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"Fingerprinting" high molecular weight RNA by two-dimensional gel electrophoresis: application to poliovirus RNA

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ABSTRACT

Conditions are described under which complete RNase T1 digests of high molecular weight RNA can be separated into numerous components by two-dimensional gel electrophoresis. Small and large oligonucleotides (n = 1 - 200) can be resolved without losses. The procedure yields fingerprints which are diagnostic for a particular species of RNA and an index of its purity as will be shown for the genomes of poliovirus type 1 and 2.

INTRODUCTION

Sequence analyses of nucleic acids depend upon efficient methods to separate fragments of the nucleic acid. These fragments may be mononucleotides, oligonucleotides or polynucleotides as long as 200 bases. Several procedures have been developed which separate fragments by adsorption, ionexchange, gel filtration, electrophoretic mobility or combinations thereof. Individual fractionation procedures, however, are usually limited to a certain size class of digestion products of the nucleic acid.

DeWachter and Fiers (1) have recently used two-dimensional gel electrophoresis to separate large fragments of high molecular weight RNA. We have modified their procedure to facilitate the simultaneous separation of mononucleotides and large oligonucleotides. As will be described below, our method is suitable for the preparation of unique "fingerprints" diagnostic for a RNA of high molecular weight. Since all separated products can be recovered with little or no loss, the method can be applied for sequence studies and the determination of the molecular weight of the RNA (2). We shall demonstrate the power of the procedure with fingerprints of the genomes of two virus strains, polio type 1 and type 2.

MATERIALS AND METHODS

Preparation of Virus and Viral [^{32}P]RNA. Poliovirus, type 1 (Mahoney) was grown in a suspension of HeLa S3 cells as previously described (3). Preparation of ^{32}P-labeled virus was carried out as published (4) except that the infected cells were incubated at a concentration of 6.7 x 10^6 cells/ml with carrier-free phosphate-32 at 240 µc/ml. Under these conditions the yield of labeled virus from 1.65 x 10^9 cells was 6 - 9 x 10^8 cpm at 5 - 7 x 10^5 cpm/µg (2). Poliovirus type 2 (vaccine strain P2l7ch2ab), which we obtained from Dr. Londberg-Holm, was labeled under similar conditions. Its purification, however, was modified due to our observation that type 2 virus aggregates and is partially degraded in the presence of 0.5% sodium dodecylsulfate (SDS) in 0.1 M NaCl. A similar observation has been reported for the Brunhilde strain of poliovirus, type 1 (5). Cytoplasmic extracts of infected HeLa cells were, therefore, made 0.3 M in NaCl and 1% in sarcosyl (5). The virus was then pelleted by centrifugation and purified in CsCl gradients (2). Viral RNA was extracted from virions with SDS at pH 3.8 (6,7) or by treatment with phenol/chloroform in 0.5% SDS at pH 7.5 and further purified by sucrose density gradient centrifugation (8). 8.5 µg of viral [^{32}P]RNA were digested with 5 units RNase T1 in 50 µl Tris·HCl, pH 7.5 for 30 min. at 37°C.

Slab gel electrophoresis: First Dimension. Electrophoresis in the first dimension was carried out in a small slab gel apparatus (Fig. 1A), and in the second dimension in a large slab gel apparatus (Fig. 1B) which was equipped with a cooling mantle. Both instruments were built in our laboratory.

Preparation of the slab gel of the first dimension (8% polyacrylamide, 0.1% bisacrylamide) was as follows: 3.2 g acrylamide (recrystallized from chloroform) and 0.04 g N, N'-Methylene bisacrylamide were dissolved in 26.6 ml 9 M urea (the urea solution was filtered through a layer of silicic acid to remove impurities from reagent grade urea). The pH of the solution was adjusted with a saturated solution of citric acid to 3.3 and distilled water was added to a total volume of 38.5 ml. The solution was then mixed with 0.15 ml of 6 mM $FeSO_4 \cdot 7H_2O$, 1.2 ml of 0.05 M ascorbic acid and 0.15 ml of 3% hydrogen peroxide and poured between the glass plates of the electrophoresis apparatus (space between glass plates: 1.6 mm; for further details, see Fig. 2A). After insertion of an appropriate spacer for slot formation (size of the slot: 2 cm deep, 1.5 cm wide) the polymerization was allowed

