Proc. Natl. Acad. Sci. USA
Vol. 74, No. 3, pp. 994–998, March 1977
Biochemistry

Rous sarcoma virus genome is terminally redundant: The 3′ sequence

(DNA sequence/RNA sequence/reverse transcription)

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ABSTRACT A sequence of 20 nucleotide residues immediately adjacent to the 3′-terminal poly(A) in Rous sarcoma virus (Prague strain, subgroup C) 35S RNA has been determined by extension of a riboguanylic acid-terminated oligothymidylic acid primer hybridized at the 5′ end of the 3′-terminal poly(A) with purified reverse transcriptase (RNA-directed DNA polymerase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) from avian myeloblastosis virus. The sequence is $^{5'}$GCCAUUUUACCAUUCACCACpoly(A)$^{3'}$. This same nucleotide sequence, excluding the poly(A) segment, has also been found at the 5′ terminus of Rous sarcoma virus RNA (W. A. Haseltine, A. Maxam, and W. Gilbert, this issue, pp. 989–993), and therefore the RNA genome of this virus is terminally redundant. Possible mechanisms for endogenous *in vitro* copying of the complete RNA genome by reverse transcriptase which involve terminally repeated nucleotide sequences are discussed.

Determination of the primary structure of the region immediately adjacent to the 3′-terminal poly(A) sequence of RNA tumor virus genomes is of considerable importance. Various models for synthesis of virus particles in infected cells suggest that the viral RNA genome is transcribed from proviral genes integrated in the host DNA (1). Thus, tumor virus RNA genomes and eukaryotic mRNA molecules may have common structural features adjacent to the 3′-terminal poly(A) sequence that represent signals for termination of transcription and possibly recognition sites for post-transcriptional addition of the poly(A) sequence. More significantly, however, this region of tumor virus RNA genomes may have an important function during early steps in the process of reverse transcription of the viral RNA molecule into proviral DNA. For a number of RNA tumor viruses, considerable evidence now demonstrates that a tRNA molecule, associated with the viral 35S RNA genome, functions *in vitro* as the primer for RNA-directed DNA polymerase (reverse transcriptase) (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) (2) and initiates synthesis of complementary DNA at a unique site located within 150 nucleotide residues from the 5′ terminus of the RNA template (3, 4). Synthesis of DNA proceeds from the 3′-hydroxyl end of the tRNA primer toward the 5′ end of the template 35S RNA.

However, there has been no direct evidence, to suggest a mechanism by which reverse transcriptase, having transcribed the 5′ end of the RNA template molecule, continues to copy the remaining major portion of the viral RNA genome which is located between the 3′-terminal poly(A) sequence and the tRNA primer binding site. Indirect evidence suggests that the DNA transcribed from the 5′ end of the viral 35S RNA molecule contains nucleotide sequences complementary to the 3′ end of the genome and that transcription of the complete viral RNA genome may occur by circularization of the template RNA molecule (5). In the present communication, a sequence of 20 nucleotide residues is reported for the region immediately adjacent to the 3′-terminal poly(A) of Rous sarcoma virus (RSV) 35S RNA. The same nucleotide sequence is also present at the 5′ end of the RNA (6), and thus the RSV RNA genome is terminally redundant.

MATERIALS

RSV (Prague strain, subgroup C) was obtained from Eugene Bernstein, University Laboratories, N.J., through Jack Gruber of the Virus Cancer Program. The RNA was extracted and the 70S and 35S RNAs were isolated as described by Stephenson *et al.* (7). Purified reverse transcriptase from avian myeloblastosis virus was obtained from Joseph Beard through the Virus Cancer Program. Oligothymidylic acid primer molecules were synthesized chemically by standard procedures (8) and purified according to chain-length by column chromatography on DEAE-cellulose in the presence of 7 M urea (9). The primer dT(pdT)$_8$prG was synthesized by terminal deoxynucleotidyl transferase-catalyzed addition of a rG residue onto the acceptor oligonucleotide d(pT)$_9$ by the method of Roychoudhury and Kössel (10) and was then purified by column chromatography on DEAE-cellulose. Random DNA oligonucleotides, used as carrier, were prepared by pancreatic DNase digestion of calf thymus DNA and had an average chain-length of 10 as measured by the ratio of terminal phosphate to total phosphate. [α-^{32}P]Deoxynucleoside triphosphates (specific activity, 50–150 Ci/mmol) were obtained from New England Nuclear Corp. Unlabeled deoxynucleoside triphosphates and terminal deoxynucleotidyl transferase were purchased from P-L Biochemicals, Inc.

