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Heterogeneity of the 3′ End of Minus-Strand RNA in the Poliovirus Replicative Form

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The 3′ terminus of the strand (minus strand) complementary to poliovirion RNA (plus strand) has been examined to see whether this sequence extends to the 5′-nucleotide terminus of the plus strand, or whether minus-strand synthesis terminates prematurely, perhaps due to the presence of a nonreplicated nucleotide primer for initiation of plus-strand synthesis. The 3′ terminus was labeled with ^{32}P using [5′-^{32}P]pCp and RNA ligase, and complete RNase digests were performed with RNases A, T_1, and U_2. ^{32}P-oligonucleotides were analyzed for size by polyacrylamide-urea gel electrophoresis. The major oligonucleotide products formed were consistent with the minus strand containing 3′ ends complementary and flush with the 5′ end of the plus strand. However, a variable proportion of the isolated minus strands from different preparations were heterogeneous in length and appeared to differ from each other by the presence of one, two, or three 3′-terminal A residues.

The replication of poliovirus (polio) RNA, a single-stranded RNA of positive polarity, requires the synthesis of an intact complementary minus strand. Such minus-strand molecules have been isolated by phenol extraction of infected cells, predominantly in double-stranded hybrids composed of one plus and one minus strand (replicative form; RF) (3, 24) or in replicative intermediates (2, 4, 5, 25). Synthesis of minus strands accounts for approximately 5 to 10% of the total synthesis of viral RNA (18).

Little is known about the mechanism of polio RNA synthesis. A replication complex composed of a viral RNA template bound to active RNA polymerase activity has been purified from infected cells and has been shown to contain only one viral polypeptide, designated NCVP 4 (13, 19). Although this activity appears to faithfully transcribe the template with which it is isolated, no initiation of new RNA chains was detected (20). More recently, this same viral polypeptide has been purified from infected cells with an activity which will transcribe polyadenylic acid [poly(A)] (12) or polio RNA (15) in the presence of an oligouridylic acid primer. In the absence of primer, polio RNA is also transcribed; however, initiation appears to occur at multiple sites, resulting in the synthesis of short fragments of complementary RNA (8). Proper initiation on polio RNA and transcription into a full-length complementary product have been reported to occur only in the presence of oligouridylic acid primer (J. B. Flanegan, T. Van Dyke, and R. Rickles, personal communication).

The requirement of a primer for RNA synthesis is unprecedented, and no biological substitute for the oligonucleotide has been identified for RNA replication in vivo. Several investigators have proposed that VP_g, the small protein covalently linked to the 5′ termini of viral RNA molecules, might serve a primer function for RNA synthesis (21, 22), although a precise conceptual picture of the protein priming model has not been presented. VP_g has been shown to occur on both plus and minus strands of polio double-stranded RNA (21, 29) and on the nascent chains of replicative intermediates (21, 22).

Recently, Wengler et al. (28) analyzed the nucleotide sequence at the 3′ end of Semliki Forest virus minus strands isolated from the double-stranded RF. Surprisingly, a single unpaired G residue was found at the 3′ terminus. The implications of this structure with respect to RNA replication are not understood. In this report, we have similarly examined the 3′-terminal oligonucleotide of polio minus-strand RNA to determine whether it is flush with the nucleotide sequence at the 5′ end of the plus strand, whose sequence is known (14, 21). We have found that the 3′-terminal sequence of the minus strand is not unique; but rather, RF molecules contain a population of minus strands, differing from one another at their 3′ ends by one, two, or three A residues. While this manuscript was in preparation, a similar study was published by Larsen et al. (17), which reported the 3′-terminal sequence of the polio minus strand to be an exact complement of the 5′ end of the plus strand. Their data, however, do show several additional minor species of 3′-terminal

oligonucleotides which coincide with those described here.

MATERIALS AND METHODS

Cells, virus, and growth. Suspension cultures of HeLa S3 cells were grown as described (7). Cells were concentrated to 5×10^6/ml and were infected with the Mahoney strain of poliovirus type 1 at a multiplicity of 50 PFU/cell at 37°C. Polio RNA was labeled with [^{3}H]uridine, [^{3}H]adenosine, or both at 4 to 10 μCi/ml in the presence of actinomycin D (2 μg/ml). Infected cells were collected by centrifugation at 8 h postinfection.

RNA extraction and LiCl precipitation. Cells were washed with an isotonic salt solution (Earle), frozen at −20°C, thawed, and suspended in RSB (10 mM Tris-hydrochloride, pH 7.4–10 mM NaCl–1.5 mM $MgCl_2$) at about 1.5×10^8 cells per ml. Cells were lysed in 1% Nonidet P-40 at 0°C, and nuclei were removed by centrifugation. The nuclei were washed with RSB containing 1% Nonidet P-40, and the combined supernatant fractions were pooled (cytoplasm).

