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Deletion Mutant of the Bratislava-77 Strain of Rous Sarcoma Virus Containing a Fusion of the Group-Specific Antigen and Envelope Genes

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The genetic compositions of two independently derived preparations of the Bratislava-77 strain (B_{77}) of Rous sarcoma virus were analyzed after each was passaged seven or more times in duck embryo fibroblasts. RNase T_1-resistant oligonucleotide fingerprint analysis of virion RNA from both preparations of duck-passaged B_{77} revealed the presence of two large noncontiguous deletions. Approximately 75% of the RNAs contained a deletion which spans oligonucleotides 304 to 4 on the viral genome (about 3,500 nucleotides) and encompasses all of the B_{77} polymerase gene. More than 90% of the RNAs also contained a deletion which spans *src*-specific oligonucleotides 6 and 5 (about 2,200 nucleotides) and is identical to the deletion observed in transformation-defective B_{77}. Virion RNA from duck-passaged B_{77} also contained two oligonucleotides (D1 and D2) not observed in the RNA of B_{77} virus grown on chicken embryo fibroblasts. Analysis of the virion RNA of duck-passaged B_{77} by denaturing agarose gel electrophoresis revealed four major subunits with molecular weights of 3.40×10^6, 2.65×10^6, 2.25×10^6, and 1.55×10^6. Whereas the 3.40- and 2.65-megadalton (Mdal) RNA species comigrated with the nondefective and transformation-defective RNAs of B_{77} propagated on chicken embryo fibroblasts, no counterparts to the 2.25- and 1.55-Mdal RNAs were observed in the RNA of B_{77} grown on chicken embryo fibroblasts. Oligonucleotide fingerprint analysis of these RNA species revealed that the 2.65-Mdal RNA contains the *src*-specific deletion and that 2.25-Mdal RNA contains the polymerase region deletion; both of these deletions were observed in the 1.55-Mdal RNA, which was the major RNA subunit species detected in duck-passaged B_{77}. The new oligonucleotides (D_1 and D_2) observed in the duck-passaged virus were present in the 2.25- and 1.55-Mdal RNA species, but not in the 2.65-Mdal RNA. The 1.55-Mdal RNA can function as an mRNA in vitro and in vivo and directs the synthesis of a 130,000-dalton protein (p130). p130 contains antigenic determinants specific for p27 (*gag* gene) and gp85 (*env* gene) but does not contain sequences which cross-react with antisera directed against the $\alpha\beta$ form of RNA-dependent DNA polymerase (*pol* gene). This RNA, therefore, is generated by a fusion of the *gag* and *env* genes of Rous sarcoma virus B_{77}.

Until recently only two classes of nonconditional mutants of avian sarcoma viruses have been well characterized. Transformation-defective (*td*) mutants of avian sarcoma viruses are generated by spontaneous deletion of avian sarcoma virus *src* gene sequences and are ubiquitous in uncloned avian sarcoma virus stocks (7, 36-38). On the other hand, the Bryan high-titer strain of Rous sarcoma virus (RSV) and the NY8 variant of Schmidt-Ruppin RSV are nonconditionally defective in their capacity for helper-independent replication (20, 35). Both of these viruses have substantial deletions in the viral gene which encodes the virion envelope glycoproteins (*env*) (11, 16, 20, 39). Although derivatives of the Bryan high-titer strain of RSV and the NY8 variant of Schmidt-Ruppin RSV which are additionally defective in the synthesis of virion RNA-dependent DNA polymerase have been obtained, the nature of these lesions, believed to be small deletions, has not been effectively ascertained (15, 20, 37).

More recently, additional nonconditional mutations in replicative functions other than the *env* gene have been described. These include a replication-defective recombinant between the Prague strain of RSV, subgroup C, and Rous-

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associated virus type 0 which has about 30% of the RNA-dependent DNA polymerase gene (*pol*) deleted (24), a replication-defective endogenous chicken virus which contains a deletion spanning the 3′ terminus of the group-specific antigen (*gag*) gene and the 5′ terminus of the *pol* gene (12, 40), and a derivative of the Prague strain of RSV, subgroup E (41), which appears to produce all the requisite viral polypeptides but fails to package virus-specific RNA (26).

