Transcription of DNA from the 70S RNA of Rous Sarcoma Virus

II. Structure of a 4S RNA Primer

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The 70S RNA of Rous sarcoma virus contains 4S RNAs which serve as primers for the initiation of DNA synthesis *in vitro* by the RNA-directed DNA polymerase of the virus. We purified these primers in three different ways—by isolation of the covalent complex between primer and nascent DNA, by differential melting of the 70S RNA, and by two-dimensional electrophoresis in polyacrylamide gels. The 4S RNAs purified by these procedures were homogeneous and possessed very similar if not identical nucleotide compositions and sequences. The RNAs were approximately 75 nucleotides long, had pG at the 5' terminus and CpCpA_{OH} at the 3' terminus, and contained a number of minor nucleotides characteristic of tRNA. In contrast to most tRNA's, the primer lacked rTp and contained Gp (ψ p, ψ p, Cp) Gp (possibly in place of the characteristic sequence GprTp ψ pCpGp). At least 50% of the 4S primers available on 70S RNA were utilized in a standard polymerase reaction *in vitro*.

The 70S RNA from RNA tumor viruses is a better template for the RNA-directed DNA polymerase of Rous sarcoma virus (RSV) than any other naturally occurring RNA tested to date (8, 9, 13). This fact may be due to a special structural feature of the 70S complex, viz., the presence of a low-molecular-weight RNA which acts as primer for the initiation of DNA synthesis (4, 12, 21). We have developed three different procedures for the purification of this primer (7, 12), using either of two sources—4S RNA which is hydrogen-bonded to 70S RNA (70Sa-4S RNA) (10, 11), and 4S RNA found free in purified virions (free 4S RNA) (3). The present communication describes features of the nucleotide composition and sequence of purified primer which indicate that it may be an unusual tRNA. In addition, our data validate the use of each of the several techniques for preparing primer RNA, and establish reference standards for the identification of primer RNA in other viruses and cells (R. C. Sawyer and J. E. Dahlberg, submitted for publication).

MATERIALS AND METHODS

Isolation of primer RNA. Viral RNA was uniformly labeled with ³²P₄³⁻ as described elsewhere (7,

18). The work in San Francisco (methods 1 and 2, see below) was carried out with subgroup A of the Schmidt-Ruppin strain of RSV harvested at intervals of 12 h, and the work in Madison (method 3) was carried out with subgroup D of the same virus strain harvested at intervals of 24 h. To date, we have found only minor differences between primers prepared in the two laboratories. Primer RNA was isolated with three different procedures, details of which have been described (7, 12). In each instance, the amount of primer recovered corresponds to approximately 0.3% of the mass of 70S RNA used in the procedure. Consequently, it is necessary to use more than 10 million counts/min of ³²P-labeled 70S RNA in order to obtain useful amounts of radioactivity in purified primer.

Method 1. The product of RNA-directed DNA synthesis with RSV 70S RNA as template includes a covalent complex between 4S primer and nascent DNA (12, 21). Primer released from this complex by hydrolysis with DNase can be purified by electrophoresis in polyacrylamide gels (2, 12).

Method 2. Primer can be separated from the bulk of 70Sa-4S RNA by sequentially heating 70S RNA at 63 and 80 C and isolating the 4S RNAs released at each temperature (2, 7). The RNA obtained at 80 C consists entirely of primer.

Method 3. Two-dimensional electrophoresis in polyacrylamide gels separates 70Sa-4S RNA of RSV into approximately eight fractions, each of which may represent a single species of tRNA (18). One of these fractions ("spot 1 RNA"; see ref. 7 and 18) has been

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tentatively identified as primer by virtue of its covalent attachment to nascent DNA (7). Since spot 1 RNA is the most abundant species of both free 4S RNA and 70Sa-4S RNA in RSV (18), it can be conveniently purified in relatively large amounts by two-dimensional electrophoresis (7).

Analysis of nucleotide composition and sequence. The purified ^{32}P -labeled RNAs (a minimum of 10,000 counts/min) were digested with T1 RNase (Sankyo) or pancreatic RNase A (Worthington Biochemical Corp.), and the resulting oligonucleotides were fractionated by electrophoresis as described by Sanger et al. (17). Cellulose acetate was purchased from Wilson Diagnostics, Glenwood, Ill., or Millipore Corp., Bedford, Mass., and DE81 paper was purchased from Reeve Angel, Clifton, N.J.