The cytoplasm was adjusted to 1% sodium dodecyl sulfate (SDS), 10 μg of polyvinyl sulfate (Sigma) per ml, 10 mM EDTA, 20 mM Tris-hydrochloride (pH 8.3), and 0.1 M NaCl and extracted with an equal volume of TNE (0.15 M NaCl–0.01 M Tris-hydrochloride, pH 8.3–5 mM EDTA)-saturated phenol at room temperature. After centrifugation the aqueous phase was removed, the phenol phase and interface were extracted with an equal volume of TNE, and the mixture was centrifuged. The combined aqueous phases were then extracted with an equal volume of TNE-saturated phenol, the mixture was centrifuged, the aqueous layer was removed and adjusted to 0.2 M sodium acetate (pH 5.5), and nucleic acids were precipitated with 2 volumes of 95% ethanol at −20°C overnight. The precipitate was collected by centrifugation at 16,000 × *g* for 30 min at 2°C and washed with 70% ethanol. On some occasions the ethanol precipitate was dissolved in 10 mM EDTA (pH 7.4)–1% SDS and fractionated in 2 M LiCl at −20°C overnight (26). The suspension was centrifuged at 16,000 × *g* for 30 min at 2°C, and the LiCl-soluble fraction, containing RF RNA, was precipitated with ethanol as described above.

Purification of polio RF. The LiCl-soluble material was chromatographed through a cellulose CF-11 (Whatman) column (21 by 0.9 cm) according to the method of Bishop and Koch (4). Occasionally, total cytoplasmic nucleic acids were fractionated directly on a cellulose CF-11 column (23 by 2.5 cm) without LiCl fractionation; this column could accommodate 40 mg of RNA without overloading, provided that samples were applied in 15% ethanol. The material eluting in salt without ethanol was pooled and ethanol precipitated. The precipitated RNA was examined analytically by rate zonal sedimentation in a 15 to 30% sucrose gradient containing 0.1 M NaCl–10 mM Tris-hydrochloride (pH 7.4)–2 mM EDTA–0.2% SDS, as described previously (23).

RNA precipitated from appropriate fractions from the CF-11 column was dissolved in 0.4 ml of 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.0)–1 mM EDTA–0.1% SDS and fractionated on a Sepharose CL-2B (Pharmacia) column (77 by 1.5 cm) at room temperature. The column was eluted at 6 ml/h with the same buffer, and 0.75-ml fractions were collected. The excluded material, containing polio RF, was pooled, and RF was collected by ethanol precipitation without carrier.

Sometimes these polio RF preparations were further purified in cesium trichloroacetate equilibrium gradients (6). Linear, preformed gradients (3.0 M to 4.8 M cesium trichloroacetate) containing 0.03 mM Tris-hydrochloride–2.5 mM EDTA (pH 7.4) were prepared, giving an average initial density of 1.717 g/cm^3, and overlaid with 0.1-ml samples. These gradients were centrifuged in an SW50.1 rotor at 43,000 rpm and 20°C for 44 to 48 h. Gradients were collected from the bottom, samples were assayed for radioactivity, and fractions banding at a peak density of 1.67 g/cm^3 were combined, diluted 1:3 with water, and ethanol precipitated without carrier.

Labeling 3′ termini of polio RF. ^{3}H-labeled polio RF ([^{3}H]RF; 14 to 27 μg) was precipitated in ethanol and dissolved in 14 μl of 1 mM EDTA. The 3′-hydroxyl termini were labeled with [5′-^{32}P]pCp, basically by the procedure of England et al. (11). Reaction mixtures (20 μl) contained polio RF, 3 μM [5′-^{32}P]pCp (1,700 Ci/mmol; New England Nuclear Corp.), 20 μM ATP, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.1), 3 mM dithiothreitol, 8 mM $MgCl_2$, 10% (vol/vol) dimethyl sulfoxide, and 5 U of T4 RNA ligase (P-L Biochemicals) in a conical polypropylene tube. Reactions were incubated at 0°C for 16 to 20 h and terminated by adjusting the mixture to 0.2% SDS and 40 mM EDTA and incubating with pronase (2 mg/ml) at room temperature for 5 min. The mixture was treated with a few beads of Chelex-100 (Bio-Rad; 200 to 400 mesh) at room temperature for 1 min and then made 50% (vol/vol) dimethyl sulfoxide (total volume now 60 μl), supplemented with 200 μl of 0.2 M LiCl–0.03 M Tris-hydrochloride (pH 7.4)–2 mM EDTA–0.1% SDS, and passed through a Sephadex G-100 column (26 by 0.9 cm) in the same buffer. Fractions in the excluded volume contained ligated polio RF and were pooled, supplemented with 40 μg of yeast RNA carrier, and ethanol precipitated. After centrifugation the RNA was dissolved in 20 μl of borate buffer (55 mM sodium borate–10 mM Na_2SO_4–1 mM EDTA, pH 8.1).