Here we report the detection and characterization of a deletion mutant of the Bratislava-77 strain (B_{77}) of RSV containing a large deletion (about 3,500 nucleotides) in the polymerase region of the viral genome. This deletion mutant accumulated after multiple passage of B_{77} RSV on Peking duck embryo fibroblasts (DEF). In each of two independently duck-passaged B_{77} stocks, more than 75% of the viral subunits contain this deletion after seven or more passages. Using RNase T_1-resistant oligonucleotide fingerprint analysis of viral RNA, direct RNA sequencing of selected oligonucleotides, and analysis of virus-specific proteins synthesized in vivo and in vitro, we determined that this deletion encompasses the 3′ terminus (about 250 nucleotides) of the *gag* gene, all of the *pol* gene, and an undetermined amount (probably less than 500 nucleotides) of the *env* gene.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of chicken embryo fibroblasts (CEF) were prepared from 10-day-old, gs-negative, chf-negative, Marek-negative embryos (SPAFAS, Norwich, Conn.). Primary cultures of DEF were prepared from 13-day-old Peking duck embryos (Truslow Farms, Chestertown, Md.). Cultures were maintained in Dulbecco modified Eagle medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% tryptose phosphate (Difco Laboratories, Detroit, Mich.) and 5% calf serum (Flow Laboratories). B_{77} RSV was provided by R. E. Smith, Duke University. An uncloned preparation of B_{77} RSV, passaged seven times in DEF [DK1-B_{77}(p7)], was provided by M. S. Collett and A. J. Faras, University of Minnesota. Secondary cultures of either CEF or DEF were routinely inoculated at a multiplicity of 0.1 to 0.5 focus-forming units of B_{77} RSV per cell or 0.1 to 0.5 ml of DK1-B_{77}(p7) per 3×10^6 cells in the presence of 2 μg of polybrene (Aldrich Chemical Co., Milwaukee, Wis.) per ml. Infected cells were subcultured every 3 to 5 days.

An independently derived duck-passaged B_{77} virus preparation was prepared by infecting DEF with B_{77} RSV as described above. After four or five subcultures, the medium from confluent cultures was removed and clarified by low-speed centrifugation. Part of this harvest [designated passage 1 virus or DK2-B_{77} (p1)] was used to reinfect new secondary cultures of DEF. The remainder was stored at −70°C. This protocol was followed seven times and resulted in the production of the DK2-B_{77}(p8) virus preparation used in these studies. Unlike DK1-B_{77}(p7), infection of DEF with DK2-B_{77}(p8) did not result in overt cellular transformation until the fifth to seventh subculture.

Preparation of radiolabeled viral RNA. The preparation of ^{32}P-labeled virus was carried out as described previously (30). In experiments in which the [^{32}P]RNA was to be used for RNase T_1-resistant oligonucleotide fingerprint analysis, the radioactive medium (containing 200 to 250 μCi of $^{32}P_i$ [New England Nuclear Corp., Boston, Mass.] per ml) was collected after 24 h and replaced with 5 ml of unlabeled, phosphate-free medium 199. After 24 h, this medium was collected, and the cells were subjected to a second 24-h labeling with ^{32}P-containing medium followed by two 24-h chase periods. The medium collected after each of the harvests was clarified at $13,000 \times g$ for 15 min at 4°C and stored at −20°C until all harvests were completed. If the [^{32}P]RNA was to be used for analysis of viral RNA subunit species, the radioactive medium was collected after 12 h and replaced with 5 ml of phosphate-free medium 199. This medium was harvested and replaced with 5 ml of phosphate-free medium 199 at 2-h intervals for the next 12 to 14 h. The medium collected after each of the 2-h chase periods was clarified by centrifugation at $13,000 \times g$ for 15 min at 4°C and stored at 4°C. For virus purification, labeled virus was sedimented by centrifugation at 42,000 rpm for 40 min at 4°C in a Beckman 45Ti rotor. The crude virus pellet was suspended in 5 ml of 100 mM NaCl–20 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA (STE buffer) containing 0.5 mg of yeast RNA. An equal volume of STE buffer-saturated phenol was added, and the aqueous suspension was adjusted to 1% sodium dodecyl sulfate (SDS). Labeled RNA was precipitated from the aqueous phase by the addition of NaCl to 0.3 M and 3 volumes of 95% ethanol. For the preparation of 60 to 70S B_{77} RNA, labeled viral RNA was layered onto a 5 to 25% sucrose gradient (containing 20 mM Tris-hydrochloride, pH 7.6, 1 mM EDTA, and 0.1% SDS) and centrifuged in an SW50.1 rotor for 80 min at 45,000 rpm and 15°C. Fractions containing RNA sedimenting at 60 to 70S were pooled, and the RNA was recovered by precipitation. The specific activity of ^{32}P-labeled 60 to 70S RNA was about 2×10^6 to 4×10^6 cpm/μg. ^3H-labeled 60 to70S viral RNA was prepared by the same procedure, except that cells were maintained in medium 199 supplemented with 5% calf serum and 100 to 150 μCi of [^3H]uridine (25 to 30 Ci/mmol; Amersham/Searle) per ml. The specific activity of ^3H-labeled 60 to 70S RNA was about 2.5×10^5 cpm/μg.