RESULTS

Determination of the nucleotide residue immediately adjacent to the 3′-terminal poly(A) sequence

The first nucleotide residue adjacent to the poly(A) in RSV 35S RNA was tentatively identified as C by a series of preliminary experiments in which RSV RNA was copied by the reverse transcriptase with d(pT)$_{10}$ as primer. Each experiment contained only three deoxynucleoside triphosphates, one of which was labeled with ^{32}P in the α position, except the controls which included all four deoxynucleoside triphosphates. The results (Table 1) indicate that the synthesis of DNA is totally dependent on the presence of added d(pT)$_{10}$ primer which initiates synthesis while hybridized to the RSV poly(A) sequence and that there is no endogenous 4S primer associated with the 35S RNA. The data also indicate that DNA synthesis is dependent on the presence of dGTP in the reaction mixture and show that a dG residue is the first nucleotide added onto the oligo-dT primer. In addition, the data suggest that the order of appearance of nucleotides in the DNA product is dG, then dA, and finally dC. It is not possible, however, to determine the order of incorporation of the first dT residue into DNA from these experiments.

Abbreviation: RSV, Rous sarcoma virus.

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Table 1. Oligo-dT primed incorporation of labeled nucleotides into acid-insoluble products*

Reaction conditions	Labeled nucleotide		
	dGTP	dATP	dCTP
Complete system	0.59	0.93	0.45
Complete system minus pT_{10}	0.008	0.015	0.008
Complete system minus dGTP	—	0.063	0.051
Complete system minus dATP	0.136	—	0.025
Complete system minus dTTP	0.04	0.081	0.012
Complete system minus dCTP	0.361	0.572	—

* The values in the table are pmol of [^{32}P]nucleotide incorporated in 20 min/10 μl reaction containing 0.012 A_{260} unit of RSV 35S RNA, 0.005 A_{267} unit of d(pT)$_{10}$, 50 mM Tris·HCl (pH 8.3), 50 mM KCl, 7.5 mM $MgCl_2$, 1.25 mM dithiothreitol, 100 μM of each unlabeled deoxynucleoside triphosphate, 2 μM [α-^{32}P]deoxynucleoside triphosphate (about 130 Ci/mmol), and 0.2 unit of reverse transcriptase. After incubation at 18° for 20 min, an aliquot was precipitated onto a Whatman no. 1 paper disc with 5% (wt/vol) trichloroacetic acid/1% tetrasodium pyrophosphate (wt/vol), washed extensively with 5% trichloroacetic acid, and then assayed by scintillation counting.

Therefore it follows that C is the first nucleotide residue adjacent to the poly(A) in RSV 35S RNA, and this is followed at some distance by U and finally by a G residue.

The presence of a C residue adjacent to the poly(A) sequence in RSV 35S RNA was confirmed in the following experiment. A large-scale polymerization containing RSV 35S RNA, d(pT)$_{10}$ primer, reverse transcriptase, and only [α-^{32}P]dGTP was carried out. Under these conditions, DNA synthesis can be initiated only by those d(pT)$_{10}$ primer molecules that are hybridized to the 10 A residues at the 5′ end of the poly(A) sequence because dTTP was omitted from the reaction mixture. The DNA products from this reaction were extracted with phenol, precipitated with alcohol, and then analyzed by standard two-dimensional fractionation procedures (11). One major oligonucleotide (80%) and one longer minor oligonucleotide (20%) were the only products, and these were subjected to nearest-neighbor analysis (12). In each case ^{32}P label was transferred to dT. These results show that the first nucleotide added onto the d(pT)$_{10}$ primer is a dG residue, and thus the first nucleotide adjacent to the poly(A) sequence in RSV 35S RNA is a C residue. In addition, analysis of the longer oligonucleotide obtained in minor yield showed transfer of ^{32}P label to dG, suggesting the presence of two or more C residues immediately adjacent to the poly(A) in RSV RNA.

