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In Vitro Synthesis of Infectious Transforming DNA by the Avian Sarcoma Virus Reverse Transcriptase

CAROL H. CLAYMAN, ENAYAT MOSHARRAFA, AND ANTHONY J. FARAS*

Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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Infectious DNA molecules, capable of transforming chicken embryo fibroblasts, can be synthesized by the Rous sarcoma virus-associated reverse transcriptase in vitro. The optimal enzymatic conditions employed for infectious DNA synthesis also facilitate maximum synthesis of genome length DNA. Analysis of the DNA product synthesized by detergent-disrupted Rous sarcoma virus under these conditions indicates that DNA complementary to viral RNA (minus-strand DNA) is genome length in size, whereas DNA complementary to genome length minus-strand DNA (plus-strand DNA) appears as subgenomic-length molecules ranging between 300 and 3,500 nucleotides in length. These features of the DNA product synthesized by the Rous sarcoma virus reverse transcriptase in vitro are similar to those identified in the cytoplasm of cells shortly after infection and lend credence to studies of the mechanism of reverse transcription in vitro and their significance to proviral DNA synthesis in vivo.

The retrovirus reverse transcriptase plays an important role in the life cycle of the virus by converting the viral RNA genome into proviral DNA shortly after infection. Studies on the nature of proviral DNA have delineated two predominant structures in virus-infected cells: a linear molecule and a covalently closed circular molecule (7, 8, 11, 28, 30). Both appear to be double stranded in nature and correspond to the length of a 35S RNA subunit of the retrovirus genome. The double-stranded linear molecule consists of a strand of genome length DNA which is complementary to the viral genome (minus-strand DNA) and pieces of DNA varying in size from 300 to 3,500 nucleotides in length which are complementary to the genome length minus DNA (plus-strand DNA) (8, 28, 30). These linear molecules represent the initial viral DNA structures found in the cytoplasm of cells shortly after infection and appear to be precursors to covalently closed circular forms of viral DNA identified in the infected cell nucleus (25). Both forms of viral DNA purified from virus-infected cells have been shown to be infectious (6).

Although the reverse transcriptase is capable of transcribing the viral RNA genome into proviral DNA in vivo and presumably could independently be responsible for the synthesis of double-stranded linear DNA molecules, no double-stranded structures similar to those observed in vivo have been identified in the DNA product made in vitro by the retrovirus reverse transcriptase. Earlier studies indicated that the enzyme could, at best, transcribe the viral genome into small pieces of viral DNA 100 to 700 nucleotides in length (10, 27). In fact, only recently have enzymatic conditions been described that promoted the transcription of the viral genome into large DNA molecules, including genome length DNA in vitro (3, 14, 18, 23, 24, 31). In this report, we demonstrate that the avian retrovirus-associated reverse transcriptase is capable of synthesizing infectious, transforming DNA under reaction conditions that facilitate a high yield of genome length DNA transcripts (ca. 7.5 to 10 kilobases). We also document the conditions required for maximum full-length DNA transcription in this system and elaborate on the similarities and differences in reaction conditions between the murine and avian systems required to facilitate this transcription. Finally, we present data indicating that the DNA product, synthesized under reaction conditions required to generate infectious viral DNA, exhibits features closely resembling the double-stranded linear forms of viral DNA found in the cytoplasm of cells shortly after infection. These latter observations further justify studies on reverse transcription in vitro as a viable means of delineating at least the initial stages of retrovirus proviral DNA synthesis in vivo. While this manuscript was in preparation, infectious DNA was synthesized by murine leukemia virus in vitro

under considerably different reaction conditions (24). The major differences between these two systems are discussed.

MATERIALS AND METHODS

Radiochemicals. [^{3}H]TTP (50 to 60 Ci/mmol) and [^{3}H]dGTP (10 to 25 Ci/mmol) were obtained from Schwarz/Mann; [^{3}H]uridine was from Nuclear Dynamics; and carrier-free ^{125}I (350 mCi/ml) was from Amersham/Searle Inc. [^{125}I]dCTP was prepared by previously published procedures (5, 15). Unlabeled deoxynucleoside triphosphates were purchased from P-L Biochemicals, and unlabeled ribonucleoside triphosphates were purchased from Sigma.

