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Terminal redundancy and the origin of replication of Rous sarcoma virus RNA

(RNA-directed DNA synthesis/nucleotide sequence analysis/RNA fingerprinting/nucleic acid hybridization)

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ABSTRACT *In vitro* synthesis of Rous sarcoma virus DNA by the virion endogenous DNA polymerase activity is initiated on a $tRNA^{trp}$ primer located near the 5′ end of the genome. A major product of such synthesis is a piece of DNA 101 nucleotides long (strong stop DNA) which can be isolated covalently bound to the tRNA primer. Here we show that the strong stop DNA is complementary to the extreme 5′ end of the genome. We also show that the 5′ and 3′ termini of the Rous sarcoma virus genome, excluding the cap and the poly(A), have the identical sequence. We propose that the function of this sequence is to facilitate elongation from the 3′ end of DNA chains initiated elsewhere on the virus genome.

RNA tumor viruses replicate through a DNA intermediate (provirus) that is synthesized shortly after infection, integrated into the host genome, and transcribed to give progeny RNA molecules (1). Viral DNA can be synthesized *in vitro* by using either the endogenous DNA polymerase activity of disrupted virions (2, 3) or systems reconstituted from pure RNA and DNA polymerase (4–6). A major fraction of the DNA products of such *in vitro* reactions, synthesized under a defined set of reaction conditions, is a set of DNA molecules of discrete length (5). The longest of these discrete molecules are transcripts of the entire genome (5–7). In the case of Rous sarcoma virus (RSV), all or most of this *in vitro* DNA synthesis is initiated with a $tRNA^{trp}$ primer (8) that is located near the 5′ end of the genome (9). The majority of the DNA products of discrete length share a common initiation sequence, and the shorter molecules are the 5′-terminal portions of the longer sequences (5). More than 20% of all the DNA molecules initiated at the tRNA primer terminate at a site 101 bases away. Here we show that this 101-nucleotide-long DNA species, called strong stop DNA, is synthesized from the extreme 5′ end of the RNA genome and is a DNA copy of the RNA from the primer binding site to the capping group (10, 11) at the end of the genome.

The observation that initiation of DNA synthesis in disrupted virions occurs near the 5′ end of the genome means that, if the entire genome is to be copied, the growing chain must jump from the 5′ end of the genome to the 3′ end. One possible mechanism by which synthesis of DNA chains could be initiated near the 5′ end of the RNA molecule and then continued from the 3′ end would make use of a short terminal redundancy in the viral genome RNA molecule (Fig. 1). Nascent DNA chains, copied from the region of the RNA between the primer and the 5′ end, would contain, at their 3′ end, a nucleotide sequence complementary to the 3′ end of the viral RNA. This complementary sequence could form a short hybrid with the 3′ sequence and thus allow DNA synthesis to continue from the 3′ end of the viral RNA.

In this report we present evidence that the high-molecular-weight genome RNA of RSV contains the identical sequence of 21 nucleotides at its 5′ and 3′ termini [excluding the poly(A) and 7mGppp of the capping group] and that this sequence is complementary to the 3′ terminus of RSV strong stop DNA.

MATERIALS AND METHODS

Viruses. Prague strain RSV, subgroup B, used as the source of labeled virus RNA, was previously described (13). Large quantities of purified virus for the preparation of DNA products were obtained from the National Cancer Institute Program of Resources and Logistics. Two-dimensional gel electrophoresis of the RNA of this virus revealed that it was largely or entirely Prague strain RSV, subgroup C (data not shown). This discrepancy does not affect the results of this study because both subgroups have the identical 5′-terminal sequence in that they both contain oligonucleotide 13 (see below) (13, 21).