Preparative CH_3HgOH-agarose electrophoresis. Low-molecular-weight material labeled with ^{32}P during ligation was removed from genome-length ^{32}P-labeled polio plus and minus strands by electrophoresis in the strongly denaturing CH_3HgOH gels described by Bailey and Davidson (1). Horizontal slab gels (20 by 13 cm) containing 1% agarose (Seakem) and 5 mM CH_3HgOH (Alfa Div.; 1.27 M stock) in borate buffer were prepared, and samples (20 μl), which were previously treated with 10 mM CH_3HgOH at room temperature for 15 to 60 min, were applied to the gel. Electrophoresis was at 90 V, 45 to 50 mA, for 6.5 to 7 h, after which nonradioactive markers of HeLa cell rRNA's were visualized by UV absorbance of RNA bands by placing the gel on PPO(2,5-diphenyloxazole)-impregnated 3MM paper and illuminating from above with UV light. The gel was exposed directly to X-ray

film (Kodak SB-5) to discern genome-length polio RNA strands. The predominant band containing full-length strands was then excised from the gel.

Elution of ^{32}P-labeled polio RNA from CH_3HgOH-agarose gels. RNA from the gel bands was extracted by modification of the procedure of Dunn et al. (10). Bands were mixed with 0.1 M NaCl-0.01 M Tris-hydrochloride (pH 7.0)-0.001 M EDTA (1.0 g of wet gel per 5.6 ml of buffer) and supplemented with 40 μg of yeast RNA carrier. The mixture was dispersed in a Dounce homogenizer and extracted with an equal volume of TNE-saturated phenol at 4°C overnight. The mixture was centrifuged, and the phenol plus interface material were extracted with an equal volume of TNE at 4°C for 2 h. The mixture was centrifuged, and the combined aqueous phases were extracted with TNE-saturated phenol and then with $CHCl_3$-isoamyl alcohol (24:1) for 5 min at room temperature. The aqueous phase was ethanol precipitated to give a final recovery of about 60% of the [^{32}P]RNA. The size of recovered [^{32}P]RNA was assessed by an analytical CH_3HgOH-agarose gel or by rate zonal sucrose sedimentation, as previously described.

Oligo(dT)-cellulose chromatography. ^{32}P-labeled strands of polio RNA were dissolved in borate buffer (100 μl) after ethanol precipitation and denatured in 10 mM CH_3HgOH at room temperature for 60 min. The solution was then adjusted to 0.5 M NaCl and supplemented with 100 μl of 0.5 M NaCl-10 mM Tris-hydrochloride (pH 7.4) and applied to an oligodeoxythymidylic acid [oligo(dT)]-cellulose column (3.5 by 0.7 cm). The column was washed with 0.5 M NaCl-10 mM Tris-hydrochloride (pH 7.4) to remove sequences lacking poly(A) and then with 10 mM Tris-hydrochloride (pH 7.4) to elute molecules containing poly(A) sequences. Fractions containing molecules lacking poly(A) sequences were pooled, supplemented with 20 μg of *Escherichia coli* tRNA carrier, and ethanol precipitated. This procedure was repeated to obtain RNA strands without poly(A) sequences (^{32}P-minus strands).

Protection of ^{32}P-minus strands with poliovirion RNA. ^{32}P-labeled polio minus strands were mixed with fragments of poliovirion RNA in 1 mM EDTA (pH 7.4) and denatured (i) with 9 volumes of 99% dimethyl sulfoxide at 53°C for 10 min, followed by diluting 1:3 with water and ethanol precipitation, or (ii) by heating at 103°C for 3 min. Virion RNA fragments were produced from 1 mg of RNA per ml in 0.04 M KOH at room temperature for 5 min, followed by neutralization with HCl and rate zonal sucrose sedimentation; the average size of the RNA fragments was 12S, and these fragments were collected by ethanol precipitation.

Denatured samples (mixtures of ^{32}P-minus strands and plus-strand fragments) were concentrated by ethanol precipitation, adjusted to 0.3 M NaCl-0.02 M Tris-hydrochloride (pH 7.4)-2 mM EDTA (30 to 75 μl), sealed in capillaries, and annealed at 75°C for 1 to 4 h. These samples were fractionated on a Sepharose CL-2B column, as described previously, and excluded material was collected by ethanol precipitation.

