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Terminally repeated sequences in the avian sarcoma virus RNA genome

(oncornavirus/RNA-directed DNA synthesis/terminal redundancy/circularization)

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ABSTRACT The initiation of DNA synthesis *in vitro* by RNA-directed DNA polymerase (deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7) of avian oncornaviruses requires a tRNA^{trp} primer molecule located close to the 5′ end of the viral RNA genome. DNA transcripts, 100 nucleotides in length, initiated on the tRNA^{trp} primer molecule contain nucleotide sequences complementary to a large (25 nucleotides) RNase T_1 oligonucleotide, T-13, located at the 5′ terminus of the avian sarcoma virus RNA genome. tRNA^{trp}-initiated DNA transcripts with a length of about 70 nucleotides contain substantially fewer nucleotide sequences complementary to this 5′-terminal oligonucleotide, suggesting that the tRNA^{trp} primer associated with the avian sarcoma virus RNA is located approximately 100 nucleotides from the 5′ end of the RNA. In addition, we present evidence to demonstrate that DNA transcribed from avian sarcoma virus RNA sequences located at the 3′ end, immediately adjacent to the poly(A), contains nucleotide sequences that are complementary to the 5′-terminal T_1 oligonucleotide T-13. These data indicate that the 5′ end of the viral genome contains nucleotide sequences that are repeated at the 3′ end of the genome. We conclude that the avian oncornavirus RNA genome is terminally redundant.

After infection of cells by RNA tumor viruses, proviral DNA is transcribed from the viral RNA genome by the virion-associated DNA polymerase and subsequently integrated into the genome of the host cell (1). Proviral DNA can be found in the form of covalently closed circular molecules that are presumably required intermediates for integration (2–4). Although molecular weight estimates suggest that proviral DNA isolated from infected cells represents a complete transcript of 35S RNA (2–4), the mechanism by which the viral RNA genome is converted into the circular form of proviral DNA is presently unknown.

The *in vitro* synthesis of DNA from avian tumor virus 70S RNA by purified RNA-directed DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) is dependent on the presence of a tRNA^{trp} primer molecule that is located near the 5′ end of the avian RNA tumor virus genome and serves as the major initiation site for DNA synthesis *in vitro* (5–9). Evidence for the 5′-terminal location of tRNA^{trp}-initiated DNA transcripts has been obtained by determining the location of nucleotide sequences in the viral RNA genome that are complementary to, and therefore transcribed into, these DNA transcripts (6, 10, 11). Recently, a large T_1-RNase-resistant oligonucleotide containing the modified structure 7mGpppG^{m}pCp has been identified as the 5′ terminus of the avian sarcoma virus (ASV) genome (11). We have used this observation to confirm the location of tRNA^{trp}-initiated DNA transcripts at the 5′ end of the viral genome and to define the approximate distance between the tRNA^{trp} primer and the 5′ terminus. In addition, we demonstrate that DNA transcripts synthesized at the 3′ terminus of the viral RNA contain nucleotide sequences complementary to genomic sequences at the 5′ end of the molecule. The possible role of this observed terminal redundancy in provirus replication is discussed.

MATERIALS AND METHODS

Reagents and Virus. The sources and preparation of most of the pertinent materials have been described (12–14). Purified avian myeloblastosis virus RNA-directed DNA polymerase was obtained from J. W. Beard of Life Sciences, Inc., St. Petersburg, FL., through the auspices of the Viral Cancer Program of the National Cancer Institute. ASV, Bratislava strain, subgroup C (B_{77}), was propagated in duck embryo fibroblasts (15, 16). ASV (Prague C) was also obtained through the Viral Cancer Program of the National Cancer Institute from University Laboratories Inc., Highland Park, NJ.

Purification of Viral RNA. RNA was extracted from virus with sodium dodecyl sulfate/phenol at room temperature and subsequently fractionated into 70S RNA and free, low molecular weight RNA by rate zonal sedimentation (13). The 35S RNA subunits, free of any 70S-associated low molecular weight RNA, were prepared as described (12, 16). To obtain genome RNA deficient in poly(A), viral RNA was heat denatured and chromatographed on poly(U)–Sepharose to separate poly(A)-containing from poly(A)-lacking RNA (6). Avian myeloblastosis virus primer RNA (tRNA^{trp}) was purified by electrophoresis in 20% polyacrylamide gels (16).