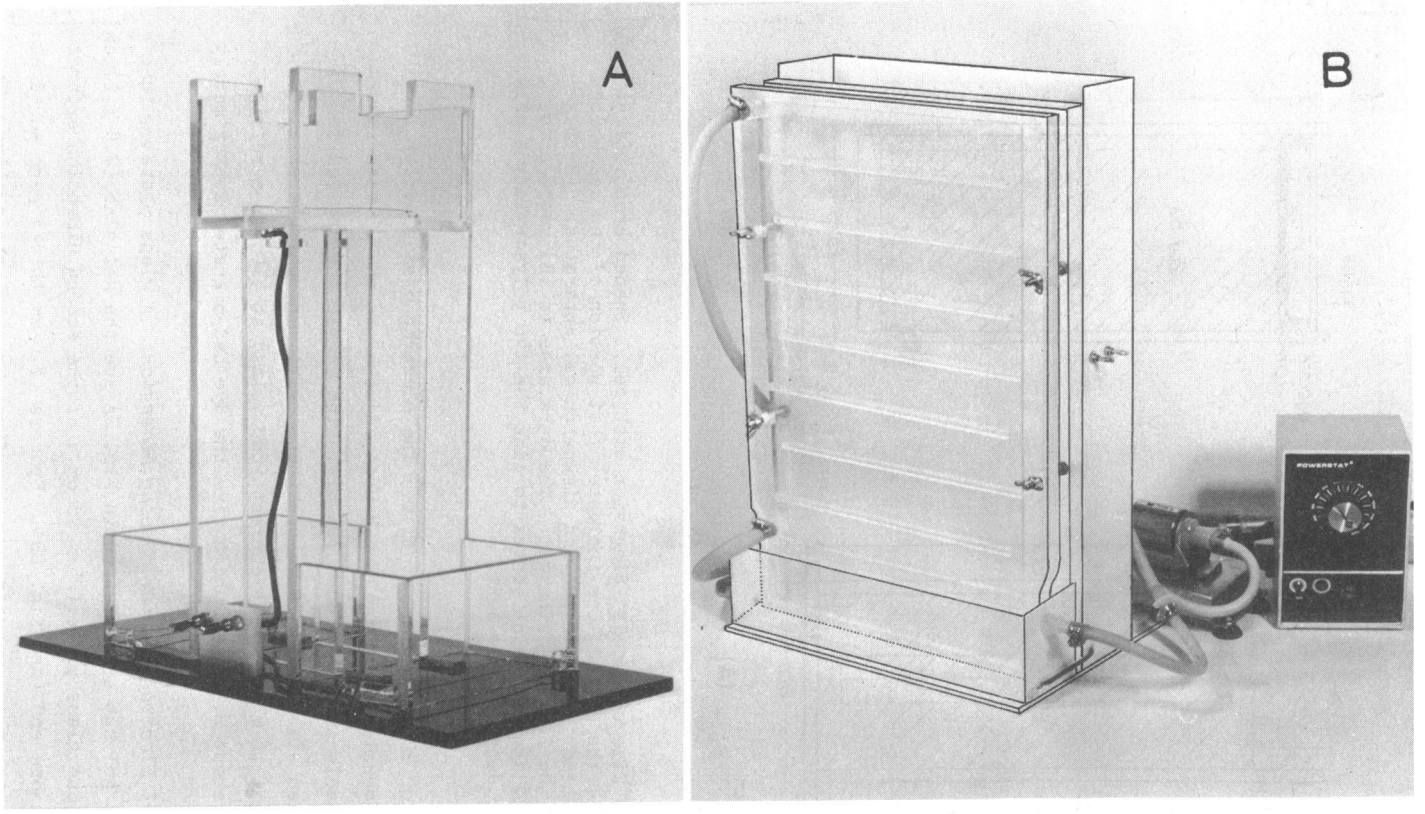

Fig. 1. (A) 1st dimension gel apparatus which can accomodate two gels. (B) 2nd dimension gel apparatus with buffer circulation pump. For dimensions of the gels see Fig. 2.