In an attempt to determine a longer nucleotide sequence adjacent to the poly(A) segment, polymerization reactions were carried out with d(pT)$_{10}$ primer and various combinations of two or more deoxynucleoside triphosphates. It was found that transcription further into the region adjacent to the poly(A) required the presence of dTTP in the reaction mixture. Under these conditions the oligo-dT primer can initiate DNA synthesis from any point on the poly(A), and sequence analysis of the DNA products becomes more difficult. Thus, an oligonucleotide primer that, in the presence of dTTP, can initiate DNA synthesis uniquely at the 5′ end of the poly(A) of RSV RNA was used for all further sequence studies.

Sequence analysis of the region adjacent to the 3′-terminal poly(A)

In order to determine the nucleotide sequence adjacent to the poly(A), synthesis of DNA transcripts with reverse transcriptase was initiated by the primer dT(pdT)$_8$prG. It was assumed that

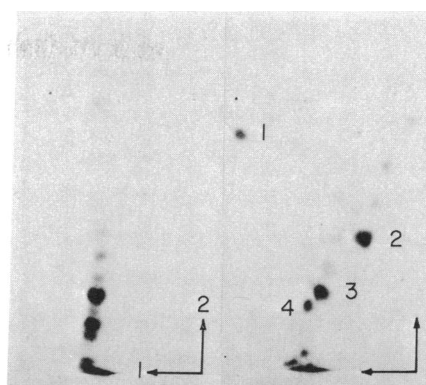

FIG. 1. Fractionation of hydrolyzed and unhydrolyzed dT(pdT)$_8$prG-primed products synthesized in the absence of dCTP. The polymerization reaction was run as described in Table 1 except that the volume of the incubation mixture was 100 μl. After 30 min at 18°, 60 μg of carrier DNA oligonucleotide was added and the mixture was extracted with phenol and precipitated with ethanol. The precipitate was further purified by a second precipitation and then divided into two samples. One aliquot was fractionated directly; the second was treated with alkali prior to fractionation. The sample was dissolved in 12 μl of 0.5 M NaOH, incubated for 18 hr at 37°, and then neutralized with 12 μl of 0.5 M acetic acid; the mixture was passed through a BioGel P2 column (0.8 × 26 cm, 100–200 mesh) and the desalted oligonucleotides were pooled and lyophilized. Fractionation of both samples in the first dimension was by electrophoresis at pH 3.5 on cellulose acetate. The second dimension was homochromatography on DEAE-cellulose (1:7.5) thin-layer plates with a 4% 30-min hydrolyzed homomixture (11). *Left.* Radioautograph of fractionation of unhydrolyzed products. *Right.* Radioautograph of fractionation of the alkali-hydrolyzed products. As a control, the experiments were repeated with actinomycin D present in the polymerization mixture to suppress synthesis of double-stranded DNA; patterns identical to those shown here were obtained.

