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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

LU-HAI WANG,* PETER DUESBERG, KAREN BEEMON, AND PETER K. VOGT

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720, and Department of Microbiology, University of Southern California, Los Angeles, California 90033*

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The large RNase T_1-resistant oligonucleotides of the nondefective (nd) Rous sarcoma viruses (RSV): Prague RSV of subgroup B (PR-B), PR-C and B77 of subgroup C; of their transformation-defective (td) deletion mutants: td PR-B, td PR-C, and td B77; and of replication-defective (rd) RSV(−) were completely or partially mapped on the 30 to 40*S* viral RNAs. The location of a given oligonucleotide relative to the poly(A) terminus of the viral RNAs was directly deduced from the smallest size of the poly(A)-tagged RNA fragment from which it could be isolated. Identification of distinct oligonucleotides was based on their location in the electrophoretic/chromatographic fingerprint pattern and on analysis of their RNase A-resistant fragments. The following results were obtained. (i) The number of large oligonucleotides per poly(A)-tagged fragment increased with increasing size of the fragment. This implies that the genetic map is linear and that a given RNase T_1-resistant oligonucleotide has, relative to the poly(A) end, the same location on all 30 to 40*S* RNA subunits of a given 60 to 70*S* viral RNA complex. (ii) Three sarcoma-specific oligonucleotides were identified in the RNAs of PR-B, PR-C and B77 by comparison with the RNAs of the corresponding td viruses. The sarcoma-specific oligonucleotides of these three sarcoma viruses had very similar compositions. Based on the map positions of these oligonucleotides, sarcoma-specific sequences of nd viral RNAs were estimated to map between 6.6 and 20% away from the poly(A) end. (iii) As far as analyzed, the oligonucleotide maps of the RNAs of nd and td PR-B were the same with the exception of the sarcoma-specific sequences, as were the maps of nd and td B77 RNAs. (iv) Comparisons of the map locations of oligonucleotides from the three nd sarcoma virus strains analyzed suggested that homologous oligonucleotides are found in certain homologous map positions of the respective RNAs. This implies that these three virus strains probably have similar gene orders. (v) The complexity in daltons of the RNA of PR-C was 3.22×10^6 and that of B77 was 3.02×10^6. (vi) The RNase T_1-resistant oligonucleotides of poly(A)-tagged RNA fragments ranging up to 15*S* from each nd/td virus pair studied here were very similar. This implies that they share a common heteropolymeric sequence at their poly(A) ends. (vii) Poly(A)-tagged 12*S* RNA fragments of all avian tumor viruses studied so far including that of RSV(−) shared one oligonucleotide, termed spot C, which mapped very near the poly(A) end. It may be part of a short terminal heteropolymeric sequence common to all avian tumor virus RNAs investigated. The following maps emerged for the poly(A) terminal sequences of the nd/td virus RNAs which were analyzed: they start with poly(A) (molecular weight = 60,000), continue with a heteropolymeric sequence shared partially or completely by nd and td viruses (molecular weight = 140,000), which is followed by sarcoma-specific sequences (molecular weight $\simeq$ 300,000 to 450,000) in the case of nd sarcoma virus RNAs. Several mechanisms are discussed to explain the generation of deletion mutants from nd viruses.

Transformation-defective (td) deletion mutants, which lack the ability to transform chicken fibroblasts, have been derived from several nondefective (nd) avian sarcoma viruses (14, 20, 26). The 30 to 40S RNA of td viruses was shown to lack about 15% of the mass and one to three large RNase T_1-resistant oligonucleotides when compared to the RNA from the corresponding nd viruses (7, 9, 16, 18, 20, 21).

The present study was initiated to investigate the location of large RNase T_1-resistant oligonucleotides on nd and td viral RNAs and, in particular, to locate sarcoma-specific oligonucleotides on the RNAs of nd viruses. Our approach is based on the following characteristics of the 60 to 70S complex of avian tumor virus RNA. (i) It consists predominantly of two 30 to 40S subunits (6, 19, 29). (ii) The 30 to 40S subunits of a cloned virus are similar or identical (3, 4, 8, 10, 11, 22). At least 66% of the 30 to 40S RNA subunits of a given virus preparation carry a poly(A) at their 3'OH end (17, 28). The 30 to 40S subunits with and without poly(A) have the same fingerprint patterns of RNase T_1-resistant oligonucleotides (23, 28). (iv) The number of RNase T_1-resistant oligonucleotides of poly(A)-tagged RNA fragments increases with increasing size of the fragment implying that the genetic map is linear and that a given hetropolymeric sequence has the same location relative to the poly(A)-terminus on both 30 to 40S subunits (28). Moreover, fingerprinting of poly(A)-tagged fragments of different sizes has indicated that two sarcoma-specific oligonucleotides of Prague Rous sarcoma virus (RSV) of subgroup B (PR-B) are located near the poly(A) end of the viral RNA (28).

The present report provides evidence that sarcoma-specific oligonucleotides of two other nd avian sarcoma viruses, PR-C and B77, are located near the poly(A) end of viral RNA. In addition, the sarcoma-specific oligonucleotides of PR-B, PR-C, and B77 were found to have very similar, possibly identical, compositions. The location of each large T_1-resistant oligonucleotide on the viral RNA, relative to the poly(A) terminus, was estimated from the length of the smallest fragment from which it could be isolated. Oligonucleotides of the ultimate heteropolymeric sequences of nd and corresponding td viruses, mapping between the sarcoma-specific oligonucleotides and the terminal poly(A) in the case of nd viruses, were found to be very similar. All avian tumor virus RNAs investigated shared at least one oligonucleotide which mapped very close to the poly(A) end.

MATERIALS AND METHODS

Reagents and materials. The following reagents were purchased: [^{3}H]uridine (40 Ci/mmol), [^{14}C]uridine (500 mCi/mmol) and [^{3}H]TTP (18 Ci/mmol) from New England Nuclear Corp., unlabeled TTP from Sigma Chemical Co., $H_3{}^{32}PO_4$ from ICN, Los Angeles, unlabeled poly(A) from Miles Laboratories, Inc., oligo(dT)$_{12\text{-}18}$ and oligo(dT)-cellulose from Collaborative Research, Inc., membrane filter (type HA) from Millipore Corp., cellulose acetate membrane strips from Schleicher and Schuell Inc., DEAE thin-layer plates (20 by 40 cm) coated to 250-μm thickness with a 1:7.5 mixture of DEAE-cellulose and cellulose for homochromatography from Analtech, Inc., DEAE-cellulose paper (DE81) from Whatman Corp., RNase A from Worthington Biochemical Corp., RNase T_1 from Calbiochem, Los Angeles, yeast RNA from P. L. Biochemical Co.

Virus. Cloned strains of Prague RSV of subgroup B (PR-B) and PR-C and B77 RSV of subgroup C were used (8). Isolation and cloning of the td viruses from their corresponding nd viruses has been described (20, 26). Viruses were propagated and purified according to published procedures (8). Replication-defective (rd) Bryan RSV which lacks glycoproteins and is termed RSV(−) was propagated in a line of transformed quail cells, grown as described previously (11).

Viral DNA polymerase assay. This was used to assay virus production. At various times after infection, 50-μl aliquots of overnight culture medium were tested for polymerase activity. The medium was spun at 5,000 × *g* for 10 min to remove cells and debris and incubated for 30 min at 38 C with an equal volume of polymerase cocktail. This cocktail was freshly mixed before the reaction from a stock reagent solution and a primer-template solution in 0.01 M Tris-hydrochloride (pH 7.4). These solutions were stored at −20 C. The reaction mixture (100 μl) contained 50 mM Tris-hydrochloride (pH 8.1), 6 mM magnesium acetate, 1 mM EDTA, 5 mM dithiothreitol, 0.1% Triton X-100 (Packard Instrument Co. Inc.), 1 μM [^{3}H]TTP (18 Ci/mmol) and a template-primer complex consisting of 2 μg of poly(A) and 0.4 μg of oligo(dT)$_{12\text{-}18}$. The reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid at 0 C. No carrier nucleic acid was added. After 15 min at 0 C the trichloroacetic acid-precipitable [^{3}H]poly(T) synthesized in the reaction was collected on a membrane filter (Millipore Corp.) and its radioactivity was assayed as described previously (27). Under these conditions media of maximally producing cells generally synthesized 20,000 to 50,000 counts/min of [^{3}H]poly(T) if infected with transforming viruses and 3,000 to 20,000 counts/min if infected with td viruses. Control media from uninfected cells incorporated 300 to 500 counts/min of [^{3}H]TMP into acid-insoluble material.

^{32}P labeling of virus. Subconfluent cultures of secondary chicken embryo fibroblasts were infected with transforming virus at a multiplicity of 0.1 to 1.0 or with td virus at a multiplicity of 1 to 5. In general, maximal virus production and confluency of cells was reached 5 to 10 days after infection with transforming virus. To attain maximum production of td viruses by

infected cultures, 1 to 2 weeks were required with the following regime: infection of primary cells was in 10-cm petri dishes; after 4 to 5 days these cultures were dissociated with trypsin, reseeded at a four-times-lower cell density and allowed to reach confluency.

Labeling of confluent cultures infected by td or nd viruses was in 150-cm^2 Corning tissue culture flasks. Two to 4 h before addition of label, the culture was changed to phosphate-free medium 199 supplemented with 2% dialyzed calf serum, 1% dialyzed chick serum, 1% Me_2SO and 0.5 μg of amphotericin B per ml (Fungizone). After incubation the medium was changed again (20 ml/flask) and 10 to 25 mCi of carrier-free $H_3{}^{32}PO_4$ was added per flask. The flask, shielded by a lucite box for radiation safety purposes, was incubated at 38 to 39 C in a Wedco humidified incubator.

Virus was collected at 12-h intervals for 2 to 3 days with an additional one or two changes with phosphate-free medium before shifting to regular medium. Under these conditions, generally 10^6 counts/min of 60 to 70*S* [^{32}P]RNA was obtained per mCi of ^{32}P input for transforming viruses. The yield of td virus [^{32}P]RNA was usually five- to 10-fold lower than that of nd viruses.

RNA preparations. Tobacco mosaic virus (TMV) RNA and [^{3}H]uridine- or [^{14}C]uridine-labeled 60 to 70*S* viral RNA were prepared according to published procedures (8, 28). ^{32}P labeling of viral RNA has been described above. [^{3}H]uridine- or ^{32}P-labeled RNA's of chicken embryo fibroblasts were prepared as described previously (28).

Gel electrophoresis. Heated (45 s, 100 C, 1 mM EDTA, 0.01 M Tris, pH 7.4, 0.2% sodium dodecyl sulfate), radioactively labeled 60 to 70*S* RNAs isolated from viruses harvested at 3- to 4-h intervals were used for electrophoresis in aqueous polyacrylamide gels as described (8).

