JOURNAL OF VIROLOGY, Jan. 1977, p. 386-395

Vol. 21, No. 1
Printed in U.S.A.

Complexity and Polyadenylic Acid Content of Visna Virus 60-70S RNA

ROBERT VIGNE, MICHEL BRAHIC,[1]* PIERRE FILIPPI, AND JACQUES TAMALET
Laboratoire de Virologie, Faculté de Médecine, Secteur Nord, 13326 Marseille, Cedex 3, France

Received for publication 25 June 1976

The genomic complexity of visna virus was measured by quantitative analysis of 18 RNase T_1-resistant oligonucleotides from 60-70S RNA. T_1-resistant oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis. Visna virus had a genomic complexity of 3.6×10^6 daltons, very close to the size of a single 30-40S RNA subunit. It was therefore concluded that the visna virus genome is largely polyploid. Visna virus 60-70S RNA polyadenylic acid segment was purified by T_1 RNase digestion followed by oligodeoxythymidylic acid-cellulose column chromatography. It contained over 99% AMP and had a size of about 200 nucleotides. The binding capacities on oligodeoxythymidylic acid-cellulose of native 60-70S RNA and purified 30-40S RNA subunits were examined. It was concluded that two out of three intact subunits contain a polyadenylic acid segment.

Visna virus, the agent of a slow neurological disease of sheep, is a ribodeoxyvirus closely related to the RNA tumor viruses in biochemical and morphological properties (19). However, this virus does not transform cells in vitro and undergoes a typical lytic cycle in sheep fibroblasts.

The visna virus genome, like that of RNA tumor viruses, consists of a 60-70S RNA (approximate molecular weight, 10^7), which can be dissociated into subunits of molecular weight 3×10^6. Recently, the genomic complexity of avian RNA tumor viruses has been examined by several authors (2, 4, 24). Based on chemical analysis, they found complexities ranging from 2.7×10^6 to 3.5×10^6 daltons, which is very close to the size of a single subunit. Moreover, the analysis of 3'-polyadenylic acid [poly(A)]-tagged subunit fragments indicates that the nucleotide sequences of all the subunits are identical (7, 28). Therefore, it is now accepted that these viruses are largely polyploid.

We decided to investigate the genomic complexity of visna virus because previous results indicated that the lytic ribodeoxyviruses had a more complex genome than their transforming counterparts. First, Cooper and Temin (8) had determined the minimal size of infectious double-stranded proviral DNA for both Rous sarcoma virus (RSV) and members of the reticuloendotheliosis viruses (REV). Although they found a minimal size of 6×10^6 daltons in the case of RSV, which is in agreement with a genomic complexity of 3×10^6 daltons, they also observed that the minimal size of infectious proviral DNA was 10×10^6 to 20×10^6 daltons in the case of REV, indicating a higher genomic complexity. Second, the analysis of visna virus proteins (17) revealed 15 different polypeptides with a combined molecular weight of 8.8×10^5. Contamination by cellular proteins was excluded by reconstruction experiments (17). This amount of protein requires a genomic complexity of about 8.8×10^6 daltons, unless extensive overlap exists between the different polypeptides.

In the present work, we have determined the genomic complexity of the K 796 strain of visna virus by analysis of the unique RNase T_1-resistant oligonucleotides according to De Wachter and Fiers (10). As a control, we also analyzed the complexity of the Prague B strain of RSV (PR-B RSV). We found that both viruses have essentially the same genomic complexity (3.6×10^6 daltons and 3.3×10^6 daltons for visna virus and PR-B RSV, respectively). We also present in this article a detailed study of the poly(A) segment present in visna virus 60-70S RNA (15).

While this manuscript was in preparation, Beemon et al. (3) reported results similar to ours. They found a genomic complexity for visna RNA of 3.5×10^6 daltons.

MATERIALS AND METHODS

Virus. The K 796 strain of visna virus was grown on sheep choroid plexus cells as previously described (6).

[1] Present address: University of California Service, Veterans Administration Hospital, San Francisco, CA 94121.

Reagents. Eagle minimal essential medium, L 15 medium, and calf and lamb sera were from Grand Island Biological Co.

[^{3}H]uridine (20 Ci/mM) and carrier-free ^{32}P were from CEA, France. [^{3}H]poly(A) (30 μCi/μmol of phosphorus) was purchased from Miles Laboratories, and oligodeoxythymidylic acid [oligo(dT)]-cellulose was from P-L Biochemicals Inc. Acrylamide and bisacrylamide were from Bio-Rad. Ethylene diacrylate was from Pfaltz and Bauer, Inc. Diethylpyrocarbonate and *N,N,N',N'*-tetramethylethylenediamine were from Eastman Organic Chemicals.

Pancreatic RNase A was purchased from Worthington. T_1 and T_2 RNases were from Calbiochem. Pronase was from Sigma Chemical Co.

Viral RNA labeling and extraction. Visna virus was labeled with [^{3}H]uridine as described (6).

For ^{32}P labeling, the cells were grown in L 15 medium supplemented with 15% of a mixture (4:1) of lamb serum and fetal calf serum. When confluent, the cells were infected at a multiplicity of infection of 3 PFU/cell and incubated in maintenance medium (L 15 supplemented with 2% lamb serum) for 48 h. The medium was then replaced by phosphate-free Eagle MEM containing 2% dialyzed lamb serum. After 6 h of incubation in phosphate-free medium, ^{32}P was added at a final concentration of 2 mCi/ml. The medium was harvested and replaced every 12 h for 3 days. ^{32}P was present throughout the harvesting period. For RNA size analysis, the harvests were repeated every 3 h.

Visna virus was purified according to published procedures (16), except for the linear density gradient centrifugation, which was omitted. RNA was extracted from purified virus by treatment with 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg of preincubated Pronase per ml for 45 min at 37°C. The incubation mixture was then layered on top of a 5 to 20% linear sucrose gradient in 0.01 M Tris-hydrochloride (pH 7.5)-0.1 M NaCl-0.001 M EDTA-0.1% SDS and centrifuged for 50 min at 50,000 rpm in an SW50 rotor. The 60-70*S* RNA was recovered from the gradient by ethanol precipitation after the addition of yeast carrier RNA.

Cellular RNAs. Sheep choroid plexus cells were labeled with [^{3}H]uridine (0.03 mCi/ml) or carrier-free ^{32}P (1 mCi/ml) for 24 h. For ^{32}P labeling, the cells were grown in L 15 medium and shifted to phosphate-free Eagle minimal essential medium as described above for viral RNA labeling. Cytoplasmic RNAs were extracted as described (6).

