The results are plotted as a function of the ratio of DNA to RNA present in each sample. Backgrounds due to the intrinsic RNase resistance of the RNA (5%) were subtracted. Symbols:* ○, *total DNA product synthesized in the presence of equimolar concentrations of TTP;* ★, *total DNA product synthesized in the presence of rate-limiting concentrations of TTP;* ■, *DNA eluted from region I;* ▲, *DNA eluted from region II;* ●, *DNA eluted from region III;* △, *DNA eluted from region IV,* □, *DNA eluted from region V.*

70S RNA to the same extent with the larger DNA transcripts than with the smaller transcripts. These data indicate that the larger-sized DNA transcripts contain a greater extent of the nucleotide sequence present in the RSV genome than do the smaller-sized DNA transcripts. The data also indicate that the RSV genome is more uniformly represented in the larger DNA transcripts than in the smaller DNA transcripts. Because of the limited availability of the DNA obtained from region V of the gels, we have been unable to perform hybridizations at DNA:RNA ratios greater than one with this size class.

Finally, we have also compared the ability of

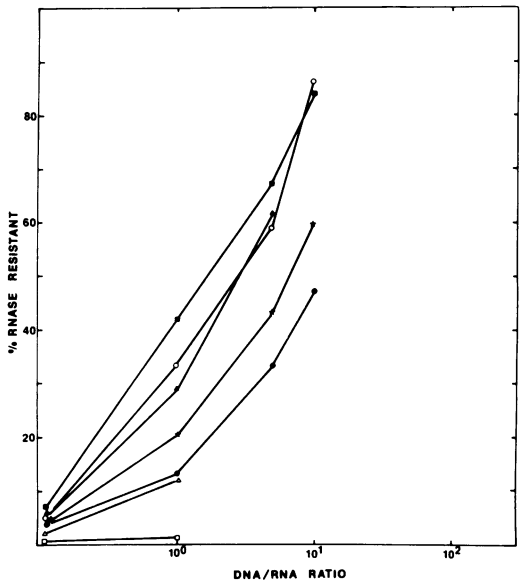

FIG. 5. *Extent of hybridization to 70S RNA by different size classes of DNA product enriched for the minus strand. Total ^{3}H-labeled DNA product synthesized in the presence of either equimolar or rate-limiting concentrations of TTP and the ^{3}H-labeled DNA transcripts eluted from the various regions of the polyacrylamide gel described in the legend to Fig. 2b were heat denatured and hybridized to an excess of viral 70S RNA. After isolation of the RNA:DNA hybrids by differential elution from hydroxyapatite and desalting by Sephadex G-50 chromatography, the RNA was hydrolyzed with alkali, and the resultant single-strand ^{3}H-labeled DNA, which was enriched for minus-strand DNA, was hybridized to ^{32}P-labeled 70S RNA. The extent of hybridization of the various DNAs to ^{32}P-labeled 70S RNA was measured as described in the legend to Fig. 4. Symbols: ★, total DNA product synthesized in the presence of rate-limiting concentrations of TTP; ○, total DNA product synthesized in the presence of equimolar concentrations of deoxyribonucleoside triphosphates; ■, DNA product eluted from region I of the 2.25% polyacrylamide gel illustrated in Fig. 2; ▲, DNA product eluted from region II; ●, DNA product eluted from region III; △, DNA product eluted from region IV; □, DNA product eluted from region V.*

total unfractionated DNA product, synthesized in reactions containing either equimolar or rate-limiting concentrations of deoxynucleoside triphosphates, to protect the RSV genome from RNase hydrolysis (Fig. 4). Although both preparations of DNA are comparable with respect to secondary structure (approximately 70%), it appears that DNA product made in the presence of equimolar concentrations of precursors contains a more uniform representation of the RSV nucleotide sequences than DNA product made in the presence of rate-limiting concentrations of precursors since lower DNA:RNA ratios are required to protect the genome to the same extent. A similar relationship is observed when the minus-strand constituent from total DNA product synthesized under equimolar or rate-limiting concentrations of deoxynucleoside precursor is employed in the hybridization mixtures (Fig. 5). These observations are consistent with the data obtained from gel electrophoresis analysis, indicating that the DNA product synthesized in the presence of equimolar concentrations of deoxynucleoside triphosphates contains a larger proportion of longer-chain length molecules than DNA made in the presence of rate-limiting concentrations of the deoxyribonucleoside precursor.