Enzymatic Synthesis of Viral-Specific DNA in Reconstructed Reactions. The conditions for enzymatic synthesis of DNA by using purified viral RNA template-primer complexes and purified viral DNA polymerase have been described (12). Briefly, reactions contained either 35S RNA·tRNA^{trp} or 35S RNA·oligo(dT)$_{12-18}$ template-primer complexes at 10 μg/ml, 0.1 M Tris·HCl at pH 8.1, 0.01 M $MgCl_2$, 1.4% (vol/vol) 2-mercaptoethanol, each deoxynucleoside triphosphate at 60 μM, and purified $\alpha\beta$ DNA polymerase (50 units/ml) from avian myeloblastosis virus. Purification of the DNA transcripts has been described (12).

Isolation of Specific DNA Transcripts. The DNA product synthesized from 35S RNA·tRNA^{trp} template-primer complexes has previously been shown to consist of several small discrete pieces of DNA (10, 12). DNA transcripts synthesized from 35S RNA·oligo(dT)$_{12-18}$ complexes exhibit a size distribution from 40 to 2000 nucleotides in length. tRNA^{trp}-initiated and oligo(dT)-initiated DNA of defined length were isolated from formamide/polyacrylamide gels (12). Oligo(dT)$_{12-18}$ initiation of DNA synthesis was confirmed by the demonstration that the purified DNA product was able to bind quantitatively to poly(A)–Sepharose in 0.1 M NaCl (data not shown). The lo-

Abbreviations: ASV, avian sarcoma virus; PrB, Prague strain, subgroup B; B_{77}, Bratislava strain, subgroup C; $NaDodSO_4$, sodium dodecyl sulfate.

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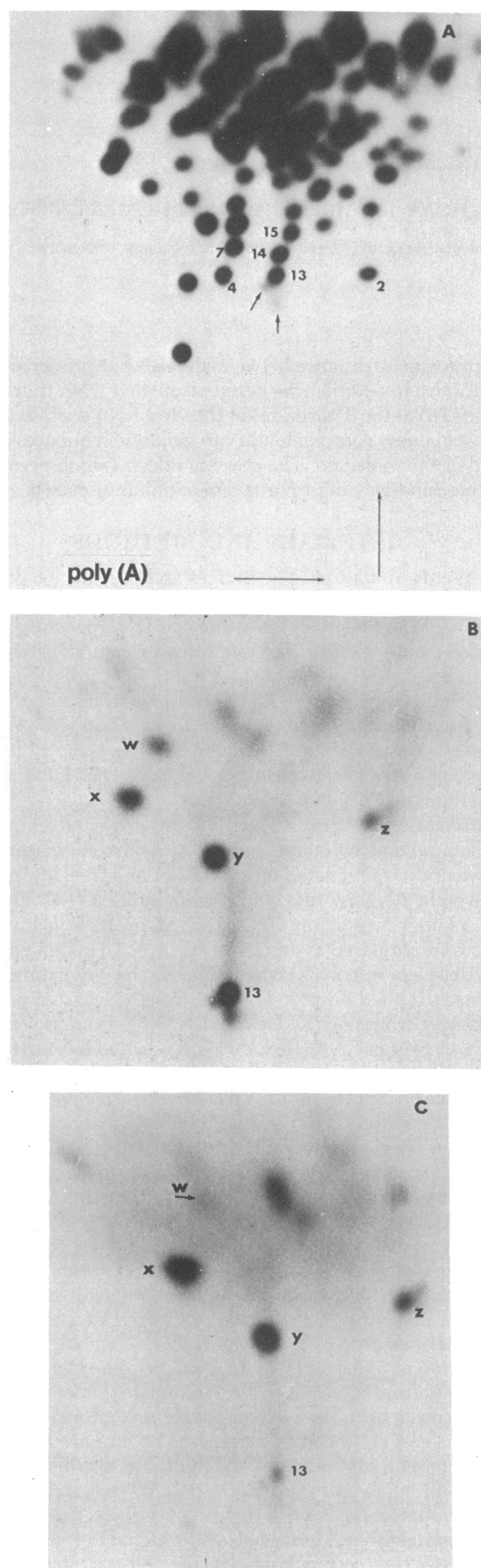