Oligonucleotides were located on the electropherograms by autoradiography (Kodak NS54-T X-ray film), and the radioactivity in individual oligonucleotides was measured before elution for further analysis. Individual T1 and pancreatic oligonucleotides were eluted from the paper and further digested with pancreatic RNase or T1 RNase, respectively, and the resulting products were separated by electrophoresis on DE81 paper at pH 3.5 (1).

Two-dimensional chromatography of 3' mononucleotides obtained by hydrolysis with either T2 RNase or alkali was performed as described by Saneyoshi et al. (16). RNase T2 was purchased from Calbiochem as RNase CB or RNase T2 and used at either 500 U/ml with 20 μg of carrier RNA in a total volume of 4 μl iters (18 h, 37 C) or 0.2 U per sample in the absence of carrier RNA (2 h, 37 C). Hydrolysis was carried out in 0.1 M sodium acetate (pH 4.5) containing 2 mM EDTA. Celplate-22 thin-layer chromatography plates were purchased from Brinkman Instruments, Inc., Westbury, N.Y.

Alkaline digestion of oligonucleotides was for 18 h at 37 C in either 0.2 or 0.3 N NaOH. The resulting products were separated by electrophoresis at pH 3.5 on Whatman paper (3MM, 540 or DE81, as indicated).

Other materials and procedures. The preceding manuscript (7) describes the source of most of our reagents, the propagation and purification of virus, the preparation of viral nucleic acids and RNA-directed DNA polymerase, and the standard conditions for enzymatic reactions.

Nomenclature. The notations for known and unknown sequences of nucleotides follow standard conventions wherever possible (14). Modified nucleotides in which the nature of the modification is unknown are represented by a symbol (eg., Cp*, Cp**, etc.), the letter identifying the base which is modified, and different modifications being distinguished by the number of asterisks. When the base is unknown, the modified nucleotides (which in some cases may be dinucleotides) are represented by the symbols Xp*, Xp**, etc. The symbols Z*, Z**, etc., are used to denote oligonucleotides with specific electrophoretic mobilities known to contain modified nucleotides, but of unknown nucleotide composition. Numerical superscripts are used throughout to indicate which T1 RNase oligonucleotide contains the unknown modified nucleotide or oligonucleotide in ques-

tion. Thus, Z^{10a}, Z^{10b}, Z^{10c}, Xp^{*10}, and Xp^{**10} are all contained in the T1 RNase oligonucleotide numbered 10 among the 17 products obtained when primer is hydrolyzed with T1 RNase (see Results, Fig. 1f, and Tables 1 and 2).

RESULTS

Oligonucleotides produced by hydrolysis of primer with T1 RNase. Primer isolated from 70Sa-4S RNA by each of three methods was hydrolyzed with T1 RNase, and the products were separated by two-dimensional electrophoresis on paper (17). The patterns of oligonucleotides obtained from the different preparations of primer are almost identical (Fig. 1a, b, and d), as outlined schematically in Fig. 1f. Similar material can also be prepared from free 4S RNA by method 3 (Fig. 1c). This observation is valuable from a logistical standpoint because of the relative abundance of free 4S RNA (7).

The small number of oligonucleotides (about 17) obtained from each preparation indicates that primer is probably a single species of 4S RNA. This conclusion conforms to the apparent homogeneity of the RNA when analyzed by two-dimensional electrophoresis in polyacrylamide gels (7). By contrast, the bulk of 70Sa-4S RNA (prepared virtually free of primer by heating 70S RNA at 63 C; see ref. 7) contains multiple species of RNA (7), and the pattern of oligonucleotides obtained from this RNA by hydrolysis with T1 RNase is correspondingly complex (Fig. 1e).