Polyacrylamide gel electrophoresis of 30-40*S* viral RNAs. Electrophoresis in 3.4 or 5% polyacrylamide gels in the presence of formamide was as described (13). Analysis of viral RNA subunits in diacrylate cross-linked, 1.8% polyacrylamide gels was performed according to the procedure of Duesberg (11).

RNA fingerprinting and oligonucleotide analysis. [^{32}P]RNA samples, containing 100 μg of yeast carrier RNA, were hydrolyzed by 50 U of T_1 RNase for 30 min at 37°C in a final volume of 8 μl. The incubation mixture contained 0.02 M Tris-hydrochloride (pH 7.5)-0.002 M EDTA. T_1 oligonucleotides were analyzed by two-dimensional polyacrylamide gel electrophoresis as described (10), with the following modifications. (i) The first-dimension gel (17 by 36 by 0.2 cm) was run at 800 V (15 mA) for 14 h at 4°C until the bromophenol blue marker had migrated 21 cm. (ii) For the second dimension, the gel (21 cm wide, 22 cm from the first-dimension strip to the top) contained 21.8% acrylamide and was run at 500 V (25 mA) for 36 h at 4°C until the bromophenol blue marker had migrated 17 cm.

After autoradiography, areas of the gel slab containing the large oligonucleotides were cut out. The oligonucleotides were eluted from the gel discs according to Billeter et al. (4). RNase T_1-resistant fragments were then digested by 2.5 μg of RNase A for 30 min at 37°C in a final volume of 10 μl. The incubation mixture contained 50 μg of carrier yeast RNA, 0.02 M Tris-hydrochloride (pH 7.5), and 0.002 M EDTA. Resistant fragments were analyzed by electrophoresis on DEAE-cellulose paper as described (1). The composition of most fragments was determined from their position on the pherogram. Some large fragments were eluted from the paper, and their base composition was determined as described (1), with one modification: T_2 RNase (50 U/ml) was used alone in a buffer containing 0.05 M ammonium acetate (pH 4.5)-0.001 M EDTA.

The ^{32}P radioactivity of undigested RNAs and of T_1 oligonucleotides recovered by cutting the gel slab was measured by Cerenkov counting. The RNA samples were transferred to conical plastic tubes which fitted vertically in conventional scintillation vials. Five milliliters of water was poured into the vials before inserting the plastic tube. In these conditions, counting efficiency was 40% when compared with the results obtained with toluene-based scintillation fluid containing 10% NCS (Amersham-Searle) and 1% water.

Poly(A) analysis. Undigested RNAs were analyzed on oligo(dT)-cellulose columns as described (27), except for the elution with a buffer of intermediate ionic strength, which was omitted.

Poly(A) tracts were prepared from ^{32}P-labeled 60-70*S* RNA by two different methods. (i) ^{32}P-labeled 60-70*S* visna RNA (0.05 ml containing 100 μg of yeast carrier RNA) was heated to 85°C for 50 s in 0.15 M NaCl-0.01 M Tris-hydrochloride (pH 7.5)-0.001 M EDTA and then quick cooled. Twenty units of T_1 RNase was added, and the mixture was incubated for 1 h at 37 C. (ii) This method was as above, except that 2.5 μg of RNase A was used instead of RNase T_1 and the buffer contained 0.3 M NaCl instead of 0.15 M.

In both cases, predigested Pronase was added to a final concentration of 0.5 mg/ml, and SDS was added to 0.5%. The mixture was further incubated at 37°C for 45 min. LiCl was added to a final concentration of 0.5 M and the solution, diluted to 0.15 ml with fixation buffer (0.01 M Tris-hydrochloride [pH 7.5]-0.5 M LiCl-0.05% SDS) was applied to an oligo(dT)-cellulose column. Nonspecifically bound materials were eluted by lowering the LiCl concentration to 0.1 M as described (27). [^{32}P]poly(A) tracts were eluted with 0.01 M Tris-hydrochloride (pH 7.5)-0.05% SDS. [^{32}P]poly(A) was recycled a second time on oligo(dT)-cellulose before being used for base composition determination.

Rous sarcoma virus RNAs. ^{3}H-labeled 60-70*S*

RNA, purified from the Schmidt-Ruppin strain of RSV subgroup A (SR-A RSV) was a gift of G. S. Martin, Imperial Cancer Research Fund, London, England. The virus had been propagated at a high multiplicity of infection and contained an almost equal amount of type a and b 30-40S RNA subunits. ^{32}P-labeled 60-70S RNA extracted from the Prague strain of RSV subgroup B (PR-B RSV) was a gift of M. A. Billeter, University of Zurich, Zurich, Switzerland.

RESULTS

Electrophoretic analysis of the subunits of visna virus 60-70S RNA. Visna virus 30-40S RNA subunits have a molecular weight of approximately 3.0×10^6, as determined by sedimentation analysis (5), gel electrophoresis (18), and contour length measurement (14). Since the size of RSV RNA subunits is well documented (9, 12, 13), we decided to compare both molecules in aqueous 1.8% polyacrylamide gels, a technique that has a high resolving power. The RSV RNA was obtained from the Schmidt-Ruppin strain, subgroup A. It was composed of almost equal quantities of type a and b subunits. Figure 1 shows the result of the experiment. The a and b subunits of RSV RNA were indeed resolved. Visna RNA subunits had a slightly lower mobility than the a subunit of SR-A RSV RNA. This was a constant finding obtained with several different RNA preparations. No evidence of the existence of several subunit classes in visna virus RNA was obtained. The difference in electrophoretic mobility between visna virus subunits and the a subunit of RSV RNA probably indicates a small difference in size between these two molecules; however, it could also reflect a difference in secondary structure.