Preparation of unlabeled DK2-B_{77}(p8) viral RNA. The unlabeled DK2-B_{77}(p8) viral RNA used to obtain RNase T_1-resistant oligonucleotides for direct RNA sequence analysis was obtained essentially as described above. The medium from 40 to 50 100-mm dishes of DK-B_{77}(p8)-infected DEF was harvested at 24-h intervals for 10 to 14 days. Viral RNA was prepared without the addition of yeast RNA and was fractionated by centrifugation on a 5 to 25% sucrose gradient without SDS at 40,000 rpm for 180 min in an SW41 rotor at 4°C. The amount of RNA present in each fraction was determined by measuring the optical density at 260 nm. The yield of 60 to 70S RNA from 2 liters of culture fluid was 60 to 80 μg. To prepare

unlabeled DK2-B_{77}(p8) 60 to 70S RNA for translation experiments, virus was harvested at 2-h intervals from roller bottles (Bellco Glass, Inc., Vineland, N. J.) containing DK2-B_{77}(p8)-infected DEF. The medium was clarified, the virus was pelleted, and unlabeled 60 to 70S RNA was purified as described above. A yield of 200 to 220 μg of 60 to 70S RNA was obtained from five roller bottles harvested for 11 days.

Preparation of poly(A)-containing viral RNA. Samples of 60 to 70S RNA were dissolved in 0.45 ml of 20 mM Tris-hydrochloride (pH 7.6)–0.5 mM EDTA (TE buffer), heated to 100°C for 1 min, and rapidly cooled on ice. The samples were adjusted to 0.5 M NaCl with 0.05 ml of 5 M NaCl and applied to a 0.2-ml oligodeoxythymidylic $acid_{12-18}$-cellulose column (T-3; Collaborative Research, Inc., Waltham, Mass.). Nonpolyadenylated RNA was removed by washing the column with seven 0.5-ml portions of 0.5 M NaCl–20 mM Tris-hydrochloride (pH 7.6)–0.5 mM EDTA. Polyadenylic acid [poly(A)]-containing RNA was then eluted by washing the column with four 0.5-ml portions of 10 mM Tris-hydrochloride (pH 7.6). The total recovery of RNA was always greater than 95%.

Agarose gel electrophoresis of viral RNA. Seakem agarose powder (ME) was obtained from Marine Colloids, Rockland, Maine. A 1 M aqueous solution of methyl mercuric hydroxide was obtained from Alfa Products. Electrophoresis of ^{32}P- and ^{3}H-labeled viral RNAs on 0.75% agarose gels containing 5 mM CH_3HgOH was performed as described by Bailey and Davidson (2). Radiolabeled RNA samples containing 20 μg of yeast RNA were collected by ethanol precipitation, washed once with 1 ml of absolute ethanol, and air dried. The samples were redissolved in 20 μl of sample buffer, loaded onto cylindrical gels (0.5 by 17 cm), and electrophoresed at 50 V (2.3 mA/gel) for 5 or 7 h. After electrophoresis the gels were sliced into 1-mm fractions.

Elution of RNA from denaturing agarose gels. Two to four 1-mm-thick gel slices were incubated with 20 μg of yeast RNA and 0.5 ml of 90% (vol/vol) formamide–50 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA–1% SDS at 60°C until the agarose slices were completely dissolved (about 5 min). STE buffer-saturated phenol (5 ml) was added at room temperature, and the samples were mixed vigorously. A 0.5-ml amount of STE buffer was added, and the mixture was extracted for 5 min at room temperature. The aqueous and phenol phases were separated by centrifugation at 13,000 × *g* for 10 min at 15°C. The aqueous phase was removed and re-extracted as described above with 5 ml of STE buffer-saturated phenol. RNA was recovered from the aqueous phase by ethanol precipitation. The recovery of RNA was generally 70 to 80% of the input, and of that amount, 65% migrated as intact molecules after re-electrophoresis on denaturing agarose gels. [^{32}P]RNA used to program a rabbit reticulocyte cell-free protein-synthesizing system was separated from carrier RNA and residual traces of CH_3HgOH or phenol by chromatography on a 1.5-ml column of Sephadex G-100 (in TE buffer) poured on top of a 0.5-ml column of Dowex chelating resin. [^{32}P]RNA recovered in the excluded volume of the column was precipitated with a solution containing 5 μg of yeast RNA, 0.06 volumes of 5 M NaCl, and 3 volumes of ethanol at −20°C.