only those dT(pdT)$_8$prG molecules hybridized to the nine A residues at the 5′ end of the poly(A) and the adjacent C residue would initiate DNA synthesis and, in addition, it was expected that sequence analysis of the DNA transcripts would be facilitated because the length of the DNA products could be decreased by removal of the ribo-terminated decanucleotide primer with alkali treatment. In this experiment, RSV 35S RNA and dT(pdT)$_8$prG primer were incubated with reverse transcriptase, [α-^{32}P]dATP, and unlabeled dGTP and dTTP (dCTP was omitted from the reaction mixture to limit synthesis of DNA to short products). The products of the reaction were divided into two portions, and one portion was hydrolyzed with alkali, neutralized, and desalted. Each sample was then analyzed by a two-dimensional fractionation procedure. Comparison of the radioautographs in Fig. 1 shows that dT(pdT)$_8$prG initiated synthesis of a small number of DNA products that could be readily separated after alkaline hydrolysis. Similar polymerization reactions containing either [α-^{32}P]dGTP, dATP, and dTTP or [α-^{32}P]dTTP, dATP, and dGTP gave patterns identical to those shown in Fig. 1 *right* with the exception of a few minor oligonucleotides present in low yields.

The sequences of the oligonucleotides corresponding to the strong spots numbered 1–4 in Fig. 1 *right* were determined by mobility shift analysis (12) of the products produced by limited digestion with exonucleases and by nearest-neighbor analysis of these products with different [^{32}P]deoxynucleoside triphosphates. Examples of limited exonuclease digestion of spots 2, 3, and 4 with both spleen and venom phosphodiesterases are shown in Figs. 2, 3, and 4, respectively; the nucleotide sequences of spots 1–4 are shown in Table 2. The minor products apparent

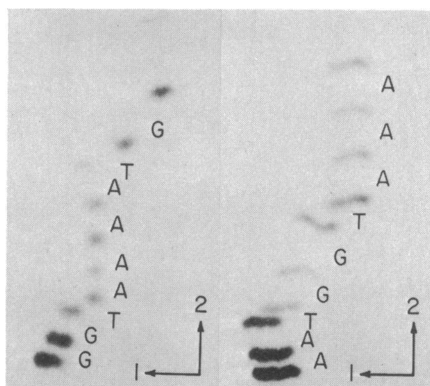

FIG. 2. Radioautographs of two-dimensional fractionations of limited exonuclease digestions of spot 2 (Fig. 1 *right*) labeled with [α-^{32}P]dGTP showing deduced nucleotides removed. *Left.* Venom exonuclease digest. *Right.* Spleen exonuclease. Conditions of digestion were as previously described (12). Fractionation was as in Fig. 1 except the second dimension was homochromatography with a combination (1:1) of 3% 30-min hydrolyzed and 4% 30-min hydrolyzed homomixtures.

in Fig. 1 *right*, obtained in variable low yields, were partially characterized by limited venom exonuclease digestion and were found to be incomplete transcripts related to the major products 2, 3, and 4. The radioactive material that remained at the second-dimension origin in Fig. 1 *right* was present in all polymerization reactions in variable yields and has not been further characterized.

Spot 1 is dT(pdT)$_8$prGp and contains label only in the 2′(3′)-phosphate of the terminal ribonucleotide. The sequence of this oligonucleotide was determined by mobility shift analysis of a limited spleen exonuclease digest and by complete digestion with the same enzyme to give radioactive Gp. Spots 2, 3, and 4 are the labeled DNA products from which the ribo-terminated primer was removed by alkaline hydrolysis. Each of these oligonucleotides has an identical 3′-terminal nucleotide sequence, which suggests that reverse transcriptase copied the template up to the first G residue in the RNA and stopped because dCTP was not present in the reaction mixture.

In contrast, the sequences at the 5′ ends of these products differ by several nucleotide residues. This unexpected 5′-terminal heterogeneity could arise by slippage of the dT(pdT)$_8$prG primer on the template, but this is considered unlikely for reasons that are discussed later. Instead, we conclude that these related transcripts are produced by selective initiation of DNA

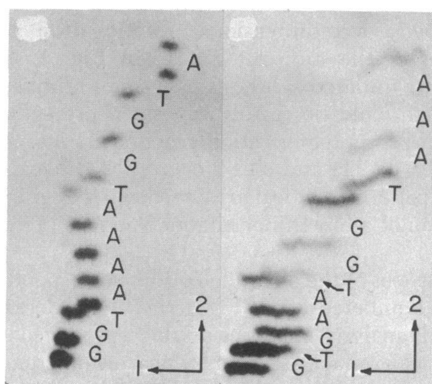

FIG. 3. Radioautographs of two-dimensional fractionations of limited exonuclease digestions of spot 3 (Fig. 1 *right*) labeled with [α-^{32}P]dATP showing deduced nucleotides removed. *Left.* Venom exonuclease. *Right.* Spleen exonuclease. Conditions of digestion and fractionation were as in Fig. 2.

