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The Nucleotide Sequence Complexity of Avian Tumor Virus RNA

(Rous sarcoma virus/T1-oligonucleotides/two-dimensional gel electrophoresis)

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ABSTRACT **The nucleotide sequence complexity of 60–70S RNA of Rous sarcoma virus was determined from the molar yields of 11 pure oligonucleotides of known chain length, obtained from [^{32}P]RNA of Rous sarcoma virus by T1 RNase digestion and two-dimensional polyacrylamide gel electrophoresis. We calculate an apparent chain length of about 9800 nucleotides, corresponding to a molecular weight of 3.4×10^6. Assuming the molecular weight of 60–70S RNA of Rous sarcoma virus to be about 10^7, our data suggest that the virus genome consists of identical, or very similar, nucleotide sequences repeated three to four times.**

Avian RNA tumor viruses contain several species of RNA, the largest of which is believed to be the genetic material (1). From the sedimentation coefficient (60–70S) and the relative electrophoretic mobility the molecular weight of this RNA was estimated to be about 10^7 (2–4). Upon denaturation it is converted into RNA with an apparent molecular weight of 3×10^6 (3–5). These observations have led to the conclusion that the genome of avian tumor viruses consists of an aggregate of three to four subunits, each with a molecular weight of about 3×10^6 (5, 6). In order to determine the coding capacity of the viral genome, it is necessary to establish whether the subunits are similar or different with regard to their nucleotide sequence. From a study of hybridization kinetics of RNA of Rous sarcoma virus (RSV) to its complementary DNA, it was concluded that the subunits are different, and that the sum of their molecular weights is about 9.3×10^6 (7).

In this paper we describe a chemical approach to the same problem. We have determined the molar yield of a number of well-characterized, pure oligonucleotides derived from 60–70S [^{32}P]RNA of RSV (Schmidt–Ruppin strain), which, on the basis of their chain length and partial sequence data, are expected to occur only once in 10^7 daltons of RNA. From these data we calculate an apparent total chain length, which in this paper will be referred to as "nucleotide sequence complexity" or "complexity." The number obtained, 9,800, implies that the nucleotide sequences of 60–70S RNA of RSV are repeated three to four times.

MATERIALS AND METHODS

Cells and Viruses. Chicken embryo fibroblast cultures were grown as described (8). Rous sarcoma virus (Schmidt–Ruppin strain, subgroup D) derived from a stock virus described by Altaner and Temin (9) was cloned (10) and passaged twice before use. Cell cultures infected with about 0.5 focus-forming unit/cell and transferred 3–5 days later (8) were used for labeling experiments.

Preparation of ^{32}P-labeled RNA. RSV [^{32}P]RNA was prepared from 5 to 10 confluent cultures of RSV-infected chicken cells essentially as described (8), except that the labeling medium contained 100 μCi/ml of [^{32}P]phosphate. Five cultures of RSV-infected chicken cells routinely yielded 2 to 3×10^6 cpm of labeled 60–70S RNA of RSV (Fig. 1) with a specific activity of about 1 to 2×10^6 cpm/μg. For the preparation of 30–40S [^{32}P]RNA of RSV, medium was harvested every 12 hr. 28S ribosomal RNA (rRNA) was isolated from ^{32}P-labeled cultures of RSV-infected cells as described (11). Bacteriophage Qβ [^{32}P]RNA was prepared essentially as described (12).

Two-dimensional Gel Electrophoresis of Labeled RNA. Unlabeled Qβ RNA was added to the sample to give a total of 100 μg. After precipitation with ethanol, the RNA was collected by centrifugation, dried, and dissolved in 200 μl of 0.1 M NaCl–50 mM Tris·HCl (pH 7.5)–5 mM EDTA. A small aliquot was removed for determination of input radioactivity. The RNA was again precipitated with ethanol, and transferred to a polyethylene sheet with several small portions of water. In some experiments two large, labeled T1 oligonucleotides derived from Qβ [^{32}P]RNA (purified by two-dimensional electrophoresis as described below) were added in known quantities to the RSV [^{32}P]RNA preparations as an internal standard for determination of the recovery of oligonucleotides. The samples were dried and taken up in a capillary in 8 μl of 20 mM Tris·HCl (pH 7.5)–2 mM EDTA containing either 50 or 12.5 units of RNase T1 (Sankyo Co., Ltd., Tokyo). After digestion for 30 min at 37°, solid urea (4 mg) and 5 μl of dye mixture [2 mg/ml each of xylene cyanol FF and bromophenol blue–50% (w/v) sucrose–6 M urea] were added. The samples were subjected to two-dimensional gel electrophoresis essentially as described by De Wachter and Fiers (13). This system, which provided excellent resolution and high reproducibility, proved to be superior for the purpose of this study as compared to commonly used fingerprinting techniques where transfer of the radioactive material from the first to the second dimension is incomplete and variable.