Preparation of poly(A)-tagged RNA fragments. These procedures were modifications of those described previously (28). (i) Partial degradation of RNA: ^{32}P-labeled 60 to 70*S* RNA in 0.3 ml of low-salt buffer (LSB) containing 0.01 M Tris-hydrochloride (pH 7.2), 0.01 M NaCl, and 1 mM EDTA was prewarmed to 50 C in a water bath. Subsequently 15 μl of 1 M Na_2CO_3 was added, which increased the pH to 10.8. After incubation at 50 C for various times (see below), the RNA solution was cooled to 0 C and neutralized with 15 μl 1 M acetic acid, and then 0.7 ml of buffer containing 0.1 M NaCl, 0.01 M Tris (pH 7.2) and 1 mM EDTA was added. RNA was precipitated with 3 volumes of 95% ethanol. (ii) Membrane filter (Millipore Corp.) binding and elution of poly(A)-tagged RNA: in a typical experiment RNA partially degraded by alkali was redissolved in 50 μl of LSB and heat denatured at 100 C for 30 s and quenched at 0 C. One milliliter of a solution containing 0.5 M KCl, 0.01 M Tris-hydrochloride (pH 7.4), and 1 mM $MgCl_2$ was added and the mixture was cooled to 0 C and passed through a membrane filter (Millipore Corp.) at room temperature using a gentle, waterpump vacuum to hasten the process of adsorption. The filter had been washed previously with 1 ml of this buffer. Recovery of filter-bound RNA was more efficient by elution under a gentle vaccum with 2 to 4 0.5-ml aliquots of 2 mM EDTA (pH 8.1) followed by two 0.5-ml aliquots of water instead of with the high pH buffer used previously (28). If necessary this elution procedure was repeated once more until elution of filter-bound RNA was greater than 80% as monitored by a Geiger counter. The RNA eluted from membrane filters was precipitated with 3 volumes of ethanol in the presence of 5 to 10 μg of TMV RNA and 0.2 M NaCl. The process of binding and subsequent eluting from membrane filters was then repeated once more. The yield of poly(A)-tagged RNA in the second cycle was 40 to 80% of that obtained in the first cycle. In a few cases an additional oligo(dT)-cellulose fractionation of poly(A)-containing RNA fragments was carried out in between the two membrane filter-binding steps, or membrane filter binding and elution was repeated with pools of poly(A)-tagged RNA fragments fractionated by sucrose gradient sedimentation (see below). However, these variations did not improve the fractionation obtained by the two cycles of membrane filter binding used in most experiments. In later experiments poly(A)-tagged RNA fragments were isolated by two cycles of binding to oligo(dT)-cellulose columns (28). As observed previously (28), poly(A)-tagged fragments bound and eluted only once from oligo(dT)-cellulose contained, besides the oligonucleotides characteristic of this fragment, all other oligonucleotides of unfractionated RNA at low concentration. A second cycle of oligo(dT)-cellulose binding eliminated this background. About 70 to 80% of the RNA bound in the first cycle was bound in the second cycle. In contrast to the membrane filter method, recovery of RNA from oligo(dT)-cellulose was 100% if elution was as described (28) and then followed by washing with two 0.5 ml-aliquots of water. An additional advantage of the oligo(dT)-cellulose selection was that the RNA recovered in the first cycle could be recycled without intermittent ethanol precipitation by adjusting the eluate to 0.5 M LiCl. (iii) Sedimentation of poly(A)-tagged RNA fragments: the poly(A)-containing RNA was dissolved in 0.5 ml of LSB, heat-denatured at 100 C for 1 min, and layered on top of a 11-ml linear sucrose gradient (10 to 25% wt/vol) containing 0.01 M Tris-hydrochloride (pH 7.2), 0.01 M NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. A buffer of low ionic strength was used here to minimize aggregation of RNA. Sedimentation was in a Spinco SW41 rotor at 40,000 rpm for 7.5 h at 20 C. A gradient containing labeled chicken cell rRNA markers was always sedimented in parallel. The gradients were divided into 0.28-ml fractions. The radioactivity of an appropriate aliquot from each fraction was measured in 5 ml of toluene-based scintillation fluid containing 10% NCS (Nuclear Chicago, Inc.) in a Tricarb (Packard Inst.) liquid scintillation counter. Sedimentation profiles of viral poly(A)-containing RNA and rRNA markers are shown in Fig. 3-5 and 7-9. RNA fragments differing from each other by 5 to 10*S* increments were pooled. The RNA of each pool was precipitated with 3 vol-

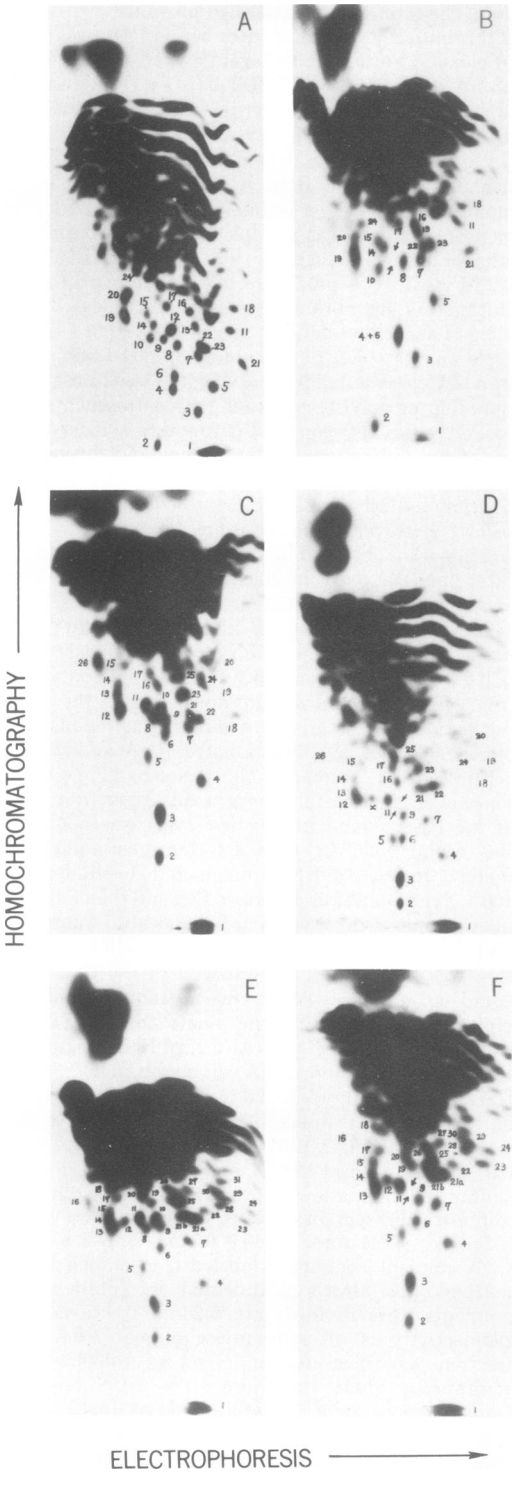


FIG. 1. *Autoradiographs of two-dimensional fingerprint analyses of RNase T_1-digested 60 to 70S [^{32}P]RNAs from different nd and td viruses: PR-B (A), td PR-B (B), PR-C (C), td PR-C (D), B77 (E), td B77 (F). Conditions for the preparation of viral [^{32}P]RNAs, digestion with RNase T_1 and fingerprinting by electrophresis and homochromatography are described in Materials and Methods. The arrows in B, D, F denote the locations where sarcoma spots would appear.*

umes of ethanol after the addition of 5 to 10 μg of TMV RNA carrier and NaCl to 0.15 M.

Fingerprinting, quantification and partial sequence analysis of RNase T_1-resistant oligonucleotides. RNase T_1 digestion of ^{32}P-labeled viral 60 to 70S RNA or of poly(A)-tagged RNA fragments and analysis of the RNase T_1-resistant oligonucleotides by electrophoresis on cellulose acetate strips and by homochromatography on DEAE-cellulose thin-layer plates followed published procedures (2, 3, 5, 10). The electrophoresis buffer used to moisten cellulose acetate strips contained 5% (vol/vol) acetic acid, 0.5% (vol/vol) pyridine, 7 M urea, and 1 mM EDTA (pH 3.6 to 3.8). The same buffer but without urea was used in the electrophoresis tank. Homochromatography was at 55 to 60 C with a 3% (wt/vol) yeast RNA homomixture b (2, 5) containing 7 M urea and 3 mM EDTA (pH 7 to 7.5). This solution had an absorbancy at 260 nm of 600 and an absorbancy at 280 nm of 300. About 12 to 15 ml of this solution was required to develop one thin-layer plate placed into a reservoir of 200 ml. The remaining homomixture was used for the next experiment after addition of fresh homomixture to 200 ml and readjustment of its pH to 7 to 7.5 with acetic acid, if necessary. RNase T_1-resistant oligonucleotides of 4 to 10S poly(A)-tagged RNA fragments and of some unfractionated viral RNAs were fingerprinted with 3% yeast RNA homomixture which had been preincubated at 50 to 60 C for 24 h to decrease the average size of polyribonucleotides. This improved the resolution of all oligonucleotides. Autoradiography of dried chromatograms was with Kodak no-screen X-ray film (NS 54T) with exposures from 0.5 to 21 days depending on the amount of radioactivity present.

RESULTS

Identification of sarcoma-specific oligonucleotides of the RNAs from nd avian sarcoma viruses. To identify sarcoma-specific oligonucleotides in the RNA of a nd sarcoma virus, its RNase T_1-resistant oligonucleotides were fingerprinted. The resulting pattern was then compared to the fingerprint pattern of a corresponding td-deletion mutant (18, 28). It can be seen in Fig. 1 that, in agreement with earlier studies (18, 28), fingerprint patterns of each nd sarcoma virus and its corresponding td virus are very similar. Therefore, identical numbers were used to desginate presumably homologous spots in corresponding patterns of nd and td viruses. Because of the different chromatographic ap-

pearance of the fingerprint patterns of nd and td PR-C RNAs, definitive matching of oligonucleotide spots was based on further compositional analyses as described below (see Table 3).

Each nd virus tested contained two spots with large oligonucleotides (numbered 9 and 12 in PR-B, 8 and 10 in PR-C, and 8 and 10 in B77) which were not present in the fingerprint pattern of the corresponding td virus. These oligonucleotide spots, which will be referred to as sarcoma specific, had homologous locations in fingerprints of each of the three sarcoma viruses compared, suggesting that they also have very similar compositions. Identication of oligonucleotides 9 and 12 of PR-B as sarcoma specific is also consistant with previous analyses of the RNAs of PR-B × RAV-3 recombinants selected from the host range marker of RAV-3 and the transforming marker of PR-B. Each of these recombinants differed from all others in some oligonucleotides, whereas all recombinants shared spots 9 and 12 (3, 10).

With regard to the remaining large RNase T_1-resistant oligonucleotides of nd and corresponding td viruses in Fig. 1, the fingerprint patterns of PR-B and td PR-B, B77, and td B77 were homologous, whereas that of td PR-C differed in one oligonucleotide spot, marked X in Fig. 1D and Table 3, from that of PR-C. This oligonucleotide has the same chromatographic location and almost the same composition as a presumably homologous oligonucleotide, no. 12, of nd/td B77 RNA (see Fig. 1E and F and Tables 2 and 3). In subsequent experiments 4 other independent isolates of td PR-C were fingerprinted. Two of these contained spot X and two did not. Spot 26 of td PR-C (Fig. 1D) was present in the original radioautography with relatively lower intensity presumably due to a technical problem (see also legend to Fig. 5) and became invisible in the photoreproduction (see also legend to Fig. 3). With regard to the other oligonucleotides their patterns were indistinguishable. Since the clones of PR-C and B77 used here have very similar fingerprint patterns (Fig. 1) and their oligonucleotides have similar maps and sequence compositions (see Fig. 6, Tables 2 and 3), the two viruses must be members of a closely related family. Thus the td PR-C analyzed here may have been derived from a variant of our PR-C stock that shares spot X with B77 (see below). Alternatively, spot X might have been acquired by mutation or by recombination with an endogeneous virus during clonal selection of the td PR-C.

The host-range markers of each td virus tested were found to be indistinguishable from those of the parental nd virus (Vogt and Duesberg, unpublished data). In addition we have shown in Fig. 2 that the mass of each td viral

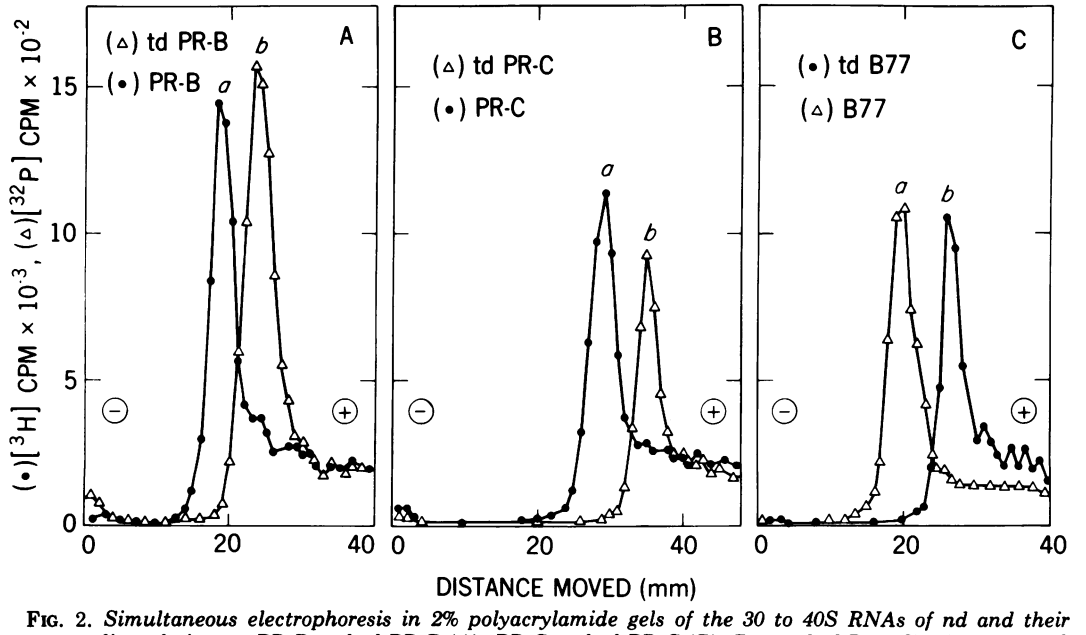


FIG. 2. *Simultaneous electrophoresis in 2% polyacrylamide gels of the 30 to 40S RNAs of nd and their corresponding td viruses: PR-B and td PR-B (A), PR-C and td PR-C (B), B77 and td B77 (C). The 30 to 40S RNA of the nd sarcoma viruses, termed class a RNA (7), had a lower electrophoretic moblity and a higher molecular weight than class b RNA typical of td viruses (7, 8). Conditions for electrophoresis have been described (Materials and Methods, [8]).*

RNA, estimated from its electrophoretic mobility in polyacrylamide gels, was smaller by the expected amount of about 15% (9, 16, 18, 21) than that of the corresponding nd viral RNA. It follows that the additional spot found in the pattern of td PR-C and other, possibly distinct, spots of the td viruses analyzed here do not reflect a larger mass but rather minor genetic variations of the respective viral RNAs as suggested above.