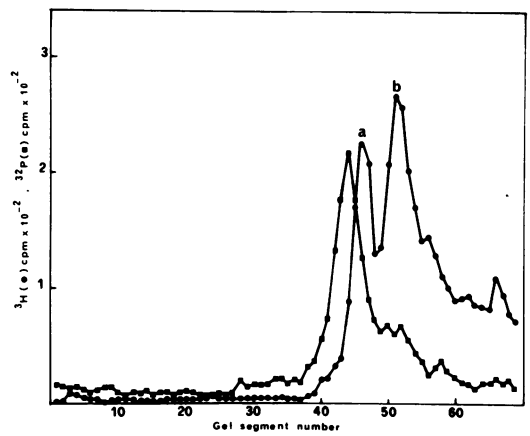

FIG. 1. *Electrophoresis in 1.8% diacrylate-cross-linked polyacrylamide gel of heat-denatured 60-70S ^{32}P RNA from visna virus (■) and 60-70S [^{3}H]RNA from SR-A RSV (●). ^{32}P-labeled visna virus 60-70S RNA was purified from virus harvested every 2 h. ^{3}H-labeled SR-A RSV 60-70S RNA was a gift of G. S. Martin. The RNAs (10,000 ^{3}H cpm, 4,000 ^{32}P cpm) were heat denatured in sample electrophoresis buffer for 50 s at 85°C. Migration (left to right) was at 40 V, 5 mA/gel, for 5.5 h with recirculation of the buffer.*

Nucleotide sequence complexities of visna virus and Pr-B RSV genomes. ^{32}P-labeled 60-70S RNAs from PR-B RSV and visna virus were digested with T_1 RNase, and the resulting oligonucleotides were separated by bidimensional gel electrophoresis as described in Materials and Methods. In this system, oligonucleotides are separated mainly according to their base composition in the first dimension and according to their length in the second dimension. Figures 3 and 4 show the fingerprints obtained form visna RNA and PR-B RSV RNA respectively. Approximately the same number of large T_1-resistant oligonucleotides was resolved for both RNAs, which already indicates that their complexities are of the same order. The visna RNA fingerprint appears characteristically different from that of PR-B RSV, most oligonucleotides being clustered in the center of the gel slab. This disposition probably reflects the peculiar base composition of visna virus 60-70S RNA (37% AMP) (Table 1).

The complexity of the viral genome can be calculated by measuring the size of a specific oligonucleotide and comparing the radioactivity present in that oligonucleotide to the radioactivity of the entire genome. This reasoning assumes that the oligonucleotide studied and the entire genome have the same specific activity, a condition that should be fulfilled by using ^{32}P as a label. The radioactivity present in a T_1-resistant oligonucleotide has to be corrected for losses occurring during the analytical procedures. The complexity, in number of nucleotides, of the entire genome will then be equal to: (counts per minute in genome × length of oligonucleotide × recovery of oligonucleotide)/(counts per minute in oligonucleotide).

The lengths of large T_1-resistant oligonucleotides were measured by partial sequencing, after RNase A digestion. This analysis also allowed a control of the purity of the oligonucleotides. The recovery of the oligonucleotides was measured by using sheep 28S rRNA as an external standard. A complete analysis of ^{32}P 28S RNA extracted from sheep cells was performed in parallel with the analysis of viral RNAs (Fig. 2, Table 2). Since the complexity of mammalian 28S RNA is known, (1.7×10^6 daltons/324 daltons = 5,250 nucleotides) (26, 25), the formula presented above could be used to measure recoveries of the T_1-resistant oligonucleo-

TABLE 1. *Base composition of RNAs*[a]

RNA	% ± standard deviation				Avg molecular weight for a bound nucleotide[b]
	CMP	AMP	GMP	UMP	
28S sheep rRNA[c]	30.6 ± 0.1	18.4 ± 0.1	34.8 ± 0.1	16.2 ± 0.1	324
60-70S visna virus RNA[d]	15.6 ± 0.7	36.6 ± 1.1	26.1 ± 0.2	21.7 ± 0.1	324
60-70S PR-B RSV RNA[e]	26.2 ± 0.7	23.8 ± 0.5	28.3 ± 0.6	21.8 ± 0.5	323

[a] RNAs were totally digested by T_2 RNase (50 U/ml, 3 h, 37°C). The resulting nucleotides were separated by high-voltage electrophoresis at pH 3.5 on Whatman no. 1 paper.
[b] Molecular weights of the four bound nucleotides are: CMP, 305; AMP, 329; GMP, 345; UMP, 306.
[c] Average of three determinations performed on the same RNA preparation.
[d] Average of four determinations performed on two different RNA preparations.
[e] According to published values (7).

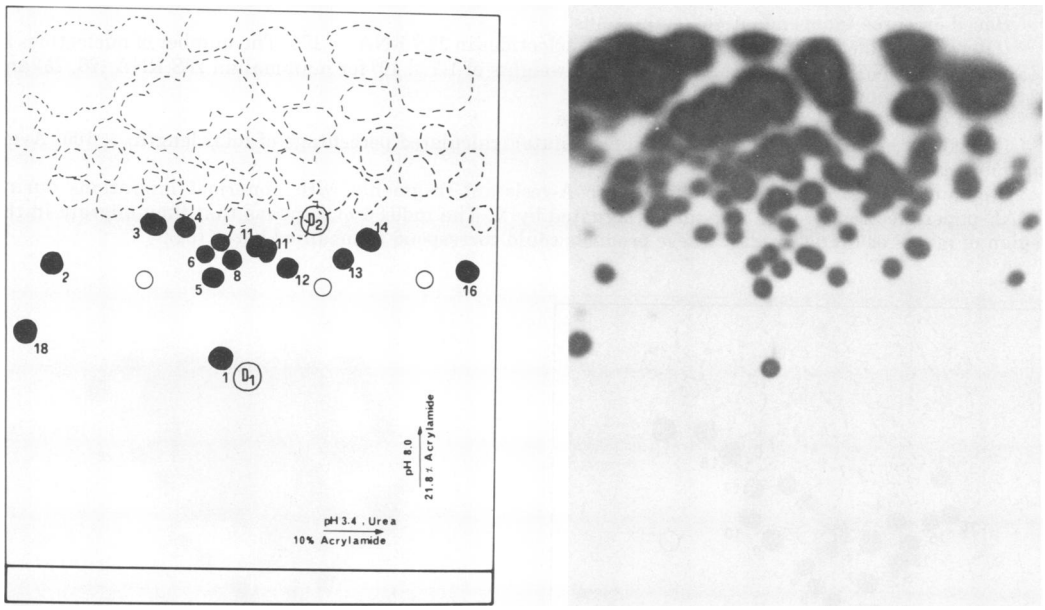

FIG. 2. *Fingerprint of RNase T_1-digested 28S sheep rRNA obtained by two-dimensional gel electrophoresis. ^{32}P-labeled 28S rRNA, 2.29 × 10^6 cpm, was digested with T_1 RNase and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. Symbol: (○) Gel areas that were cut out for background determination. D_1 and D_2 give the position of xylene cyanol and bromophenol blue markers, respectively. The horizontal bar at the bottom shows the position of the first-dimension gel strip.*

tides. For the experiments presented in this paper (Fig. 2–4, Tables 2–4) the average recovery calculated using 11 T_1-resistant 28S RNA oligonucleotides was 59.4 ± 6.5%. Table 3 presents the results of the complexity analysis of visna virus 60–70S RNA. Eighteen T_1-resistant oligonucleotides, which were considered pure, were analyzed. The average complexity of this RNA was 11,200 ± 1,600 nucleotides. Assuming an average molecular weight of 324 for a bound nucleotide (Table 1), this corresponds to a molecular weight of 3.63 × 10^6 ± 5.18 × 10^5. Another independent determination performed on a different visna RNA preparation gave a complexity of 11,000 ± 1,300 nucleotides or 3.56 × 10^6 ± 4.21 × 10^5 daltons.