Cells and virus. B77 strain (subgroup C) of Rous sarcoma virus (RSV) was propagated on gs$^-$chf$^-$ chicken and Peking duck embryo fibroblast cells (20). Virus was harvested from infected cultures at 24-h intervals and purified by previously published procedures (1).

Synthesis of genome length viral DNA. Virus-specific DNA was synthesized in standard endogenous reaction mixtures (1 ml) containing 4 to 5 mg of virus protein; 0.05 M Tris-hydrochloride, pH 8.1; 0.01 M $MgCl_2$; 6×10^{-5} M unlabeled deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP); 0.025% Nonidet P-40 (Shell); 1.4% (vol/vol) 2-mercaptoethanol; 1 mM of each ribonucleoside triphosphate (ATP, CTP, GTP, UTP); and 1 μCi of [^{125}I]dCTP. Reaction mixtures were incubated for 16 h at 41°C and were terminated by adding 0.01 M EDTA, sodium dodecyl sulfate to 0.5%, and Pronase (500 μg/ml). The reaction mixtures were further incubated for 30 min at 37°C, extracted twice with phenol, and precipitated with ethanol. The total nucleic acid product was next separated from free nucleoside triphosphates by chromatography on G-50 Sephadex. Hydrolysis of RNA from RNA·DNA hybrids was performed either by incubation in 0.3 N NaOH for 4 h at 37°C followed by neutralization and ethanol precipitation or by incubation with RNase A (Worthington) at 100 μg/ml in 20 mM Tris-hydrochloride, pH 7.4 and 10 mM EDTA (TE) for 45 min at 37°C, followed by phenol extraction and ethanol precipitation.

Transfection assay. Transfection of virus-specific DNA was performed by the calcium phosphate coprecipitation technique as outlined by Krzyzek et al. (17). Briefly, DNA was dissolved in *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonate (HEPES)-saline-phosphate buffer at 2 to 20 μg/ml, adjusted to 0.125 M $CaCl_2$, and allowed to precipitate for 30 min at room temperature. Aliquots (0.5 ml) of the calcium phosphate-DNA precipitates were added to chicken secondary fibroblasts (0.5×10^6 cells per 60-mm tissue culture plate) previously washed with HEPES-saline-phosphate buffer. After a 30-min incubation at room temperature, 2.5 ml of growth medium containing 10% calf serum but without tryptose-phosphate broth was added to the cultures and incubated for 4 h at 37°C. The DNA-calcium phosphate-containing medium was then removed and replaced with culture media containing 10% calf serum and tryptose-phosphate broth. Positive transfections were scored by visible foci of transformed cells and/or the ability of transfected cultures to either incorporate [^{3}H]uridine into virus particles with a characteristic density of 1.16 g/cm^3, or exhibit reverse transcriptase activity (16).

Hybridization for RSV-specific DNA. ^{125}I-labeled DNA was hybridized to unlabeled 70S viral RNA as follows. Genome length DNA (1,200 cpm, 1.2 ng) was heated to 100°C for 2 min and then mixed with increasing amounts of 70S RNA in buffer containing 0.6 M NaCl-0.02 M Tris-hydrochloride (pH 7.4)-0.001 M EDTA in the presence of 250 μg of carrier yeast tRNA per ml. The mixture was incubated at 68°C for 40 h to achieve final C_rt (concentration of RNA [moles/liter] × time [h]) values ranging from 2×10^{-2} to 2×10^1 (19). Hybrid formation was determined with the single-strand-specific S1 nuclease (19, 22).