Mapping sarcoma-specific and other large RNase T_1-resistant oligonucleotides on viral RNAs. From analyses of poly(A)-tagged fragments of PR-B RNA, it has been deduced that sarcoma-specific oligonucleotides map near the 3′-poly(A) end of the RNA and that other unique oligonuclotides also have a distinct location on the RNA relative to the poly(A) end (28). We are asking here whether the sarcoma-specific oligonucleotides of PR-C and B77 (Fig. 1) are also located near the poly(A) end of the RNA. Further, it is asked whether a map could be derived for all unique oligonucleotides from fingerprint analyses of poly(A)-tagged RNA fragments ranging in size from 4S to intact 30 to 40S RNA. To answer these questions, [^{32}P]RNAs of PR-B, td PR-B, PR-C, td PR-C, B77, and td B77 were fragmented by alkali (28). Poly(A)-tagged fragments were selected by binding and subsequent elution from membrane filters (Millipore Corp.) and fractionated by sucrose gradient sedimentation (Fig. 3–5). Fragments differing in size by 5 to 10S were selected from such gradients, and each distinct size class of RNA fragments was fingerprinted (Fig. 3–5). The resulting fingerprints demonstrate clearly that an approximately linear relation exists between the size of poly(A)-containing fragments and the number of RNase T_1-resistant oligonucleotides they contain.

In principle, the location of a given oligonucleotide relative to the poly(A) terminus was directly deduced from the length of the smallest fragment from which it could be obtained. The resolution of this method depends on the amount and the purity of a given fragment. The yield of a specific poly(A)-tagged fragment generated by random degradation decreases with increasing length of the fragment. Therefore, the size range of RNA fragments pooled to obtain enough material for a fingerprint is greater for the large than for the small RNA fragments (Fig. 6; a 5S cut between 25 and 30S represents a much larger size range of RNA fragments than a 5S cut between 5 and 10S [25]). As a consequence pools of large fragments are more heterogeneous than those of small fragments. Such heterogenous pools of large fragments will contain some oligonucleotides at nonequimolar ratios. Thus, quantification of the molarities of oligonucleotides (using their known molecular weights; see Tables 1–3) was used to help in mapping oligonucleotides within a pool of RNA fragments. The higher the molarity of an oligonucleotide that first appeared within a given sedimentation cut, the closer it was placed toward the poly(A) end of the map distance first covered by this fragment.

In addition, fragments of a given hydrodynamic pool may be contaminated by diffusion of neighboring fragments, thus increasing the heterogeneity of a given pool. Nevertheless, large oligonucleotides of PR-B, td PR-B, PR-C, B77, and td B77 have been tentatively mapped by this method on the basis of the fingerprints of poly(A)-tagged RNA fragments shown in Fig. 3–5 and 7–9 as well as from others not shown here to yield the schematic maps shown in Fig. 6. It can be seen in Fig. 3–6 that the two sarcoma-specific oligonucleotides of each of the nd

FIG. 3. *Autoradiographic fingerprint analyses of poly(A)-tagged PR-B RNA fragments of various sizes after exhaustive digestion with RNase T_1 as described for Fig. 1. Approximately 25 × 10^6 to 30 × 10^6 counts/min of 60 to 70S [^{32}P]PR-B RNA were partially degraded with Na_2CO_3 at pH 10.8 and 50 C for 8 min to yield RNA fragments with a peak at 12S (G) and for 4 min to yield RNA fragments with a peak at 18S (H). Poly(A)-tagged fragments were selected by two consecutive cycles of binding to and eluting from membrane filters (Millipore Corp.) (Materials and Methods). About 7.3% of the radioactive RNA was recovered as poly(A)-tagged fragements if degradation was for 4 min (H), and 5.2% of the radioactive RNA was recovered if degradation was for 8 min (G). Poly(A)-tagged RNA fragments selected from the 8-min (G) and 4-min degradation mixture (H) were fractionated according to size by sedimentation in sucrose gradients as described in Materials and Methods. The radioactivity was determined from an appropriate aliquot of each gradient fraction (G, H). The poly(A)-tagged viral RNA fragments were divided into several discrete pools (I → VI) indicated by horizontal bars in (G) and (H). Sedimentation coefficients of these pools were estimated from the positions of 28S, 18S and 4S chicken cell RNA standards analyzed in a parallel gradient and indicated in (G) and (H) by arrows. Pools of RNA fragments from the two gradients with the same range of sedimentation coefficients were combined, and the RNA of each pool was fingerprinted. The fingerprints of pools I → VI are shown in A → F, respectively. Some oligonucleotide spots which were still detectable and were numbered in the original autoradiographs are no longer visible in the reproductions shown here and in Fig. 4, 5, 7–9.*

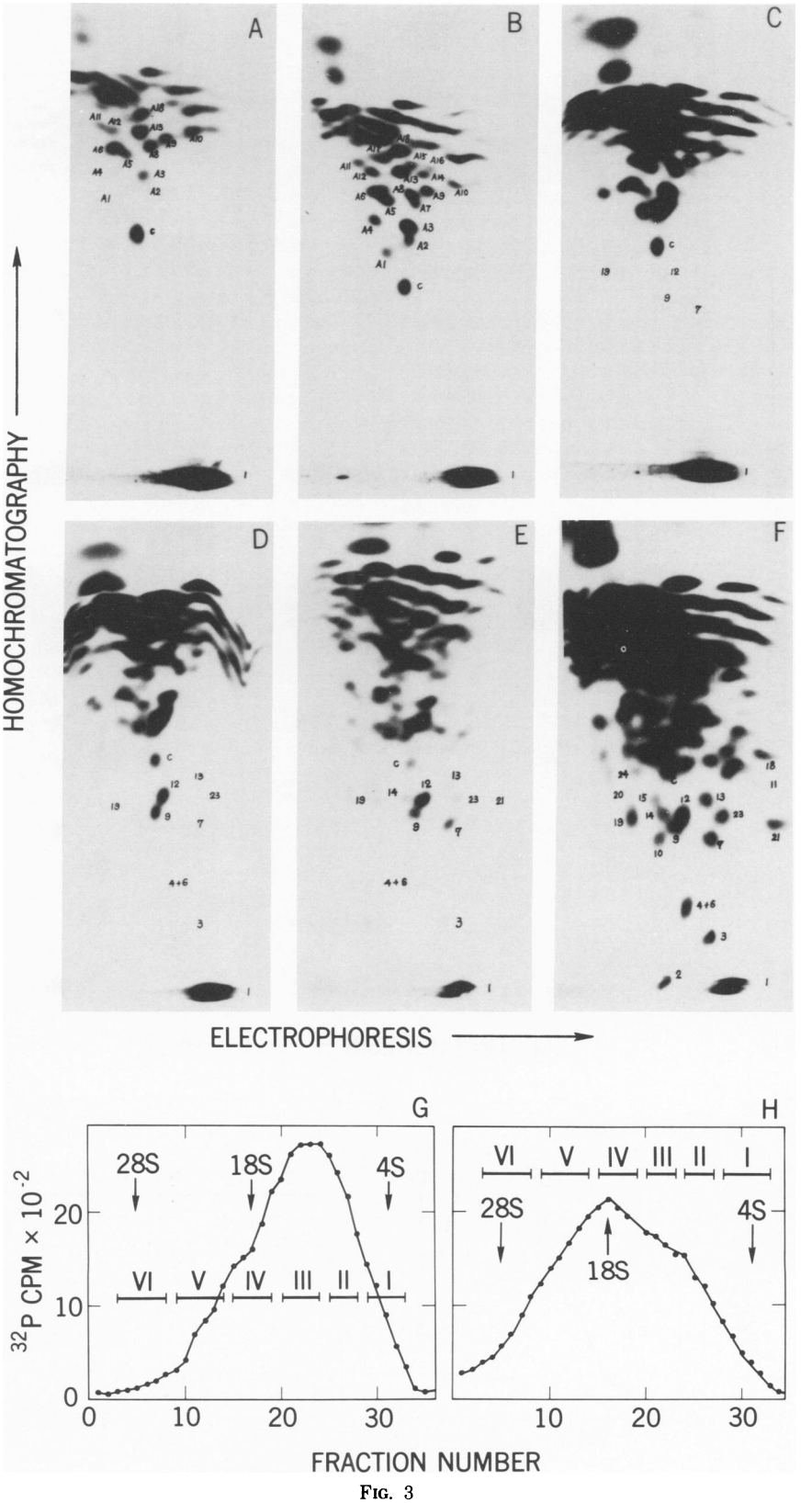
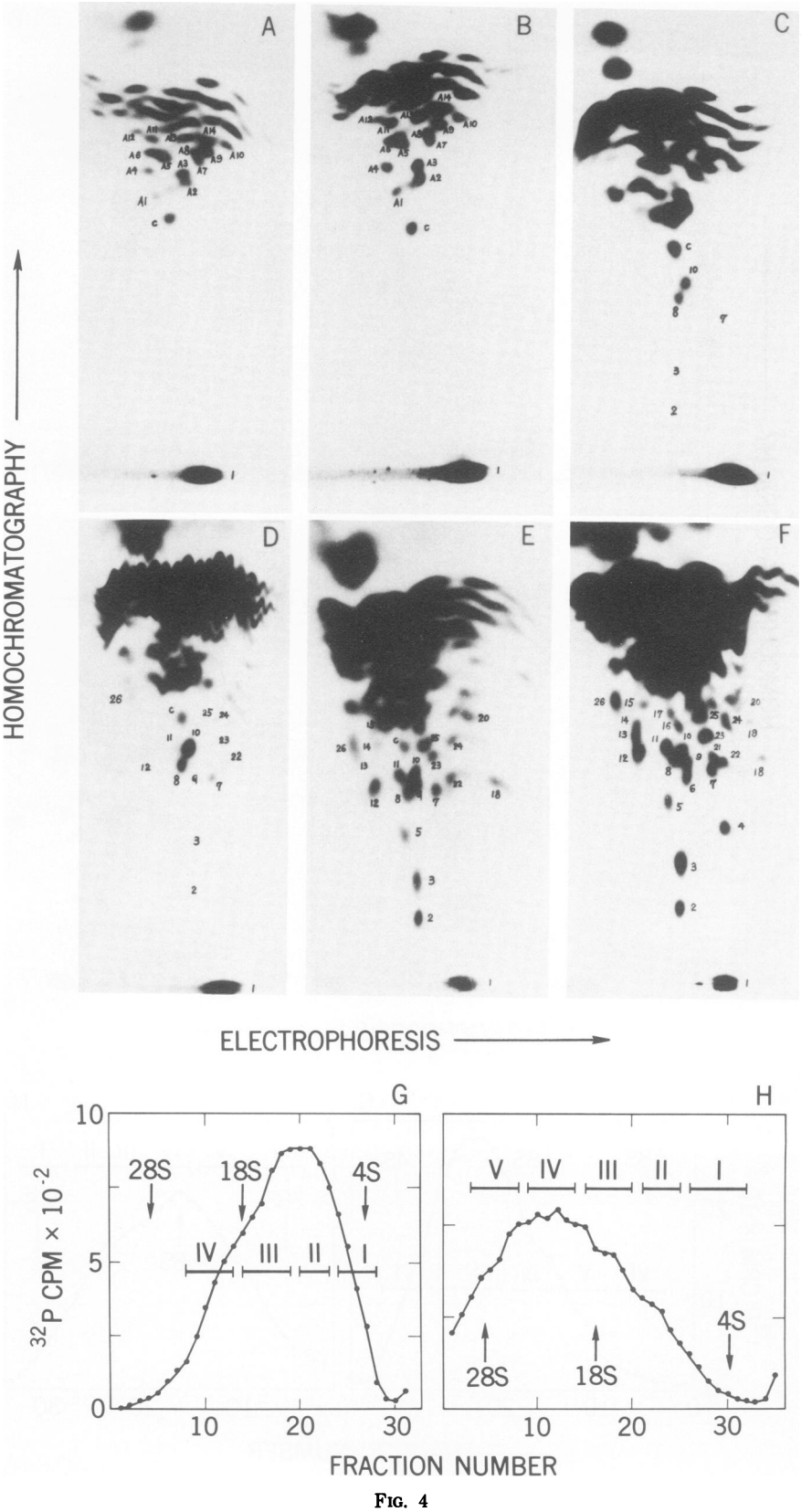