In the same experiment, PR-B RSV 60–70S RNA was also examined as a control, since the genetic complexities of several strains of RSV have been determined by different authors (2, 4, 7, 24, 28). Figure 4 and Table 4 give the results of the experiment. The partial sequences of the T_1 oligonucleotides were not determined in this experiment, since they were known from the work of Coffin and Billeter (7). The nomenclature adopted for the T_1 oligonucleotides in Fig. 4 and Table 4 is the same as the one used by these authors. The average com-

TABLE 2. *Sheep ribosomal 28S RNA: recoveries of T_1 RNase-specific oligonucleotides*

Oligonucleotide spot no.[a]	Composition (RNase A digestion products)[b]	No. of nucleotides	Calculated % of total length[c]	Measured % of total cpm[d]	Recovery[e]
1	$U_6C_7G(AU)_2(A_2C)(A_3C)(A_3U)$	29	0.552	0.333	60
2	$UC_{14}G(AC)_2$	20	0.381	0.255	67
3	$U_2C_{12}G$	15	0.286	0.170	59
4	$U_3C_3G(AC)(A_2C)_2$	15	0.286	0.160	56
5[f]	$U_4C_4(X_1)_2(AC)_2(AU)(AG)$	18	0.343	0.230	67
6	$U_2C_3G(AC)_2(A_2C)(A_3C)$	16	0.305	0.166	54
7	$U_4C_4(AU)(A_2G)(A_3C)$	17	0.324	0.165	51
12	$U_3C_5(AU)_2(AG)(A_2U)$	17	0.324	0.195	60
13[f]	$U_4C_5G(X_2)(AU)_2$	16	0.305	0.181	59
16	U_8C_5G	14	0.267	0.134	50
18	$U_2C_{23}G(AC)_3$	32	0.610	0.425	70

[a] As indicated in Fig. 2.

[b] Based on three independent determinations.

[c] (Oligonucleotide chain length)/(number of nucleotides in 28*S* RNA) × 100. The number of nucleotides in 28*S* ribosomal RNA is 5,250, based on molecular weights of 1.7×10^6 for mammalian 28*S* RNA (26, 25) and 324 for a bound nucleotide (Table 1).

[d] Total of 2.29×10^6 cpm of RNA was analyzed.

[e] (Measured percentage of total counts per minute)/(calculated percentage of total length) × 100. Average, 59.4 ± 6.5.

[f] Oligonucleotides 5 and 13 contain RNase A-resistant fragments with abnormal migrations during DEAE-paper electrophoresis. They are designated by X. The index 1 or 2 means that they migrate in the region of mono- or dinucleotides. These products could correspond to modified bases (22).

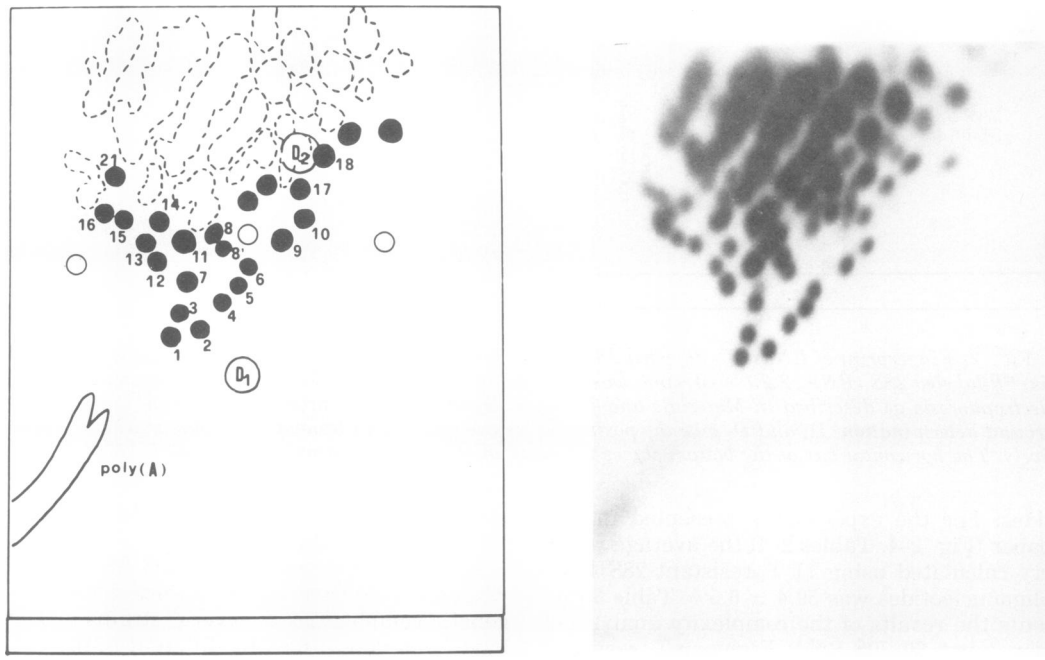

FIG. 3. *Fingerprint of RNase T_1-digested 60-70S RNA from visna virus. A total of 2.32×10^6 cpm of [^{32}P]RNA was analyzed.*

plexity calculated for the PR-B RSV genome was 10,200 ± 1,300 nucleotides or $3.29 \times 10^6 \pm 4.19 \times 10^5$ daltons.

Size of the poly(A) tract present in the visna virus genome. The technique that we used for two-dimensional analysis of T_1 RNase-resistant oligonucleotides indicates the presence of poly(A) tracts in the visna virus genome (Fig.