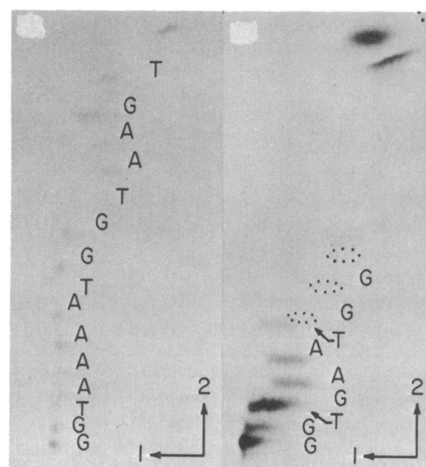

FIG. 4. Radioautograph of two-dimensional fractionation of limited exonuclease digestions of spot 4 (Fig. 1 *right*) labeled with [α-^{32}P]dGTP showing deduced nucleotides removed. *Left.* Venom exonuclease. *Right.* Spleen exonuclease. Conditions of digestion and fractionation were as in Fig. 2.

synthesis by dT(pdT)$_8$prG on three populations of RSV 35S RNA that differ slightly in their nucleotide sequences immediately adjacent to the poly(A). Therefore, assuming correct base pairing during DNA synthesis by reverse transcriptase, the three populations of RSV 35S RNA have the 3′-terminal nucleotide sequences shown in Table 3. Independent confirmation of this, however, must await sequence analysis by other techniques.

DISCUSSION

The nucleotide sequence of the region immediately adjacent to the 3′-terminal poly(A) has been determined by selective copying of RSV 35S RNA by reverse transcriptase. Three different short DNA oligonucleotides were obtained when synthesis was conducted in the absence of dCTP and when transcription was initiated with the site-specific primer, dT(pdT)$_8$prG. Sequence analysis of these DNA fragments allows the following 3′-terminal nucleotide sequences to be written for RSV 35S RNA, provided that the primer did not slip during initiation of transcription and that normal Watson-Crick base pairing prevailed during synthesis:

$^{5'}$. . . (G)CCAUUUUACCAUUCApoly(A)$^{3'}$ [1]

$^{5'}$. . . (G)CCAUUUUACCAUUCACCApoly(A)$^{3'}$ [2]

$^{5'}$. . . (G)CCAUUUUACCAUUCACCACApoly(A)$^{3'}$ [3]

Table 2. Deoxyribooligonucleotides isolated from dT(pdT)$_8$prG-primed synthesis in the absence of dCTP*

Oligo-nucleotide (Fig. 1 *right*)	Sequence
1	$^{5'}$T-T-T-T-T-T-T-T-T-rGp$^{3'}$
2	$^{5'}$A-A-T-G-G-T-A-A-A-A-T-G-G$^{3'}$
3	$^{5'}$G-T-G-A-A-T-G-G-T-A-A-A-A-T-G-G$^{3'}$
4	$^{5'}$(T)-G-G-T-G-A-A-T-G-G-T-A-A-A-A-T-G-G$^{3'}$

* The nucleotide sequence common to the 3′ ends of oligonucleotides 2, 3, and 4 is underlined. The nucleotide in parentheses is an additional T residue not seen in Fig 4 *right* (see Note Added in Proof).