FIG. 1. Two-dimensional gel analysis of RNase T_1 oligonucleotides of viral RNA and tRNA^{trp} DNA·RNA hybrids. B_{77} RNA was labeled with [^{32}P]orthophosphate (7) and the 70S RNA was isolated as described in *Materials and Methods*. Numbers and letters identify oligonucleotides. (*A*) RNase T_1 oligonucleotides of 70S RNA: 4×10^6 cpm of B_{77} [^{32}P]RNA was digested with RNase T_1 and subjected to two-dimensional gel electrophoresis as described in *Materials and Methods*. In all of the gels presented, electrophoresis in the first dimension (10% acrylamide/6 M urea citrate, pH 3.5) was from left to right, and in the second dimension (21.0% acrylamide/40 mM Tris citrate, pH 8.0) it was from bottom to top. Arrows identify minor components. (*B*) RNase T_1 oligonucleotides of tRNA^{trp}-initiated DNA$_{100}$-[^{32}P]RNA hybrid: DNA transcripts initiated from tRNA^{trp} were synthesized and purified as described in *Materials and Methods*. The DNA product was subjected to polyacrylamide/formamide gel electrophoresis and the material migrating at 100 nucleotides was eluted from the gel as previously described (12). the trp-DNA$_{100}$ (50 ng) was then hybridized to B_{77} 70S [^{32}P]RNA (17.6×10^6 cpm, 5 μg) in 30 μl of hybridization buffer (0.6 M NaCl/0.02 M Tris-HCl, pH 7.4/0.01 M EDTA/80 μg of yeast RNA) at 68° for 4 hr. The hybridization mixture was diluted into 1 ml of 0.3 M NaCl/0.03 M sodium citrate and treated with 4 μg of T_1 RNase for 30 min at 37°. After sodium dodecyl sulfate–Pronase treatment and phenol extraction, the nucleic acid was precipitated with ethanol, and RNA·DNA hybrids were resolved from low molecular weight digestion products by chromatography on a Sephadex G-100 column (0.9×20 cm) in 0.5 M NaCl/0.02 M Tris-HCl, pH 7.4/0.01 M EDTA/0.2% sodium dodecyl sulfate. [^{32}P]RNA recovered from the excluded region of the Sephadex G-100 column was heat denatured, digested with RNase T_1, and subjected to two-dimensional polyacrylamide gel electrophoresis as described in *Materials and Methods*. (*C*) RNase T_1 oligonucleotides of tRNA^{trp}-DNA$_{70}$ [^{32}P]RNA hybrids: tRNA^{trp}-initiated DNA with a length of approximately 70 nucleotides was obtained as in *A* and hybridized with B_{77} [^{32}P]RNA (17.6×10^6 cpm). [^{32}P]RNA·DNA hybrids were isolated as described in *A* and analyzed by two-dimensional gel electrophoresis.

calization of dT$_{12\text{-}18}$-initiated DNA at the 3′ end of ASV RNA was established by showing that oligo(dT)$_{12\text{-}18}$-initiated DNA transcripts hybridized to greater than 90% to small (*ca* 200–400 nucleotides) fragments of poly(A)-containing ASV RNA that lacked nucleotide sequences complementary to cDNA transcripts representing other portions of the viral genome (i.e., cDNA$_{sarc}$, cDNA$_{env}$).

Hybridization of DNA to ASV[^{32}P]RNA and Analysis of RNase T_1-Resistant Oligonucleotides. Hybridization reactions were carried out as described in the legend to Fig. 1. For the analysis of RNase T_1-resistant hybrids, ^{32}P-labeled RNA·DNA hybrids in 20 mM Tris-HCl, pH 7.4/10 mM EDTA were heat denatured and incubated for 30 min at 37° in the presence of 100 μg of yeast RNA and 40 units of RNase T_1 (Calbiochem). Samples were subjected to two-dimensional polyacrylamide gel electrophoresis as described (14). After electrophoresis, oligonucleotides were localized by autoradiography and cut out. The labeled RNA in gel discs was eluted with 1.0 M NaCl (14) and their radioactivity measured by counting Cherenkov radiation. The nucleotide composition of the individual oligonucleotides was determined by using RNase A (Calbiochem) as described by Adams *et al.* (17).