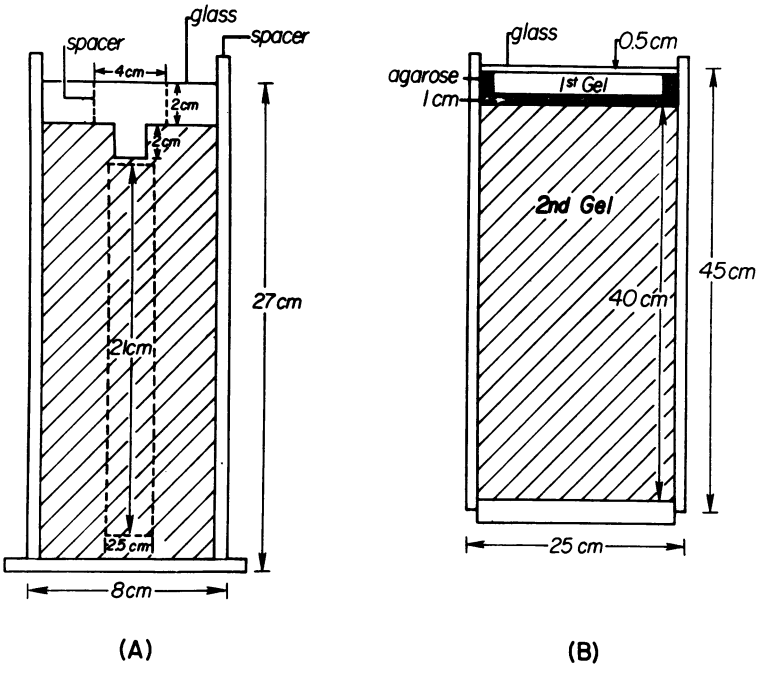

Fig. 2. (A) Size of the 1st dimension slab gel. Shaded area indicates gel; the dotted line indicates that portion of the gel which is transferred to the 2nd dimension gel apparatus. (B) Size of the 2nd dimension gel. The dark area in (B) represents 0.8% agarose which serves to link the two polyacrylamide gels. For details, see text.

to proceed for 20 to 30 min. at 37°C. The electrophoresis buffer for the first dimension consisted of 6 M urea adjusted to pH 3.3 with saturated citric acid (total volume: 700 ml). The sample was applied in 40% glycerol mixed with 3 µl of 0.2% bromphenol blue. Electrophoresis was carried out at 16 mA constant current (600 volts) at 4°C (in a refrigerator) for about 2.5 hr by which time the dye had migrated approximately 12 cm.

Slab gel Electrophoresis: Second Dimension. A glass plate was then removed, the gel cut as indicated in Fig. 2A and the gel slab (2.5 x 21 cm) placed onto one glass plate (25 x 45 cm.) of the second dimension apparatus, 0.5 cm. from one end (see Fig. 2B). Spacers (1.6 mm thick) were added and the second glass plate mounted onto the first by steel paper clips. The

assembly was made leak proof (see below) through application of small amounts of silicon grease to the spacers prior to their positioning. (Silicon grease can easily be removed with 70% ethanol).

The slab gel for the second dimension (22% acrylamide, 1.5% bisacrylamide) was prepared as follows: 44 g acrylamide and 3 g bisacrylamide were dissolved in 20 ml 0.4 M Tris·citrate, pH 8.1, and the solution brought to 200 ml with distilled water. 0.14 ml N,N,N',N'-tetramethylethylene diamine and 0.14 ml 10% ammonium persulfate were added. The solution was then poured between the two glass plates up to the level of 1 cm from the first dimension gel (1st-D gel; Fig. 2B). The glass plates were placed into a waterbath (tap water temperature, approximately 14°C), the water in the bath being level with the solution between the glass plates. The waterbath prevents leakages and assures uniform polymerization by preventing convection (due to the exothermic reaction) during gel formation. Polymerization was complete in 20 to 30 min. Before polymerization 1 ml water is layered onto gel forming solution.

Finally, the space surrounding the 1st-D gel (Fig. 2B) was filled with a freshly boiled solution of 0.8% agarose in 0.04 M Tris·citrate, pH 8. The agarose provides excellent junction between the two slab gels (under our conditions, the solution of the 2nd-D gel did not polymerize well in the area of junction with the 1st-D gel presumably because of the urea in the 1st-D gel).

Electrophoresis was carried out at 700 V (constant voltage), 40 mA for 16 hr. at 14°C, after which time the dye had migrated 20 cm. Electrophoresis buffer for the 2nd dimension was 2200 ml of 0.04 M Tris·citrate, pH 8. Migration of nucleotides may be upwards or downwards. We now run the gels routinely downwards. The buffer was circulated by a small pump (Smith Appliance Corp.).

Autoradiography: One of the two glass plates was removed and three ^{32}P-labeled markers (3 mm long polyethylene tubings [Intramedic, I.D. 0.06 inch] filled with 6 - 9 x 10^3 cpm of ^{32}P-containing solution, sealed on each end with hot tweezers) were placed into three corners of the gel. The gel was covered with a polyethylene film ("Saran wrap") and subsequently with X-ray film (Kodak SB 54). Autoradiography was carried out at 4°C for various lengths of time depending upon the amount of radioactivity applied to the gel. 5 x 10^6 cpm did produce good autoradiographs after 16 hr. Small oligonucleotides do not diffuse appreciably even after 3 days.