Table 3. 3′-Terminal sequences of RSV 35S RNA*

(1)(G)-C-C-A-U-U-U-U-A-C-C-A-U-U-C-A-poly(A)
(2)(G)-C-C-A-U-U-U-U-A-C-C-A-U-U-C-A-C-C-A-poly(A)
(3)(G)-C-C-A-U-U-U-U-A-C-C-A-U-U-C-A-C-C-A-C-A-poly(A)

* The nucleotide sequence common to the 3′ ends of the three possible populations of RSV 35S RNA is underlined.

Concurrently, Haseltine, Maxam, and Gilbert (this issue, pp. 989–993) report the sequence of 101 nucleotides of RSV strong-stop DNA (5) and that the 5′-terminal sequence of the RSV RNA genome is $^{5'}$m^{7}G(5′)ppp(5′)G^{m}CCAUUUUAC-CAUUCACCACA. . .$^{3'}$. Therefore, the RSV genome has an identical nucleotide sequence at both the 5′ end and the 3′ end and is terminally redundant. Comparison of the 3′-terminal sequences 1, 2, and 3 with the 5′-terminal sequence indicates that the terminally redundant unit consists of 16, 19, or 21 nucleotide residues, respectively.

It can be argued that the presence of three apparent populations of RSV 35S RNA is an artifact and might result from slippage of the dT(pdT)$_8$prG primer on the template RNA; however, we think that this is not the case for the following reasons. First, the sequence heterogeneity observed next to the poly(A) in spots 2, 3, and 4 is not random because the addition of several nucleotides in going from spot 2 to spot 3 or 4 actually increases the terminal redundancy. Second, we have synthesized two oligonucleotide primers, dT(pdT)$_8$pdGprA and dT(pdT)$_8$pdGprG (Weith and Schwartz, unpublished data), that would be less likely to slip and should selectively initiate DNA synthesis on the different populations of RSV 35S RNA. For example, dT(pdT)$_8$pdGprA should prime DNA synthesis only on RSV RNA molecules having sequence 1, and dT(pdT)$_8$pdGprG should prime DNA synthesis only on RSV RNA molecules having sequence 2. Both primers have been used to initiate synthesis of short DNA transcripts as described in Fig. 1 *right,* and each primer gave a simple pattern. In the case of dT(pdT)$_8$pdGprA, several major oligonucleotide products were examined by limited digestion with spleen phosphodiesterase. Each oligonucleotide contained the 5′-terminal sequence $^{5'}$ATGGTA. . .$^{3'}$ as expected. Therefore, dT(pdT)$_8$pdGprA is initiating DNA synthesis on those RSV 35S RNA molecules having sequence 1.

If multiple populations of RSV 35S RNA are not an artifact, then the question remains as to the manner in which nucleotide sequence heterogeneity adjacent to the 3′-terminal poly(A) might occur. Such sequence heterogeneity could arise in several ways: (*i*) the provirus genome might be integrated at multiple sites in the host DNA; (*ii*) the virus used in these studies has not been recently cloned and might contain mutants; (*iii*) post-transcriptional processing of the viral RNA may not be an exact process; and (*iv*) the viral RNA may be slightly frayed at its 3′ end, either during termination of transcription from the integrated proviral genome or by an exonuclease prior to addition of the poly(A).

The presence of identical nucleotide sequences at both ends of RSV RNA suggests several possibilities concerning the initial steps in the replication of the RSV genome. It has been previously shown that DNA synthesis by reverse transcriptase is initiated at a site near the 5′ end of the RSV genome (3). In the accompanying paper, this site of initiation is shown to be 101 nucleotides from the capped 5′ end. As a consequence, after initiation of transcription, reverse transcriptase can proceed only a short distance before it reaches the 5′ end of the template, and therefore continuation of DNA synthesis presumably involves association of the initial DNA product with the terminally redundant complementary sequence at the 3′ end of the genome by one of the following mechanisms. The exonuclease activity associated with reverse transcriptase (ribonuclease H) could remove a section of the 5′ terminal RNA to generate a DNA "sticky end" which could then hybridize to its complementary sequence adjacent to the poly(A) of the same or another genome. Alternatively, the transcription complex could switch to the 3′ end of the same genome or another genome by means of dissociation of the DNA from the RNA template or by a direct strand displacement mechanism. Once transcription has switched to the 3′ end of an RSV genome by any of the above mechanisms, the synthesis of a complete DNA copy of the RNA genome could take place. It is this DNA which is then presumably converted into a covalently closed circular DNA molecule characteristic of the unintegrated provirus (13).