RESULTS

Oligonucleotide T-13, obtained by complete RNase T_1 digestion of Prague B (PrB) ASV RNA (18) has been shown to be the 5′ terminus of the PrB genome by virtue of the fact that it contains the structure 7mGpppG^{m}pCp (11). Fig. 1*A* shows a two-dimensional polyacrylamide gel pattern of oligonucleotides obtained by complete digestion of B_{77}ASV 70S[^{32}P]RNA with RNase T_1. Oligonucleotide T-13 of B_{77}ASV RNA (Fig. 1*A*) contained a 7mGpppG^{m}pCp cap structure (Table 1) and therefore was the 5′ terminus of B_{77} RNA. Additionally, two minor components (small arrows, Fig. 1*A*) were resolved and

Table 1. Composition of RNase T_1 oligonucleotides

	Oligonucleotide*	Chain length (nucleotides)	Composition†
70S B_{77} ASV RNA			
	4	22	1 G, 1 A_3C, 1 AU, 1 AC, 8 C, 5 U
	7	18	1 A_2G, 1 A_5N, 5 C, 4 U
	13	25	1 G, 3 AU, 3 AC, 5 C, 5 U, 1 Cap‡
	14	19	1 G, 1 A_2C, 1 A_2U, 2 AU, 1 AC, 3 C, 3 U
	15	17	1 G, 1 AU, 8 C, 6 U
trp-DNA_{100}·RNA hybrid			
	13	25	1 G, 3 AU, 3 AC, 5 C, 5 U, 1 Cap‡
	w	6	1 G, 1 AC, 2 C, 1 U
	x	8	1 G, 1 A_2C, 1 AC, 1 C, 1 U
	y	11	1 G, 1 A_2C, 1 AU, 3 C, 2 U
	z	–	N.D.§

* T_1 oligonucleotides similar in composition to those characterized by Joho *et al.* (19) were assigned the same number.
† Compositions of T_1 oligonucleotides were determined by RNase A digestion as described in *Materials and Methods*.
‡ Cap ($7mGpppG^mpCp$) was characterized by its position on the DEAE-cellulose column as described (20, 21).
§ Insufficient amounts of radioactivity were recovered to allow accurate analysis of this oligonucleotide.

were similar to oligonucleotide T-13 in nucleotide composition but lacked the complete methylated cap structure.

To characterize the RNA sequences transcribed during $tRNA^{trp}$-primed DNA synthesis and to localize the position of the $tRNA^{trp}$ primer on the avian tumor virus genome, two discrete size classes of DNA synthesized from 35S RNA·$tRNA^{trp}$ template·primer complexes (about 100 nucleotides and 70 nucleotides, respectively) were isolated from denaturing formamide/polyacrylamide gels (12). The two classes of DNA transcripts, trp-DNA_{100} and trp-DNA_{70}, were annealed with B_{77} 70S [^{32}P]RNA. The [^{32}P]RNA sequences complementary to the DNA transcripts were isolated by treatment of the hybridization reactions with RNase T_1 (under conditions that did not disrupt hybrid structures) followed by chromatography on Sephadex G-100 (11). The DNA·RNA complexes recovered from the excluded region of the column were then denatured, the RNA moiety was completely digested with RNase T_1, and the [^{32}P]oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis (14).

Hybridization of B_{77} ASV [^{32}P]RNA with trp-DNA_{100} converted about 1.0–1.4% of the initial [^{32}P]RNA to a RNase T_1-resistant hybrid. Analysis of the T_1 oligonucleotides obtained from the RNA moiety of this hybrid by two-dimensional gel electrophoresis is shown in Fig. 1*B*. Five large T_1 oligonucleotides were observed in addition to a number of smaller components. We have identified the largest of these oligonucleotides as the 5′-terminal oligonucleotide T-13 on the basis of its nucleotide composition (Table 1) and position in the gel. Comparison of the nucleotide composition of the other large oligonucleotides (*w*, *x*, *y*) (Table 2) with the direct sequence data of the DNA_{100} transcripts (22) suggests that these oligonucleotides are also derived from the 5′ terminal 100 nucleotides of B_{77} RNA.