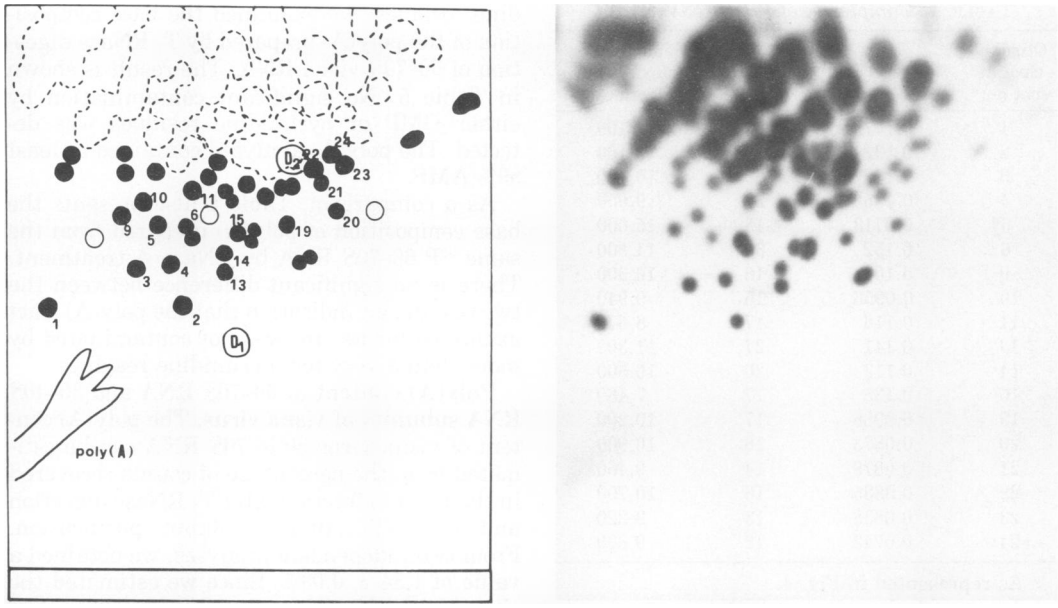

FIG. 4. *Fingerprint of RNase T_1-digested 60-70S RNA from PR-B RSV. A total of 1.85 × 10^6 cpm of [^{32}P]RNA was analyzed.*

TABLE 3. *Complexity of visna virus RNA*

Oligonucleotide spot no.[a]	% of total cpm[b]	Composition (RNase A digestion products)	No. of nucleotides	Genomic complexity (nucleotides)[c]
1	0.130	$U_4C_4(AC)_2(AU)_2(A_3U)(A_6G)$	27	12,300
2	0.118	$U_4C_5G(AU)_2(A_2U)(A_3U)(A_4U)$	26	13,100
3	0.135	$U_3C_2(AC)(AU)_3(A_5C)(A_6G)$ or $U_3C_2(AC)(AU)_3(A_6C)(A_5G)$	26	11,400
4	0.117	$U_3C_3(AC)(AU)_4(A_2G)(A_3U)$	23	11,700
5	0.135	$U_7C_5G(AC)(AU)(A_2U)(A_4U)$	25	11,000
6	0.105	$U_4C_3G(AC)_2(AU)_5(A_2U)$	25	14,200
7	0.130	$U_3C_2G(AC)_2(A_2U)(A_7U)$	21	9,590
8	0.226	$U_7C_7G_2(AC)_3(AU)_4(A_2C)(A_3U)$(mixture)	37	9,740
8	0.0944	$U_3C_2G(AC)(AU)_2(A_5C)$	18	11,300
9	0.0944	$U_5C_3G(AC)(AU)_2(A_2U)$	18	11,300
10	0.0796	$U_4(AU)_5(A_2U)(A_2G)$	20	14,900
13	0.0927	$UC_4(AU)(A_2C)(A_3U)(A_3G)$	18	11,500
14	0.106	$UC_6(AC)(AU)_2(A_3G)$	17	9,550
15	0.0921	$UC_4(AC)(A_2C)(A_2U)(A_2G)$	16	10,300
16	0.0968	$C(AC)(A_6U)(A_5G)$ or $C(AC)(A_5U)(A_6G)$	16	9,820
17	0.0933	$U_3C_2G(AC)(AU)_2(A_2U)$	15	9,550
18	0.0873	$U_2C_2G(AU)_3(A_2U)$	14	9,520
21	0.0843	$C_3(AC)_2(AG)(A_2U)(A_3C)$	16	11,300

[a] As shown in Fig. 3.

[b] Total of 2.32 × 10^6 cpm of RNA was analyzed.

[c] For each nucleotide, the total genomic complexity is given by: 59.4/(percentage of total counts per minute) × (length of oligonucleotide), where 59.4 is the percentage of recovery measured for 11 28S RNA oligonucleotides (Table 2). Average genetic complexity was 11,200 ± 1,600.

3), but does not allow a precise analysis of this sequence.

Therefore, we decided to examine the length of the poly(A) tract on polyacrylamide-formamide gels. Poly(A) was isolated by T_1 RNase digestion of ^{32}P 60–70S visna virus RNA as described in Materials and Methods. Briefly, heat-denatured ^{32}P 60–70S RNA was digested

TABLE 4. *Complexity of PR-B RSV RNA*

Oligonucleotide spot no.[a]	% of total cpm[b]	No. of nucleotides	Genomic complexity (nucleotides)[c]
1	0.147	30	12,100
2	0.132	29	13,100
3	0.148	26	10,400
4	0.135	22	9,650
5[d]	0.0713	18	15,000
6[d]	0.152	38	14,800
9	0.104	18	10,300
10	0.0956	16	9,940
11	0.116	17	8,670
13	0.141	27	11,300
14	0.112	20	10,600
15	0.135	17	7,460
19	0.0986	17	10,200
20	0.0873	16	10,900
21	0.0879	14	9,460
22	0.0885	16	10,700
23	0.0838	13	9,220
24	0.0743	12	9,600

[a] As represented in Fig. 4.

[b] A total of 1.85×10^6 cpm of RNA was analyzed.

[c] For each nucleotide, the total genomic complexity is given by: 59.4 (percentage of total counts per minute) × (length of oligonucleotide), where 59.4 is the percentage of recovery measured for 11 28S RNA oligonucleotides (Table 2). Average genetic complexity was 10,200 ± 1,300.

[d] Oligonucleotides 5 and 6 are derived from sequences present only in class a RNA of PR-B RSV (7). The virus population used for this experiment presumably contained some class b RNA due to the spontaneous segregation of transformation-defective PR-B RSV. Therefore, these oligonucleotides are not expected to be present at equimolar concentration with the others, and they were not used in the calculation of the average complexity.

with T_1 RNase, followed by treatment with SDS-Pronase and purification of the poly(A) tracts by chromatography on oligo(dT)-cellulose. The use of T_1 RNase alone for the preparation of poly(A) has been advocated by Wang and Duesberg (27) to minimize nonspecific cleavages. Figure 5 shows the result of the analysis of the visna virus poly(A) segment on polyacrylamide-formamide gels. A single, but rather heterogeneous, peak was obtained. Using 18S ribosomal RNA and 4S RNA as molecular-weight markers (0.7×10^6 and 2.5×10^4, respectively) (26), the average size of the visna 60–70S RNA poly(A) tract was estimated as 7×10^4, which corresponds to approximately 200 AMP residues (Fig. 6).