Analysis of RNase T_1-resistant oligonucleotides. ^{32}P-labeled viral RNA was incubated in 10 μl of TE buffer containing 100 μg of yeast RNA and 20 U of RNase T_1 (Calbiochem, La Jolla, Calif.) at 37°C for 30 min. Samples were subjected to two-dimensional gel electrophoresis by a modification of the procedure of Billeter et al. (4). Electrophoresis in the first dimension on 10% polyacrylamide gels containing 6 M urea and 25 mM citric acid (pH 3.5) was at 600 V until the bromophenol blue dye marker had migrated 17.5 cm (4.5 to 5.5 h). The second-dimension gel solution consisted of 21.8% acrylamide (reagent grade; 1× recrystallized and deionized; Eastman Organic Chemicals, Rochester, N. Y.), 0.71% bisacrylamide (Eastman), 100 mM Tris-citrate (pH 8.0), 0.06% (wt/vol) ammonium persulfate, and 0.015% (vol/vol) *N,N,N',N'*-tetramethylenediamine. Electrophoresis was carried out at 300 V until the bromophenol blue dye had migrated 17 cm (about 25 to 30 h). Oligonucleotides were located by autoradiography, using a DuPont Lightning Plus intensifying screen, and excised from the gel with a cork borer. RNA was eluted from the gel disks and precipitated with isopropanol as described by Coffin and Billeter (7). The nucleotide composition of the individual oligonucleotides was determined by digestion with RNase A (Calbiochem) as described by Adams et al. (1). Selected oligonucleotides were also characterized by RNase U2 digestion by incubating the oligonucleotide for 2 h at 37°C in 10 μl of a solution containing 50 mM sodium acetate (pH 4.5), 2 mM EDTA, 0.1 mg of bovine serum albumin (freed of nuclease by autoclaving) per ml, and 2 U of RNase U2 (Calbiochem) per ml. RNase U2 digestion products were resolved by ionophoresis on DEAE-cellulose paper (DE81; Whatman). Base compositions of RNase U2 digestion products were determined as described by Cashion et al. (6).

Preparation of ^{32}P-labeled RNase T_1-resistant oligonucleotides for direct RNA sequence analysis. ^{32}P-labeled DK2-B_{77}(p8) 60 to 70S RNA (20 μg; specific activity, 30,000 cpm/μg) was incubated in TE buffer with 4 U of RNase T_1 for 30 min at 37°C. RNase T_1-resistant oligonucleotides were resolved by two-dimensional gel electrophoresis, as described above. Oligonucleotides were eluted from the gel without added carrier RNA. The eluant was diluted with 20 volumes of 50 mM Tris-hydrochloride (pH 8.0) and applied to a 0.2-ml DEAE-cellulose column (DE52; Whatman). Bound RNA was eluted with 150 μl of elution buffer and desalted by gel filtration on columns of Sephadex G-50 (120 to 150 mesh) equilibrated with 0.1 mM Tris-hydrochloride (pH 8.1). RNA eluting from the column was concentrated by lyophilization. Recoveries of oligonucleotides at this stage ranged from 35 to 60% of the amount present in the original gel slice. Each oligonucleotide (0.5 to 2 pmol) was labeled with 1 U of polynucleotide kinase (Boehringer Mannheim Corp., New York, N. Y.) and [γ-^{32}P]ATP (20 pmol; specific activity, 3,000 Ci/mmol; Amersham/Searle) in a final volume of 20 μl as described by Maxam and Gilbert (27), except that the pH was 8.1 rather than 9.5. Each oligonucleotide was then repurified by electrophoresis on 20% polyacrylamide gels containing 7 M urea, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA (27). RNA

was eluted from the gel in elution buffer containing 25 μg of yeast RNA, purified by DEAE-cellulose chromatography, and precipitated with an equal volume of isopropanol at −20°C. Since oligonucleotides D1 and D2 (see Fig. 1) were found to be contaminated at this stage with a small amount of oligonucleotide 15, oligonucleotides D1 and D2 were further purified by electrophoresis on 10% polyacrylamide–6 M urea–25 mM citric acid (pH 3.5) gels.

Direct RNA sequence analysis of ^{32}P-labeled RNase T_1-resistant oligonucleotides. Partial enzymatic hydrolysis of [5′-^{32}P]RNA was performed as described by Gupta and Randerath (13) and Donis-Keller et al. (10). Electrophoresis was performed on 20% polyacrylamide slab gels containing 50 mM Tris-borate (pH 8.3), 7 M urea, and 1 mM EDTA, as described by Donis-Keller et al. (10). After electrophoresis gels were autoradiographed at −20°C by using DuPont Lightning Plus intensifying screens.

Cell-free protein synthesis. Staphylococcal nuclease-treated rabbit reticulocyte cell-free lysates were prepared essentially by the method of Pehlam and Jackson (31). [^{32}P]RNA precipitates were redissolved in 22 μl of lysate supplemented with 5.5 μCi (4 pmol) of [^{35}S]methionine (1,200 to 1,400 Ci/mmol; Amersham/Searle) and were incubated for 2 h at 30°C. Reaction mixtures were adjusted to 10 mM EDTA and 100 μg of RNase A per ml and incubated for another 30 min at 37°C. Samples were diluted into 100 μl of sample buffer (0.07 M Tris-hydrochloride, 11% glycerol, 3% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, pH 6.8) and heated for 3 min at 100°C, and a 30-μl portion was removed for analysis by SDS-polyacrylamide slab gel electrophoresis as described below.