Preparation of Viral RNA and DNA. ^{32}P-Labeled 70S RNA was prepared as described (13, 14). 3′ ends were prepared from this RNA by either partial digestion with alkali (0.05 M Na_2CO_3 for 15 min at 50°) to yield fragments about 300 nucleotides long or by complete digestion with RNase T_1 (Calbiochem, Los Angeles, CA) at a ratio of 20 units/100 μg of RNA for 30 min at 37°. The poly(A)-containing fragments were isolated by two cycles of chromatography on poly(U)-Sephadex G-10 as described (13), followed, in the case of RNase T_1 digested material, by chromatography on Sephadex G-50 (Pharmacia, Uppsala, Sweden). Strong stop DNA was synthesized *in vitro* and isolated as previously described (15).

Nucleic Acid Hybridization. RNA·DNA hybridizations were carried out in 100 μl of 0.5 M NaCl/100 mM Tris·HCl, pH 7.5, containing 20 μg of yeast RNA, 0.1–1 μg of RSV 70S [^{32}P]RNA (or 0.01–0.1 μg of 3′ ends), and 0.01–0.1 μg of strong stop DNA. Annealing with total RNA or fragments produced by alkaline hydrolysis was at 66° for 20–24 hr, to a C_0t value‡ (uncorrected) of 0.02 mol·sec·liter^{-1} or greater. 3′-terminal fragments obtained after RNase T_1 digestion were annealed at 37° in 1 M NaCl for 22 hr. The resulting hybrids were isolated by a modification of the method of Parsons *et al.* (16). After annealing, the hybridization mixture was incubated with 5 μg of RNase A (Worthington Biochemical Corp., Freehold, NJ) and 5 units of RNase T_1 for 30 min at 37°. The mixture was adjusted to 0.2% (wt/vol) in Na dodecyl sulfate and chromatographed in a 0.6 × 10 cm column of Sephadex G-100 in 0.5 M NaCl/0.2% Na dodecyl sulfate/10 mM Tris·HCl, pH 7.5. The peak fractions eluting with the void volume (from about 0.7 to 1.2 ml) were pooled, adjusted to 100 μg of yeast RNA, and extracted twice with an equal volume of redistilled phenol. The RNA was precipitated from the aqueous phase with 2.5 volumes of ethanol, dissolved in 20 mM Tris·HCl, pH 7.5/5 mM EDTA, denatured by heating in a boiling water bath for 30 sec, and

Abbreviation: RSV, Rous sarcoma virus.

‡ C_0t = initial concentration (mol/liter) of total DNA × time (sec).

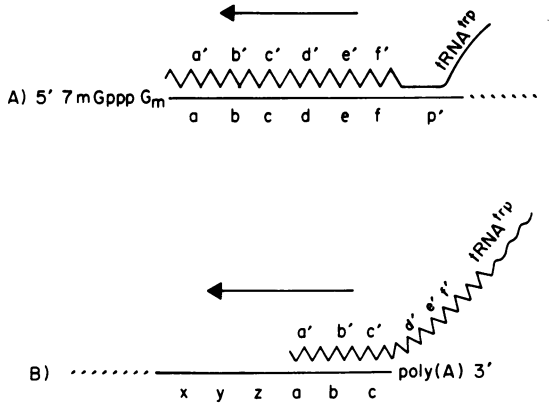

FIG. 1. Possible mechanism for the synthesis of RNA tumor virus DNA, using a terminally redundant sequence. (*A*) DNA synthesized between the $tRNA^{trp}$ primer and the 5′ end of the genome RNA contains a sequence (a′b′c′) at its 5′ end complementary to a terminally redundant sequence (abc). (*B*) After separation of the hybrid so formed [or degradation of the copied RNA by RNase H (12)] the nascent DNA–polymerase complex can align itself with the 3′ end by base pairing of a′b′c′ with the abc sequence adjacent to the poly(A). Symbols: —, RNA; ⁓, DNA; d–z, unique sequences; p′, primer binding site.