The methods described in this paper should be of general application to sequence analysis of nucleotide regions adjacent to the 3′-terminal poly(A) in viral RNA and mRNA. By the use of a simple oligonucleotide primer such as d(pdT)$_8$prG it is possible to phase transcription by an RNA-directed DNA polymerase to produce DNA products which, after removal of the primer by treatment with alkali, can be fractionated directly and then analyzed by conventional means. While this manuscript was in preparation, Cheng *et al.* (14) reported that (dpT)$_{10}$pdGpdC was used to initiate DNA synthesis by DNA polymerase I selectively at the junction between the heteropolymeric RNA sequence and the poly(A) in chicken ovalbumin mRNA. It should be noted, however, that this oligonucleotide primer contains only deoxyribonucleotide residues and therefore, in contrast to the ribo-terminated primer described in this paper, cannot be removed from the DNA transcripts by simple treatment with alkali.

We have compared the nucleotide sequence of the region adjacent to the poly(A) in RSV 35S RNA with the corresponding regions in rabbit and human α-globin and β-globin mRNAs, mouse light-chain immunoglobulin mRNA, and chicken ovalbumin mRNA (15). There are no apparent similarities except that a C residue is adjacent to the 3′-terminal poly(A) sequence in RSV 35S RNA and in five of the six mRNAs. It will be of interest to see if more extensive sequence analysis of the region adjacent to the 3′-terminal poly(A) in RSV 35S RNA uncovers any features in common with the eukaryotic mRNAs already studied.

Note Added in Proof. After examination of the 5′-terminal sequence of RSV 35S RNA reported by Haseltine *et al.* (6), it occurred to us that if spot 4 (Fig. 1 *right*) had a dT residue at its 5′ terminus, the length of the terminally redundant unit would be extended to 21 nucleotide residues. The presence of a 5′-terminal dT residue in spot 4 is not apparent from the mobility shift analysis shown in Fig. 4 *right*. Therefore, we have identified the 5′-terminal nucleotide in spots 2, 3, and 4 (Fig. 1 *right*) by 5′-end labeling with polynucleotide kinase. Briefly, a polymerization was conducted as described in Fig. 1 *right* except that unlabeled deoxynucleoside triphosphates were used. The products were hydrolyzed with alkali, labeled with ^{32}P at the 5′ end, and then passed through a small oligo (dA)-cellulose column to remove the large excess of labeled primer. Fractionation of the products gave a pattern similar to Fig. 1 *right* except that spot 1 corresponding to the primer was missing. The products corresponding to spots 2, 3, and 4 were digested to completion with snake venom phosphodiesterase and the resulting 5′-mononucleotides were fractionated by paper electrophoresis at pH 3.5 (11). It was found that spots 2, 3, and 4 gave labeled pdA, pdG, and pdT, respectively, and thus spots 2 and 3 have the sequences shown in Table 2 and spot 4 has the sequence: $^{5'}$TGGTGAATGGTAAAATGG$^{3'}$. Consequently some RSV 35S RNA molecules have the 3′-terminal sequence: (G)CCAUUUUACCAU-UCACCACApoly(A).

We thank Dr. M. L. Stephenson for help and advice during the preparation of the RSV 35S RNA. This is Publication no. 1513 of the Cancer Commission of Harvard University. This work was supported by Contract no. N01 CP 3366 of the Virus Cancer Program, National Cancer Institute, by Grant no. 315 from the Jane Coffin Childs Fund, by ERDA Contract no. EY-76-S-02-2403, and by the Burroughs Wellcome Foundation.

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