Immunoprecipitation of labeled cell proteins. Rabbit antisera directed against p27 and gp85 of the Prague strain of RSV, subgroup C, were a gift from D. Bolognesi, Duke University. Goat antiserum directed against αβ polymerase was a gift from D. Grandgenett, St. Louis University Medical School. Labeling of cells with [^{35}S]methionine was carried out as follows. Cells were washed one time with labeling medium (Dulbecco modified Eagle medium minus methionine) containing 5% calf serum and incubated for 2 h in the same medium. Cells were incubated for 2 h in fresh labeling medium containing 100 μCi of [^{35}S]methionine (1,200 to 1,400 Ci/mmol; Amersham/Searle) per ml.

The preparation of cell extracts and immunoprecipitates has been detailed elsewhere (S. J. Parsons, S. C. Riley, E. E. Mullen, E. J. Brock, D. C. Benjamin, W. M. Kuehl, and J. T. Parsons, J. Virol., in press), except that cell extracts were incubated with 5 μl of anti-p27 serum, 10 μl of anti-gp85 serum, or 15 μl of anti-polymerase serum. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis as described below.

Analysis of proteins by polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out by using the discontinuous buffer system described by Laemmli (22). The stacking gel contained 4.5% acrylamide and 0.12% bisacrylamide, pH 6.8. The separating gel contained 9.0% acrylamide and 0.24% bisacrylamide, pH 8.8. Gels were stained in 0.1% Coomassie brilliant blue–50% methanol–7.5% acetic acid and destained in 5% methanol–7.5% acetic acid. Photofluorography was performed as described previously (5).

RESULTS

Detection of deletion mutants in the DK1-B_{77}(p7) virus stocks. Infection of secondary cultures of DEF with B_{77} RSV which had been previously passaged multiple times on DEF cells [DK1-B_{77}(p7) virus] resulted in complete transformation of cells after three to four subcultures (10 to 14 days). Since alterations in the primary sequence of viral RNA upon passage of the virus in DEF might be detectable by the presence of one or more new large RNase T_1-resistant oligonucleotides, ^{32}P-labeled 60 to 70S RNA was prepared from virions harvested from either DK1-B_{77}(p7)-infected DEF or CK-B_{77}-infected CEF (CK-B_{77} denotes virus propagated only on CEF) and digested to completion with RNase T_1, and the oligonucleotides were resolved by two-dimensional gel electrophoresis. A comparison of the two fingerprints (Fig. 1) revealed that all oligonucleotides present in the RNA of CK-B_{77} (Fig. 1A) were also observed in the 60 to 70S RNA of the duck-passaged virus (Fig. 1B). However, two features were noticeably different in the fingerprint of DK1-B_{77}(p7). First, a large number of oligonucleotides were present in substantially lower amounts (Fig. 1C, shaded circles), and second, DK1-B_{77}(p7) contained two new oligonucleotides, D1 and D2 (also in reduced yield), not detected in the CK-B_{77} viral RNA (Fig. 1C, solid circles, and Fig. 1B and inset, arrows).

Since the reduced yield of some oligonucleotides in DK1-B_{77}(p7) indicated that selected regions of the genome were disproportionately represented in the DK1-B_{77}(p7) stock, a quantitative determination of the yield of each oligonucleotide was made by taking 9.8×10^6 cpm (Cerenkov radiation) of ^{32}P-labeled CK-B_{77} 60 to 70S RNA and 9.1×10^6 cpm of DK1-B_{77}(p7) 60 to 70S RNA and fingerprinting each as described above. Each oligonucleotide was excised from the gel and counted, and its recovery (expressed as percentage of input radiolabel recovered) was plotted as a function of its location on the physical map of the B_{77} RSV genome (19). The results (Fig. 2) clearly demonstrate that the DK1-B_{77}(p7) virus contains two classes of deletions, one spanning oligonucleotides 304 to 4 and one encompassing oligonucleotides 6 and 5. From the measured recoveries of oligonucleotides 403 to 11 (738 ± 58 cpm/nucleotide) and 109 to 10 (729 ± 30 cpm/nucleotide), we estimated that approximately 75 to 80% of the subunits in this preparation contained deletions spanning oligonucleotides 304 to 4 (168 ± 34 cpm/nucleotide)