RESULTS

Optimum conditions for the synthesis of genome length DNA by RSV in vitro. We have previously demonstrated the effect of increasing the concentration of deoxyribonucleoside triphosphates on the length of the DNA product synthesized by preparations of detergent-disrupted RSV in vitro (3). More recent studies from our laboratory have indicated that high concentrations of deoxyribonucleoside triphosphates not only dramatically affect the percent of the total DNA product that is greater than 5,000 nucleotides in length (ca. 20%), but that which is genome length (ca. 7%) in size as well (C. Clayman, E. Mosharrafa, and A. Faras, unpublished data). Under optimum conditions of enzymatic synthesis of DNA (Nonidet P-40, deoxyribonucleoside triphosphates, temperature, etc.) by detergent-disrupted RSV, the addition of 1 mM each of the four ribonucleoside triphosphates to the reaction mixture consistently facilitated the synthesis of high yields of genome length DNA molecules (Fig. 1). This effect was observed irrespective of whether the deoxyribonucleoside triphosphate concentration was 60 μM or 2 mM and thus provided a means by which genome length DNA could be synthesized without significantly reducing the specific activity of the DNA product. The effect of high concentrations of ribonucleoside triphosphates on the synthesis of genome-length DNA by detergent-disrupted preparations is consistent with the observations of Myers et al. (21) employing reconstructed reactions containing 35S viral RNA·oligodeoxythymidylic acid template·primer complexes and purified reverse transcriptase. Presumably the presence of high concentrations of ribonucleoside triphosphates during enzymatic synthesis reduces nicking of RNA, thus allowing uninterrupted transcription of the

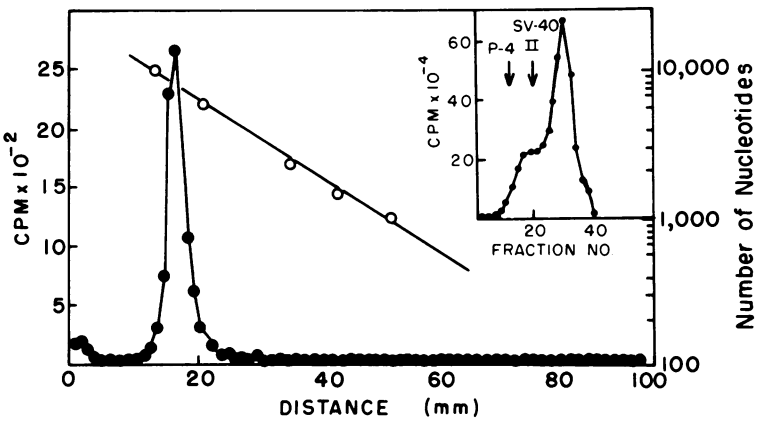

FIG. 1. *Isolation and polyacrylamide gel electrophoresis of genome length DNA transcripts. DNA transcripts synthesized by detergent-disrupted RSV under optimum conditions for genome length DNA synthesis as described in the text was subjected to rate-zonal sedimentation in 5 to 20% alkaline sucrose gradients (see inset). ^{3}H-labeled P4 DNA ($M_r \sim 7.26 \times 10^6$) and ^{14}C-labeled simian virus 40 form II DNA ($M_r \sim 3.30 \times 10^6$) were sedimented in parallel gradients as markers and are denoted by the arrows. Those fractions containing molecules of approximately 7,500 to 10,000 nucleotides in length were pooled and subsequently analyzed by electrophoresis under denaturing conditions in 98% formamide-3.5% polyacrylamide cylindrical gels by procedures previously outlined (9, 14). Nucleotide length calibration on the gels was determined by parallel electrophoresis of P4 DNA, φX-174 DNA, and the Hae III restriction endonuclease fragments of φX-174 DNA (2, 4).*

viral genome to occur.

In contrast to the murine leukemia virus system (22, 23), the ratio of divalent cation to deoxyribonucleoside triphosphate concentration did not have to be reduced to promote synthesis of high yields of genome length DNA from detergent-disrupted preparations of RSV. Two other parameters that did, however, exhibit varying effects on the yields of genome length DNA were temperature and incubation time. The optimal conditions routinely employed for the synthesis of consistently high yields of genome length DNA by detergent-disrupted preparations of RSV are as follows: 0.025% Nonidet P-40, 6×10^{-5} M deoxyribonucleoside triphosphates, 1 mM ribonucleoside triphosphates, 10 mM Mg^{2+}, 0.02 M Tris:hydrochloride, 1.4% (vol/vol) β-mercaptoethanol, and incubation for 16 h at 41°C.