Elution of Radioactive Materials. Individual spots were cut out

with surgical blades; the gel was pulverized in a 5 ml tissue culture tube (Falcon) with a glass rod, mixed with 1 ml H_2O, vigorously shaken (on a "Vortex" mixer) for 15 min. and sedimented at 2000 rpm for 5 min ("Sorvall" Centrifuge). Extraction of the pellet was repeated once or twice. Yields were 95 - 97%. For further analyses, the aqueous solution was lyophilized to dryness.

Determination of the Base Composition of Oligonucleotides. Eluted oligonucleotides were incubated with RNase A (0.1 mg/ml) in 30 µl 0.05 M Tris-HCl for 30 min. at 37°C. The products were separated by electrophoresis (4V/cm for 4 hr) on DEAE-paper at pH 3.5 (9). Products were located by autoradiography; their identity was determined by their distance of migration on DEAE-paper and/or by complete hydrolysis with RNase T2 at pH 4.5 followed by electrophoresis on Whatman paper 3 MM at pH 3.5 (9) using appropriate markers.

RESULTS AND DISCUSSION

Separation of a complete RNase T1 digest of poliovirus type 1 RNA (pv 1 RNA) is shown in Fig. 3A. The digestion products are separated

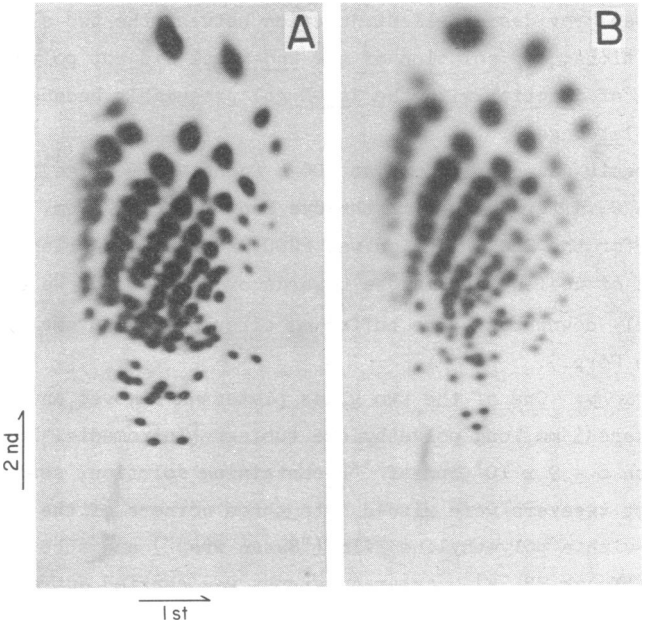

Fig. 3. Fingerprints of genome RNA of (A) poliovirus type 1 and (B) poliovirus type 2. [^{32}P]RNAs were digested exhaustively with RNase T1 and the products separated by 2D-gel electrophoresis as described in the text.

into numerous spots. The separation of the large oligonucleotides is highly reproducible. Fingerprints which have been prepared from different RNA samples at different times are superimposable if the standard conditions are followed. In the upper portion of the electropherogram, the oligonucleotides of small molecular weight fall onto lines according to base composition and size. In the lower part of the gel the large oligonucleotides appear to be randomly distributed. This area of the electropherogram is diagnostic for a particular species of high molecular weight RNA and an index of its purity (10). No radioactive material remains in the first dimension of the gel if the chain length of the fragments are below 200 nucleotides. The material which streaks over a wide range in the lower left corner represents poliovirus RNA linked poly(A) which is heterogeneous in size with an average chain length of 89 A residuse (8). When retrovirus RNA was analysed under these conditions, its poly(A) appeared as a dark spot close to the origin (11). This reflects the fact that retrovirion-poly(A) is larger and less heterogeneous than poliovirus-poly(A) (12).

As discussed previously by DeWachter and Fiers (1), the migration in the first dimension is governed by chain length and base composition. Under our conditions of a very low concentration of bisacrylamide the effect of the charge of the oligonucleotides exceeds that of their chain length. The presence of urea and the low pH facilitate dissociation of aggregates and reduce secondary structure of oligonucleotides. Lowering the pH from 3.5 (1) to 3.3 resulted in a wider separation of the nucleotides and shortened the required running time from 4 to 2.5 hr.