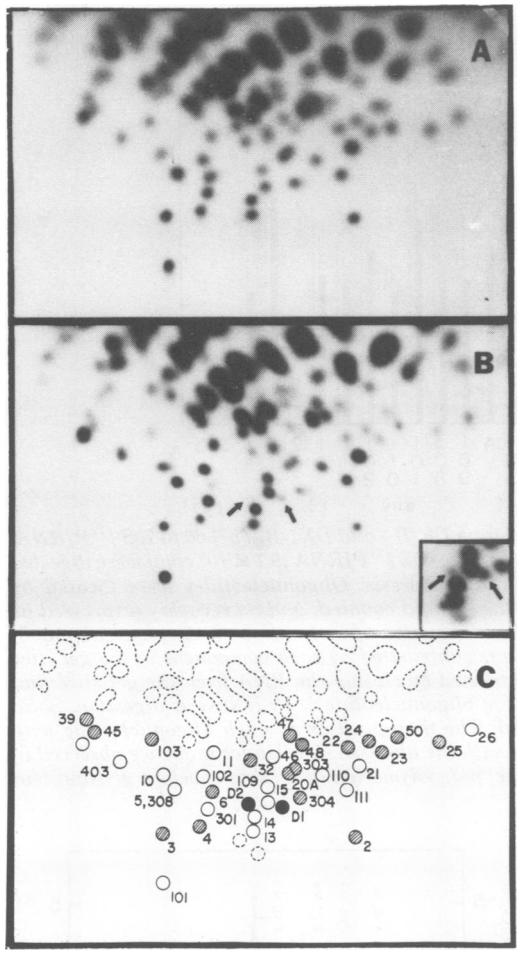

FIG. 1. *Two-dimensional gel analysis of RNase T_1-resistant oligonucleotides of CK-B_{77} RSV and DK1-B_{77}(p7) RSV RNA. B_{77} viral RNA was labeled with $^{32}P_i$, and the 60 to 70S RNA was isolated as described in the text. The identity of each oligonucleotide numbered in (C) was established by analysis of the products released after secondary digestion with RNase A. (A) RNase T_1-resistant oligonucleotide fingerprint of CK-B_{77} 60 to 70S RNA. A total of 1.5×10^6 cpm of CK-B_{77} 60 to 70S [^{32}P]RNA was digested with RNase T_1 and subjected to two-dimensional gel electrophoresis as described in the text. Electrophoresis in the first dimension was from left to right, and in the second dimension it was from bottom to top. Autoradiography was for 4 days at 4°C. (B) RNase T_1-resistant oligonucleotide fingerprint of DK1-B_{77}(p7) 60 to 70S [^{32}P]RNA (3.6×10^6 cpm). Exposure was for 2 days at 4°C. Arrows identify oligonucleotides not present in CK-B_{77} 60 to 70S RNA. (C) Schematic representation of the oligonucleotide fingerprint of DK1-B_{77}(p7). Shaded circles identify oligonucleotides present in reduced yield. Solid circles identify oligonucleotides not present in CK-B_{77}. Dashed circles indicate two oligonucleotides which were analogs of oligonucleotide 13. These components were present in variable quantities in all B_{77} stocks analyzed. RNase T_1-resistant oligonucleotides similar in composition to those characterized by Joho et al. (19) were assigned the same number.*

and that more than 90% contained deletions spanning oligonucleotides 6 and 5 (<50 cpm/nucleotide). The recoveries of the new oligonucleotides D1 and D2 were 270 and 170 cpm/nucleotide, respectively. In three separate viral RNA preparations the percentage of subunits containing the deletion spanning oligonucleotides 304 to 4 varied between 63 and 82%. In a fourth preparation only 38% of the subunits were found to contain a deletion of this region.

The recoveries of all oligonucleotides in CK-B_{77}, with the exception of oligonucleotides 6 and 5, were approximately the same, with a mean measured recovery of 538 ± 70 cpm/nucleotide. This constitutes approximately 55% of the theoretical yield and is in good agreement with previous estimates, for which a similar gel system was used (4). The measured recoveries of oligonucleotides 6 and 5 were only about 25% of the level observed for the remainder of the genome, indicating that *td* B_{77} was present in the CK-B_{77} stock at about a threefold excess over transforming virus.

The sequences located between oligonucleotides 304 and 4 appear to fall within the region of the genome coding for RNA-dependent DNA polymerase and, in addition, may encompass the 3′ end of the *gag* gene as well as the 5′ end of the *env* gene. The linear arrangement of RNase T_1-resistant oligonucleotides shown in Fig. 2 was derived by Joho et al. (19) and has been modified in the present work to accommodate the data shown above by reversing the order of oligonucleotides 303 and 304 and oligonucleotides 109 and 4 (see below). Since the boundaries of the viral genes shown in Fig. 2 are also based in part upon data presented below, a complete justification of these assignments appears below.