There are several detectable variations among the preparations of primer obtained with the three methods. (i) Oligonucleotide number 2 (see Fig. 1f) was absent from primer isolated by method 1 (Fig. 1a). This oligonucleotide was located at the 3' terminus of the primer (see below, Tables 1 and 2), and in primer isolated by method 1 it was attached to oligodeoxynucleotides of various lengths (13). Consequently, the radioactivity of the oligoribonucleotide was distributed among multiple products of the hydrolysis by T1 RNase, none of which was detectable in the electropherogram. The terminal addition of oligodeoxynucleotides to primer gave an analogous result (see below, Table 5). (ii) The oligonucleotides labeled A, B, and C occurred in variable and usually small amounts. A is the dinucleotide ApGp, a prominent product when most RNAs are hydrolyzed with T1 RNase. The large variation in its recovery from preparations of primer suggests that it is derived from contaminating RNAs rather than from primer. Oligonucleotide B occurred most frequently in primer prepared by method 3. It resulted from incomplete hydrolysis by T1 RNase and contained both oligonucleotide 16

FIG. 1. Oligonucleotides produced by hydrolysis of 4S RNAs with T1 RNase. (a) Primer purified with method 1; (b) primer purified with method 2; (c) primer purified with method 3 from free 4S RNA; (d) primer purified with method 3 from total 70Sa-4S RNA; (e) 4S RNA released from 70S RNA at 63 C; (f) schematic presentation of the electropherograms and the numbering system. The isolation of ^{32}P -labeled RNAs, hydrolysis with T1 RNase, and separation of the products by two-dimensional electrophoresis were carried out as in Materials and Methods. Panels (a) through (e) are radioautographs of the electropherograms.

and a modified Gp (unpublished data). Oligonucleotide C also contained internal Gp and probably resulted from incomplete hydrolysis of a phosphodiester bond between oligonucleotides 10 and 11 (unpublished data).

The relative amount of radioactive phosphorous in each oligonucleotide was determined by cutting out the appropriate portion of the electropherogram and counting it in a low-background gas-flow counter. These data were normalized for the number of phosphates in each oligonucleotide as described in Table 1 and are summarized in Tables 1 and 2. Approximately the same molar ratios of individual oligonucleotides were obtained for RNA isolated by either method 2 or 3. We have no quantitative data for primer isolated by method 1 because the amounts available were inadequate for proper analysis.

Identification of modified nucleotides in 4S RNAs. Modified nucleotides were identified by two-dimensional thin-layer chromatography

after complete hydrolysis of RNA with either alkali or T2 RNase (16). Typical results are illustrated in Fig. 2, using as prototypes the 70Sa-4S RNAs released by sequential heating at 63 (Fig. 2a) and 80 C (Fig. 2b). A schematic diagram of the chromatograms is given in Fig. 2c. The identification of modified nucleotides is tentative because it is based solely on their positions in the chromatograms relative to positions of known nucleotides (16).

Table 3 summarizes the modified nucleotides contained in the 70Sa-4S RNAs of RSV. The primer lacked two of the nucleotides (rTp and Cp**) which were present in the remaining 70Sa-4S RNA. Since rTp occurs in the nucleotide sequence of virtually all known tRNA's (the exceptions are discussed below), its absence from primer provides a useful indication of purity.

Structure of oligonucleotides produced by hydrolysis of primer with T1 RNase. Tables 1 and 2 summarize our current data regarding the

TABLE 1. Oligonucleotides obtained by T1 RNase digestion of primer prepared by method 2^a