A
B
C
D
E
F
HOMOCHROMATOGRAPHY
ELECTROPHORESIS
G
H
28S
18S
4S
VI
V
IV
III
II
I
^{32}P CPM × 10^{-2}
FRACTION NUMBER

FIG. 3


A
B
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HOMOCHROMATOGRAPHY
ELECTROPHORESIS
G
H
28S
18S
4S
I
II
III
IV
V
^{32}P CPM x 10^{-2}
FRACTION NUMBER

FIG. 4

viruses first appeared in 10 to 15S RNA fragments which have an approximate molecular weight of 200,000 to 400,000 (25). If isolated from a 15 to 20S RNA fragment with an approximate molecular weight of 400,000 to 800,000 (25) or from any larger fragment, the sarcoma-specific oligonucleotides had a constant ratio to reference spots, associated with smaller fragments, such as spot C. Thus, these spots map about 10 to 25% or 300,000 to 800,000 daltons away from the poly(A) terminus of nd viral RNA, which has an estimated total molecular weight of 3×10^6.

Further, it appears that homologous oligonucleotides of the corresponding nd/td pairs of PR-B and B77 also have the same map position on the respective viral RNAs. This implies that these nd/td virus pairs probably have the same gene order, with the exception of the sarcoma-specific sequences.

Compositional analyses of sarcoma-specific and other large oligonucleotides of PR-B, PR-C, and B77. Identification of sarcoma-specific and other oligonucleotides in the experiments described above was based on chromatographic homology between spots of different fingerprint patterns. Assignment of homology between such spots (as in Fig. 1, 3-5) is subject to error, as two different fingerprints of the same RNA never run identically. In addition, homologous oligonucleotide spots of two different RNAs may have different sequences, although they are known to have the same composition and size (2, 5). If an autoradiographic spot contains two oligonucleotides, both oligonucleotides would be mapped at the position of the oligonucleotide closest to the poly(A) end by our method, unless the composition of the respective spot for the presence of one or two oligonucleotides is determined in each size class of RNA fragments. Therefore most large oligonucleotides of viral RNAs and of RNA fragments were subjected to a partial sequence analysis for a more direct identification.

Each oligonucleotide to be analyzed was eluted from the DEAE-cellulose thin layer and digested with RNase A (2, 3, 5, 10). Overlapping regions of adjacent spots were not eluted for such analyses. The RNase A-resistant fragments were resolved by electrophoresis on DEAE paper and, after autoradiographic location, were quantitated in a scintillation counter (3, 10). The RNase A-resistant fragments of the large oligonucleotides of PR-B, PR-C, td PR-C, and B77 are shown in Tables 1-3. The data summarized in these tables served several purposes and allowed the following conclusions to be drawn. (i) All assignments of homology between oligonucleotides made on the basis of their chromatographic properties (compare Fig. 1, 3-5) have been checked and verified. When chromatographic identification of homologous oligonucleotides was uncertain, due to experimental variations between fingerprint patterns as in the case of PR-C and td PR-C RNA (Fig. 1), definitive homologies were determined from the data shown in Table 2.

Moreover, oligonucleotides of relatively small poly(A)-tagged RNA fragments could only be definitively identified by this method. Identification by triangulation with neighboring spots was difficult because of the lack of sufficient marker spots in these fragments. For instance, the sarcoma-specific spots and their neighbors from the short poly(A)-tagged RNA fragments shown in Fig. 3-5 were all checked by this method to confirm their chromatographic identification. Since no discrepancies were observed in compositional analyses of oligonucleotide spots from RNA fragments or from complete viral RNA, the composition of each spot is reported only once (Tables 1-3).

(ii) The sarcoma-specific oligonucleotides no. 9 of PR-B, 8 of PR-C, and 8 of B77 were found to have very similar, perhaps identical, compositions. The same was true for spots 12 of PR-B, 10 of PR-C, and 10 of B77. These latter spots contained two oligonucleotides, albeit at ratios which were not exactly equimolar (Table 1-3). The compositional similarity of these spots is in good agreement with their similar chromatographic distributions in the fingerprints shown in Fig. 1, 3-5. It would follow that the sarcoma-specific sequences of the three viruses tested are very similar and contain three RNase T_1-resistant oligonucleotides.

(iii) With the exception of the sarcoma-

FIG. 4. *Fingerprint analyses of poly(A)-tagged PR-C RNA fragments of various sizes after exhaustive digestion with RNase T_1 as described for Fig. 3. Approximately 20×10^6 counts/min of 60 to 70S [^{32}P]RNA was degraded for 2.5 min yielding 7.5% of the initial radioactivity as poly(A)-tagged RNA fragments selected by membrane filter binding (Fig. 3, Materials and Methods) with a peak at 22S (H). In a second experiment about 8×10^6 counts/min of RNA was degraded for 8 min (Fig. 3) and yielded 5% of the starting radioactivity as poly(A)-tagged RNA fragments with a peak at 12S (G). Five pools of poly(A)-tagged RNA fragments were prepared from each of the two gradients according to their sedimentation ranges as detailed for Fig. 3. Fingerprints of pools I → V are shown in A → E, respectively. (F) shows a fingerprint of undegraded PR-C RNA.*

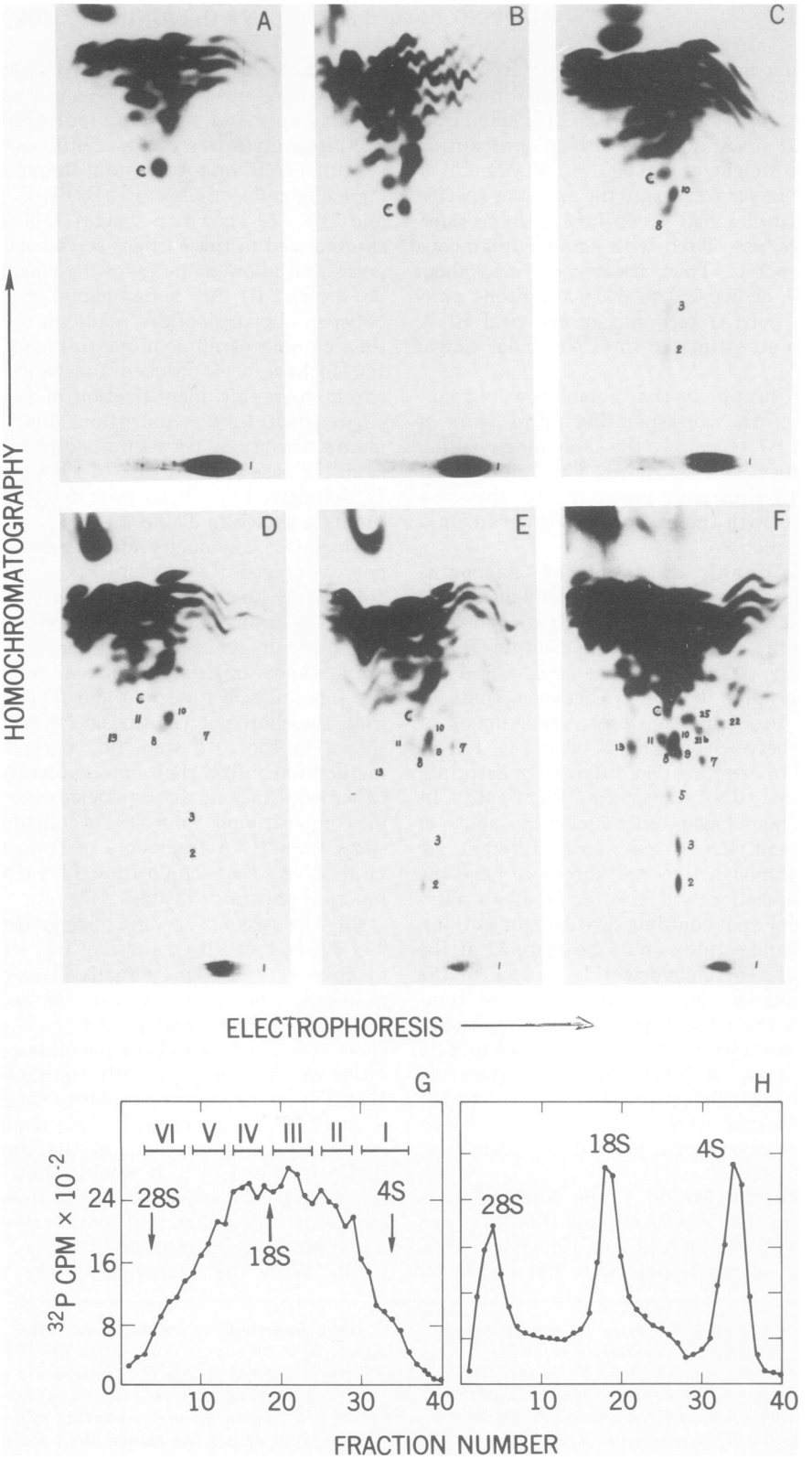
A
B
C
D
E
F
HOMOCHROMATOGRAPHY
ELECTROPHORESIS
G
H
VI
V
IV
III
II
I
28S
18S
4S
^{32}P CPM × 10^{-2}
24
16
8
0
10
20
30
40
FRACTION NUMBER

FIG. 5

specific oligonucleotides all other large oligonucleotides of the nd/td virus pairs of PR-B and B77 which were studied had the same distinctive location on the respective viral RNAs relative to the poly(A) terminus (Fig. 6). Because td viruses yield 5 to 10 times less radioactive RNA than corresponding nd viruses, mapping of td-viral oligonucleotides was based on lesser amounts of radioactivity. Thus, oligonucleotide mapping of td viral RNA was less certain and has not been extended yet to the 5'OH-terminus of the RNA. Nevertheless, it appears that the oligonucleotide maps of the nd and corresponding td viruses, as far as analyzed here, are the same (Fig. 6).