Extraction and Characterization of Oligonucleotides. After electrophoresis, the oligonucleotides were located by autoradiography and cut out with a 7.5-mm diameter corkborer. Each gel disc was placed in a tube containing 0.5 ml of 1 M NaCl, and the radioactivity was determined (see below). After 1–2 days the solution was filtered through glass wool

Abbreviation: RSV, Rous sarcoma virus.

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and the gel disc was rinsed with 0.3 ml of 1 M NaCl. Yeast RNA (50 μg; from British Drug House, Ltd.; repurified by phenol extraction) and 0.8 ml of isopropanol were added to the combined washes. After 12 hr at −20°, the precipitate was collected by centrifugation, washed with 60% isopropanol, transferred to a polyethylene sheet, and dried as above. An average of 80% of the radioactivity was recovered from the gel disc. The samples were analyzed after digestion with pancreatic RNase (Worthington, Freehold, N.J.) as described by Adams *et al.* (14).

Radioactivity Measurements. Radioactivity of dried samples was determined by liquid scintillation counting (15). Radioactivity in gel discs was measured by counting of Cherenkov radiation (16). The reproducibility and proportionality of this method was equal to that of liquid scintillation counting, but the counting efficiency was 2.6-fold lower. Input radioactivities were determined by Cherenkov counting of samples in tubes containing 0.5 ml of 1 M NaCl and a blank gel disc. The counting efficiency was the same whether the radioactivity was inside the gel or in the surrounding solution. The actual "input radioactivities" given in the figures and tables were obtained by subtracting from the measured input all losses accounted for (material remaining behind at the various transfers and on both sides of the gel strip cut out for the second dimension). The values subtracted varied between 10 and 20% of the input.

RESULTS

Fingerprint Analysis of ^{32}P-labeled RSV RNA, 28S rRNA, and Qβ RNA. 60–70S RSV [^{32}P]RNA (see Fig. 1A) was digested with RNase T1, and the resulting oligonucleotides were separated by two-dimensional gel electrophoresis (13). In this system oligonucleotides are resolved mainly according to their nucleotide composition in the first dimension and to their chain length in the second dimension. In Fig. 2 the fingerprint of 60–70S RSV [^{32}P]RNA (Fig. 2 B) is compared with those of 28S [^{32}P]rRNA (Fig. 2 A) and Qβ [^{32}P]RNA (Fig. 2 C). If we consider only the well-resolved spots, designated by numbers, it is apparent that RSV RNA yields at most twice as many spots as Qβ or 28S rRNA. The total number of T1 oligonucleotides of any size class obtained from an RNA is proportional to its complexity. Assuming that in most cases each numbered spot corresponds to one oligonucleotide, it would seem that RSV RNA has a complexity about twice that of Qβ or 28S rRNA. 60–70S [^{32}P]RNA of Rous associated virus-49 and avian myeloblastosis virus gave fingerprints indicating a similar complexity (data not shown).

Additionally, it should be noted that the fingerprints of 30–40S and 15–30S RNA of RSV (see Fig. 1B) obtained after heat-denaturation were identical to those of 60–70S RSV [^{32}P]-RNA (data not shown). Therefore, none of the characteristic oligonucleotides appeared to be derived from the 4S RNA species known to be associated with high-molecular-weight avian tumor virus RNA (17). Furthermore, it seems that 15–30S RNA is not a separate species, but more likely a degradation product of 30–40S RNA.

Quantitative Determination of the Complexity of RSV RNA. To determine the sequence complexity of RSV RNA as outlined in the introduction it was necessary (*i*) to characterize each large T1[^{32}P]oligonucleotide with regard to its purity and chain length and (*ii*) to determine its yield, corrected for losses incurred during the analytical procedure.