Hybridization of B_{77} [^{32}P]RNA with trp-DNA_{70} significantly decreased the amount of oligonucleotide T-13 recovered in the RNA·DNA hybrid (Fig. 1*C;* Table 2), indicating that trp-DNA_{70} transcripts were deficient in sequences complementary to the 5′-terminal oligonucleotide 13. The yield of oligonucleotide *w* was also significantly decreased compared to the yields of oligonucleotides *x*, *y*, and *z* (Fig. 1*C;* Table 2).

Previous experiments have suggested that DNA sequences complementary to the 5′ end of ASV RNA could also hybridize to a limited extent with short RNA sequences (less than 500 nucleotides) derived from the 3′ end of ASV RNA (10). To provide direct evidence for the existence of repeated RNA sequences at the termini of the ASV genome, DNA was transcribed from the 3′ end of the viral genome and the viral RNA sequences complementary to those 3′-terminally located DNA transcripts were purified after RNase T_1 digestion and subjected to analysis as described above. The synthesis of DNA complementary to the 3′ end of the B_{77} RNA genome was accomplished by using RNA-directed DNA polymerase, purified 35S RNA, and, as the only source of primer, oligo$(dT)_{12-18}$, which binds to the poly(A) sequences present at the 3′ end of the virus genome. Oligo $(dT)_{12-18}$-initiated DNA transcripts (dT-DNA) no greater than 450 nucleotides in length were isolated and hybridized to B_{77} [^{32}P]RNA. After hybridization of dT-DNA with B_{77} [^{32}P]RNA, a number of T_1 oligonucleotides were resolved (Fig. 2*A*) including small amounts of an oligonucleotide with a mobility similar to that of oligonucleotide T-13.

To determine whether the low level of oligonucleotide T-13 observed reflected preferential nucleation of the dT_{12-18} region of the dT-DNA transcripts to the 3′-terminal poly(A) sequences

Table 2. Relative distribution of RNase T_1 oligonucleotides in trp-DNA hybrids*

Oligonucleotide	cpm† trp-DNA_{70}	cpm† trp-DNA_{100}	Relative recovery‡ trp-DNA_{70}/trp-DNA_{100}
13	99 (4)	1811 (65)	0.06
w	102 (17)	709 (118)	0.14
x	747 (93)	1315 (164)	0.6
y	941 (86)	1276 (116)	0.7
z	321 (36)	304 (34)	1.0

* Oligonucleotides as designated in Fig. 1*B* and *C* were cut from gels and the radioactivity was measured. Background radioactivity (42 cpm) was determined by counting a blank area of the gel and was subtracted from all values.
† Numbers in parentheses represent the ratio of cpm recovered in oligonucleotide to chain length (Table 1).
‡ The ratio of the recovered counts per phosphate of the oligonucleotides protected by trp-DNA_{70} and those protected by trp-DNA_{100} was determined.

of the viral genome during annealing, dT-DNA was hybridized with [^{32}P]poly(A)-deficient viral RNA. The amount of oligonucleotide T-13 found in dT-DNA·RNA hybrids increased substantially (Fig. 2*B*). Similarly, saturation of the poly(A) sequences of 70S RNA with oligo(dT)$_{12-18}$ prior to hybridization with the dT-DNA also increased the yield of oligonucleotide T-13, indicating that more of the dT-DNA was available for hybridization to the 5′-terminal sequences (data not shown). To support the identification of this large T_1 oligonucleotide as oligonucleotide 13, T_1 oligonucleotides derived from the RNA moiety of ^{32}P-labeled poly(A)-lacking B_{77} RNA and dT-DNA hybrids were analyzed by two-dimensional gel electrophoresis in the presence of three large T_1 oligonucleotides of known composition and mobility. Comparison of the relative positions of oligonucleotides 2, 4, and 7 with those of the oligonucleotides protected by dT-DNA confirmed that the protected oligonucleotide was the 5′-terminal oligonucleotide T-13 (data not shown).