Analysis of the RNA subunit composition of DK1-B_{77}(p7) by denaturing agarose gel electrophoresis. To determine the RNA subunit composition of DK1-B_{77}(p7), 60 to 70S RNAs prepared from virions harvested at 2- to 3-h intervals from cultures of DK1-B_{77}(p7)-infected DEF (labeled with $^{32}P_i$) and CK-B_{77}-infected CEF (labeled with [^{3}H]uridine) were mixed and electrophoresed on 0.75% agarose gels containing 5 mM methyl mercuric hydroxide as a denaturing agent (2). Four major subunits were detected in the DK1-B_{77}(p7) RNA, with molecular weights of 3.40×10^6, 2.65×10^6, 2.25×10^6, and 1.55×10^6 (Fig. 3). Although the two largest subunits comigrated with the nondefective (*nd*)

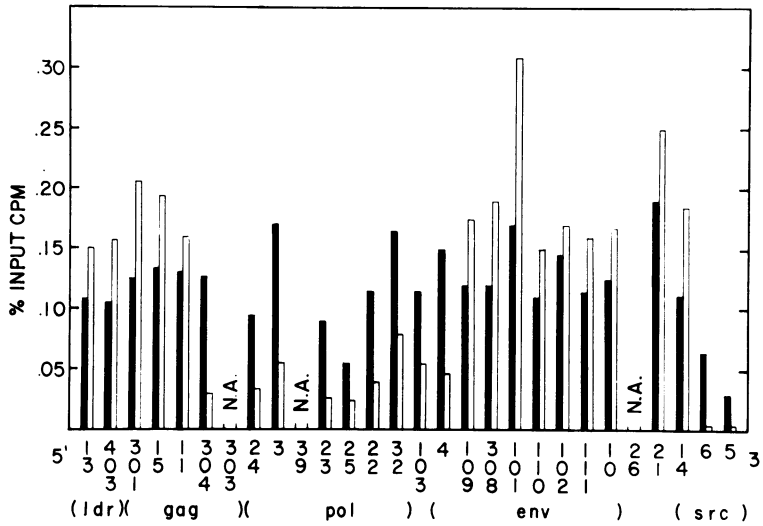

FIG. 2. *Recovery of RNase T_1-resistant oligonucleotides from CK-B_{77} and DK1-B_{77}(p7) 60 to 70S [^{32}P]RNA. CK-B_{77} 60 to 70S [^{32}P]RNA (9.8 × 10^6 cpm) and DK1-B_{77}(p7) 60 to 70S [^{32}P]RNA (9.1 × 10^6 cpm) were digested with RNase T_1 and subjected to two-dimensional gel electrophoresis. Oligonucleotides were located by autoradiography, each oligonucleotide was excised from the gel and counted, and its recovery (expressed as the percentage of input radiolabel recovered) was plotted as a function of its location on the physical map of the B_{77} RSV genome (19). The proportion of the total counts contributed by each component in the gel slice containing oligonucleotides 5 and 308 (Fig. 1C) was determined by eluting the RNA from the gel slice and determining the amount of A_3C (indicative of the amount of oligonucleotide 5) by RNase A digestion. Solid bars, CK-B_{77}; open bars, DK1-B_{77}(p7). N.A., Not assayed. The map position of each oligonucleotide with respect to the individual viral genes (see text) is shown beneath the abscissa: ldr, leader sequence observed in subgenomic viral mRNA's; gag, group-specific antigen gene; pol, polymerase gene; env, envelope glycoprotein gene; src sarcoma gene.*

and *td* RNA subunits, respectively, of CK-B_{77}, no counterparts to the 2.25- and 1.55-megadalton (Mdal) subunits were observed in the B_{77} stock propagated on CEF (Fig. 3). Based on the molecular weight of the *nd* RNA (3.4 × 10^6), we estimate that the 2.65-, 2.25-, and 1.55-Mdal RNA species contain deletions of about 21% (about 2,200 nucleotides), 34% (about 3,500 nucleotides), and 54% (about 5,600 nucleotides) of the *nd* B_{77} RSV genome, respectively. These data are consistent with the hypothesis that the 2.65-Mdal subunit contains an *src* deletion spanning oligonucleotides 6 and 5 and the 2.25-Mdal subunit contains a *pol* region deletion spanning oligonucleotides 304 to 4. Since the 1.55-Mdal subunit contains a deletion of approximately 5,600 nucleotides, this subunit, which represents the major component of DK1-B_{77}(p7), appears to be missing sequences from both of these regions. The data of Fig. 3 (see also Fig. 5D) permit an approximation of the molar ratio of the individual subunits which are present in DK1-B_{77}(p7) RNA, about 0.1:1.0:0.3:5.0 (3.40-Mdal/2.65-Mdal/2.25-Mdal/1.55-Mdal RNAs, respectively). Based on the assignments of deletions made above, we estimate approximately 85% of the subunits contain a deletion in the *pol* region