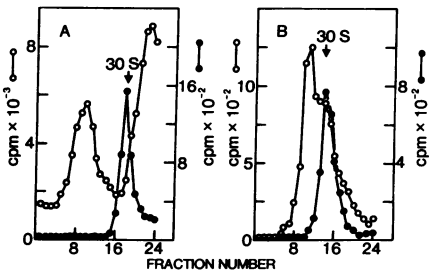

FIG. 1. Sucrose gradient centrifugation of RSV [^{32}P]RNA. (A) RSV [^{32}P]RNA (10^5 cpm; O) and Qβ [^{3}H]RNA (6,000 cpm; ●) were mixed and applied to a 5–23% sucrose gradient containing 50 mM Tris·HCl (pH 7.5)–1 mM EDTA–0.1% (w/v) sodium dodecyl sulfate and centrifuged in a Spinco SW 65 rotor for 75 min at 55,000 rpm at 4°. Fractions were collected directly onto paper filters, and the radioactivity was determined. (B) 60–70S RSV [^{32}P]RNA (9,000 cpm; O) and Qβ [^{3}H]RNA (3,000 cpm; ●) were mixed and heated in 100 μl of 20 mM Tris·HCl (pH 7.5)–0.5 mM EDTA for 1 min at 100°, quickly cooled, and analyzed by sucrose gradient centrifugation (90 min at 58,000 rpm and 4°). Fractions were analyzed for ^{32}P and ^{3}H radioactivity as above. Fractions from analogous preparative gradients corresponding to fractions 7–13 (60–70S) of A and 8–12 (30–40S) and 13–17 (15–30S) of B were pooled for fingerprint analysis.

(*i*) Characterization of T1 oligonucleotides: Each typical T1 oligonucleotide contains only one guanosine residue, located at the 3′ end. Degradation of T1 oligonucleotides with pancreatic RNase yields only the nucleoside 3′-monophosphates, guanosine-, uridine-, and cytidine-3′*P*, and oligonucleotides containing one or more adenosine residues followed by guanosine-, uridine-, or, cytidine -3-′*P* (secondary oligonucleotides). A T1 oligonucleotide was considered impure if, after treatment with pancreatic RNase, (*a*) more than one guanosine containing product was present or (*b*) a secondary oligonucleotide occurred in a nonintegral molar ratio (i.e., deviating by more than 0.2 mole) as compared to the guanosine-containing product. Since the quantitation of mononucleotides by the method used entails an error of ± 30%, nonintegral ratios of cytidine and uridine monophosphate were not considered to be indicative of impurity.

The chain length was calculated as the sum of all nucleotides recovered after pancreatic RNase digestion (Table 1), and is estimated to be accurate to ±10%. The interpretation of the nucleotide analyses is based on the assumption that the RNA was homogeneously labeled. This was confirmed by the fact that the same nucleotide composition was obtained whether RSV [^{32}P]RNA was digested with a mixture of RNase T1 and T2 and pancreatic RNase (which yields nucleoside 3′-phosphates) or snake venom phosphodiesterase (which gives nucleoside 5′-phosphates).

(*ii*) The losses of T1 oligonucleotides incurred during the analytical procedures were corrected for by either external or internal standardization. For external standardization, RNase T1 digests of labeled Qβ RNA or 28S rRNA were fingerprinted in parallel with the RSV RNA. The oligonucleotides from the external standards were characterized with regard to chain length and purity, as in the case of the RSV oligonucleotides. Assuming a chain length of 4500 for Qβ RNA (18) and of 4600 for 28S rRNA (from ref. 19), the calculated recovery of oligonucleotides varied from 54–60% in three different experiments. Internal standardization was carried out by adding Qβ [^{32}P]RNA oligonucleotides 1 and 11 (which have mobili-