Oligo-nucleotide no.	Molar ratio ($\pm$ SD)	Pancreatic RNase products ^b	RNase T2 or alkaline digestion products ^c	Structure deduced ^d
1	5.2 $\pm$ 0.7	Gp	Gp	Gp
2	1.1 $\pm$ 0.3	ApCp, Cp ₁₋₂ , Up	Ap, Cp ₂₋₃ , Up	Up (ApCp, Cp ₁₋₂) A ^o OH
3	3.5 $\pm$ 0.7	Cp, Gp	Cp, Gp	CpGp
4	1.0 $\pm$ 0.2	ApApGp	Ap ₂ , Gp	ApApGp
5	1.1 $\pm$ 0.07	ND	Up, Gp	UpGp
6	1.0 $\pm$ 0.1	Up, Cp, Gp	Up, Cp, Gp	(Up, Cp) Gp
7	1.0 $\pm$ 0.1	Dp, ApGp	Dp, Ap, Gp	DpApGp
8	1.0 $\pm$ 0.1	Up, Z ⁹ , Gp	Cp, Up, Gp, m ⁷ Gp	(m ⁷ GpCp, Up) Gp
9	1.0 $\pm$ 0.1	Cp, Z ⁹ , ApApCp	Cp ₂ , Ap ₂ , GmpGp	(Cp, ApApCp) GmpGp
10	0.7 $\pm$ 0.2	Z ^{10a} , Z ^{10b} , Z ^{10c}	Ap ₂ , Xp ^{*10} , Xp ^{**10} , Gp [*]	(Ap ₂ , Xp ^{*10} , Xp ^{**10}) Gp [*]
11	1.3 $\pm$ 0.1	Z ¹¹	Ap ₂ , Gp, Xp ^{***11}	(Ap ₂ , Xp ^{***11}) Gp
12	0.8 $\pm$ 0.1	ApCp, Cp ₂ , Up, Gp	Ap, Up, Cp ₂ , Cp ^{*12} , Gp	(ApCp, Cp, Cp ^{*12} , Up) Gp
13	0.7 $\pm$ 0.04	ApCp, Z ¹³ , Cp, Gp	m ¹ Ap, Ap ₁₋₂ , Cp ₂ , Up, Gp	(ApCp, Cp, Ap, m ¹ Ap, Up) Gp
14	1.8 $\pm$ 0.2	Up, ψ p, Cp, Gp	Up, ψ p, Cp, Gp	(Up, ψ p, Cp) Gp
15	0.6 $\pm$ 0.2	Z ¹⁵	pGp	pGp
16	ND	ND	ND	ND
17	0.5 $\pm$ 0.1	ND	ND	ND
A	ND	ApGp	Ap, Gp	ApGp

^aHydrolysis with T1 RNase and electrophoresis in two dimensions were performed as described in Materials and Methods. Molar ratios are relative to an average of 1.0 copy each of spots 4, 6, and 7, all of which are trinucleotides. The total radioactivity in these three oligonucleotides was summed and divided by 9 to give the average counts per minute per phosphate. Radioactivity in each oligonucleotide was normalized for the number of phosphates contained in it, as deduced from its composition. The numbers are the mean $\pm$ the standard deviation of the mean of four separate determinations.

^bIndividual T1 RNase oligonucleotides were hydrolyzed with pancreatic RNase, and the products were separated by electrophoresis on DE-81 paper at pH 3.5. The products were identified by their mobility relative to a standard mixture of unmodified oligonucleotides and by their alkaline digestion products.

^cEvery T1 RNase oligonucleotide was digested with alkali and/or T2 RNase, and the resulting mononucleotides were separated by electrophoresis on Whatman 540 or 3MM paper at pH 3.5. The products of hydrolysis with T2 RNase were also separated by two-dimensional thin-layer chromatography. In cases where the identity of modified nucleotides is uncertain, a symbol is given to the T2 RNase product as explained in the text and summarized in Table 3. The positions of these unidentified compounds on the chromatography plate are shown schematically in Fig. 2c.

^dSystem of notation is described under Materials and Methods. The symbols Cp₁₋₂, Ap₂, etc., indicate 1 to 2 mol of Cp, 2 mol of Ap, etc.; ND indicates "not done".

^eSnake venom phosphodiesterase digestion of oligonucleotide 2 produced 2 to 3 mol of pC and 2 mol of pA. Thus, the 5' and 3' ends of this oligonucleotide are Up and A^oOH, respectively.

nucleotide composition and sequence of the individual oligonucleotides illustrated in Fig. 1b and c. The results for primer purified by either method 2 or 3 were identical except for the following. (i) The recovery of oligonucleotides 16, 17, B, and C from method 2 was inadequate for either quantitative analysis or determination of structure. (ii) The Up in oligonucleotide 14 from method 2 was replaced by ψ p in the case of method 3. As discussed below, this discrepancy could be attributed to a difference in the extent of modification of a single Up in oligonucleotide 17.