Enzyme digestions. [^{32}P]RNA plus carrier *E. coli* tRNA were heated in 1 mM EDTA at 103°C for 3 min in capillaries, transferred to conical polypropylene tubes, and lyophilized. For RNase T_1 digestion, the mixture was adjusted to 10 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA and treated with RNase T_1 (Calbiochem) at 1 μg of RNase T_1 to 20 μg of RNA at 37°C for 1 h. For RNase A digestion, the RNA mixture was adjusted to 0.2 M NaCl-10 mM Tris-hydrochloride (pH 7.4) and incubated at 1 μg of RNase A (Worthington) to 20 μg of RNA at 37°C for 1 h. For RNase U_2 digestion, the RNA mixture was adjusted to 0.05 M sodium acetate (pH 4.5) and incubated at 1 U of RNase U_2 (Calbiochem) to 20 μg of RNA at 37°C for 1 h. To obtain a [^{32}P]3′-AMP marker, ^{32}P-labeled polio plus strands or poly(A) (Miles) ligated with [5′-^{32}P]pCp were adjusted to 0.05 M ammonium acetate (pH 5.0) and treated with RNase T_2 (Calbiochem) at 0.2 U of RNase T_2 per ml at 37°C for 30 min. All reactions were halted by cooling in ice.

Gel electrophoresis and autoradiography. Complete enzyme digests or partial alkali digests (10 μl) were mixed with an equal volume of 50% (vol/vol) glycerol, 0.1% bromophenol blue, and 0.1% xylene cyanol and fractionated in a polyacrylamide gel (0.1 by 40 by 22 cm; 0.1 by 1 by 1 cm sample wells) containing 20% or 25% acrylamide-0.7% methylenebisacrylamide-7 M urea-50 mM Tris-borate (pH 8.2)-1 mM EDTA-0.053% ammonium persulfate (9). Gels were prerun for a minimum of 2 h at 450 V. Samples were then loaded and were electrophoresed at 1,000 V until the bromophenol blue dye had migrated about 20 cm.

After electrophoresis, the glass plates were removed, the gel was covered with Saran Wrap, and the dye positions were marked. The gel was autoradiographed on Kodak SB-5 film at −70°C (to prevent diffusion) for appropriate exposure times.

RESULTS

Preparation of polio minus strands. Polio [^{3}H]RF was obtained from the cytoplasm of polio-infected HeLa cells, and this [^{3}H]RF was used as the source of polio minus strands. The strategy of purification included (i) steps to obtain preparations of polio RF essentially free of contaminating nucleic acids, (ii) specific tagging of free 3′-hydroxyl termini with a ^{32}P label, and (iii) isolation of RNA strands which were equivalent in size to full-length poliovirion RNA after tagging and which were free of poly(A) segments (3′ ends of plus strands).

Initially, the cytoplasmic nucleic acids were fractionated in 2 M LiCl to obtain the LiCl-soluble fraction, which includes RF, small RNAs and fragments, and DNA. This material was fractionated on a cellulose CF-11 column (Fig. 1A) to remove small RNA and residual DNA, and RF molecules were recovered in the fraction eluted with 0% ethanol (bracketed fractions). Although this procedure yielded material with a high degree of purity, only low yields of polio RF were obtained, since considerable polio RF was trapped in the LiCl-insoluble material. Thus, occasionally, LiCl fractionation was eliminated and cytoplasmic nucleic acids were di-