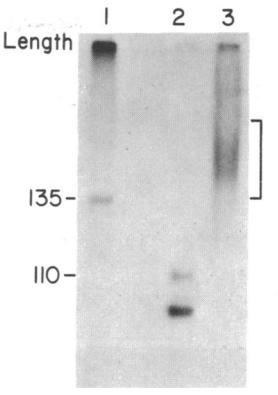

FIG. 2. RSV RNA protected by strong stop DNA. ^{32}P-Labeled RSV 70S RNA (10^7 cpm; 5.7×10^6 cpm/μg) or 3′-terminal fragments (about 300 nucleotides long) from the same RNA (5×10^5 cpm) were annealed with 0.1 μg of strong stop DNA, and the hybrids (10^5 cpm in each case) were obtained after treatment with RNase A and T_1 as described in *Materials and Methods*. These hybrids were denatured by heating to 95° for 2 min and then were loaded onto a 10% polyacrylamide gel. Lane 1, single-stranded DNA segments of known length [obtained from an endogenous DNA polymerase reaction using Moloney murine leukemia virus (5)] served as size markers; the 110-nucleotide-long marker band cannot be seen here but was visible in the original autoradiograph. Lane 2, hybrid derived from total RSV RNA. Lane 3, hybrid derived from fragmented 3′-poly(A)-containing RSV RNA bracket = poly(A).

precipitated again with ethanol before further manipulations.

Partial Sequence Analysis. Characterization of RNA fractions by two-dimensional gel electrophoresis and analysis of the oligonucleotides was as described (13, 14, 17, 18).

The $7mGpppG_m$ capping group in RNA fractions was detected as described by Rose (19).

To analyze the 5′ end of the poly(A)-containing T_1 oligonucleotide, 2 μg of unlabeled RSV RNA was digested with 0.3 unit of RNase T_1 in 10 μl of 5 mM Tris·HCl, pH 7.5. After incubation at 37° for 30 min, the total reaction mixture was lyophilized and redissolved in 10 μl of 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/5% (vol/vol) glycerol/5 mM dithiothreitol containing 2 nmol of [α-^{32}P]ATP (specific activity, 1000 Ci/mmol; New England Nuclear Corp., Boston, MA). After 30 min at 37°, the reaction was terminated by the addition of ammonium acetate (pH 5.4) to a final concentration of 0.2 M and 20 μg of yeast tRNA. The 5′-end-labeled oligonucleotides were precipitated twice with ethanol and the 3′ ends were isolated by chromatography on poly(U)-Sephadex as above.

RESULTS

Analysis of DNA fragments synthesized *in vitro*

The hypothesis for the elongation of RSV DNA outlined in the *Introduction* predicts that DNA complementary to the 5′ terminus of the RSV RNA genome should contain, at its 3′ end, a region complementary to the 3′ end of the genome. To test this prediction, we analyzed the major defined product of *in vitro* DNA polymerase reactions of RSV virions to determine what portions of the genome are complementary to it. The products of an endogenous DNA polymerase reaction using disrupted virions of RSV under defined conditions of synthesis can be separated by polyacrylamide gel electrophoresis into a number of size classes. These fractions represent elongation products of various lengths, at the 3′ end, of a common nulceotide sequence covalently attached to the $tRNA^{trp}$ primer (5). One of these fractions, called strong stop DNA, comprises more than 20% by mass of the total reaction product. The nucleotide sequence of this DNA fragment has been determined (15).

To determine which part of the RSV genome serves as template for this DNA, unlabeled strong stop DNA was annealed with ^{32}P-labeled RSV RNA. The resulting hybrid was isolated after treatment with RNase A and T_1, and analyzed in several different ways.

Fig. 2 (lane 2) shows the result of polyacrylamide gel electrophoresis of this material. The bulk of the RNA from the hybrid migrated in a single band with an apparent length of approximately 100 nucleotides, identical to that of the strong stop DNA. The faint slower migrating band probably represents reannealed DNA·RNA hybrid. Occasionally, material moving faster than the 100-nucleotide band was observed. This material may have been produced by overdigestion of the hybrids.