To eliminate the possibility that T_1 RNase digestion was not complete and to ensure that the absence of RNase A treatment did not significantly contaminate the poly(A) by pyrimidine residues, we examined the base composition of the poly(A) prepared by T_1 RNase digestion of 60–70S visna RNA. The result is shown in Table 5. No significant contamination by either GMP or pyrimidine residues was detected. The poly(A) analyzed contained at least 99% AMP.

As a comparison, Table 5 also presents the base composition of poly(A) prepared from the same ^{32}P 60–70S RNA by RNase A treatment. There is no significant difference between the two results, an indication that the poly(A) tract examined for its size was not contaminated by more than a very few pyrimidine residues.

Poly(A) content of 60–70S RNA and 30–40S RNA subunits of visna virus. The poly(A) content of visna virus 60 to 70S RNA can be estimated from the percentage of counts recovered in the poly(A) fraction after T_1 RNase digestion and oligo(dT)-cellulose column purification. From two independent analyses, we obtained a value of 1.84 ± 0.03%. Since we estimated the size of a 30–40S RNA subunit as 10,000 nucleotides and that of the poly(A) tract as 200 nucleotides, the measured percentage of poly(A) (1.8%) is compatible with the presence of one poly(A) tract in nearly all 30–40S RNA subunits. This was further investigated by measuring the oligo(dT)-binding capacity of 60–70S

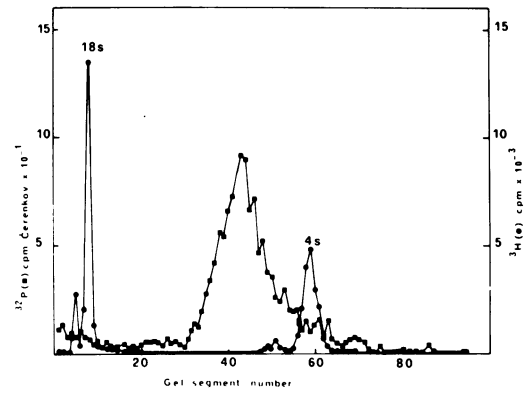

FIG. 5. *Electrophoresis in a 5% polyacrylamide-98% formamide gel of the poly(A) segment present in visna virus 60–70S RNA. Approximately 3 × 10^5 cpm of [^{32}P]60–70S RNA was used for preparing poly(A) by T_1 RNase digestion (see Materials and Methods). A total of 6 × 10^3 cpm was recovered in the poly(A) fraction after oligo(dT)-cellulose chromatography. The poly(A) was ethanol precipitated in the presence of 100 μg of yeast carrier RNA, dissolved in phosphate-buffered formamide, and applied to the gel. Electrophoresis was at 120 V for 3.5 h. ^3H-labeled 18S rRNA and 4S RNA were subjected to electrophoresis in parallel in another gel. Sections, 1 mm, of the gels were counted for radioactivity. Migration was from left to right.*

TABLE 5. *Base composition of visna virus 60-70S RNA poly(A)*

Poly(A)	CMP		AMP		GMP		UMP	
	cpm[a]	%	cpm[a]	%	cpm[a]	%	cpm[a]	%
T_1[b]	10	0.27	3,607	99.04	0	0	25	0.69
A[c]	4	0.12	3,441	99.53	11	0.32	1	0.03

[a] After subtraction of blank and machine backgrounds.

[b] Poly(A) prepared by T_1 RNase A digestion as described in Materials and Methods. A total of 4.5×10^5 cpm of 60-70S RNA was used.

[c] Poly(A) prepared by RNase A digestion as described in Materials and Methods. A total of 4.0×10^5 cpm of 60-70S RNA was used.

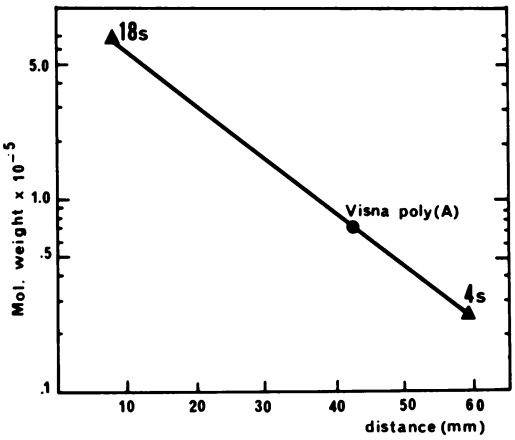

FIG. 6. *Plot of molecular weight versus electrophoretic mobility. The data are taken from Fig. 5.*

and 30-40S RNAs. Table 6 summarizes the results obtained with native 60-70S RNA, heat-denatured 60-70S RNA, and 30-40S RNA subunits purified on a preparative sucrose gradient. ^{32}P 28S RNA and commercial [^{3}H]poly(A) were also used as standards for this experiment. Their respective binding capacities were 0.7 and 97.3%. In experiments not presented in the table, the extent of rebinding of the viral RNA fractions was examined. Nearly 100% of the RNA that previously bound to oligo(dT) was retained again by the column; conversely, none of the RNA that failed to bind in the first cycle was retained during a second cycle of chromatography. Table 6 shows that 80% of native and 50% of heat-denatured 60-70S RNA bound to oligo(dT). Since denaturation of 60-70S RNA yields subunits contaminated by degradation products, the lower binding capacity obtained with heat-denatured 60-70S RNA does not necessarily mean that some subunits do not contain poly(A). Therefore, we examined the binding capacity of subunits that were purified on a sucrose gradient (not shown). The contamination of the purified subunits by degradation products was examined on polyacrylamide-formamide gels. Figure 7 shows that a single peak was obtained with no evidence of contamination. As shown by Table 6, 62% of these purified subunits could bind to an oligo(dT)-cellulose column, which indicates that approximately two out of three subunits contain a poly(A) tract.