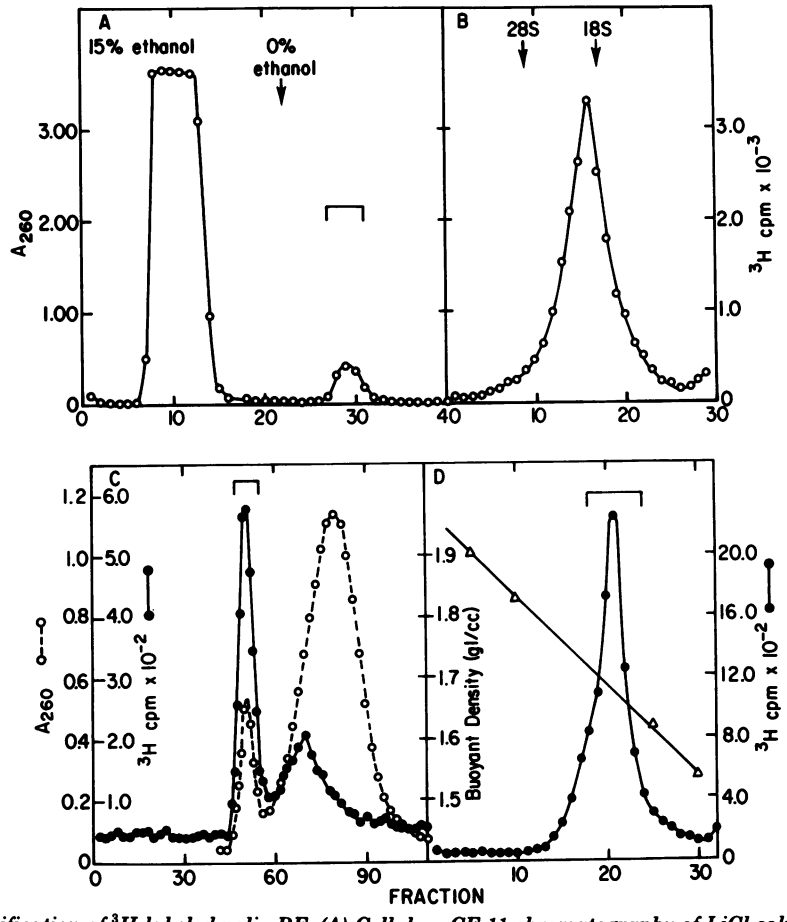

FIG. 1. *Purification of ^{3}H-labeled polio RF. (A) Cellulose CF-11 chromatography of LiCl-soluble fraction. Sample was applied in 15% ethanol-TSE (0.1 M NaCl-0.01 M Tris-hydrochloride, pH 7.0-1 mM EDTA), and RF (bracketed material) was eluted in TSE. (B) An analytical analysis of the bracketed material from (A) was examined by 15 to 30% sucrose rate zonal sedimentation in an SW41 rotor at 40,000 rpm, 20°C, for 6 h. Arrows indicate the positions of HeLa [^{3}H]rRNA in external marker gradients. (C) Sepharose CL-2B chromatography of the bracketed material from (A). The material was applied and processed, and the excluded material (brackets) was collected as described in the text. (D) Cesium trichloroacetate equilibrium centrifugation of the bracketed material from (C). Preparation of gradients, centrifugation, and gradient analysis were as described in the text. The material banding as duplex RNA (brackets) was collected and used as the source of [^{3}H]RF.*

rectly fractionated on cellulose CF-11 to obtain RF and replicative intermediate molecules in considerably better yield. Figure 1B shows an analysis of the RNA recovered from the CF-11 column by velocity sedimentation in sucrose gradients. A predominant peak sedimenting at 20S, characteristic of polio RF molecules, was observed. This material was then fractionated on a Sepharose 2B column to eliminate any remaining single-stranded RNA (Fig. 1C). The excluded material was occasionally further purified in cesium trichloroacetate density gradients, and the RNA was ethanol precipitated from those fractions with a density of 1.67 g/cm^3 (Fig. 1D). This procedure was designed to eliminate any residual protein remaining bound to the RNA (29). Analysis of the isolated RNA in a CH_3HgOH-agarose gel showed that the preparation contained exclusively intact strands of the same size as those extracted from purified virions.

The 3'-hydroxyl termini of polio RF were labeled with [5'-^{32}P]pCp by RNA ligase. Size analysis of the product after ligation in a CH_3HgOH-agarose gel (Fig. 2A) showed a predominant intact, virion-length RNA band, but also a considerable amount of low-molecular-weight RNA which may or may not be polio RNA. Detection of small contaminants by this method after ligation is markedly facilitated, since labeled species score on a molar basis instead of on a mass basis, as is the case with internally labeled species. Thus, we felt it necessary to perform pre-

parative CH_3HgOH-agarose gel fractionation of the [^{32}P]RNA to select for full-length polio strands. RNA was eluted from the excised band by phenol and $CHCl_3$ extractions. Size analysis of the extracted RNA in a CH_3HgOH-agarose gel showed that it remained predominantly intact (Fig. 2B). Molecules containing the 3′ ends of plus strands were removed from the ^{32}P-labeled minus strands by passage over an oligo(dT)-cellulose column after denaturation of the RNA in CH_3HgOH (Fig. 2C and D). Two cycles through the oligo(dT)-cellulose were used to ensure complete elimination of poly(A)-containing RNA.