To localize the hybridized RNA on the genome, [^{32}P]RNA·DNA hybrid was prepared as above and analyzed by complete digestion with RNase T_1 and two-dimensional gel electrophoresis (14, 17). Four principal large oligonucleotides were found among the digestion products (Fig. 3*B*). The largest of these corresponded precisely in position and composition of digestion products with RNase A (Table 1) to oligonucleotide 13 of Prague-strain RSV (13) which contains the $7mGpppG_m$ capping group and 23 additional nucleotides (21, 22). These results are similar to those of Cashion *et al.* (21) who analyzed RSV RNA protected by total DNA products (of less than 200 nucleotides in length) at low DNA/RNA ratios and found the same oligonucleotides.

These results indicate that strong stop DNA protects a piece of RNA of identical length and complementary nucleic acid sequence at the 5′ end of the RSV RNA molecule. This conclusion was confirmed by quantitative analysis of the capping group in the RNA protected by strong stop DNA. When the total protected RNA was digested to completion with a mixture of RNase A, T_1, and T_2, 4.0 ± 0.2% (average determinations on two preparations) of the total radioactivity recovered was in the $7mGpppG_mpCp$ sequence (data not shown). Because this sequence has five phosphate groups, the total sequence complexity of the protected RNA must be 125 ± 7. In another experiment, the capping group comprised 4% of the ^{32}P label in the 100-nucleotide-long RNA from the band shown in Fig. 2.

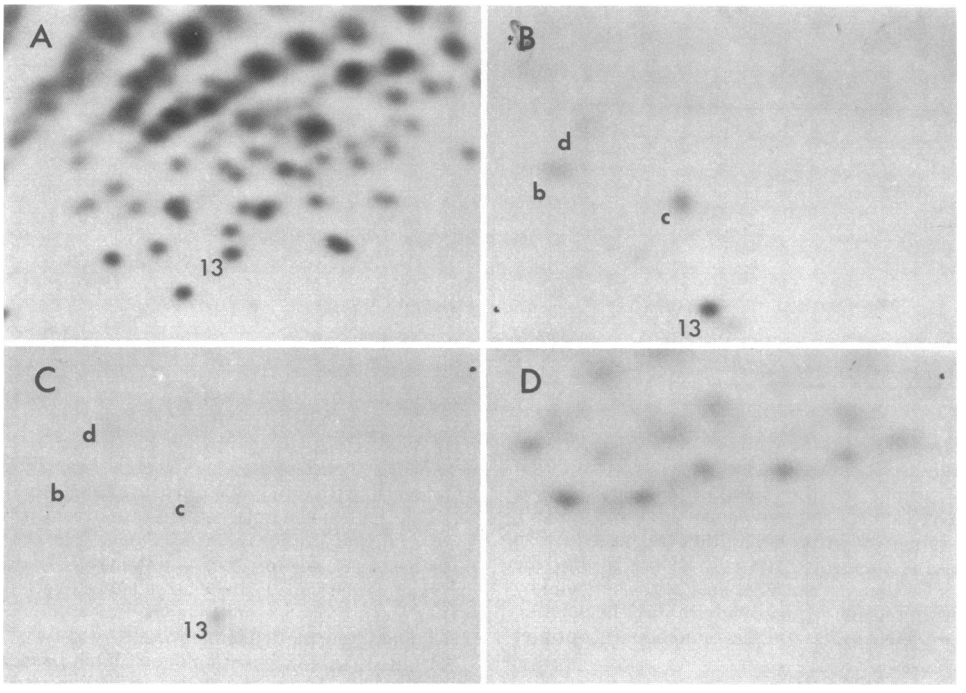

FIG. 3. Analysis of RSV RNA and fractions. Samples derived from ^{32}P-labeled 70S RNA from Prague strain RSV, subgroup B, were digested with RNase T_1 and analyzed by two-dimensional gel electrophoresis as described (13, 14, 17). (*A*) Total unfractionated RNA. (*B*) Total 70S RNA annealed with strong stop DNA from the experiment shown in Fig. 2. After further digestion with RNase A or U_2, the spot below and to the right of oligonucleotide 13 yielded products identical to those from oligonucleotide 13 except for the capping group, which had an altered electrophoretic mobility, and was not further characterized. The spot below and to the left of oligonucleotic c was in much less than equimolar yield to the other oligonucleotides and yielded too little material for further analysis. (*C*) RNA eluted from the most intense (100-nucleotide-long) band of the experiment shown in Fig. 2. (*D*) 3′-Terminal fragments (total, not hybridized) as described in the legend to Fig. 2.