Infectious nature of DNA synthesized by detergent-disrupted RSV in vitro. We have analyzed the DNA product synthesized under optimum conditions for genome length DNA production for infectivity by the $Ca_3(PO_4)_2$ coprecipitation method of Graham and van der Eb (9). The infectivity studies were initially performed on total extracted viral nucleic acids consisting of viral RNA and DNA products to optimize the conditions for maximum transfection with the in vitro-synthesized DNA product. Because our purified virus preparations employed for the synthesis of infectious DNA contained a mixture of both transforming virus and transformation-defective virus, assays were developed to determine the production of virus particles as well as transformed foci. Supernatants were assayed for virus by both [^{3}H]uridine labeling and reverse transcriptase procedures (16). In our hands, virus production as assayed by either of these techniques could be detected before overt transformation of cells became evident. As seen in Fig. 2, virus production could be detected in those cell cultures transfected with the total RSV nucleic acid product but not in mock-infected cultures. B77 RSV-infected duck cell DNA was routinely employed as a positive control for maximum virus production under optimum transfection conditions.

The kinetics of transfection are shown in Fig. 3A. Virus detection routinely occurred by 12 days posttransfection and only after the cell cultures were passaged at least twice to facilitate secondary virus infection of surrounding nontransfected cells. If sufficient concentrations of viral DNA were employed in the transfection experiments, transformed foci appeared in a similar time period. The minimum concentration of total DNA product required to reproducibly initiate transfection was 5 μg/ml. Chicken cell cultures transfected with both high and low concentrations of viral DNA product are shown in Fig. 4. Although both transfected cell cultures actively produce RSV, only the higher concentrations of DNA product were sufficient to in-

duce morphological transformation (Fig. 4). The dependence of transformation on the concentration of viral DNA employed in the transfection assay presumably reflects the presence of transformation-defective variants which are present in our preparations and arise during continuous propagation of large amounts of RSV for these studies. The virus obtained from chicken cell cultures transfected with viral DNA product was analyzed for its host range on several avian cell types and was found to exhibit properties of subgroup C viruses of which B77 RSV, the virus employed to generate infectious DNA in these studies, was a member.

Nature of infectious RSV DNA product. Although purified RSV RNA is not infectious when assayed with the $Ca_3(PO_4)_2$ technique (M. Smith and A. Faras, unpublished data), it was nevertheless necessary to determine which component of the total viral nucleic acid isolated from virions after enzymatic synthesis of DNA was responsible for the observed transfection of chick embryo fibroblast cells. Therefore, sodium dodecyl sulfate–Pronase–phenol-extracted total reaction product was assayed for infectivity after either RNase or DNase treatment (Fig. 3). The most dramatic effect on infectivity was exhibited by DNase hydrolysis because both virus production and transformation were completely eliminated by such treatment. On the other hand, RNase treatment, under conditions of hydrolysis sufficiently exhaustive to completely solubilize RNA, including that which was complexed with DNA, did not eliminate infectivity. In fact, RNase-treated product accelerated the rate of transfection. Because a constant amount of nucleic acid was employed in the transfection assays, we attribute the accelerated rate of transfection observed after RNase hydrolysis to an increase in the number of infectious DNA molecules present per microgram of product because of the removal of the noninfectious RNA component. Alternatively, RNase hydrolysis may liberate infectious DNA molecules complexed in noninfectious DNA-RNA hybrids. Nevertheless, these data demonstrate that infectivity is dependent upon DNA alone and not DNA-RNA or DNA-protein complexes. Alkaline hydrolysis of the total in vitro-synthesized virus-specific nucleic acid product did not significantly affect infectivity, although a slight, but reproducible, lag was observed subsequent to alkaline hydrolysis, suggesting that denaturation may affect the infectivity of at least a portion of the molecules present in the DNA product. The implications of these findings with regard to the possible structure of the infectious DNA molecules are discussed below.