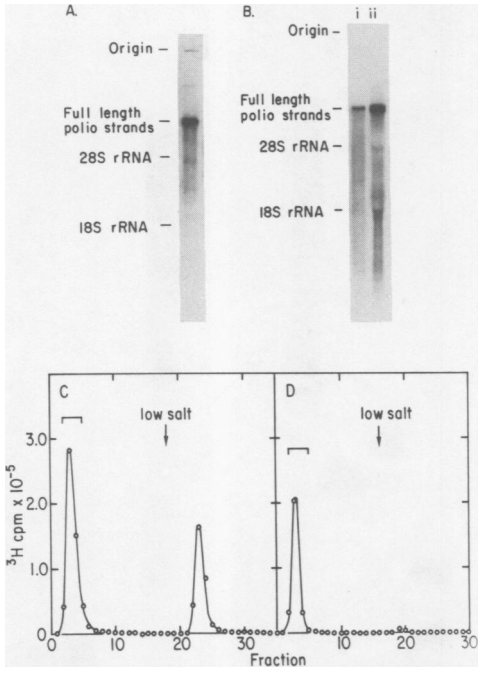

FIG. 2. *Isolation of polio ^{32}P-minus strands. (A) Autoradiography of CH_3HgOH-agarose gel of polio [^{32}P]RF after ligation with [5′-^{32}P]pCp and exclusion from Sephadex G-100. The positions of 28S and 18S HeLa rRNA's were determined by absorbance, as described in the text. (B) (i) Autoradiography of CH_3HgOH-agarose gel of polio [^{32}P]RNA strands eluted from the region in (A) corresponding to full-length polio strands by phenol and chloroform extraction and ethanol precipitation. (ii) Marker ^{32}P-labeled polio full-length plus strands. (C) The initial fractionation on an oligo(dT) cellulose column of the polio [^{32}P]RNA strands analyzed in (B). Sample was denatured in 10 mM CH_3HgOH and applied directly to the column in 0.5 M NaCl–10 mM Tris-hydrochloride (pH 7.4); molecules containing poly(A) sequences were eluted in 10 M Tris-hydrochloride (pH 7.4) (low salt). (D) The bracketed fractions in (C) were combined and rechromatographed on oligo(dT)-cellulose, as described for (C) and in the text. The bracketed fractions were combined as the source of polio minus strands.*

In some experiments, the minus strands were annealed with a vast excess of poliovirion RNA fragments (plus strands) to assure selection only of polio-specific RNA. The protected minus strands were separated from residual RNA on Sepharose 2B and collected from the excluded fractions. These procedures provided convincing assurance that the final labeled product represented the 3′ end of polio minus strands.

Analysis of the 3′ end of polio minus strands. Polio minus strands labeled with 3′-^{32}P were digested to completion with RNases A, T_1, and U_2, and the products of these reactions were analyzed in polyacrylamide gels which separate oligonucleotides according to size (9). Under the conditions of the enzyme digestions, RNase A produced oligonucleotides with cytidine phosphate or uridine phosphate at the 3′ end, RNase T_1 produced oligonucleotides with guanosine phosphate at the 3′ end, and RNase U_2 produced oligonucleotides with guanosine cyclic phosphate or adenosine cyclic phosphate at the 3′ end. Autoradiographs of the distribution of ^{32}P-labeled oligonucleotides resulting from each of these enzyme digestions of one preparation of RF are shown in Fig. 3. Surprisingly, no single unique 3′ oligonucleotide was detected. Digestion with RNase A yielded predominantly oligonucleotides of three and four nucleotides in length and lesser amounts of species of one and two nucleotides in length. (Longer oligonucleotides observed in this digest, as well as those digested with RNases T_1 and U_2, apparently resulted from some incomplete digestion and were eliminated in subsequent preparations; e.g., see Fig. 4). Digestion with RNase T_1 yielded major bands of ^{32}P-oligonucleotides of seven and eight nucleotides in length and minor bands of five and six nucleotides in length. RNase U_2 digestion yielded a major band of mononucleotide and weaker bands migrating at positions corresponding to 2.5 and 5.5 residues.

Five additional preparations of ^{32}P-minus strands yielded qualitatively the same results, but showed different ratios of the same four species. In all cases, an RNase T_1 oligonucleotide of seven nucleotides was a predominant product, representing from 30 to 70% of the total termini. (Each of the additional species comprised from 0 to 40% of the total, in different experiments.) This would be the expected ligated 3′-terminal oligonucleotide complementary to the known 5′ end of the plus strand (14, 21) and is in fact consistent with the sequence obtained by Larsen et al. (17) for the major ligated 3′-terminal T_1 oligonucleotide obtained from their RF preparations, ... UUUUAAp*Cp.