The complexity of the total protected RNA was therefore nearly identical to its length. (The higher complexity is probably due to a small amount of random contamination with other sequences.) Because the length and complexity of the hybridized genome RNA are approximately the same as those of strong stop DNA, this DNA must hybridize only to the 101-nucleotide-long sequence between the primer and the 5′ end of the RSV genome. This conclusion is also supported by the observation that an excess of strong stop DNA protected only 1% of the total RSV RNA from nuclease digestion (see legend, Fig. 2).

Because nuclease does not remove the capping group from the RNA hybridized to strong stop DNA, the strong stop sequence must extend to within at least one nucleotide of the cap sequence. We therefore conclude that strong stop DNA is a complementary copy of the 101-nucleotide sequence of RNA between the primer binding site and the inverted 7mGppp at the 5′ end of the RSV genome. A similar conclusion can be drawn from the 3′ end of the sequence of strong stop DNA (15). The complement of this sequence (Table 1) is consistent with the partial sequence of oligonucleotide 13 previously reported (21). The present results strengthen this conclusion because it is possible that a sequence with the same digestion products occurs elsewhere in the genome.

Table 1 also shows all T_1 oligonucleotides of four or more nucleotides expected from the sequence of strong stop DNA (15). The four smaller oligonucleotides from the hybrid of RSV 70S [^{32}P]RNA and strong stop DNA yielded, on digestion with RNase A, products consistent with the nucelotide sequence of strong stop DNA from nucleotides 22 to 29 (product b), 35 to 45 (product c), and 67 to 72 (product d). One expected oligonucleotide (product a), which extends from the second nucleotide from the 3′ end of the primer to the ninth nucleotide

Table 1. Analysis of oligonucleotides protected by hybridization of strong stop DNA with RSV [^{32}P]RNA

Product* found	RNase A digestion products†	Predicted from strong stop DNA (15)	
		Expected sequence	Position‡
a	Not observed	CUUCAUUUG§	−2 to 7
b	AAC, AC, C ≃ U, G	AACACCUG	22 to 29
c	AU, AAC, C ≃ U, G	AUUCCCUAACG	35 to 45
d	AC, C > U, G	CACCUG	67 to 72
13	7mGpppG$_m$C, AU ≃ AC, G, C ≃ U	7mGpppC$_m$CCAUUUU-ACCAUUCACCACAUUG	78 to 101

* The letters correspond to those in Fig. 3*B* and are ordered according to their position in the strong stop DNA.
† Identity and relative yields estimated from electrophoretic mobility and relative intensity on the autoradiograph.
‡ In the strong stop DNA with the 5′-deoxynucleotide adjacent to the primer = 1.
§ The 3′-terminal UG is derived from the complement of the sequence of the tRNA^{trp} primer. Some ambiguity exists in regard to the identity of the penultimate U (20).

Table 2. Nucleotide sequences adjacent to the 5′ and 3′ ends of RSV RNA

RNase U_2 products*				RNase A products*			
		Poly(A) associated				Poly(A) associated	
Product	Oligonucleotide 13	Total	Hybrid with strong stop DNA†	Product	Oligonucleotide 13	Total	Hybrid with strong stop DNA†
7mGpppG$_m$CCA	1	—	—	Poly(A)‡	—	(200) [29]§	(500) [10]§
U_4A	1	1	1	7mGpppG$_m$C	1	—	—
(U_2, C)A	1	(1) [29]	(1) [9]	AU	3	2–3	2
UUG	(1) [60]	—	—	AC	3	2–3	2
CCA	2	3	4	G	(1) [116]	—	—
CA	1	1	2	C	4	3–5	3
A plus poly(A)‡	—	200	500	U	5	2–4	3

* Rounded molar yield relative to the product given in parentheses. The numbers in brackets give the amount of radioactivity, in cpm/nucleotide, used for this calculation. All values are corrected for a background of about 30 cpm obtained by counting five blank areas from the same electropherogram.