(iv) Comparisons of the map locations (Fig. 6) and the compositional analyses (Tables 1-3) of certain oligonucleotides from the different virus

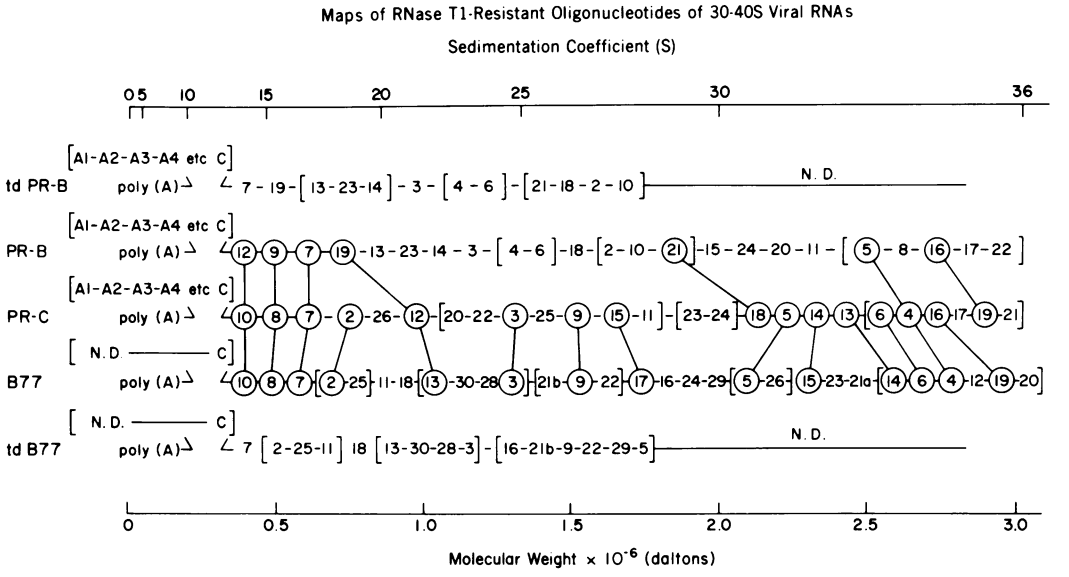

FIG. 6. *Maps of RNase T_1-resistant oligonucleotides of 30 to 40S viral RNAs. RNase T_1-resistant oligonucleotides of td PR-B, PR-B, PR-C, B77, and td B77, numbered as in the fingerprints shown in Fig. 1, 3-5, and 7-9, were arranged linearly relative to the poly(A) end. The map position of a given oligonucleotide was determined from the smallest poly(A)-tagged RNA fragment from which it could be obtained. The relative positions of several oligonucleotides, which first appeared together within a give 5 to 10S cut of poly(A)-tagged fragments (Fig. 3-5), were estimated by quantitating their molarities. The higher the molarity of a given oligonucleotide, the closer it was placed toward the poly(A) end of a given size cut (see text). Oligonucleotides whose relative map positions within a certain sedimentation range could not be estimated are in parentheses. ND, Not determined. The top scale represents the size of the viral RNA or RNA fragments in S values and the bottom scale represents a molecular weight scale derived from the respective S values by Spirin's formula: molecular weight = $1{,}550 \cdot S^{2.1}$ (25). Those oligonucleotides of the three nd sarcoma viruses that have very similar or identical compositions and sequences (Fig. 1, 3-5; Tables 1-3) are circled and connected by vertical lines. The portions of these maps which include the poly(A) segments and the terminal heteropolymeric sequences of about 140,000 daltons, containing the oligonucleotides A1 → A4 and C (in parenthesis), are not strictly proportional to the scales of the remaining RNA segments.*

FIG. 5. *Fingerprint analyses of poly(A)-tagged B77 RNA fragments of various sizes after exhaustive digestion with RNase T_1 as described for Fig. 3. Approximately 15×10^6 counts/min of 60 to 70S [^{32}P]RNA was degraded for 4 min (Fig. 3) and yielding 18% of the starting radioactivity, after one cycle of binding and elution from membrane filters (Millipore Corp.), as poly(A)-tagged fragments with a broad peak of about 16S (G). Six pools of poly(A)-tagged fragments of different sedimentation ranges were prepared as described (Fig. 3) using the chicken cell RNA standards shown in (H). Each poly(A)-tagged RNA pool was subjected to an additional cycle of binding to and elution from membrane filters as described in Materials and Methods. Fingerprints of pools I → VI are shown in A → F, respectively. These fingerprints were done with homomixture not preincubated at 60 C, thus the resolution of smaller oligonucleotides is not as good as in Fig. 3 and 4 where preincubated homomixture was used. Presumably for technical reasons spot C and also spot 8 appeared faint in fragments D and E if compared to the other fragments of this experiment.*

TABLE 1. *RNase T1-resistant oligonucleotides of PR-B*[a]

Oligonucleotide spot no.[b]	RNase A digestion products[c]
1	Poly(A)
2	$UC_2G(AC)_3(AAU)(A_4C)_3$
3	$U_5C_7G(AC)(AU)_2(AAC)(AAU)_2$
4	$U_3C_7(AC)_2(AU)(AAC)(AAG)$
5	$U_4C_4G(AC)_3(AU)_3(A_3C)$
6	$U_5C_6G(AC)(AU)(A_3C)$
7	$U_5C_3G(AC)(AU)(AAC)(AAU)$
8	$U_3C_6(AAG)(A_5C)$
9	$U_4C_6G(AC)_2(AU)(A_3C)$
10	$U_2C_6G(AC)_2(AU)(AAC)$
11	$U_6C_7G(AU)$
12[d]	$U_5C_8G(AC)_4(AU)_2(AG)$
13	$U_6C_3(AC)(A_3C)(A_3G)$
14	$U_2C_4G(AC)_2(AU)(AAC)$
15	$UC_2(AC)_2(AU)(AG)(AAC)$
16	$U_6C_8G(AU)$
17	$U_4C_4(AC)(A_4G)$
18	$U_6C_3G(AU)(AAU)$
19	$UC_7G(AC)_4(AU)(AG)(AAC)(A_4C)$
20	$UC_5(AC)_3(AAG)$
21	$U_6C_2G(AU)_4(AAU)$

[a] Most of these data have been described in an earlier publication (3, 10).

[b] Numbers refer to oligonucleotide spots of the fingerprints shown in Fig. 1A and 3.

[c] Conditions for the elution of RNase T_1-resistant oligonucleotides from DEAE-cellulose with 30% (vol/vol) triethylammonium bicarbonate (pH 10), digestion with RNase A and electrophoretic analysis of the digest on DEAE paper have been described (2, 3, 5, 10).

[d] This spot contained 0.5 to 0.8 (AG) and 1.5 to 2 (AU) residues per G in different experiments.

strains analyzed suggest that homologous, in some cases identical, oligonucleotides are found in homologous map positions of the respective RNAs. Oligonucleotides which fall into this category are connected in Fig. 6. This implies that the virus strains analyzed here probably have very similar gene orders. A particularly close relationship is suggested between the clones of PR-C and B77 used here on the basis of their fingerprints, oligonucleotide compositions, and maps. This is consistent with the known biological (12) and RNA sequence (13, 18, 30) relationships of these viruses.

(v) Finally, the complexities of the RNAs of PR-C and B77 were also determined from the quantitative and qualitative analyses of their oligonucleotides, summarized in Tables 2 and 3, by the methods described earlier (3, 10). The average complexity of PR-C was found to be 3.22×10^6 daltons and that of B77 to be 3.02×10^6 daltons. This is in good agreement with complexity estimates obtained for other strains of avian tumor viruses (3, 4, 10, 22).

Minor compositional variations in oligonucleotides, such as those encountered between presumably homologous spots of PR-C and td PR-C or between the sarcoma-specific spots of the three nd viruses, are within the limits of our experimental accuracy. Hence, these variations may not reflect genuine differences among the oligonucleotides from which they were derived.

The stoichiometry of RNase A-resistant fragments of a few T_1-resistant oligonucleotides was consistently found to be poor. For example, the homologous sarcoma-specific oligonucleotides, no. 12 (PR-B) and no. 10 (PR-C, B77), all contained less than two (AU) and less than one (AG) per G residue. This may be due to the presence of two oligonucleotides in this spot, one of which carries an (AG) sequence partially digested by RNase A, or a (GN) sequence partially resistant to RNase T_1.

Heteropolymeric sequences at the poly(A) terminus of three strains of nd and corresponding td viruses are similar. While fingerprinting poly(A)-tagged fragments of tumor virus RNA of decreasing size and complexity, we observed that as the number of unique large oligonucleotides decreased, a new class of oligonucleotides of intermediate size began to form unique patterns. The largest of these, termed spot C in a previous study (28), and four others termed A1–A4 were associated with poly(A)-tagged fragments of PR-B RNA ranging from 4 to 11*S* (Fig. 7). These fragments lacked both the large sarcoma-specific oligonucleotides described above. Very similar patterns were obtained when analogous poly(A)-tagged fragments of PR-C were subjected to the same type of analysis (Fig. 8). Poly(A)-tagged RNA fragments of B77 RNA also contained spot C, but the resolution of the fingerprints shown in Fig. 9 was not sufficient to determine whether the smaller oligonucleotides were homologous to A1, A2, A3, and A4 seen in Fig. 7 and 8.

These fingerprints (Fig. 9) were the first analyses done with small poly(A)-tagged RNA fragments and were obtained with homomixture not preincubated at 60 C (which improves resolution of all oligonucleotides). The composition of spot C in RNase A-resistant fragments is reported in Table 4.

Control experiments, originally designed to confirm the absence of sarcoma-specific oligonucleotides in poly(A)-tagged fragments of td-viral RNA, indicated that small 4 to 11*S* fragments of td PR-B, td PR-C, and td B77 also contained spot C (Fig. 7–9). Further analyses showed that td PR-B and td PR-C also contained oligonucleotides of intermediate size, which appeared to be homologous to those found in the patterns of nd-viral RNA fragments which had been termed A1–A4 (Fig. 7, 8). The fingerprints of poly(A) fragments of td B77 also contained spot C, but due to insufficient

TABLE 2. *RNase T_1-resistant oligonucleotides of B77*

Oligonucleotide spot no.[a]	Counts/min per spot in total digest of 2.08×10^{6} [b]	RNase A digestion products[c]	No. of nucleotides	Genome complexity (daltons) ($\times 10^{-6}$)[d]
1	18,000	Poly(A)		
2	4,800	$U_6C_9G(AC)_3(AAC)$	25	3.51
3	8,100	$U_6C_{14}G(AC)_3(AU)_2(AAC)$ (AAG) (AAAC)	41	3.41
4	4,000	$U_4C_5G(AC)_3(AU)_3(AAAC)$	26	4.38
5	3,990	$U_3C_7G(AC)_3(AU)$	19	3.21
6	4,070	$U_4C_5(AAG)$ (A_4N)	17	2.85
7	4,500	$U_4C_3G(AC)$ $(AU)_2(AAC)$ (AAU)	20	2.99
8	5,400	$U_3C_5G(AC)_2(AU)$ (A_3C)	17	2.12
9	4,800	$U_2C_3(AC)$ (AAG) (A_4N)	15	2.10
10[e]	5,170	$U_6C_9G(AC)_4(AU)_2(AG)$	30	3.78
11	4,600	$U_2C_3G(AC)_3(A_3N)$	16	2.34
12	3,700	$U_2C_5G(AC)_2(AU)$ (AAC)	17	3.09
13	4,600	$C_3(AC)_4(AU)_{1\text{-}2}$,(AG) (AAC) ($A_4N$)	28	4.10
14	4,200	$UC_6G(AC)_3(A_4N)$	19	3.05
15	4,160	$UC_6(AC)_3(AAG)$	16	2.59
16	5,020	$C_9G(AC)_4(AAC)$	20	2.68
17	3,950	$C_3(AU)$ (AG) (A_5N)	13	2.22
18	7,900	$UC_{10}G_2(AC)_5(AAU)$	26	2.22
19	3,370	$U_3C_4(AC)$ (A_2G)	13	2.60
23	2,970	$U_6C_2G(AC)_2(AU)$ (AAC)	18	4.08
24		$U_6C_{10}G(AU)$		
28		$U_5C_3G(AU)$ (AAU)		
29		$U_5C_2G(AU)$ $(AAU)_2$		

[a] Numbers refer to oligonucleotide spots of the fingerprints shown in Fig. 1E and Fig. 5.

[b] One of two parallel fingerprints was used to determine the total radioactivity of each spot and the total radioactivity present on the DEAE thin-layer plate as detailed previously (3, 10).

[c] The other fingerprint was used to analyze the RNase A-resistant fragments of large oligonucleotides as described (Table 1 and references 3, 10).

[d] Complexity of viral RNA was calculated from each oligonucleotide with the following formula described earlier (3, 10). Complexity RNA (in daltons) = [size oligonucleotide (daltons)·input counts/min of RNA digest]/[counts/min of oligonucleotide]. Average complexity = 3.02×10^{6} daltons.

[e] This spot contained 0.5 to 0.85 (AG) and 1.5 to 2.0 (AU)-residues per G in different experiments.