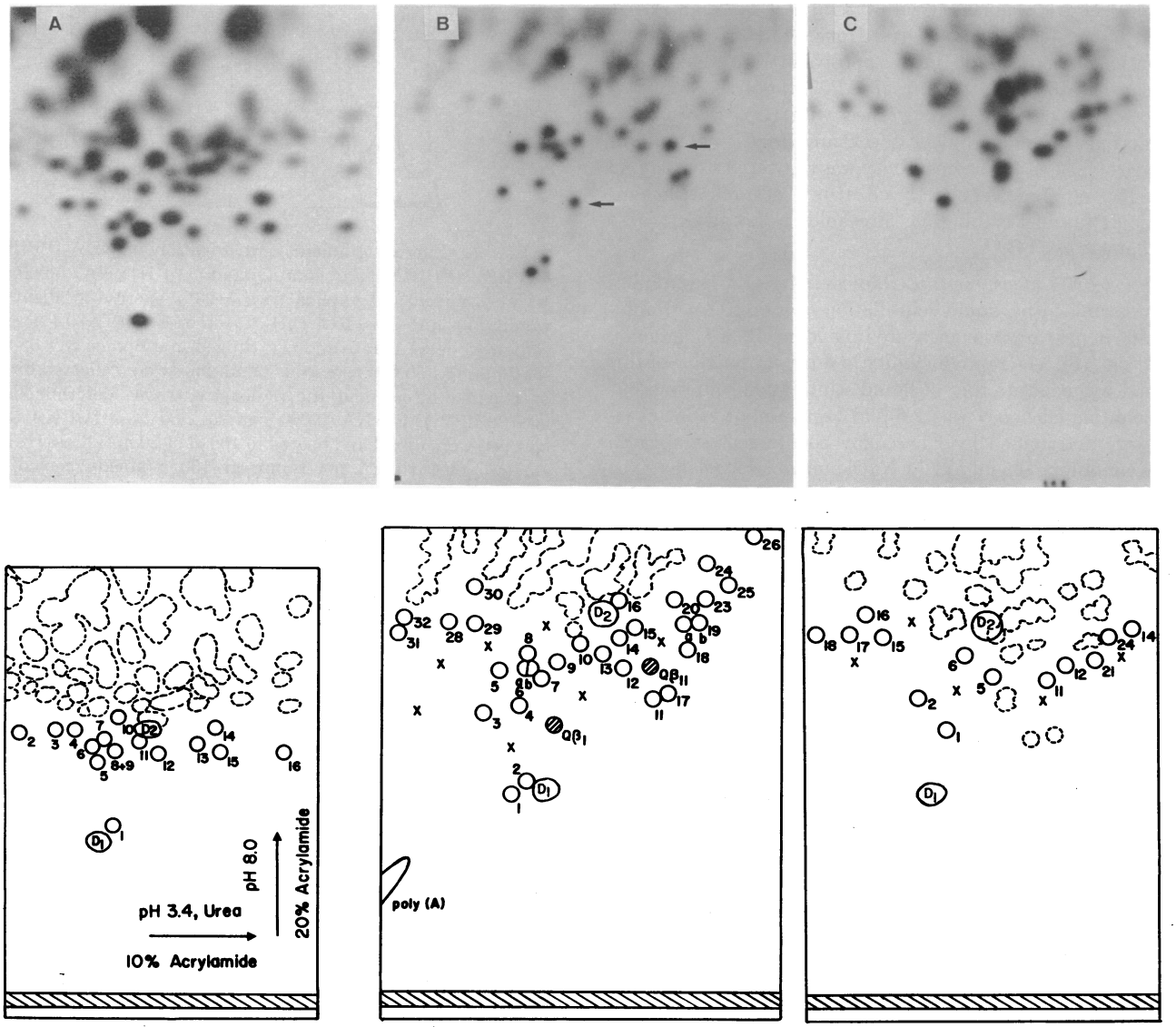

FIG. 2. Two-dimensional gel electrophoretic fingerprints of ^{32}P-labeled 28S rRNA, RSV RNA, and Qβ RNA. RNase T1 digests of (A) 28S [^{32}P]rRNA (2.6 $\times$ 10^6 cpm), (B) 60–70S RSV [^{32}P]RNA (Schmidt-Ruppin-D strain) (1.22 $\times$ 10^6 cpm) containing Qβ oligonucleotides 1 (3,270 cpm) and 11 (3,900 cpm), and (C) Qβ [^{32}P]RNA (1.87 $\times$ 10^6 cpm) were analyzed by two-dimensional gel electrophoresis. The first dimension gel (17 $\times$ 36 $\times$ 0.2 cm) was run at 500 V (30 mA) for 20 hr at 4° until the faster dye had migrated 21 cm. Strips of 1 cm width, comprising the area from 9 to 30.3 cm from the origin, were cut out and embedded in the second dimension gels (21.5 cm wide, 22 cm from strip to top). Electrophoresis was at 350 V (40 mA per gel) for 14.5 and 18 hr until the faster dye had migrated 13.5 cm (gel A) and 17 cm (gels B and C, respectively). Under these conditions many small oligonucleotides were lost from the top of the gel. The internal standard oligonucleotides from Qβ RNA are marked with arrows. Areas marked with X were cut out to measure background, which in all cases was less than 10% or the radioactivity found in neighboring spots. Bars at the bottom of the drawings show the location of the first dimension gel strip. D_1 and D_2 give the position of the dye markers. Autoradiographs were exposed for 1 day.

ties different from any of the RSV oligonucleotides, Fig. 2) to the RSV [^{32}P]RNA preparation before RNase T1 digestion. After fingerprinting, their recoveries were determined (Table 1), and their purity was established by digestion with pancreatic RNase and analysis of the products.