Oligonucleotides released from primer by hydrolysis with pancreatic RNase. Primer purified by methods 2 and 3 was hydrolyzed with pancreatic RNase, and the products were

fractionated by two-dimensional electrophoresis. The results were similar in the two cases (Fig. 3a and b), although the preparation from method 2 contained a number of oligonucleotides not seen with method 3. The amounts of these additional oligonucleotides varies from one preparation to another and were usually not found in primer prepared with method 3. Fig. 3c is a schematic summary of the oligonucleotides which occur in reproducible amounts in primer prepared by either technique. We assume that the additional oligonucleotides found with method 2 are derived from contaminating RNA. The relative yields and nucleotide compositions of the oligonucleotides are given in Table 4. We have not identified all of the modified nucleotides in individual oligonucleotides.

TABLE 2. Oligonucleotides obtained by T1 RNase digestion of primer prepared by method 3^a

Oligo-nucleotide no.	Molar ratio	Pancreatic RNase products	RNase T2 products	Structure deduced
1	4.7	Gp	Gp	Gp
2	0.9	ApCp, Cp ₂ , Up	Ap, Cp, Up	UpCpApCpCpA _{OH} ^b
3	2.7	Cp, Gp	Cp, Gp	CpGp
4	1.0	ApApGp	Ap ₂ , Gp	ApApGp
5	1.3	Up, Gp	Up, Gp	UpGp
6	1.0	Up, Cp, Gp	Up, Cp, Gp	(Up, Cp) Gp
7	1.0	Dp, ApGp	Dp, Ap, Gp	DpApGp
8	1.0	Up, Gp, Z ^a	m ⁷ Gp, Cp, Up, Gp	(m ⁷ GpCp, Up) Gp
9	0.6	Cp, ApApCp, Z ^a	Cp ₂ , GmpGp, Ap ₂	(Cp, ApApCp) GmpGp
10	1.1	Z ^{10a} , Z ^{10b} , Z ^{10c}	Ap ₂ , Xp ^{**10} , Xp ^{***10} , m ¹ Gp	(Ap ₂ , Xp ^{**10} , Xp ^{***10}) m ¹ Gp
11	1.1	ApGp, Z ¹¹	Ap ₂ , Xp ^{***11} , Gp	(Ap ₂ , Xp ^{***11}) Gp
12	0.9	ApCp, Cp ₂ , Up, Gp	Ap, Up, Cp ₂ , Gp	(ApCp, Cp ₂ , Up) Gp
13	0.6	ApCp, Cp, Gp, Z ¹³	m ¹ Ap, Ap, Cp ₂ , Up, Gp	(ApCp, Cp, Ap, m ¹ Ap, Up) Gp
14	1.5	ψp ₂ , Cp, Gp	ψp ₂ , Cp, Gp	(ψp ₂ , Cp) Gp
15	1.0	Z ¹⁵	pGp	pGp
16	0.3	Up, Gp ^{***16}	Up, Gp ^{***16}	UpGp ^{***16}
17	0.8	Up, Cp ₂ , Gp, Z ¹⁷	Up ₂ , Cp ₂ , X ^{****17} , Gp	(Cpm ₂ ² GpUp, Up, Cp) Gp
A	ND	ApGp	ND	ApGp
B	ND	Up, Z ^B	Up, Gp ^{***16} , Gp	(UpGp ^{***16} , Gp)
C	variable ^c	ApGp, Z ^{10a} , Z ^{10b} , Z ^{10c}	Ap ₂ , Xp ^{**10} , Xp ^{***10} , Xp ^{***11} , m ¹ Gp, Gp	10 + 11

^a RNA was prepared from free 4S RNA of RSV and was then analyzed as described in the legend to Table 1. The use of free 4S RNA (rather than 70Sa-4S RNA) was a convenience justified by the apparent identity of primer prepared from different sources with method 3. The molar ratios are the average of determinations made on RNA isolated from five different preparations of virus.

^b Sequence of oligonucleotide 2 was deduced from venom phosphodiesterase digestion which yielded pA and pC in the ratio 2:3 and RNase U₂ digestion which produced (Up, Cp) Ap and CpCpA_{OH}.

^c Oligonucleotide C is a partial digestion product, containing oligonucleotides 10 and 11, as discussed in the text. The yield of this product varied between 0 and 0.9 mol, depending on the particular conditions of T1 RNase digestion. Likewise, the yields of oligonucleotides 10 and 11 varied in inverse proportions to the yield of this product.