TABLE 3. *RNase T_1-resistant oligonucleotides of PR-C and td PR-C*

Spot no[a]	Counts/min per spot in total digest of 5.78×10^{5} [b]	RNase A digestion products[c] (PR-C)	No. of nucleotides	Genome complexity in daltons ($\times 10^{-6}$)[d]	Spot no.[a]	RNase A digestion products (td PR-C)
1	7353	Poly(A)	—	—	1	Poly(A)
2	1340	$U_6C_9G(AC)_3(AAC)$	25	3.48	2	$U_6C_{11}G(AC)_3(AAC)$
3	2290	$U_9C_{17}G(AC)_3(AU)_2(AAC)$ (AAG) (A_3C)	47	3.83	3	$U_9C_{15}G(AC)_2(AU)_2(AAC)$ (AAG) (A_3C)
4	1200	$U_4C_4G(AC)_3(AU)_3(A_3C)$	25	3.89	4	$U_4C_4G(AC)_3(AU)_3(A_3C)$
5	875	$U_3C_7G(AC)_3(AU)$	19	4.05	5	$U_3C_6G(AC)_3(AU)$
6	1100	$U_5C_6(AAG)$ (A_4N)	19	3.22	6	$U_5C_6(AAG)(A_5N_2)$
7	1130	$U_4C_3G(AC)$ $(AU)_3(AAC)$ (AAU)	20	3.30	7	$U_3C_3G(AC)(AU)_2(AAC)$ (AAU)
8	1410	$U_4C_5G(AC)_2(AU)$ (A_3C)	20	2.65		
9	870	$U_2C_2(AC)$ (AAG) (A_4N)	15	2.86	9	$U_2C_2(AC)(AAG)(A_3N)$
10[e]	1200	$U_6C_9G(AC)_4(AU)_2(AG)_{1/2}$	19	2.96		
11	1570	$U_3C_4G(AC)_3(AU)_2(AAC)$ (A_3N)	25	2.85	11	$U_2C_4G(AC)_3(AU)_2(AAC)(A_4N)$
12	2050	$C_6(AC)_4(AU)_2(AG)$ (AAC) (A_4N)	28	2.55	ND	
13	1590	$UC_6G(AC)_3(A_3N)$	18	3.87	ND	
14		$UC_5(AC)_3(AAG)$	15		ND	
15	920	$C_3(AU)$ (AG) (A_5N)	13	2.64	ND	
16	720	$U_4C_4(AC)$ (A_4G)	15	2.68	16	$U_3C_4(AC)(A_3G)$
17		$UC_2G(AC)$ (AU) (AAU) (A_3C)	15		17	$UC_2G(AC)$ (AU) (AAU) (A_3C)
18		$U_6C_2G(AU)_4(AAU)$	20		18	$U_5CG(AU)_3(AAU)$
19	900	$U_7C_8G(AU)$	15	3.11	19	$U_5C_7G(AU)$
20	680	$U_4C_3G(AU)$ (AAU)	13	3.57	X	$UC_4G(AC)_2(AU)$ (AAC)

[a] Numbers refer to oligonucleotide spots of the fingerprints shown in Fig. 1C, D, Fig. 4, and Fig. 8. Other footnotes are the same as those for Table 2.

[b c e] See footnotes *b* and *c* of Table 2.

[d] Same as footnote *d* of Table 2, except that the average complexity of PR-C RNA = 3.22×10^{6} dalton.

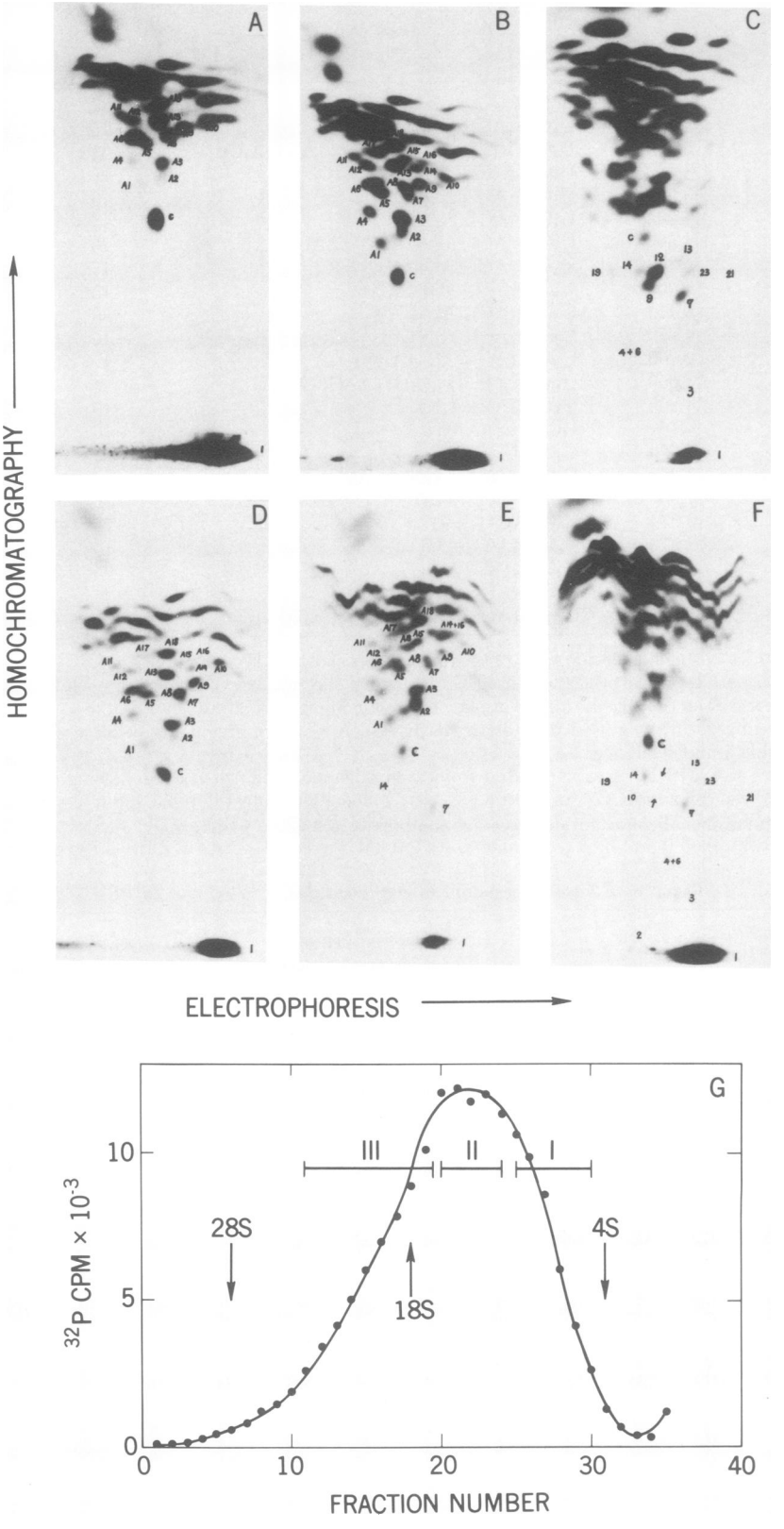
A
B
C
D
E
F
HOMOCHROMATOGRAPHY
ELECTROPHORESIS
G
III
II
I
28S
18S
4S
^{32}P CPM × 10^{-3}
FRACTION NUMBER
0
5
10
0
10
20
30
40

FIG. 7

resolution (see above) smaller oligonucleotides could not be recognized. Moreover, the fingerprint patterns of the remaining, very small RNase T_1-resistant oligonucleotides of 4 to 11S poly(A)-tagged fragments of the nd and td viruses investigated appeared very similar (Fig. 7–9). The chromatographically homologous oligonucleotides termed C, A1 → A4 shared by the 4-11S poly(A)-tagged fragments of PR-B, td PR-B, PR-C and td PR-C also had very similar or identical RNase A-resistant fragments (Table 4).

It follows that the RNAs of the nd and corresponding td viruses investigated here have very similar if not identical heteropolymeric sequences at the poly(A) end. In addition, there is considerable similarity among the RNAs of the three virus pairs analyzed with regard to their poly(A)-terminal oligonucleotides.

Heteropolymeric sequences at the poly(A) end of RSV(−) RNA. To test whether the great similarity of the poly(A)-terminal heteropolymeric sequences between each of the three nd/td virus pairs described above and the considerable similarity of these oligonucleotides among the three pairs is due to a close relationship among these strains or is typical for all avian tumor viuses, the RNA of RSV(−) propagated in quail cells (11) was investigated. It can be

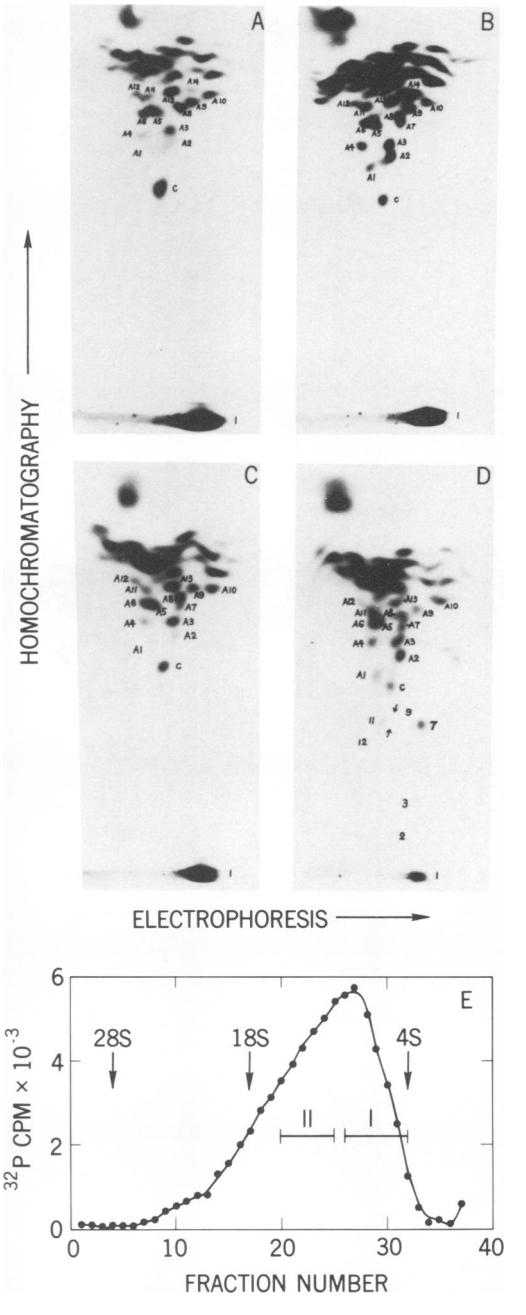

FIG. 8. *Fingerprint analyses of RNase T_1-resistant oligonucleotides of small poly(A)-tagged fragments of PR-C and td PR-C RNAs. 60 to 70S [^{32}P]PR-C RNA was degraded for 8 min with Na_2CO_3 and analyzed as described for Fig. 3. Poly(A)-tagged fragments sedimenting between 4 to 8S (A) and 8 to 13S (B) were fingerprinted after digestion with RNase T_1. About 6 × 10^6 counts/min of 60 to 70S td PR-C [^{32}P]RNA was degraded for 8 min as described for Fig. 3, and 7.3% of the starting radioactivity was recovered after two cycles of binding and elution from membrane filters (Millipore Corp.) (Fig. 4, 7). Sedimentation of the poly(A)-tagged fragments is shown in (E). Two pools of poly(A)-tagged fragments were prepared, I (4 to 10S) and II (10 to 16S). Fingerprint analyses of RNase T_1-digested pools I and II are shown in C and D, respectively. The arrows in (D) indicate the locations where sarcoma-specific spots would appear. Homomixture b was as described for Fig. 7.*