The specificity of the above hybridization-protection experiments has been confirmed by demonstrating that short DNA transcripts synthesized from an internal site on the viral RNA genome (M. S. Collett and A. J. Faras, unpublished data) did not protect the 5′-terminally located oligonucleotide T-13. Similarly, hybridization of B_{77} [^{32}P]RNA with calf thymus DNA fragments did not result in the protection of oligonucleotide T-13. These experiments demonstrate that oligonucleotide T-13 is present in RNA·DNA hybrids because of its sequence homology with tRNA^{trp}-initiated DNA and dT$_{12-18}$ initiated DNA.

DISCUSSION

DNA transcripts approximately 100 nucleotides in length initiated on a tRNA^{trp}-35S ASV RNA complex contain sequences complementary to several large RNase T_1 oligonucleotides, one of which (oligonucleotide T-13) is located directly at the 5′ terminus of B_{77} ASV RNA genome. A smaller species of tRNA^{trp}-initiated DNA transcripts (70 nucleotides) contains significantly fewer sequences complementary to the 5′-terminal oligonucleotide T-13. These data suggest that the tRNA^{trp} primer site for RNA-directed DNA polymerase is located approximately 100 nucleotides from the 5′ end of the ASV genome. A similar conclusion has been reached recently on the basis of direct DNA sequence data (22). The sequence data obtained by Shine *et al.* (22) for ASV trp-DNA$_{100}$ predict five RNase T_1 oligonucleotides having a length of six nucleotides or greater. We find, in addition to oligonucleotide T-13, four large T oligonucleotides (*w, x, y, z;* Fig. 1*B*) that are complementary to tRNA^{trp}-initiated DNA. The nucleotide compositions of three of the oligonucleotides (*w, x,* and *y;* Table 1) are consistent with their being derived from the 5′-terminal 100 nucleotides of B_{77} RNA.

DNA transcribed from the 3′ end of the avian oncornavirus genome also contains nucleotide sequences complementary to oligonucleotide T-13 located directly at the 5′ terminus of the RNA genome. Therefore, nucleotide sequences present at the 5′ end of the viral RNA are also present at the 3′ end. We conclude that the avian oncornavirus genome is terminally redundant. Although these experiments establish the presence of terminally repeated sequences in the viral RNA, they do not rigorously define the extent of this terminal repetition. Analysis of dT-DNA·RNA hybrids indicates that oligonucleotide *y*, which is thought to be located at position 57–67 from the 5′ end, is not present in a significant amount (compare Fig. 2*B*), suggesting that the extent of terminal repetition is not greater than 40 to 50 nucleotides. The nucleotide composition of olig-