DISCUSSION

It was previously thought that the DNA product synthesized by the RNA-directed DNA polymerase of RNA tumor viruses consisted entirely of small pieces of DNA varying between 75 and 300 nucleotides in length. In this communication, we report the identification of DNA chains ranging between 1,500 and 4,500 nucleotides in length in the DNA product synthesized by detergent-disrupted preparations of RSV. Although these larger DNA chains represent less than 5% of the total DNA product synthesized under standard reaction conditions (rate-limiting concentrations of TTP), under certain conditions (equimolar concentrations of precursors), approximately 20% of the total DNA product exhibits a sedimentation coefficient of $\geq$ 10*S* in alkaline sucrose gradients. This larger DNA product appears to be a more uniform representation of the RSV 70*S* RNA genome than the smaller DNA product since it protects a larger proportion of the 70*S* RNA genome from RNase hydrolysis at very low DNA:RNA ratios.

The results presented in this communication also appear to resolve the current paradox that exists between the number of primer sites present per 70*S* RNA genome and the genetic complexity and previously reported size of the DNA product (19, 32). For example, although the DNA transcribed by the RNA-directed DNA polymerase in vitro was originally thought to contain DNA molecules of only 75 to 300 nucleotides in length, it could be demonstrated that most, if not all, of the nucleotide sequences present in the 70*S* RNA genome are transcribed into DNA (5, 17). This implies that DNA synthesis initiates at a large number of sites on the RNA template. However, from previous

studies (4; Taylor et al., submitted for publication; Faras, in preparation) it has been determined that there are insufficient primers on any single 70S molecule to provide the required number of initiation sites. Therefore, the identification in this report of DNA chains representing 20 to 50% of the RSV genome appears to reconcile this discrepancy. Further protection experiments, involving the smaller-sized classes of DNA product, are in progress to determine the extent of the RSV genome represented by these DNA transcripts and, hopefully, to provide additional information concerning this paradox. In addition, we plan to determine whether a portion of the DNA product synthesized under our reaction conditions is initiated upon primer molecules other than the principle RSV primer molecule since at DNA: RNA ratios greater than 1:1 a small portion (<20%) of the DNA product exhibits more of the nucleotide sequences of the RSV genome than is predicted by its size (Fig. 5).

The identification of larger RSV-specific DNA transcripts is also pertinent to our understanding of the mechanism of RNA-directed DNA synthesis since it provides the first demonstration that the RNA-directed DNA polymerase is capable of transcribing large heteropolymeric regions of 70S RNA in vitro. While this manuscript was in preparation, Efstratiadis et al. reported the in vitro synthesis of full-length DNA transcripts of globin mRNA by the avian myeloblastosis virus DNA polymerase (6). Complete transcription of the mRNA species was dependent upon the concentration of deoxyribonucleotide triphosphates in the reaction mixture, an observation consistent with those presented in this communication. We are currently attempting to increase the size and proportion of the larger DNA transcripts relative to the total DNA synthesized by the RNA-directed DNA polymerase in vitro. Preliminary results indicate that increasing the concentration of deoxynucleoside triphosphates in the reaction mixtures to 2.7 mM appears to exhibit no additional effect on the size distribution of the DNA product synthesized by detergent-disrupted virions of RSV. Furthermore, the DNA product synthesized by frequently harvested virus (harvested at 4-h intervals), whose genome consists of intact, homogeneous 30–40S RNA subunits, exhibits a size distribution similar to DNA synthesized by virus harvested at 24-h intervals (Fig. 2c; Collett and Faras, unpublished data). It is, therefore, conceivable that additional requirements, including the correct structural configuration of the 70S RNA genome and/or a host cell factor(s), may be needed to facilitate the complete transcription of the RSV genome in vitro. In regard to the latter requirement, several investigators have recently demonstrated a dependence on a cytoplasmic factor(s) for the synthesis of polyoma DNA in vitro (15, 25).

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