FIG. 7. *Fingerprint patterns of RNase T_1-resistant oligonucleotides of small poly(A)-tagged fragments of PR-B and td PR-B RNAs. 60 to 70S [^{32}P]PR-B RNA was degraded for 8 min with Na_2CO_3 and analyzed as described for Fig. 3. Poly(A)-tagged fragments sedimenting between 4 to 7S (A), 7 to 10S (B), and 15 to 20S (C) were fingerprinted after digestion with RNase T_1. About 9 × 10^6 counts/min of 60 to 70S td PR-B [^{32}P]RNA was partially degraded with Na_2CO_3 at 50 C for 8 min. About 6.6% of the starting radioactivity was recovered after two cycles of binding and eluting from membrane filters (Materials and Methods). Sedimentation of the poly (A)-tagged fragments is shown in (G). Three pools of poly(A)-tagged fragments were prepared: I (4 to 7S), II (8 to 13S), and III (13 to 23S). Fingerprint analyses of RNase T_1-digested pools I → III are shown in D → F. The arrows in F denote the locations where the sarcoma-specific spots would appear. The homomixture b used here was preincubated at 60 C for 24 h.*

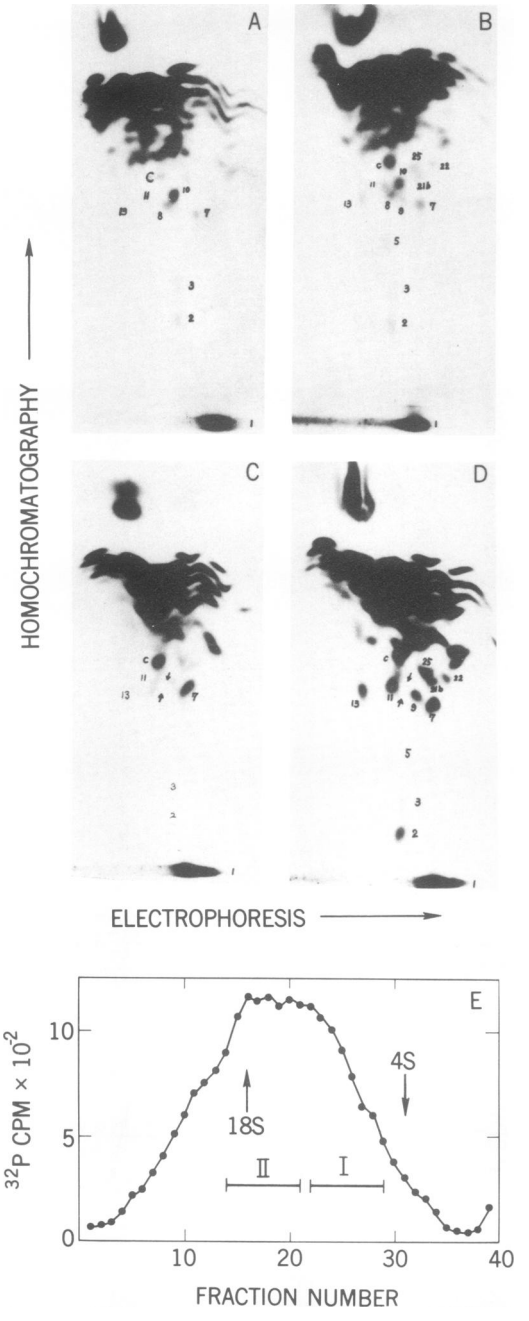

FIG. 9. *Fingerprint patterns of RNase T_1-resistant oligonucleotides of small poly(A)-tagged fragments of B77 and td B77 RNAs. 60 to 70S [^{32}P]B77 RNA was degraded with Na_2CO_3 for 8 min and analyzed as described for Fig. 3. Poly(A)-tagged fragments sedimenting between 15 to 20S (A) (same as Fig. 5D) and 20 to 25S (B) were fingerprinted. About 2.2 x 10^6 counts/min of 60 to 70S [^{32}P]td B77 RNA was partially degraded with Na_2CO_3 at 50 C for 5 min, and 30% of the starting radioactivity was recovered after one cycle of binding and eluting from membrane filters (Materials and Methods). This RNA was sedimented in a sucrose gradient as described for Fig. 3 and two pools, I (5 to 12S) and II (12 to 20S), were prepared (E). The RNA of each pool was ethanol-precipitated and further separated from fragments not containing poly(A) by an additional cycle of binding and eluting from membrane filters (Millipore Corp.). Fingerprint analyses of RNase T_1-digested pools I and II are shown in C and D. Regarding the relatively low intensity of spots C and 8 in frame (A) see comment to Fig. 5. The arrows in (D) indicate the locations where sarcoma-specific spots would appear.*

seen in Fig. 10 that the fingerprint patterns of a 4 to 7S and a 7 to 12S poly(A)-tagged fragment of RSV(−) RNA differ from those of PR-B, PR-C, or B77 (Fig. 7-9). However, a spot homologous to spot C of the three nd/td virus pairs analyzed above is present in the poly(A) fragments of RSV(−) RNA (Fig. 10). Analysis of its RNase A-resistant fragments reported in Table 4 confirmed that this spot was identical or very similar to spot C of the above viruses. The two spots seen on the northeast line above spot C had the following compositions: the lower one termed B (Fig. 10A) contained [U,C,G, (AC), $(AAU)_2$] and the upper one termed A contained [U_3,C_2,G,(AC), (AAU)]. Hence these spots are not related to those termed A1–A4 in the RNAs of the viruses studied above. Spots homologous to the sarcoma-specific oligonucleotides found at low molar ratio in the 10 to 15S poly(A) fragments of the nd viruses described above were not seen in the 7 to 12S fragment of RSV(−) RNA.

We conclude that RSV(−) shares one poly(A)-terminal oligonucleotide, termed C, with the RNAs of nd/td PR-B, PR-C, and B77. The other poly(A)-terminal oligonucleotides appear to be different. [Since there is no corresponding td RSV(−) for comparison we can not deduce from our results which oligonucleotide(s) of RSV(−) is sarcoma-specific.]

DISCUSSION

Conclusions and comparisons concerning oligonucleotide maps of different viral RNAs. (i) Probably all large RNase T_1-resistant oligonucleotides have a distinct location on the viral 30 to 40S RNA relative to the poly(A) terminus. (ii) The three sarcoma-specific oligonucleotides of the three nd viruses analyzed have very similar compositions and map near, but not right at, the poly(A) terminus of viral RNA. They are separated from the poly(A) end by heteropolymeric sequences which they share, partially or completely, with corresponding td viral RNAs. It may be argued that the sarcoma-specific oligonucleotides do not derive from the

sarcoma-specific sequences of nd viral RNA but reflect mutations that affected sequences of td virus RNA which have homologous counterparts in nd virus RNA. This appears unlikely for three reasons. First, a consistently positive correlation was observed between the presence of class *a* RNA in many clonal isolates of several nd virus strains here and earlier (18, 28) and the presence of additional oligonucleotides which were absent from corresponding td viruses. Only in one case (Fig. 2C, and D) did we find an oligonucleotide in a td viral RNA not present in the corresponding nd viral RNA. This was thought to be the result of a mutation. Second, mutations are unlikely to eliminate the same three oligonucleotides from all td viral RNAs which have been studied here. They are equally unlikely to affect only the map distance occupied by sarcoma-specific oligonucleotides. Third, in the case of mutations td virus-specific spots would be expected to occur with equal probability as sarcoma-specific spots. This was not observed. (iii) All homologous RNase T_1-resistant oligonucleotides of nd and corresponding td viruses which were tested also have the same relative location on the respective viral RNAs relative to the poly(A) terminus. This is consistent with the very close genetic relationship between these viruses (14, 18, 20, 21, 26). (iv) Some oligonucleotides of three different nd sarcoma viruses (PR-B, PR-C and B77), which map in similar positions on the respective viral RNAs, also have similar or identical chromatographic properties and chemical compositions, suggesting that these strains of avian tumor viruses have the same gene order. It appears from the data summarized in Fig. 6 that homologous oligonucleotides shared between PR-C and B77 show an approximately random distribution over the respective RNAs. Comparisons between the maps of PR-B and those of B77 and PR-C show a gap of homologous oligonucleotides at a map distance from about 1×10^6 to 1.7×10^6 daltons from the poly(A) end. Considering that PR-C and B77 belong to subgroup C and Pr-B to subgroup B of avian tumor viruses, it may be speculated that subgroup-specific oligonucleotides might map in this map distance. This is compatible with preliminary experiments in which envelope-specific oligonucleotides were mapped between 0.8×10^6 to 1.6×10^6 daltons away from the poly(A) end of Schmidt-Ruppin RSV RNA. (L. H. Wang, P. H. Duesberg, S. Kawai, and H. Hanafusa, unpublished data). (v) The poly(A) terminal heteropolymeric oligonucleotides of each nd/td virus pair studied here are very similar. In addition terminal oligonucleotides of these three nd/td virus pairs are similar to each other. (vi) Poly(A)-tagged fragments of up to 12*S* from RSV(−), propagated in quail cells, shared only oligonucleotide C with the analogous poly(A)-terminal fragments of the three nd/td virus pairs described above. In preliminary experiments we have observed spot C also in Schmidt-Ruppin RSV and in an envelope-defective (11) and a td deletion mutant of this virus (L. H. Wang, P. H. Duesberg, S. Kawai, and H. Hanafusa, unpublished data). Therefore it is likely that spot C derives from a very terminal heteropolymeric sequence shared by all avian tumor viruses tested so far.

Location of sarcoma-specific sequences on the RNAs of nd viruses and the size of the poly(A)-terminal heteropolymeric sequences shared by nd and td viruses. The 10*S* poly(A)-tagged RNA fragments of the nd/td viruses which were studied here were indistinguishable by fingerprinting. Such a 10*S* poly(A)-tagged viral RNA fragment consists of about 60,000 daltons of poly(A) and 140,000 daltons of heteropolymeric sequences. It cannot be decided

TABLE 4. *Composition[a] of RNase T_1-resistant oligonucleotides of small poly(A)-tagged viral RNA fragments*

Virus	Spot no.[b]				
	C[c]	A1	A2	A3	A4
PR-B	G(AC) (AU) (AAU) (A_3C)	UC_3(AG) (AAU)	$U_4C_4G_2(AC)_2(AAU)_2(A_3C)$	$U_4C_6G_2(AC)_3$(AU) (AAU)$_2$ (AAG)	C_3(AAG) (A_3N)
td PR-B	G(AC) (AU) (AAU) (A_3C)	UC_3(AG) (AAU)	$U_3C_3G_2(AC)_2(AAU)_2(A_3C)$	$U_4C_6G_2(AC)_3$(AU) (AAU)$_2$ (AAG)	C_3(AAG) (A_3N)
PR-C	G(AC) (AU) (AAU) (A_3C)	UC_3(AG) (AAU)	$U_4C_3G_2(AC)_2(AAU)_2(A_3N)$	$U_4C_6G_2(AC)_2(AAU)_2$	C_2(AAG) (A_3N)
td PR-C	G(AC) (AU) (AAU) (A_3N)	UC_4(AG) (AAU)	$U_2C_3G_2(AC)_2(AU)_3$(AAC) (AAU)	$U_6C_6G_2(AC)_3(AU)_2(AAU)_2$ (AAG)	C_3(AAG) (A_3N)
B77	G(AC) (AU) (AAU) (A_3N)				
RSV(−)	G(AC) (AU) (AAU) (A_3N)				

[a] Compositions of RNase T_1-resistant oligonucleotides are reported as RNase A-resistant fragments obtained as described in Table 1.

[b] Spot numbers refers to oligonucleotide spots of the fingerprints shown in Fig. 7–10.

[c] Spot C may contain two oligonucleotides.