Migration in the second dimension depends upon the chain length. Thus, spot 3 ($N_{32}Gp$) in Fig. 4 is larger than spot 2 ($N_{29}Gp$) or spot 1 ($N_{25}Gp$) (Table 1). In some cases, however, the base composition and/or structure of the oligonucleotide appears to influence the mobility also. For example, spot 12 [$(C_{14},U_4,A_5)Gp$] is larger than spot 26 [$(C_6,U_5,A_{11})Gp$] (Table 1) yet migrates faster in the 2nd dimension.

As compared to the procedure by DeWachter and Fiers (1) we use a relatively high concentration of bisacrylamide in the 2nd dimension. This has the advantage that (i) a better separation of the large oligonucleotides is achieved, (ii) oligonucleotides or even mononucleotides do not diffuse during prolonged periods of autoradiography (e.g. 3 days for 5×10^5 cpm of a digest of poliovirus or retrovirus RNA), (iii) gel pieces can be readily pulverized for the elution of

radioactive components, and (iv) the glass plates do not "stick" to the gel and can be easily removed.

Moreover the high concentration of cross-linker in the 2nd dimension gel prevents small oligonucleotides from migrating out of the gel while simultaneously all large RNase T1 fragments are well separated. Thus, *all* products of exhaustive RNase T1 digestion can be recovered from the gel. This is significant for two reasons. Firstly, the molar yields of oligonucleotides which have been completely separated from other components can be determined without the need of an internal standard (13). This can be achieved by cutting the gel into equal squares which are separately counted. The counts recovered from unique spots are then compared to the counts contained in all other spots. Secondly, the gel can be used for the simultaneous purification of large (n = 30-50) and small (n > 10) oligonucleotides. This is a particular advantage for sequence studies of high molecular weight RNA fragments.

Clearly, the gel system described here can also be used for fingerprinting small molecular weight RNA [$(Np)_{200}$] which would presumably yield no large oligonucleotides after digestion with RNase T1. This may appear to be laborious as compared to other procedures. However, 2D-gels may be advantageous over the 2D-paper ionophoresis (9) when U-rich oligonucleotides are present in digests of small molecular weight

Table 1. Base composition of RNase T1 products eluted from the gel.

Spot No.	Composition after digestion with RNase A	Base composition*	Chain length
1	1 A_3C, 5 AC, 5 C, 6 U 1 G	$C_{11}A_8U_6G$	26
2	1 A_4C, 1 A_3U, 1 A_2U, 1 A_2C 2 AU, 2 AC, 5 C, 1 U, 1 G	$C_9A_{15}U_5G$	30
3	1 A_3U, 1 A_3C, 1 A_2G, 2 AU, 2 AC, 10 C, 4 U	$C_{13}A_{12}U_7G$	33
12	1 A_2G, 3 AC, 11 C, 4 U	$C_{14}A_5U_4G$	24
26	1 A_3G, 1 A_3U, 1 A_2U, 1 AU, 2 AC, 4 C, 2 U	$C_6A_{11}U_5G$	23

*Results of 4 experiments

RNA or when complete transfer of materials from the 1st to 2nd dimension of separation is required.

The fastest running material in the first dimension is U_4Gp, in the 2nd dimension Gp. Most spots of oligonucleotides in the upper portion of the gel are mixtures of 2 to 3 oligonucleotides (Lee and Wimmer, unpublished) whereas all spots (spots 1 through 28) in the lower portion represent unique oligonucleotides. The latter is based on sequence analyses (2) and on the observation that the spots in the lower portion of the gel do not separate further upon prolonged electrophoresis (Fig. 4).

A fingerprint of an RNase T1 digest of poliovirus type 2 [^{32}P]RNA is shown in Fig. 3B. The upper portion of the fingerprint is very similar to but not identical with the upper portion of Fig. 3A which is not surprising since a large number of digestion products of similar base composition and chain length can be expected from two different RNAs of high molecular weight. It should be pointed out, however, that the upper portion of the gels reveal unique patterns with decreasing size of the RNA as we have found with fingerprints of 28S and 18S HeLa rRNAs. Many of the small oligonucleotides of 18S rRNA are completely resolved (Nomoto, Lee and Wimmer, unpublished). The lower portion of the fingerprint in Fig. 3B is totally different from that in Fig. 3A. If the fingerprints are superimposed, the genomes of pv 2 and pv 1 do not share any large RNase T1 fragments which was confirmed by analyses of base compositions (2). This result was unexpected since it has been estimated on the basis of hybridization studies that the genomes of both virus strains are 35-50% homologous in sequence (14). It should be emphasized, however, that due to wobble in the genetic code, differences in the fingerprints do not necessarily reflect differences in gene products.