TABLE 3. Modified nucleotides in 70Sa-4S RNA and primer^a

Oligonucleotide source ^b	Symbol ^c	Probable identification ^d
7	Dp	5,6-Dihydrouridine-3'-monophosphate
8	m ⁷ Gp	7-Methylguanosine-3'-monophosphate
9	Gmp	2'-O-methylguanosine-3'-monophosphate
10	Xp*	Possible dinucleotide (AmpCp)
10	Xp**	Possible dinucleotide (CmpUp)
10	Gp*	Possible (m ¹ Gp): 1-methylguanosine-3'-monophosphate
11	Xp***	Possible dinucleotide
12	Cp*	Unknown
13	m ¹ Ap	1-Methyladenosine-3'-monophosphate
14	ψp	Pseudouridine-3'-monophosphate
15	pGp	3',5'-Guanosine diphosphate
16	Gp**	Unknown
17	Xp****	Possible (m ₂ ² Gp): N ² ,N ² -dimethylguanosine-3'-monophosphate
70Sa-4S (63 C) ^e	rTp	5-Methyluridine-3'-monophosphate
70Sa-4S (63 C) ^e	Cp**	Unknown

^a Table includes all of the modified nucleotides shared by both total 70Sa-4S RNA and primer purified by either method 2 or 3. The modified nucleotides are those indicated in Fig. 2c.

^b These numbers correspond to those in Tables 1 and 2 and denote the T1 oligonucleotides from which each modified nucleotide was obtained.

^c System of notation is described under Materials and Methods.

^d Determined by comparison with the positions of known modified nucleotides in two-dimensional chromatography (16).

^e Modified nucleotides rTp and Cp** were detected only in 4S RNA released from 70S RNA at 63 C, not in primer isolated by either method 2 or 3.

FIG. 2. Analysis of T2 RNase hydrolysates by two-dimensional thin-layer chromatography. Purification of the ^{32}P -labeled RNAs, hydrolysis with T2 RNase, and separation of the products by two-dimensional thin-layer chromatography were carried out as in Materials and Methods. Panels (a) and (b) illustrate radioautographs of the chromatograms. (a) 4S RNA from RSV 70S RNA heated to 63 C; (b) primer isolated from RSV 70S RNA with method 2; (c) schematic diagram of the chromatograms showing the

Extent of utilization of 4S primer. For each mole of 70S RNA there are 2 to 4 moles of 70Sa-4S RNA with the structural features of primer (7). To determine what proportion of these 4S RNAs actually serves for the initiation of DNA synthesis *in vitro*, we carried out the following experiment. RSV 70S [^{32}P]RNA was incubated with purified RSV polymerase under reaction conditions which attach ^3H -labeled pdA and pdApdA to the 3' termini of primer but permit no further DNA synthesis (12). Primer was then isolated by method 2 and hydrolyzed with T1 RNase, and the products were separated by two-dimensional electrophoresis (Fig. 4). The resulting pattern of oligonucleotides is identical to that observed previously (Fig. 1b) except for the appearance of two additional oligonucleotides (X and Y), both of which contain [^3H]dA as well as [^{32}P]ribonucleotides (Table 5). From the position of the oligonucleotides X and Y in the electropherogram (Fig. 4), we conclude that they represent the 3' terminal oligonucleotide (here labeled number 2, as in Fig. 1f and Table 1), the electrophoretic mobility of which has been retarded by the addition of deoxynucleotides. The distribution of ^{32}P among the three oligonucleotides (2, X, and Y) indicates that DNA synthesis *in vitro* initiated on at least one-half of the primer isolated by method 2.

DISCUSSION

We have isolated an RNA molecule which serves as primer for the transcription of DNA from the 70S RNA of RSV *in vitro*. Three distinct preparative techniques based upon different properties of the primer all yield very similar if not identical molecules, as judged from the pattern of oligonucleotides produced by hydrolysis with T1 and pancreatic RNase and from analyses of nucleotide composition and sequence. Only method 1 provides RNA which is entirely linked to nascent DNA prior to isolation (12) and which therefore can be rigorously identified as primer. Consequently, proof of sequence identity will be essential to further studies because the logistical limitations of method 1 make it desirable to use method 2 or 3, or both, for isolation of primer.