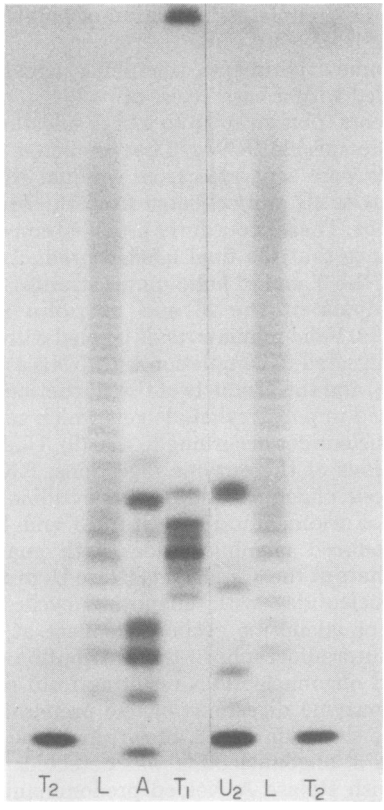

FIG. 3. *Polyacrylamide-urea gel electrophoresis of complete RNase digests of ^{32}P-minus strands visualized by autoradiography. Lanes labeled T_2 were RNase T_2 digests of [3'-^{32}P]poly(A) to yield adenosine [^{32}P]phosphate. Lanes labeled L contained partial carbonate digests of [3'-^{32}P]poly(A) (9). Lanes labeled A_1, T_1, and U_2 were complete pancreatic RNase A, RNase T_1, and RNase U_2 digests, respectively, of 3'-^{32}P-labeled polio minus strands.*

Figure 4A shows an autoradiogram of the oligonucleotide products of RNase digestion of another preparation of polio minus strands. Purification of the ^{32}P-minus strands included hybridization to unlabeled polio plus strands with subsequent isolation of double-stranded material, to insure that all of the species were derived from polio RNA. Again, multiple bands resulted from each enzyme digest. These results show that polio minus strands isolated from intracellular RF have heterogeneous ends. Assuming that the 3' end of the minus strand is complementary to the 5' end of the plus strand ($VP_gUUAAAACAG$) (14, 21), the enzymatic digestions of the minus strand are consistent with 3' sequences terminating in: (i) ...GUUUUAAA$_{3'\text{-OH}}$ (= RNase A or T_1 oligonucleotide 1; Fig. 4A); (ii) ...GUUUUAA$_{3'\text{-OH}}$ (= RNase A or T_1 oligonucleotide 2; Fig. 4A); (iii) ...GUUUUA$_{3'\text{-OH}}$ (= RNase A or T_1 oligonucleotide 3; Fig. 4A); (iv) ...GUUUU$_{3'\text{-OH}}$ (= smallest RNase A or T_1 oligonucleotide; Fig. 3).

Confirmation of these deduced sequences was obtained by secondary digestion with RNase A of each of the RNase T_1 oligonucleotide products recovered from the gel shown in Fig. 4A. Figure 4B shows the analysis of the secondary digestion products. Pyrimidine-specific cleavage yielded a dinucleotide (Xp*Cp) from T_1 oligonucleotide 3,

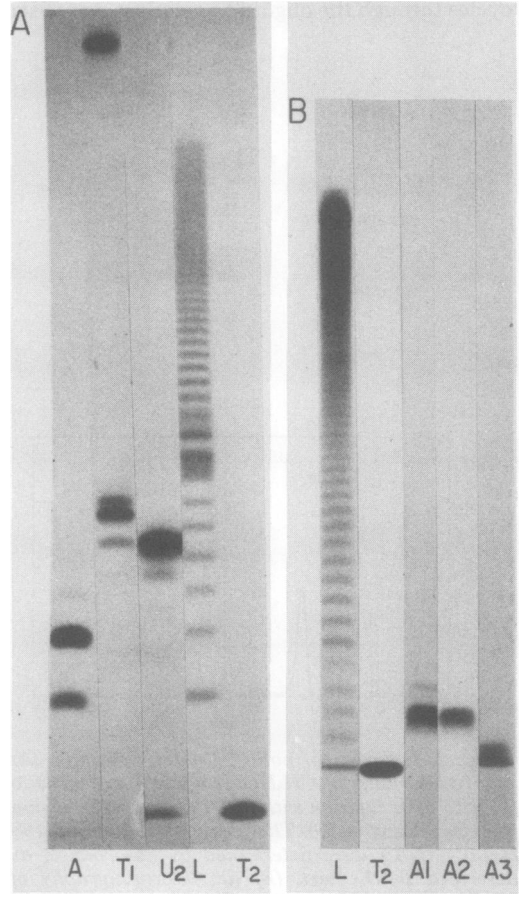

FIG. 4. *Polyacrylamide-urea gel electrophoresis of complete RNase digests of ^{32}P-minus strands visualized by autoradiography. Lanes are labeled as in Fig. 3. (A) Complete digests with RNases A, T_1, and U_2. (B) Each RNase T_1 band in (A) was excised, and oligonucleotides were eluted, filtered, extracted with phenol, and precipitated with ethanol. Each ^{32}P-oligonucleotide was then digested with RNase A and reanalyzed in a polyacrylamide-urea gel. Lanes labeled A1, A2, and A3 refer to the top, middle, and bottom bands, respectively, for RNase T_1 in (A). The diffuseness of these bands, particularly A3, is attributable to the large amount of carrier RNA in these samples, which was necessary for ethanol precipitation of the RNase T_1 oligonucleotides.*

a trinucleotide (XpXp*Cp) from T_1 oligonucleotide 2, and a tetranucleotide (XpXpXp*Cp) from T_1 oligonucleotide 1. (The latter sample was contaminated with the major oligonucleotide 2 during excision from the gel.) Identification of the nucleotide adjacent to the ligated C as an A residue was accomplished by nearest-neighbor analysis (data not shown). The results of these analyses are summarized in Table 1.