TABLE 6. *Binding capacity of visna virus RNA to oligo(dT)-cellulose*

RNA	% cpm not bound to the column[a]	% cpm bound to the column
[^{3}H]poly(A)[b]	2.7	97.3
28S rRNA[b]	99.3	0.7
Native 60-70S RNA[c]	19.2	80.8
Heat-denatured 60-70S RNA[c]	52.7	47.3
Purified 30-40S RNA subunits[c]	37.9	62.1

[a] ^{3}H radioactivity eluted from the column was measured by precipitation with 5% trichloroacetic acid, filtration, and counting in toluene-based scintillation mixture.

[b] [^{3}H]poly(A) was purchased from Miles Laboratories. ^{3}H-labeled 28S rRNA was prepared from cultured sheep choroid plexus cells and purified by sucrose gradient centrifugation.

[c] Visna RNA was prepared, as described in Materials and Methods, from [^{3}H]uridine-labeled virus harvested every 3 h. Approximately 2,000 cpm was used for each binding assay.

DISCUSSION

The molecular weights of visna virus (5, 18, 14) and RSV (12, 13, 9) RNA subunits have been estimated by sedimentation analysis, polyacrylamide gel electrophoresis, and contour length measurement. For both viruses, a value of approximately 3×10^6 daltons has been obtained. This is in agreement with the result shown in Fig. 1, which indicates that visna virus RNA subunits could be slightly heavier than the SR-A RSV a subunit.

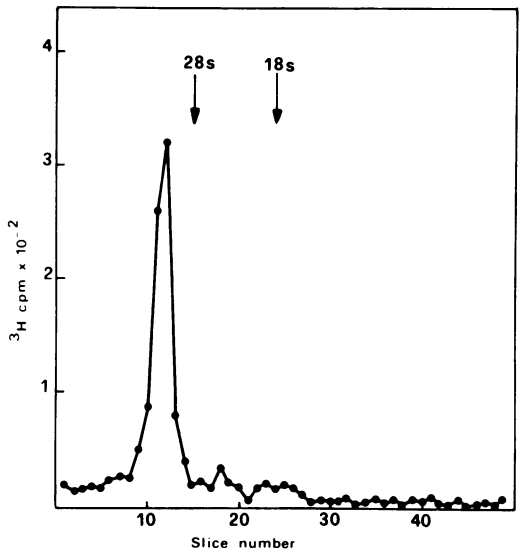

FIG. 7. *Electrophoresis in 3.4% polyacrylamide-98% formamide gel of purified ^{3}H-labeled 30–40S RNA subunits. ^{3}H-labeled 60–70S RNA was purified from visna virus harvested every 3 h, heat denatured, and sedimented on a 5 to 20% sucrose gradient. Fractions corresponding to the leading half of the 30–40S peak were pooled, and the RNA was precipitated with ethanol. Approximately 1,000 cpm of this RNA was dissolved in buffered formamide, together with markers ^{32}P-labeled 28S and 18S rRNA's, and applied to the top of the gel. Electrophoresis was for 8 h at 120 V. Sections, 1 mm, of the gel were counted for radioactivity. Migration was from left to right.*

Our estimation of visna virus genome complexity is 3.6×10^6 daltons. Errors on this value could have arisen from the following sources: (i) contamination of 60–70*S* RNA by 28*S* rRNA (the most likely contaminant). This would increase the measured complexity by raising the total radioactivity analyzed without changing that recovered from the large T_1 oligonucleotides. In our measurement this error was probably very small, since we did not observe, on the 60–70*S* RNA fingerprints, any minor spots corresponding to 28*S* T_1 RNase oligonucleotides (compare Fig. 2 and 3). (ii) Incomplete T_1 RNase digestion or degradation of the oligonucleotide would lower the recoveries of T_1 oligonucleotides, again resulting in abnormally high complexities. (iii) Contamination, incomplete digestion, or degradation of the oligonucleotides of the 28*S* RNA used as external standard would lower the measured complexities by lowering the recovery factor used in the calculation of complexities. We think that these errors were small, since the fingerprints did not give evidence of important contaminations or incomplete digestion. In addition, it should be noted that our parallel measurement of PR-B RSV RNA complexity gave a value of 3.3×10^6 daltons, in very good agreement with the published value obtained by a different technique (2). Therefore, we think that our measurement of visna virus RNA complexity (3.6×10^6 daltons) is reasonably accurate. Since the size of one single 30–40*S* subunit is close to 3×10^6 daltons, we conclude that the visna virus genome is largely polyploid.

As already discussed by different authors (2, 4), this result does not necessarily mean that all subunits are made of identical nucleotide sequences. It is also compatible with sequence permutations between the different subunits or with sequence redundancies within subunits. These two possibilities can be represented by (a b c d) (b c d a) and (a b a b) (c d c d), in which parentheses stand for subunits. Recently, results were published on transfection by visna proviral DNA (20), which demonstrated that the kinetics of transfection is two hit, and the minimal size of infectious DNA is 6×10^6 daltons of double-stranded DNA. These results are consistent with the sequences being distributed between the subunits according to the second model presented above and symbolized by (a b a b) (c d c d). The distribution of the large T_1 oligonucleotides among poly(A)-tagged fragments of 30–40*S* RNA subunits is presently being investigated to clarify this point.

As mentioned in the introduction of this article, it was reported that the combined molecular weight of visna virus proteins is 8.8×10^5, which requires a genomic complexity of 8.8×10^6 (17). This is considerably higher than the value obtained by chemical analysis (3.6×10^6 daltons). These results can be reconciled either by the presence of overlaps in the sequences of several viral proteins or by the presence in the virion of proteins that are not virus coded.

Our results show that visna virus and RSV genomic complexities are of the same order. Therefore, there is no indication that a lytic ribodeoxyvirus (visna virus) is genetically more complex than the prototype RNA tumor virus (RSV). In agreement with this, Beemon et al. (3) recently reported that REV (a lytic avian ribodeoxyvirus) has a genomic complexity of 3.5×10^6 daltons.

It is interesting to note (Fig. 1) that heat denaturation of visna virus 60–70*S* RNA gives rise to a single subunit class. We have never been able to obtain evidence of the existence of several subunit classes with our strain of visna virus (K 796). It should be emphasized that this strain has never been cloned and that the same observation was made even after several delib-

erate passages at a high multiplicity of infection (R. Vigne, unpublished observation).

Although RSV and visna virus have comparable genomic complexities, the base composition of their RNAs is very different (Table 1). Visna virus RNA is very rich in AMP. This is not due to a particularly long poly(A) tract, as shown in this paper. This peculiar base composition is reflected in our partial sequence analysis of the large T_1-specific oligonucleotides. Table 3 shows a high number of A_nX sequences with n equal to 5, 6, or 7. Therefore, it is likely that the large number of AMP residues present in visna RNA are more or less evenly distributed along the genome.