The primer isolated by either method 2 or 3 appears to be homogeneous. The number of

positions of modified nucleotides. The symbols used are defined in Materials and Methods and described in Table 3. All of the symbols (except X*, X**, X****, X****, and pGp) represent nucleoside-3'-monophosphates, i.e., ψ is ψp , D is Dp, etc.

FIG. 3. Oligonucleotides produced by hydrolysis of primer with pancreatic RNase. Purification of the ^{32}P -labeled RNAs, hydrolysis with pancreatic RNase A, and separation of the products by two-dimensional electrophoresis were carried out as in Materials and Methods. (a) RNA isolated by method 2; (b) RNA isolated by method 3; (c) schematic representation of the electropherograms and the numbering system used for the oligonucleotides.

TABLE 4. Oligonucleotides obtained by pancreatic RNase digestion of primer prepared by method 3^a

Oligonucleo- no.	Molar ratio	RNase T1 products	RNase T2 products	Structure deduced
100	ND	Up	Up, ψ p	Up, ψ p
101	8.7	Cp	Cp	Cp
102	1.2	Z ^{10c}	Xp ^{*10}	Xp ^{*10}
103	1.6	ApCp	Ap, Cp	ApCp
104	0.7	ApApCp	Ap ₂ , Cp	ApApCp
105	2.0	Gp, Cp	Gp, Cp	GpCp
106	1.0	ApGp, Cp	Ap, Gp, Cp	ApGpCp
107	1.6	Z ^B	Gp ^{**16} , Gp, Cp	(Gp ^{**16} , Gp) Cp
108	1.0	Z ¹⁷	Xp ^{****17}	Xp ^{****17}
109	4.2	Gp, Up, ψ p	Gp, Up, ψ p	GpUp and Gp ψ p
110	0.7	Z ¹⁵ , ApCp	pGp, Ap, Cp	pGpApCp
111	1.3	Gp, Z ^{10a}	Gp, Ap, Xp ^{*10}	GpApXp ^{*10}
112	1.2	Gp, Z ¹³	Gp, Ap, m ¹ Ap, Up	Gpm ¹ ApApUp
113	0.7	ApGp, ApApGp, Z ^a	Ap ₃ , Gp ₂ , m ⁷ Gp, Cp	(ApGp, ApApGp) m ⁷ GpCp
114	0.9	Z ^{10b} , Z ¹¹	Ap ₂ , Xp ^{****11} , Xp ^{**10}	Ap ₂ , Xp ^{****11} , Xp ^{**10}
115	ND	ND	ND	ND
116	1.0	Z ⁹ , Dp	GmpGp, Dp	GmpGpDp
117	ND	Gp ₂ , Dp	Gp ₂ , Dp	GpGpDp
118	0.9	Gp ₄ , Up	Gp ₃₋₄ , Up	GpGpGpGpUp
119	ND	ND	ND	ND

^aRNA was prepared from free 4S RNA of RSV and digested with pancreatic RNase. The resulting oligonucleotides were purified as shown in Fig. 3b. The numbering system is that shown in Fig. 3c. Molar ratios were determined relative to an assumed 1.0 mol (3 phosphates) of oligonucleotide 116 per mol of RNA. The system of notation is described under Materials and Methods.

oligonucleotides obtained after T1 RNase digestion (about 17) and pancreatic RNase digestion (about 17), together with their compositions, indicates that the molecule is approximately 75 nucleotides long. This is compatible with a single 4S RNA of well-defined structure.