We have also prepared fingerprints from poliovirus RNAs digested with pancreatic RNase. Due to the specificity of the enzyme these fingerprints are much less complex when compared with RNase T1 fingerprints (data not shown).

2D-gel electrophoresis of exhaustive enzymic digests should prove to be a valuable procedure for structural analyses of high molecular weight RNA. Determination of molar yields of oligonucleotides in digests permits one to establish the sequence complexity of the RNA from which the oligonucleotides were derived. This approach has been used by Billeter et al. (13) for the analysis of the sequence complexity

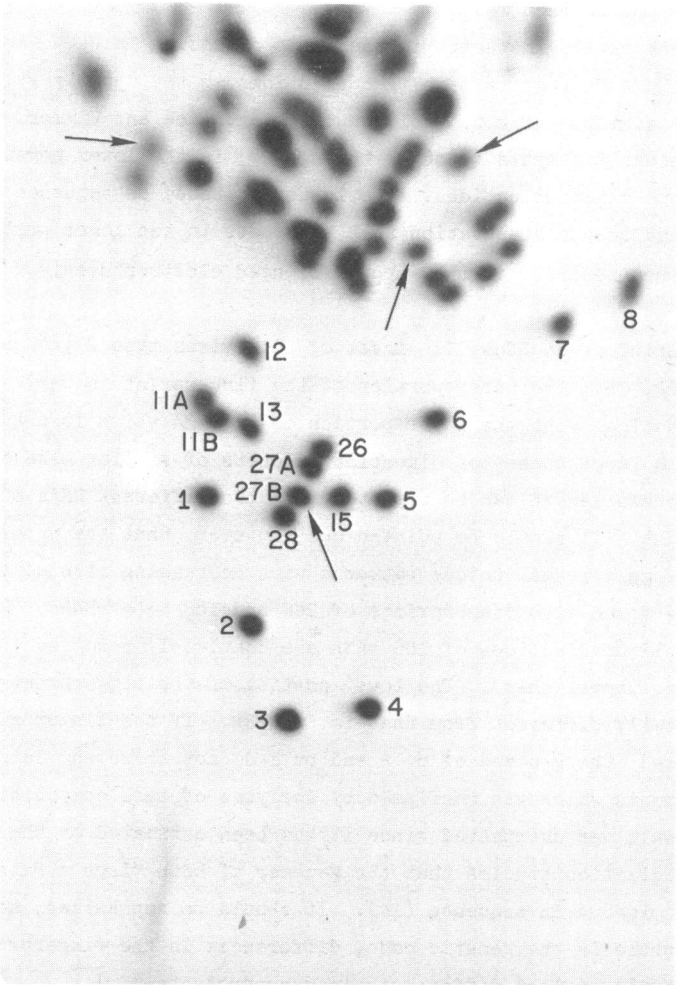

Fig. 4. Separation of a digest of [^{32}P]RNA of poliovirus type 1 by 2D-gel electrophoresis. Conditions were as described in the text except electrophoresis was prolonged. Most of the small oligonucleotide (on top of fingerprint) eluted. Spots marked with an arrow are those which are absent in fingerprints of poliovirus defective interfering particles RNA.

of Rous sarcoma virus RNA (Schmidt-Ruppin strain). These authors used the conditions of DeWachter and Fiers (1) under which small oligonucleotides elute from the gel during 2nd dimension electrophoresis. This makes it necessary to introduce an internal standard (13). We have used

the method as described here to characterize the genome of Rous sarcoma virus (strain B77) and its transformation defective segregant (11). We determined the sequence complexities of B77 RNA (11), of pv 1 RNA and of pv 2 RNA (2), and identified poliovirus specific mRNA (10) by the same method. Coffin and Billeter (15) have applied 2D-gel electrophoresis to construct a physical map of the Rous sarcoma virus genome by locating large oligonucleotides relative to the poly(A) segment of the RNA. Since fingerprints of all poliovirus defective interfering particle ("DI") RNAs (16) were found to lack several large oligonucleotides (see Fig. 4; Nomoto, Lee and Wimmer, unpublished) we are currently employing a similar approach to map the deletion in pv DI RNA.

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