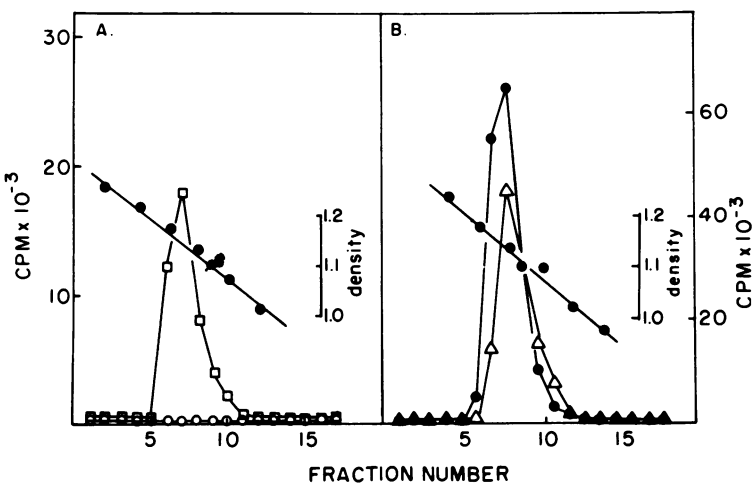

FIG. 2. *^{3}H-uridine labeling of RSV from transfected chicken embryo fibroblasts. Chicken embryo fibroblast cultures were labeled overnight with 20 μCi of [^{3}H]uridine per ml at 30 days posttransfection. Supernatants were collected, clarified by centrifuging at 10,000 × g for 10 min at 4°C, and layered onto 5-ml sucrose gradients consisting of 25 to 55% sucrose (wt/vol) in 0.1 M NaCl–0.02 M Tris-hydrochloride (pH 7.4)–0.01 M EDTA (STE) overlayed with 3 ml of 15% sucrose (wt/vol) in STE. The gradients were centrifuged for 2.5 h at 40,000 rpm in an SW 41 rotor at 4°C. Fractions were collected, and the refractive index was determined. Each fraction was assayed for [^{3}H]uridine incorporation by trichloroacetic acid precipitation as described previously (28). Data are presented as total [^{3}H]uridine contained within the 1.15- to 1.16-g/cm^2 region of the gradient. Symbols: (A) □, 0.48 μg of RSV-DNA product per 60-mm plate; ○, mock-infected cultures. (B) △, 4.8 μg of RSV-DNA product per 60-mm plate; ●, 5 μg of RSV transformed Peking duck cell DNA per 60-mm plate prepared by previously published methods (12).*