† These numbers are somewhat inaccurate because of the low amount of radioactivity recovered. A control annealing with no DNA yielded only products containing Ap in the "hybrid." The higher molar yield of poly(A) is probably the result of incomplete hybridization of the adjoining sequences.

‡ Includes all products yielding only Ap after digestion with RNase A, T_1, and T_2.

§ In the case of the RNase A digests of poly(A)-associated material, no product was considered likely to be present only once. The A in the RNase U_2 digests must have derived from poly(A), since no products containing two or more A residues followed by a pyrimidine were found in the RNase A digests. Therefore, the amounts of the various RNase A products were calculated relative to the amount of poly(A) determined as the sum of all RNase U_2 products containing only A.

of the strong stop DNA, was not found. The sequence of this oligonucleotide is predicted from the complement of the strong stop DNA sequence plus the last two nucleotides of the $tRNA^{trp}$ primer, although it is uncertain whether the terminal A of the primer is base-paired with the genome RNA (20).

Terminally redundant sequences in RSV

If the hypothesis for elongation of strong stop DNA from the 3′ end of the genome shown in Fig. 1 is correct, the identical nucleotide sequence should be present at both ends of the RSV genome RNA. Such a sequence must include all or part of the sequence of oligonucleotide 13. To test for the presence of all of oligonucleotide 13 near the 3′ end, RSV RNA was partially digested with alkali and the 3′-end fragments (about 300 nucleotides long) were isolated as described in *Materials and Methods*, digested with RNase T_1, and separated. A number of small oligonucleotides were found (Fig. 3*D*), but, after digestion with pancreatic RNase, none of these oligonucleotides yielded products consistent with its being identical to number 13 without the capping group. Furthermore, when the same 3′-terminal fragments were annealed with strong stop DNA, which contains the complementary sequence of oligonucleotide 13, and the hybrid analyzed, no oligonucleotide, except the poly(A) (and adjoining A, C, and U residues) was found. Similarly, polyacrylamide gel electrophoresis of such protected 3′ material showed only a broad band of size consistent with the poly(A) (Fig. 2, lane 3 indicated by bracket) and no discrete smaller bands. The reason for the absence of such a poly(A) band in the hybridization experiment shown in lane 2 is not known. It is possible that the poly(A) sequence isolated from poly(U)-Sephadex columns is protected from a small amount of overdigestion by RNase by traces of poly(U) eluted from the column. The pooled fractions from the poly(A) band in slot 3 contained no oligonucleotides except the poly(A) (data not shown). These results show that, if a terminal redundancy exists, it must be shorter than 23 nucleotides (the length of oligonucleotide 13).

If a redundant sequence shorter than 23 nucleotides is present at the ends of the RSV genome, then it would contain no internal G residue and would not be separated from the poly(A) by digestion with RNase T_1. Therefore, it would not appear as a large oligonucleotide. For this reason, the 3′ end of the RSV RNA was analyzed for the presence of such a sequence.

RSV 70S [^{32}P]RNA was digested with RNase T_1, and the poly(A) sequence with its adjoining nucleotides was isolated. This material was digested with either RNase A or RNase U_2 and the digestion products were compared with those of oligonucleotide 13 isolated from total RSV RNA (Table 2). As previously reported (21), and as expected from the sequence of strong stop DNA (15), digestion of oligonucleotide 13 with these nucleases gave products consistent with the sequence:

7mGpppG$_m$CCA [(2CCA, (U_2,C)A, U_4A, CA)]UUG.