An example of complexity determination with use of internal standardization is shown in Table 1. The complexity, as determined separately for each of 11 oligonucleotides, varied from 6,700 to 10,700. Table 2 summarizes the results of four different experiments. The complexity of the 60–70S RNA of RSV was calculated to be 9,800 $\pm$ 2,500 nucleotides, corresponding to a molecular weight of (3.4 $\pm$ 0.86) $\times$ 10^6.

DISCUSSION

In evaluating the results of our studies, several possible sources of error should be considered. We have assumed that the recovery of oligonucleotides from RSV RNA was similar to that of the external and internal standards. This might not be the case if, for instance, cleavages at residues other than guanosine, which are known to occur (especially at high RNase T1-to-RNA ratios), had selectively decreased the yield of oligonucleotides of one of the RNAs. However, experiments in which both sample and external standard were digested at a lower enzyme-to-RNA ratio yielded essentially the same

TABLE 1. *Calculation of the nucleotide sequence complexity of RSV RNA from the yield of pure T1 oligonucleotides*[a]

Oligonucleotide no.[b]	Composition[c]	Chain length[d] (nucleotides)	Radioactivity (cpm)	Complexity of RSV RNA[e] (nucleotides)
1	1.1 A_3C, 1.0 A_2U, 1.1 A_2C, 1.0 AC, 1 G, 9.9 U, 11.0 C	34	2,130	10,500
3	1 A_2G, 0.9 A_2C, 1.1 AU, 1.8 AC, 7.8 C, 4.0 U	24	1,770	8,940
4	1.1 A_3C, 1.0 AU, 0.9 AC, 1 G, 7.7 C, 4.6 U	21	1,450	9,500
11	0.9 AU, 1 G, 5.5 C, 7.6 U	16	1,330	7,900
13	0.9 A_2C, 1.0 AU, 1.0 AC, 1 G, 2.6 C, 4.2 U	15	1,470	6,710
18	0.9 A_2U, 0.9 AU, 1 G, 2.7 C, 4.8 U	14	1,090	8,430
25	1 AG, 1.1 AU, 2.1 C, 6.1 U	12	737	10,710
28	2.1 AC, 1 G, 3.6 C, 1.5 U	10	844	7,790
29	1.2 A_4C, 1.2 AU, 2.0 AC, 1 G, 1.0 C, 1.7 U	15	936	10,540
31	1.0 A_3C, 1.9 AC, 1 G, 3.7 C, 1.5 U	14	1,120	8,190
32	1 A_2G, 3.1 AC, 4.7 C, 1.2 U	15	1,060	9,270
Average ± SEM				8,950 ± 1,290

[a] The fingerprint for this experiment is shown in Fig. 2.

[b] Some oligonucleotides (numbers 2, 9, 17, 20, 26, and 30, Fig. 2B) were probably pure, but were excluded from this list either because they were analyzed in only one experiment or because they marginally failed to meet the strict purity criteria stated in the text. If these oligonucleotides were included in the sequence complexity calculations, the average value was only 3% different from the average value given above.

[c] Average of determinations from two or three experiments.

[d] Chain length is given as the total number of nucleotides in secondary oligonucleotides (each rounded to the nearest integer) plus the rounded sum of the mononucleotides.

[e] $\frac{\text{Input cpm} \times \text{oligonucleotide chain length} \times \text{estimated recovery}}{\text{cpm in oligonucleotide}}$, where input = 1.22×10^6 cpm and estimated recovery = 53.9%, based on the average of two Qβ oligonucleotide internal standards (Qβ1 = 49.0%, Qβ11 = 58.8%).

results (Table 2). In any event, even if the recovery of RSV oligonucleotides had been essentially complete, while that of the standards was 54–60%, the complexity of RSV RNA would not exceed 18,000 nucleotides.

Contamination of 60–70S RNA of RSV by other labeled RNA species would have led to an overestimate of the complexity. No oligonucleotides due to a minor RNA species were detected by exposing autoradiographs for 10 times longer than usual. Furthermore, the similarity of the background values found in the fingerprints of both Qβ and RSV RNA (Fig. 2) indicated no significant contamination of the RSV RNA with a complex mixture of minor RNAs. Since low yield of oligonucleotides and contamination with other RNA is more likely for RSV RNA, our complexity estimate of 9,800 ± 2,500 nucleotides is more likely to be too high than too low.