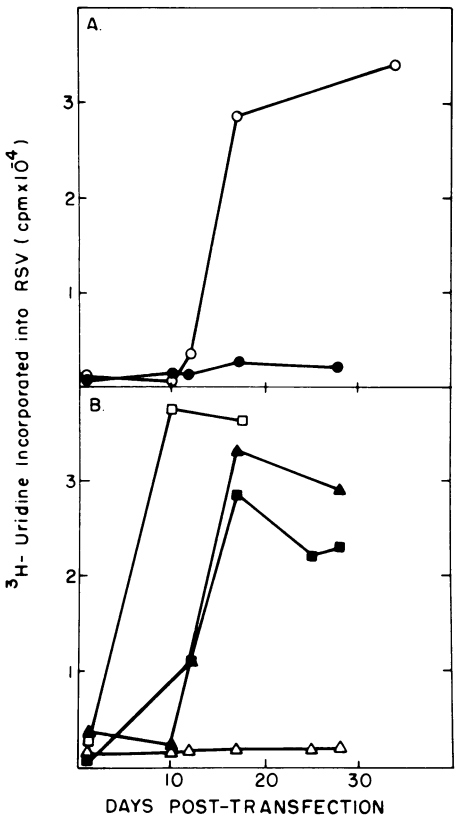

FIG. 3. *Kinetics of transfection with DNA synthesized by detergent-disrupted RSV in vitro. The kinetics of transfection with DNA synthesized by detergent-disrupted RSV were determined by assaying for virus production at distinct time intervals posttransfection by methods outlined in the legend to Fig. 2. Symbols: (A) ○, chicken embryo fibroblasts transfected with total DNA isolated from RSV-transformed duck cells: ●, mock-infected cells. (B) Total nucleic acid was isolated from reactions after synthesis of DNA by detergent-disrupted virus as described in the text and employed in transfection experiments after either no treatment (■); RNase A (100 μg/ml) treatment (□) in 3 mM EDTA for 45 min at 37°C; alkaline treatment (▲) in 0.3 M NaOH for 4 h at 37°C; or DNase (2 μg/ml) treatment (△) in 0.2 M Tris-hydrochloride (pH 7.4)-0.01 M $MgCl_2$ buffer for 2 h at room temperature. In each case 5 μg of product was added to the tissue culture dishes.*

In an effort to determine whether similarities exist between viral DNA synthesized in vitro and that isolated from virus-infected cells in vivo, we have analyzed the structure of the DNA product synthesized under our reaction conditions in vitro. In these studies viral DNA was sedimented in alkaline sucrose, and the size and polarity of the DNA were determined (Fig. 5). Reassociation of the DNA present in each fraction indicated that DNA strands of both polarities could be observed in the lower-molecular-weight region of the gradient (Fig. 5A) ranging between 300 and 3,500 nucleotides in length. However, very few double-stranded molecules were found after reassociation of the genome length region of the gradient, indicating that the bulk (ca. 90 to 95%) of DNA molecules of that size class contained either minus- or plus-strand molecules but not both. The polarity of genome length DNA was directly established by demonstrating that these molecules could completely hybridize to the viral RNA genome (Fig. 5B). These data suggest that viral plus-strand DNA molecules synthesized under our reaction conditions consist of subgenomic length DNA transcripts. To directly prove this contention, we have reassociated single-stranded, purified, genome length minus-strand DNA to each fraction of an alkaline sucrose gradient and assayed for conversion to S1-resistant duplex molecules. The results of such an experiment are shown in Fig. 5A and demonstrate that DNA molecules complementary to the genome length minus-strand DNA molecules can only be found in the range of 300 to 3,500 nucleotides in length. Furthermore, because the subgenomic length plus-strand DNA renders the bulk (>80%) of genome length minus-strand DNA resistant to S1-nuclease, most, if not all, of the viral genomic sequences must be transcribed into plus-strand DNA. We conclude that the structure, size, and polarity of DNA synthesized under enzymatic conditions that facilitate synthesis of infectious DNA molecules are compatible with the structure of duplex molecules identified in vivo consisting of intact genome length minus-strand DNA and subgenomic length plus-strand DNA molecules.

DISCUSSION

In this communication we demonstrate that infectious, transforming DNA can be synthesized by the RSV reverse transcriptase in vitro under reaction conditions that promote maximum synthesis of genome length DNA transcripts. It appears that neither RNA nor protein is required for infectivity because neither RNase nor proteinase treatment affect the ability of the in vitro-synthesized DNA to transfect chicken embryo fibroblasts.

The reaction conditions required for the maximum synthesis of RSV infectious DNA are considerably different from those reported for murine leukemia virus (22, 23). In our hands the Mg^{2+} concentration does not have to be kept below that of the deoxyribonucleoside triphosphate concentration for maximum genome