Digestion of the 3′ end with RNase A and U_2 yielded products consistent with the sequence:

G[(3CCA, (U_2,C)A, U_4A, CA)]A_{200}.

Some additional products were always observed, but digestion of these with RNase A, T_1, and T_2 yielded only A, indicating that these products are the result of underdigestion with RNase U_2 or overdigestion with RNase A. The purity of the 3′ material in this experiment is shown by the fact that no G-containing product was detected. However, in some experiments, a higher ratio of CA and two additional products [UA and (U,C)G] were observed in U_2-digested 3′ ends, and a few additional products, including Gp, were found after RNase A digestion. These additional products were in roughly equimolar yield with the products listed in Table 2. In such cases, strong stop DNA protected *only* the products shown in Table 2. Therefore, the additional products were probably contaminating sequences from another part of the genome.

To further characterize the 3′ sequence, unlabeled RSV 70S RNA was digested with RNase T_1, the digestion products were labeled at their 5′ ends with polynucleotide kinase, and the 3′ end was isolated. Digestion of this material with RNase A and

U_2 yielded pCp and pCCAp, respectively, as the predominant products [along with about one-third as much [^{32}P]poly(A) and pAp, which were probably the result of labeling of free poly(A)]. Therefore, the 3′-terminal sequence can be partially deduced as:

GCCA [(2CCA, (U_2,C)A, U_4A, CA)]A_{200}.

These results strongly indicate that there could be terminal redundancy of 21 nucleotides in the RSV genome. To confirm this supposition, the 3′ end of RSV RNA was isolated by digestion with RNase T_1 and poly(U)-Sephadex chromatography as above and annealed with strong stop DNA, and the hybrids formed were isolated after digestion with RNase A and T_1. All the products characteristic of the 3′ end were found after digestion of the RNA from isolated hybrids with RNase A or U_2 (Table 2). Using the sequence data of Haseltine *et al.* (15), we conclude that the 21 nucleotide sequence,

GCCAUUUUACCAUUCACCACA,

adjacent to the 7mGppp at the 5′ end is also present adjacent to the poly(A) at the 3′ end.

DISCUSSION

The experiments described here indicate that the genome RNA of Prague strain RSV, subgroup B, contains a sequence of 21 nucleotides adjacent to the cap at the 5′ end that is identical to a sequence at the 3′ end adjacent to the poly(A). A similar conclusion can be drawn from a comparison of the results of sequence analysis of strong stop DNA (15) and from direct sequence analysis of the products of oligo(dT)-primed DNA synthesis on Pr-RSV-C RNA templates (23). The complement of this sequence forms the 5′ end of the major *in vitro* product of the endogenous RSV RNA polymerase reaction.

It has previously been reported by Collett and Faras (24) that labeled DNA chains initiated near the 5′ end of the RSV genome could be hybridized to fragments derived from the 3′ end of the genome. However, these authors did not show that such hybridization was the result of a redundant sequence rather than of reading across the union of 5′ and 3′ ends. Furthermore, their claim that primer-containing DNA fragments shorter than 100 nucleotides could hybridize with the 3′ end is inconsistent with the results presented here.

These results are consistent with the mechanism of elongation of RSV DNA proposed in Fig. 1 but do not prove it. Although such a mechanism accounts for the elongation of newly synthesized tumor virus DNA from the 5′ to the 3′ end of the genome, a number of additional questions arise. These include details of how such a transfer actually takes place. For example, it is not known whether the 5′ end of the RNA is degraded by the viral DNA polymerase-associated RNase H (12) (which would leave the DNA still attached to the template by the hydrogen bonding of primer RNA), or whether the DNA·RNA hybrid formed by synthesis at the 5′ end is denatured and the DNA hybridized to the 3′ sequence. Furthermore, it is not known whether the 5′-to-3′ transfer of the nascent DNA occurs from one subunit RNA molecule to another or from one end to the other end of the same subunit.

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