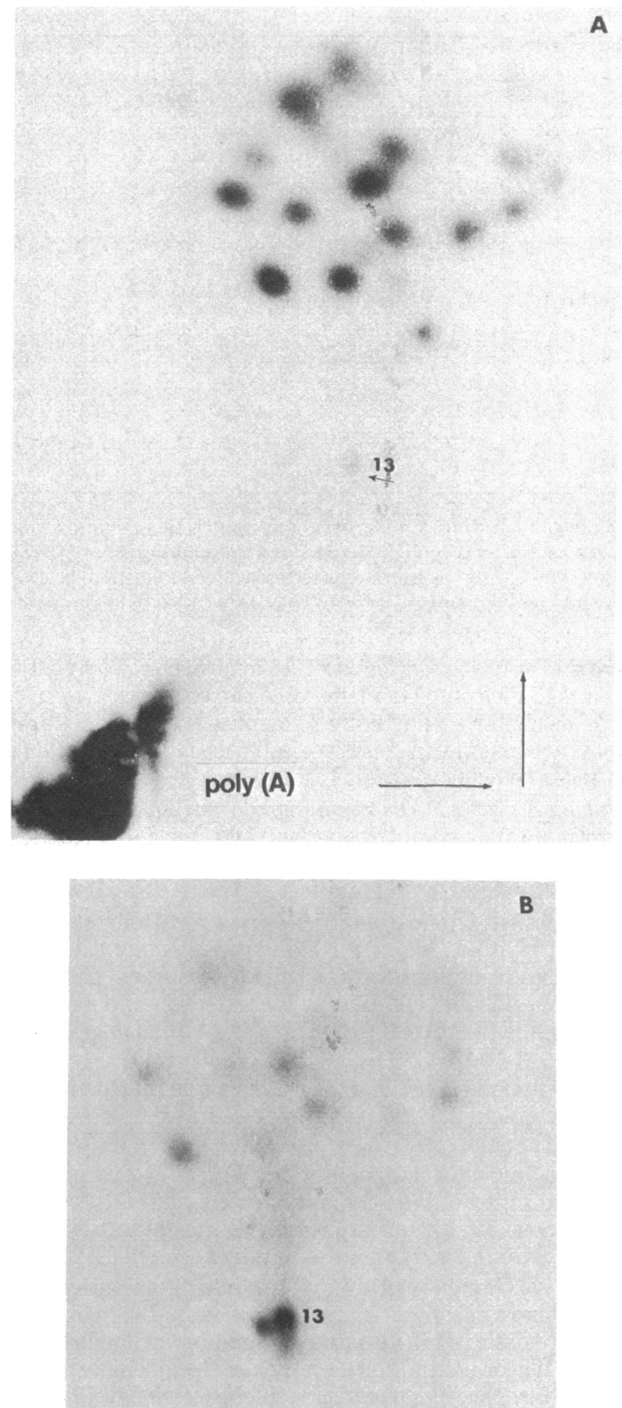

FIG. 2. Two-dimensional gel analysis of RNase T_1 oligonucleotides of oligo(dT)$_{12-18}$ RNA·DNA hybrids. (*A*) RNase T_1 oligonucleotides of oligo(dT)-DNA-[^{32}P]RNA hybrid. Oligo(dT)$_{12-18}$-initiated DNA was synthesized and purified as described in *Materials and Methods*. Material no greater than 450 nucleotides in length was isolated from polyacrylamide/formamide gels. dT-DNA (43 ng) was annealed with B_{77} ASV 70S [^{32}P]RNA (17.6 × 10^6 cpm, 5 μg) and the RNA·DNA hybrid structures were isolated as described in the legend to Fig. 1. RNase T_1 oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis as described in *Materials and Methods*. (*B*) RNase T_1 oligonucleotides of oligo(dT)-DNA·[^{32}P]poly(A)-lacking RNA hybrids. Oligo(dT)$_{12-18}$-initiated DNA (60 ng) was annealed with ^{32}P-labeled poly(A)-minus B_{77} ASV RNA (7 × 10^6 cpm, 2 μg) and RNA·DNA hybrids were isolated as described in Fig. 1. RNase T_1 oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis as described in *Materials and Methods*.

onucleotide T-13 indicates a length of about 25 nucleotides. It is unlikely that the entire sequence of oligonucleotide 13 is repeated at the 3′ end of the 35S RNA because RNase T_1 digestion of short ^{32}P-labeled poly(A)-containing RNA pieces does not yield an oligonucleotide with composition similar to that of oligonucleotide T-13 (ref. 23; unpublished data). However, if the repeated sequence were adjacent to the poly(A) and did not contain the 3′ G residue of oligonucleotide T-13, it would not be released from the poly(A) by RNase T_1 digestion and would not be resolved by two-dimensional gel electrophoresis.

Our previous studies (12) of RNA-directed DNA synthesis *in vitro* have suggested that two major reactions occur during the early stages of proviral DNA synthesis. The first involves initiation of DNA synthesis on the $tRNA^{trp}$ primer molecule located near the 5′ end of the avian oncornavirus genome (5, 6) followed by transcription to the 5′ terminus of the template. The second reaction involves continuation of DNA synthesis, presumably at the 3′ end of the template. The terminally redundant nature of the avian oncornavirus genome provides a mechanism by which the virus RNA genome could circularize shortly after DNA synthesis has begun. This circularization would involve the initial DNA product, transcribed from the 5′ terminus of the viral RNA, base-pairing with complementary sequences at the 3′ end of the viral RNA. The circularization step would therefore lead to a continuous transcription of viral RNA sequences and obviate any requirement for the DNA polymerase to jump a gap from one end to the other of the RNA subunit during proviral DNA synthesis.

Note Added in Proof. While this manuscript was being considered for publication, the nucleotide sequence of DNA transcripts synthesized from the 5′ (refs. 22 and 24) and the 3′ (ref. 25) ends of the RSV genome was reported. The data from these independent studies confirm the existence and nature of the terminally repeated sequences in the RSV genome.

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