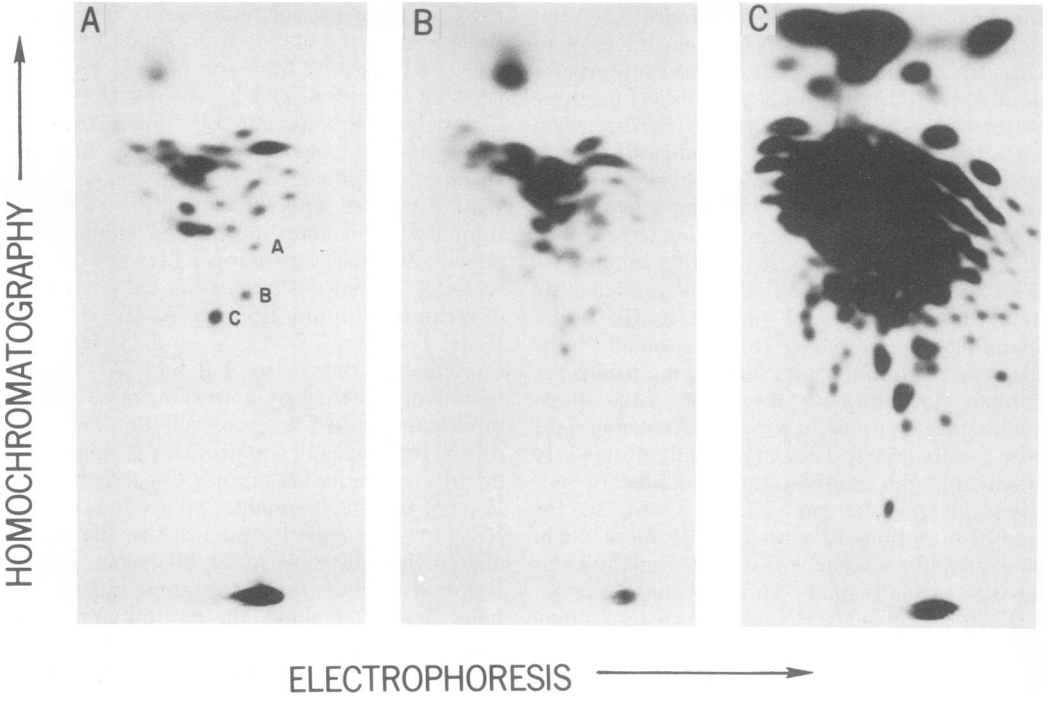

FIG. 10. *Autoradiographic fingerprint analyses of RSV(−) RNA: (A) 4 to 7S, (B) 7 to 12S poly(A)-tagged RNA fragments, (C) 60 to 70S viral [^{32}P]RNA after digestion with RNase T_1. About 3.3 × 10^6 counts/min of 60 to 70S [^{32}P]RNA were degraded with Na_2CO_3 for 8 min as described for Fig. 4. 7.5% of the starting radioactivity was recovered after two cycles of binding and eluting from membrane filters (Materials and Methods). The RNA fragments was resolved by sucrose gradient sedimentation as described for Fig. 3–5. A 4 to 7S pool (A) and 7 to 12S pool (B) were fingerprinted after exhaustive digestion with RNase T_1. A, B, C denote three oligonucleotides in frame (A) which were eluted to determine their RNase A-resistant fragments (see text). The fingerprint shown in (C) is from a previous publication (11).*

from our data whether these terminal heteropolymeric sequences of nd viruses are completely or only partially identical to those of corresponding td viruses.

Sarcoma-specific oligonucleotides were first recovered from 10 to 15*S* poly(A)-tagged RNA fragments of PR-B, PR-C, and B77 and were fully recovered from 15 to 20*S* RNA fragments of these viruses. Thus, sarcoma-specific oligonucleotides are associated with 12 to 20*S* poly(A) fragments ranging from 300,000 to 800,000 daltons. Given a mass of 300,000 to 450,000 daltons for sarcoma-specific sequences of nd viruses (9, 16, 18, 21) and assuming that all sarcoma-specific sequences are clustered together (as the three sarcoma-specific oligonucleotides are), we estimate that the sarcoma-specific sequences start at about 200,000 daltons (6.6%) and extend to about 600,000 daltons (20%) from the poly(A) end of nd viral RNA.

Nature of the poly(A)-terminal sequences shared by nd and corresponding td viruses. The finding of at least one common oligonucleotide, C, in the poly(A)-terminal RNA fragments of three nd/td virus pairs propagated in chicken cells and of RSV(−) propagated in quail cells (and in Schmidt-Ruppin RSV, unpublished observation), suggests that this oligonucleotide may be part of a sequence common to avian tumor viruses. It could function as a specific signal for the addition of poly(A) to the RNA. It may also be complementary to a specific viral integration site of cellular DNA.

Besides oligonucleotide C, the poly(A)-terminal fragments of the two nd/td virus pairs had some but not all other oligonucleotides in common (Table 4). However, the oligonucleotides of an analogous poly(A) fragment of RSV(−) were different from those of the nd/td virus paris. This suggests that the terminal heteropolymeric sequences of the avian tumor viruses tested consist of strain- or subgroup-specific components, in addition to possible group-specific components containing oligonucleotide C. Obviously more virus strains propagated in different host cells have to be investigated to obtain a definite answer to these questions.

How are deletion mutants generated? Nonconditional deletion mutants defective in transforming functions have been derived frequently

from nd sarcoma viruses, whereas those defective in replicative functions (termed rd viruses) have been obtained only in a few cases (11, 15). One possible explanation of this postulates that different deletion mechanisms account for the generation of nonconditional rd and td viruses. Since sarcoma-specific sequences are located near the poly(A) end of the nd viral RNAs tested, internal initiation of RNA-dependent proviral DNA synthesis or early termination of DNA-dependent viral RNA synthesis may be possible mechanisms to delete sarcoma-specific sequences. If either one of these transcriptional processes could terminate or initiate at random, defective viral RNAs ranging from 30 to 40*S* to small polynucleotides would be expected. The finding that the known td viral RNAs have nevertheless a 30 to 40*S* RNA of rather uniform size, termed class b RNA (7), could be explained by additional assumptions: transcriptional deletions may be frequent and may generate a wide spectrum of defectives. Nd viruses with partially deleted sarcoma sequences, containing RNAs smaller than nd but larger than td viruses, may be subject to repeated cycles of deletions. Subsequent selection favors defectives which can replicate unaided by helper viruses, e.g., the td viruses. Thus their RNA may define the lower limit of a self-replicating avian tumor virus which is consistent with the observation that td viruses contain the smallest RNAs of independently replicating avian tumor viruses (7, 8, 20).

However, in view of the observations described here that nd and corresponding td viruses terminate in a very similar, perhaps identical, heteropolymeric sequence, additional assumptions have to be made to explain transcriptional deletion of sarcoma sequences. One possible assumption would postulate that the ultimate heteropolymeric sequences of nd and td viruses are cellular sequences, representing a specific cellular integration site, which is compatible with some recent observations on the RNA of B77 propagated in different hosts (24).

Alternatively, some or all of the terminal heteropolymeric sequences, common to nd and corresponding td viruses, could represent a repeated intercistronic signal which separates the major functional genes (e.g., gag, pol, env, and onc, [1]) of avian tumor viruses. This sequence would then occur several times in tumor virus RNA. This would explain most readily the relative ease with which deletions of transforming functions occur (14, 20, 26) and why nd and corresponding td viruses terminate in the same heteropolymeric sequences. However, it remains to be determined whether the ultimate heteropolymeric sequences of nd and their td viruses are indeed identical or only similar and whether these sequences are represented repeatedly in viral RNA.

In contrast to the transcriptional mechanisms discussed above, deletion of transforming functions could follow a completely different mechanism perhaps involving recombinational events of proviral DNA. Further analyses of the RNAs of different avian tumor viruses should help to decide between these possibilities.

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LITERATURE CITED

1. Baltimore, D. 1974. Tumor viruses: 1974. Cold Spring Harbor Symp. Quant. Biol. **39**:1187-1200.
2. 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 and Row, New York.
3. 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.
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. Brownlee, G. G., and F. Sanger. 1969. Chromatography of ^{32}P-labeled oligonucleotides on thin layers of DEAE-cellulose. Eur. J. Biochem. **11**:395-399.
6. 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.
7. Duesberg, P. H., and P. K. Vogt. 1970. Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses. Proc. Natl. Acad. Sci. U.S.A. **67**:1673-1680.
8. Duesberg, P. H., and P. K. Vogt. 1973. RNA species obtained from clonal lines of avian sarcoma and from avian leukosis virus. Virology **54**:207-218.
9. 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 RNA species. J. Virol. **12**:594-599.
10. Duesberg, P. H., P. K. Vogt, K. Beemon, and M. Lai. 1974. Avian RNA tumor viruses: mechanism of recombination and complexity of the genome. Cold Spring Harbor Symp. Quant. Biol. **39**:847-857.
11. Duesberg, P. H., S. Kawai, L. H. Wang, P. K. Vogt, H. M. Murphy, and H. Hanafusa. 1975. RNA of replication-defective strains of Rous sarcoma virus (RSV). Proc. Natl. Acad. Sci. U. S. A. **72**:1569-1573.
12. Duff, R. G., and P. K. Vogt. 1969. Characteristics of two new tumor virus subgroups. Virology **39**:18-30.
13. Kang, C-Y., and H. M. Temin. 1973. Lack of sequence homology among RNAs of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and chicken endogenous RNA-directed DNA polymerase activity. J. Virol. **12**:1314-1324.
14. Kawai, S., and H. Hanafusa. 1972. Genetic recombination with avian tumor viruses. Virology **49**:37-44.

15. Kawai, S., and T. Yamamoto. 1970. Isolation of different kinds of non-virus producing chick cells transformed by Schmidt-Ruppin strain (subgroup A) of Rous sarcoma virus. Jpn. J. Exp. Med. **40**:243-256.
16. Kung, H. J., J. M. Bailey, N. Davidson, P. K. Vogt, M. O. Nicolson, and R. M. McAllister. 1974. Electron microscope studies of tumor virus RNA. Cold Spring Harbor Symp. Quant. Biol. **39**:827-834.
17. Lai, M. M-C., and P. H. Duesberg. 1972. Adenylic acid-rich sequences in RNAs of Rous sarcoma virus and Rauscher mouse leukaemia virus. Nature (London) **235**:383-386.
18. Lai, M. M-C., P. H. Duesberg, J. Horst, and P. K. Vogt. 1973. Avian tumor virus RNA: a comparison of three sarcoma viruses and their transformation-defective derivatives by oligonucleotide fingerprinting and DNA-RNA hybridization. Proc. Natl. Acad. Sci. U.S.A. **70**:2266-2270.
19. Mangel, W. F., H. Deluis, and P. H. Duesberg. 1974. Structure and molecular weight of the 60-70S RNA and the 30-40S RNA of the Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **71**:4541-4545.
20. Martin, G. S., and P. H. Duesberg. 1972. The *a*-subunit in the RNA of transforming avian tumor viruses. I. Occurrence in different virus strains. II. Spontaneous loss resulting in nontransforming variants. Virology **47**:494-497.
21. Neiman, P. E., S. E. Wright, C. T. McMillin, and D. MacDonnel. 1974. Nucleotide sequence relationships of avian RNA tumor viruses: measurements of the deletion in a transformation-defective mutant of Rous sarcoma virus. J. Virol. **13**:837-846.
22. Quade, K., R. E. Smith, and J. L. Nichols. 1974. Evidence for common nucleotide sequences in the RNA subunits comprising Rous sarcoma virus 70S RNA. Virology **61**:287-291.
23. Quade, K., R. E. Smith, and J. L. Nichols. 1974. Polyriboadenylic acid and adjacent nucleotides in Rous sarcoma virus. Virology **62**:60-70.
24. Shoyab, M., P. D. Markham, and M. Baluda. 1975. Host-induced alteration of avian sarcoma virus B77 genome. Proc. Natl. Acad. Sci. U.S.A. **72**:1031-1035.
25. Spirin, A. S. 1963. Some problems concerning macromolecular structure of RNA. Progr. Nucleic Acid. Res. **1**:301-345.
26. Vogt, P. K. 1971. Spontaneous segregation of nontransforming viruses from cloned sarcoma viruses. Virology **46**:939-946.
27. Wang, L. H., and P. H. Duesberg. 1973. DNA polymerase of murine sarcoma-leukemia virus: lack of detectable RNase H and low activity with viral RNA and natural DNA templates. J. Virol. **12**:1512-1521.
28. Wang, L. H., and P. H. Duesberg. 1974. Properties and location of poly(A) in Rous sarcoma virus RNA. J. Virol. **14**:1515-1529.
29. Weissmann, C., J. T. Parsons, J. W. Coffin, L. Rymo, M. A. Billeter, and H. Hofstetter. 1974. Studies on the structure and synthesis of Rous sarcoma virus RNA. Cold Spring Harbor Symp. Quant. Biol. **39**:1043-1056.
30. Wright, S. E., and P. E. Neiman. 1974. Base-sequence relationships between avian ribonucleic acid endogenous and sarcoma viruses assayed by competitive ribonucleic acid-deoxyribonucleic acid hydridization. Biochemistry **13**:1549-1554.