The composition of primer RNA indicates that it is probably a tRNA. It contains pG at its 5' terminus and GpUp (ApCp, Cp, Cp) A_{OH} at its 3' terminus. It also contains modified nucleotides, including Dp, ψ p, Gmp, and m⁷Gp, and several as yet unidentified nucleotides. There is

FIG. 4. Oligonucleotides produced by hydrolysis of primer with T1 RNase after initiation of DNA synthesis. Purified DNA polymerase of RSV and ^{32}P -labeled RSV 70S RNA were incubated for 1 h in a standard polymerase reaction mixture, except that dTTP was omitted and replaced by uracil arabinoside triphosphate (10^{-5} M). The other precursors were dCTP and dGTP (both 10^{-5} M) and ^3H dATP (10^{-5} M). Under these conditions, the principal enzymatic products are pA and pApdA covalently linked to the 3' terminus of primer (J. M. Taylor et al., submitted for publication). Uracil arabinoside monophosphate is also incorporated into the product, but at levels too low to affect the present procedures. Method 2 was used to isolate primer, which was then hydrolyzed with T1 RNase, and the resulting oligonucleotides were separated as in Fig. 1.

no detectable rTp in the primer RNA even though rTp occurs in most tRNA's (15, 19, 20). Recent reports indicate that initiator tRNA's from several eukaryotes lack both rTp and ψp (15, 19, 20), and that the sequence GpTp ψp CpGp characteristic of tRNA's has been replaced by GpApUpCpGp (15, 19, 20). By

TABLE 5. Initiation of DNA synthesis on primer RNA^a

Oligo-nucleotide	Tentative composition	^{32}P (counts/ 10 min)	^3H -dA (counts/ 10 min)
2	Up (ApCp, Cp ₂) A _{OH}	210	0
X	Up (ApCp, Cp ₂) ApdA _{OH}	140	520
Y	Up (ApCp, Cp ₂) ApdApdA _{OH}	120	670
	Paper blank	20	0

^a Oligonucleotides 2, X, and Y from the electropherogram illustrated in Fig. 4 were cut out and counted separately in a scintillation spectrometer. Machine backgrounds have been subtracted from all data. The paper blank was cut from a portion of the electropherogram which contained no nucleotides detectable by autoradiography. The tentative composition is deduced from the known composition of oligonucleotide 2 (Table 2) and from the positions of X and Y in the electropherogram.

contrast, the 4S primer which we have isolated from RSV contains Gp (ψp_2 , Cp) Gp. Therefore, we consider it unlikely that the primer RNA is identical to any presently identified initiator tRNA of eukaryotes.

The small differences in nucleotide composition between the primer of method 2 and that of method 3 (Table 1 and 2) are of uncertain significance, because the RNAs used in these two instances were prepared and analyzed in different laboratories (method 2, San Francisco; method 3, Madison). The only significant difference between the compositions of these RNAs involves oligonucleotide 14, which is (Up, ψp , Cp) Gp for method 2 and (ψp_2 , Cp) Gp for method 3. This difference could be attributed to incomplete modification of oligonucleotide 17—(Cpm₂²GpUp, Up, Cp) Gp in the RNA of method 2 (San Francisco). The unmodified form of 17, (CpGpUp, Up, Cp) Gp, is further split by RNase T1 digestion into two oligonucleotides, CpGp and (Up, Up, Cp) Gp; the latter would migrate with oligonucleotide 14 of method 3, (ψp_2 , Cp) Gp. If these were present in equal amounts, the result would be the apparent composition (Up, ψp , Cp) Gp, i.e., oligonucleotide 14 of method 2. The other possible difference between the RNAs of methods 2 and 3 also involve modification (oligonucleotide 12). Consequently, we suggest that RSV RNA prepared and analyzed in different laboratories may display variations in the extent of modification of at least certain nucleotides. Experiments to test this possibility are in progress.

The primer prepared with method 3 for compositional analysis (Table 2) was obtained from the free 4S RNA of RSV rather than from 70Sa-4S RNA. Although our results are presently incomplete, all available data indicate

that the 4S RNAs prepared from these two sources with method 3 are identical. This is consistent with the view that both primer per se and 70Sa-4S RNA in general are acquired from the population of free 4S RNA during maturation of genome subunits into their final 70S form (5, 6).

We have also used method 2 to prepare primer from the 70S RNA of B77 RSV (subgroup C) propagated in either chicken or duck cells. In both instances, the pattern of oligonucleotides released by hydrolysis with T1 RNase and the content of modified nucleotides were identical to those of Schmidt-Ruppin RSV as illustrated in Fig. 1b and 2b. It seems likely that all avian leukosis viruses have the same 4S RNA as primer and that this RNA is derived from the host cell (R. C. Sawyer and J. E. Dahlberg, submitted for publication).

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