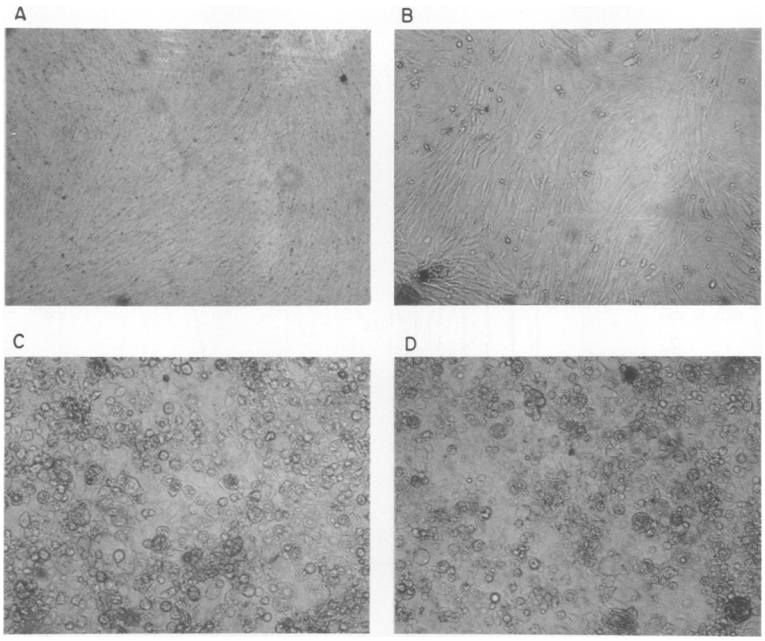

FIG. 4. *Morphology of chicken embryo fibroblasts after transfection with DNA product synthesized by detergent-disrupted RSV in vitro. Chicken embryo fibroblasts were transfected with DNA product synthesized by detergent-disrupted RSV in vitro as described in the text. (A) Mock-infected cells; (B) 0.48 μg of DNA product per 60-mm plate; (C) 4.8 μg of DNA product per 60-mm plate; (D) 5 μg of DNA from RSV-transformed duck cells per 60-mm plate. All photographs were taken at ×80 magnification.*

length DNA synthesis. However, the significance of this observation is not clear, because although the rate of DNA synthesis is maximum with Mg^{2+} for RSV, Mn^{2+} appears the preferred divalent cation by murine leukemia virus (10). Furthermore, we have found that the concentration of deoxyribonucleoside triphosphates can be significantly reduced without any appreciable affect on the yields of genome length DNA if ribonucleoside triphosphates are present during enzymatic synthesis. The advantage of this maneuver is that genome length DNA can be synthesized without affecting the extent to which the molecules can be labeled with radioactive precursors. The yields of genome length DNA under our conditions of enzymatic synthesis range between 5 and 15% of the total DNA product and are appreciably better than those reported for RSV by Junghans et al. (14), employing somewhat different conditions.

From preliminary structural studies on the nature of the infectious DNA product synthesized by RSV, it is clear that genome length DNA is of the minus polarity, whereas the plus-strand DNA product is found predominantly as subgenomic pieces ranging from 300 to 3,500 nucleotides in length. These findings suggest that enzymatic reactions containing detergent-disrupted RSV may be capable of synthesizing in vitro structural forms of proviral DNA similar to the DNA species found in the cytoplasm of infected cells. Linear, duplex viral DNA isolated from RSV-infected cells appears to contain genome length minus-strand DNA and subgenomic-length plus-strand DNA of the approximate size range as that identified in our in vitro product (25, 28, 30). Our analyses, however, do not rule out the possibility of the presence of a small portion of duplex molecules containing intact plus- and minus-strand DNA components (see Fig. 5). Furthermore, because double-stranded linear DNA molecules isolated from virus-infected cells are infectious (6, 13, 26), it is conceivable that the infectious molecules synthesized in vitro represent structures very similar to those identified in vivo. Our transfection assays employing RNase-treated and DNase-treated product synthesized in vitro clearly demonstrate that the DNA component is infectious. We believe that the accelerated rate of transfection observed after RNase hydrolysis is due to an increase in the total number of infectious molecules present per microgram of reaction product because of the removal of the noninfectious RNA genome and other virion-associated low-molecular-weight RNA species. On the other hand, alkaline hydrolysis which results in a slight decrease in the efficiency of transfection