Our study of the poly(A) tract present in the visna virus genome confirms and extends previous results (15) obtained by hybridization of visna RNA with [^{3}H]poly(U). The size of the poly(A) segment and its base composition are very similar to those of RSV poly(A) (23, 27). The presence of poly(A) in only two out of three subunits has also been reported for avian (27) and murine (21) RNA tumor viruses.

ACKNOWLEDGMENTS

We wish to thank M. A. Billeter for a gift of ^{32}P-labeled PR-B RNA and for introducing one of us (R.V.) to bidimensional gel electrophoresis techniques, G. S. Martin for a gift of ^{3}H-labeled SR-A RSV RNA and B. R. Jordan for providing us with [^{3}H]poly(A) and several reagents. We are grateful to N. Sauze and to G. Vestris for their excellent technical assistance. This work was supported by grants A 651 2119 and 99 57 17 from CNRS.

LITERATURE CITED

1. **Barrell, B. G.** 1971. Fractionation and sequence analysis of radioactive nucleotides, p. 751-795. *In* S. L. Cantoni and D. R. Davis (ed.), Procedures in nucleic acid research, vol. 2. Harper & Row Publishers, New York.
2. **Beemon, K., P. Duesberg, and P. Vogt.** 1974. Evidence for crossing-over between avian tumor viruses based on analysis of viral RNAs. Proc. Natl. Acad. Sci. U.S.A. 71:4254-4258.
3. **Beemon, K. L., A. J. Faras, A. T. Haase, P. H. Duesberg, and J. E. Maisel.** 1976. Genomic complexities of murine leukemia and sarcoma, reticuloendotheliosis, and visna virus. J. Virol. 17:525-537.
4. **Billeter, M. A., J. T. Parsons, and J. M. Coffin.** 1974. The nucleotide sequence complexity of avian tumor virus RNA. Proc. Natl. Acad. Sci. U.S.A. 71:3560-3564.
5. **Brahic, M., J. Tamalet, P. Filippi, and L. Delbecchi.** 1973. The high molecular weight RNA of visna virus. Biochimie 55:885-891.
6. **Brahic, M., and R. Vigne.** 1975. Properties of visna virus particles harvested at short time intervals: RNA content, infectivity, and ultrastructure. J. Virol. 15:1222-1230.
7. **Coffin, J. M., and M. A. Billeter.** 1976. A physical map of the Rous sarcoma virus genome. J. Mol. Biol. 100:293-318.
8. **Cooper, G. M., and H. M. Temin.** 1974. Infectious Rous sarcoma virus and reticuloendotheliosis virus DNAs. J. Virol. 14:1132-1141.
9. **Delius, H., P. H. Duesberg, and W. F. Mangel.** 1974. Electron microscope measurements of Rous sarcoma virus RNA. Cold Spring Harbor Symp. Quant. Biol. 39:835-844.
10. **De Wachter, R., and W. Fiers.** 1972. Preparative two-dimensional polyacrylamide gel electrophoresis of ^{32}P labelled RNA. Anal. Biochem. 49:184-197.
11. **Duesberg, P. H.** 1968. The RNA's of influenza virus. Proc. Natl. Acad. Sci. U.S.A. 59:930-937.
12. **Duesberg, P. H.** 1968. Physical properties of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 60:1511-1518.
13. **Duesberg, P. H., and P. K. Vogt.** 1973. Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular species. J. Virol. 12:594-599.
14. **Friedmann, A., J. E. Coward, D. H. Harter, J. S. Lipset, and C. Morgan.** 1974. Electron microscopic studies of visna virus ribonucleic acid. J. Gen. Virol. 25:93-104.
15. **Gillespie, D., K. Takemoto, M. Robert, and R. C. Gallo.** 1973. Polyadenylic acid in visna virus RNA. Science 179:1328-1330.
16. **Haase, A. T., and W. Levinson.** 1973. Inhibition of RNA slow viruses by thiosemicarbazones. Biochem. Biophys. Res. Commun. 51:875-880.
17. **Haase, A. T., and J. R. Baringer.** 1974. The structural polypeptides of RNA slow viruses. Virology 57:238-250.
18. **Haase, A. T., A. C. Garapin, A. J. Faras, J. M. Taylor, and J. M. Bishop.** 1974. A comparison of the high molecular weight RNAs of visna virus Rous sarcoma virus. Virology 57:259-270.
19. **Haase, A. T.** 1975. The slow infection caused by visna virus. Curr. Top. Microbiol. Immunol. 72:101-156.
20. **Haase, A. T., B. L. Traynor, P. E. Ventura, and D. W. Alling.** 1976. Infectivity of visna virus DNA. Virology 70:65-79.
21. **Ihle, J. N., K. L. Lee, and F. T. Kenney.** 1974. Fractionation of 34S ribonucleic acid subunits from oncornaviruses on polyuridylate-sepharose columns. J. Biol. Chem. 249:38-42.
22. **Khan, M. S. N., and B. E. H. Maden.** 1976. Nucleotide sequences within the ribosomal ribonucleic acids of HeLa cells, Xenopus laevis and chick embryo fibroblasts. J. Mol. Biol. 101:235-254.
23. **Lai, M. M. C., and P. H. Duesberg.** 1972. Adenylic acid-rich sequence in RNAs of Rous sarcoma virus and Rauscher mouse leukaemia virus. Nature (London) 235:383-386.
24. **Quade, K., R. E. Smith, and J. J. Nichols.** 1974. Evidence for common nucleotide sequences in the RNA subunits comprising Rous sarcoma virus 70S RNA. Virology 61:287-291.
25. **Schibler, U., T. Wyler, and O. Hagenbüchle.** 1975. Changes in size and secondary structure of the ribosomal transcription unit during vertebrate evolution. J. Mol. Biol. 94:503-517.
26. **Staynov, D. Z., J. C. Pinder, and W. B. Gratzer.** 1972. Molecular weight determination of nucleic acids by gel electrophoresis in non-aqueous solution. Nature (London) New Biol. 235:108-110.
27. **Wang, L. H., and P. H. Duesberg.** 1974. Properties and location of poly(A) in Rous sarcoma virus RNA. J. Virol. 14:1515-1529.
28. **Wang, L. H., P. Duesberg, K. Beemon, and P. K. Vogt.** 1975. Mapping RNase T_1-resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation-defective viruses are at the poly(A) end. J. Virol. 16:1051-1070.