Taylor *et al.* (7), from RNA·DNA hybridization kinetics, have estimated the complexity of RSV to be about 30,000 nucleotides. At present, we have no explanation for these contradictory estimates.

Our results, taken together with the molecular weight estimate of the total genome, lead us to the conclusion that the nucleotide sequences of RSV RNA are repeated three to four times. While it is presently not possible to decide whether such repeated sequences are identical or whether there is some variability, no evidence has been found so far to support the latter possibility. Fig. 3 shows several models for the organization

TABLE 2. *Summary of the complexity measurements of RSV RNA*

Exp.[a]	Input RSV [^{32}P]RNA (cpm × 10^{-5})	Determination of yield[b]		Calculated complexity[c] (nucleotides)
		Method of standardization	Recovery (%)	
1[d]	7.85	External (Qβ RNA)	60 ± 9.1	8,900 ± 1,700 (9)
2[d]	5.68	External (28S rRNA)	53.6 ± 9.3	11,490 ± 4,300 (8)
3[e]	26.7	Internal	28.5[f]	10,200 ± 1,700 (11)
4[e]	12.2	Internal	53.9	8,900 ± 1,300 (11)
		External (Qβ RNA)	55.5 ± 5.6	9,100 ± 1,300 (11)

[a] Three of the fingerprints used for experiments 2 and 4 are shown in Fig. 2.

[b] Based on the average recovery of 11 pure oligonucleotides from 28S rRNA or 12 pure oligonucleotides from Qβ RNA in parallel fingerprints (external standard) or on the average recovery of Qβ oligonucleotides 1 and 11 from the RSV RNA fingerprint (internal standard). Values are averages ± SEM.

[c] Determined from the number of pure oligonucleotides given in parentheses. Values are averages ± SEM.

[d] A ratio of 50 units of RNase T1 to 100 μg of RNA was used.

[e] A ratio of 12.5 units of RNase T1 to 100 μg of RNA was used.

[f] The low yield is due to partial loss of the preparation.

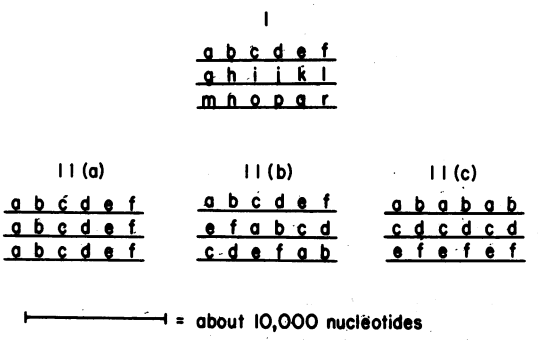

FIG. 3. Possible structures of the avian tumor virus genome. Each letter represents an arbitrary section of the genome and serves only to orient the parts of the genome relative to each other. One unit (6 letters) would represent about 3×10^6 daltons, according to the molecular weight estimate of Duesberg and Vogt (6).

of the genome of an RNA tumor virus which, for the purpose of this discussion, is assumed to consist of three subunits. Model I implies a complexity of 30,000 nucleotides and is inconsistent with the results of this study. Model II shows three of the possible ways in which an aggregate RNA of 30,000 nucleotides could have a sequence complexity of 10,000 nucleotides: (*a*) all subunits are identical; (*b*) each subunit contains the same set of sequences but in different (for example, permuted) arrangement; and (*c*) each subunit contains different sets of sequences.

Models II*a* and *b* readily account for the generation of heterozygotes (20) by simple mixing of progeny subunits from different parents, as well as for the occurrence of mutants containing apparent deletions in all subunits (21–23). Recombination (24, 25) between markers could occur at the DNA level or, in the case of model II*b*, by transcription of progeny RNA from tandemly integrated parental DNA molecules. Model II*c* would allow for a high frequency of recombination between certain markers by simple reassortment of subunits. However, formation of heterozygotes would only be possible by partial diploidy, and the occurrence of deletion mutants in which the size of all subunits is reduced would be difficult to explain with such a model.

A genome with a complexity of 10,000 nucleotides contains sufficient information to code for different proteins of a total molecular weight of about 300,000. If the majority of the virion proteins (total molecular weight about 365,000; refs. 26 and 27) were specified by the viral genome, this would account for most or all of the available coding capacity of the virus.

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