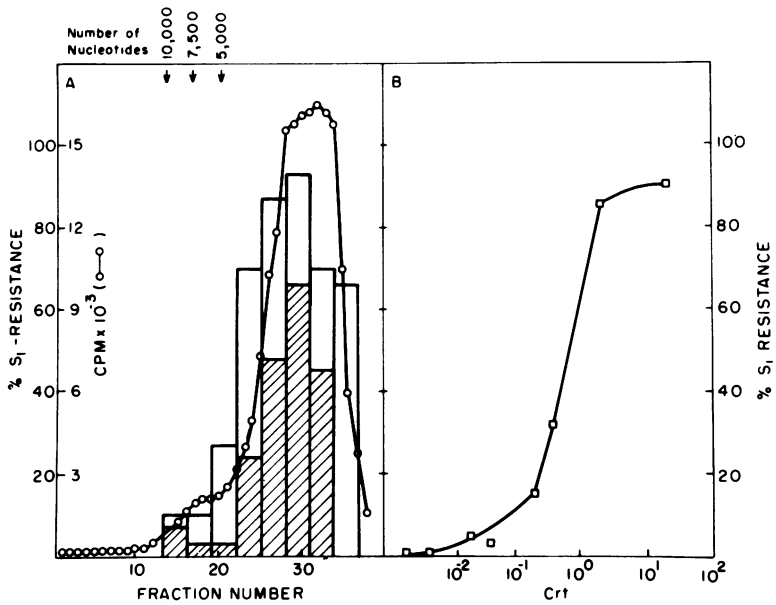

FIG. 5. *Nature of viral DNA synthesized under optimum conditions for infectious transforming DNA and genome length DNA. Viral DNA product was synthesized by detergent-disrupted RSV under conditions for maximum genome-length DNA and subjected to alkaline sucrose gradient sedimentation subsequent to alkaline hydrolysis (A) as described in the legend to Fig. 1. The resultant profile is indicated by the open circles and the position of the various nucleotide length markers denoted by the arrows. Every four fractions of the gradient were pooled, neutralized, and ethanol precipitated. The precipitates were pelleted and resuspended in 0.02 M Tris-hydrochloride, pH 7.4, and a portion from each region was heated at 100°C for 2 min and quenched on ice. The salt concentration for each sample was adjusted to 0.6 M NaCl, and the DNA was incubated at 68°C for 60 h (C_0t 3.33 × 10^{-1}) (29) to promote reassociation of duplex DNA. The percent double-stranded DNA in each pooled gradient fraction was determined by the resistance of the reassociated portions to digestion with the single-strand-specific nuclease S1 (19) and is represented by the shaded bar graph. The complexity and polarity of each size class of DNA was ascertained by hybridizing unlabeled DNA from each region of the gradient to labeled, single-stranded genome length DNA which is of minus polarity (see B). Briefly, 1,200 cpm of genome length DNA (1.2 ng) was mixed with unlabeled DNA from each size class and allowed to reassociate to a C_0t of 3.33 × 10^{-1} as outlined above. Duplex formation was determined by resistance to S1 nuclease digestion and denoted by the open bar graph. Intrinsic S1 nuclease resistance of the genome length DNA was 9.0%. (B) Complexity and polarity of the genome length DNA. Labeled DNA pooled from the alkaline sucrose gradient in the range of 7,500 to 10,000 nucleotides was hybridized to varying amounts of viral 70S RNA by procedures outlined in the text. Extent of hybridization was determined by S1 nuclease hydrolysis.*

not only removes viral RNA but denatures any double-stranded DNA present in the reaction product as well. This relative lag in the rate of transfection after alkaline hydrolysis suggests that the predominant infectious species is a fully or partially double-stranded DNA molecule. This interpretation is compatible with the preliminary observation that double-stranded DNA synthesized under conditions which facilitate genome length DNA synthesis exhibits greater infectivity than single-stranded DNA which is only weakly infectious (Clayman and Faras, unpublished data). Consistent with this interpretation is the demonstration that the infectivity of linear double-stranded DNA from avian RNA tumor virus-infected cells is also affected by alkaline denaturation (6, 13, 26). Further studies are currently under way to elucidate the exact nature of the infectious DNA molecules as well as the alkaline-sensitive DNA present in these preparations.

Finally, although these studies indicate that virions of RSV contain all the necessary components for the synthesis of infectious DNA and it is therefore tempting to speculate on the significance of these observations with respect to the relevance of proviral DNA synthesis in vivo, further studies confirming the restriction endonuclease maps of the in vitro product with the in vivo structures will be required before we can unequivocally conclude that in vitro transcription accurately reflects the mechanism of pro-

viral DNA synthesis in vivo. Such studies are currently in progress in our laboratory.

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