The reproducible heterogeneity observed at the 3′ end of polio minus strands could not have resulted from extensive nicking of the RNA before or during ligation so as to expose internal sites for ligation, since only full-length molecules remaining after ligation were selected for enzyme digestion analysis. A further concern, however, was the possibility that impurities in the [^{32}P]pCp might have produced more than one ligation product from a homogeneous RNA, or that trace exonucleolytic activity in the RNA ligase preparation might have produced ragged ends. These concerns were eliminated by analysis of the enzymatic digestion products of similarly ligated 28S rRNA from HeLa cells. The ligated 28S RNA was purified by preparative CH_3HgOH-agarose gel electrophoresis. The ^{32}P-labeled digestion products of RNase A and T_1 (Fig. 5) yielded a single oligonucleotide species for each enzyme. This result, together with a nearest-neighbor analysis of the ^{32}P-labeled 28S rRNA of 88% U, 4.2% G, 3.9% A, and 3.5% C, suggested that 28S rRNA molecules are unique and terminate in .... $GU_{3'\text{-}OH}$.

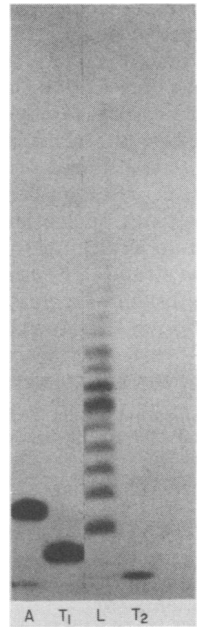

FIG. 5. *Polyacrylamide-urea gel electrophoresis of complete RNase A and T_1 digests of ^{32}P-labeled HeLa 28S rRNA visualized by autoradiography. Lanes are as described in the legend to Fig. 3.*

DISCUSSION

Isolation of 3′-terminally labeled oligonucleotides produced by RNase digestion of polio minus strands yielded four products differing from one another by the presence of no, one, two, or three terminal A residues. The relative quantities of each species varied in different preparations of RF from different infections, although a major product in all cases was the oligonucleotide, which is complementing and flush with the 5′ end of the plus strand. This 3′ terminus was the one recently isolated and sequenced by Larsen et al. (17). Their data do show minor amounts of additional RNase T_1 digestion products, which are consistent with those described here. A possible explanation for the relative enrichment of heterogeneous minus strands in our preparations compared with those described by Larsen et al. (17) is that we purified RF molecules from cells harvested 8 h postinfection rather than 5 h postinfection. Thus, additional incubation in the cell may have permitted additional template-independent A addition or removal, or the accumulation of prematurely terminated species.

TABLE 1. *Oligonucleotides from complete RNase digests of 3′-^{32}P-labeled polio minus strands*

Minus-strand terminus	Oligonucleotide[a]	
	RNase T_1	RNase A
1	Py-p-Py-p-Py-p-Py-pApApAp*Cp	ApApAp*Cp
2	Py-p-Py-p-Py-p-Py-pApAp*Cp	ApAp*Cp
3	Py-p-Py-p-Py-p-Py-pAp*Cp	Ap*Cp

[a] Py, Pyrimidine.

It is not known whether all of the intracellular RF molecules contain minus strands which represent functional templates for plus-strand synthesis. For example, it could be imagined that only minus strands which can form flush ends with the 5′ terminus of plus strands are functional, and that minus strands with incomplete or extra residues accumulate as useless, dead-end products. Existing evidence is consistent with a unique and homogeneous 5′-nucleotide terminus on plus strands isolated from virions (14, 21). It would be of major interest to determine whether the minus strands in replicative intermediates are unique or are also heterogeneous. The results presented neither implicate nor exclude VP_g or VP_g covalently joined to one or two U residues as primer in the replication reaction.

There is some precedence for the addition of

A residues to RNA molecules in vivo in a nonreplicative manner. Phage R17 RNA has an extra A at the 3′ end of the plus strand; removal of this extra A has no effect on the infectivity of the RNA molecule, but removal of the next nucleotide abolishes infectivity (16). Qβ RNA has an extra A at the 3′ end of both plus and minus strands (27). No significance has been assigned to these extra nucleotides. Finally, the recent finding of an additional unpaired G residue on the minus strand of Semliki Forest virus RNA (28) raises further interest regarding the mode of replication of these viral genomes.

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