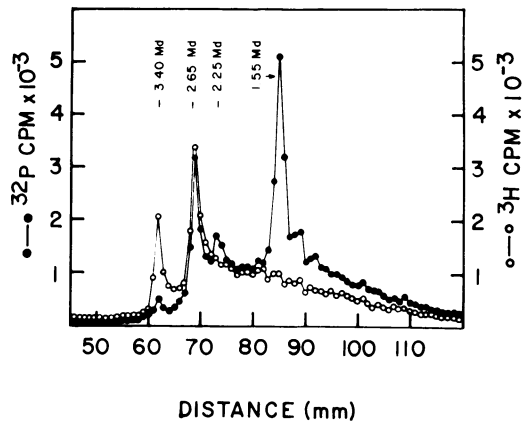

FIG. 3. *Analysis of CK-B_{77} and DK1-B_{77}(p7) 60 to 70S RNAs by denaturing agarose gel electrophoresis. DK1-B_{77}(p7) 60 to 70S [^{32}P]RNA (●; 89,000 cpm) and CK-B_{77} 60 to 70S [^{3}H]RNA (○; 42,000 cpm) were prepared from radiolabeled virions harvested at 2-h intervals, mixed, and electrophoresed on 0.75% agarose gels containing 5 mM methyl mercuric hydroxide. Electrophoresis was performed at 50 V (2.4 mA/gel) for 7 h. The molecular weight of individual RNA species was estimated relative to the position of chicken 28 and 18S [^{3}H]rRNA electrophoresed in a parallel gel. Md, Megadaltons.*

(oligonucleotides 304 to 4) and that approximately 95% of the subunits contain a deletion in *src* (oligonucleotides 6 and 5). This result is in good agreement with the estimates obtained by RNase T_1 oligonucleotide fingerprinting (Fig. 1 and 2).

Polymerase deletions in two independently derived preparations of duck-passaged B_{77} RSV. To determine whether a second preparation of B_{77} RSV with properties similar to those of DK1-B_{77}(p7) could be generated by independently passaging CK-B_{77} in DEF, and to examine the rate at which the defective subunits accumulate, a cloned stock of CK-B_{77} was passaged multiple times in DEF as described above. Virus obtained after eight passages on DEF [designated DK2-B_{77}(p8)] differed from the DK1-B_{77}(p7) virus in that infection of DEF with DK2-B_{77}(p8) virus frequently did not result in overt cellular transformation until 3 to 5 weeks postinfection. However, when cultures of DK1-B_{77}(p7)- and DK2-B_{77}(p8)-infected DEF were analyzed for virus production after subculturing three to five times, both appeared to be producing approximately equivalent amounts of virus, as judged by the amount of labeled 60 to 70S RNA recovered from culture fluids (data not shown).

The RNase T_1-resistant oligonucleotide fingerprint of ^{32}P-labeled DK2-B_{77}(p8) 60 to 70S RNA is shown in Fig. 4. A comparison of the fingerprints shown in Fig. 4 and 1 clearly shows that not only was DK2-B_{77}(p8) RNA deficient in the same oligonucleotides as DK1-B_{77}(p7), but that it also contained two new oligonucleotides, again in reduced yield, with the same relative electrophoretic mobilities as D1 and D2 (Fig. 4A and inset, arrows, and Fig. 4B, solid circles). Although an oligonucleotide with the apparent mobility of D2 is not readily visible in Fig. 4A, it could be observed on the original autoradiograph and has been readily detected in subsequent preparations (e.g., see Fig. 6F). Secondary digestion of oligonucleotides D1 and D2 with RNase A yielded the same digestion products as were obtained from oligonucleotides D1 and D2 derived from DK1-B_{77}(p7) (Table 1). By these criteria, the RNA composition of DK2-B_{77}(p8) appears remarkably similar to that of DK1-B_{77}(p7).

To compare the RNA subunits of the two DK-B_{77} virus preparations, ^{3}H-labeled poly(A)-containing RNA isolated from DK2-B_{77}(p8) virions was mixed with similarly prepared ^{32}P-labeled virion RNA derived from DK1-B_{77}(p7)-infected DEF (Fig. 5D) and electrophoresed on denaturing agarose gels. DK2-B_{77}(p8) was composed primarily of two RNA subunits, which comigrated with the 2.65- and 1.55-Mdal RNA subunits of DK1-B_{77}(p7). The molar ratio of the subunits in DK2-B_{77}(p8) was approximately the same as that observed in DK1-B_{77}(p7) (1:9 and 1:6, respectively; Fig. 5D). Viral RNA species comigrating with the 3.40- and 2.25-Mdal RNA subunits observed in DK1-B_{77}(p7) were not readily detectable in DK2-B_{77}(p8) virion RNA, although in subsequent preparations small amounts of both subunits were observed (data not shown).

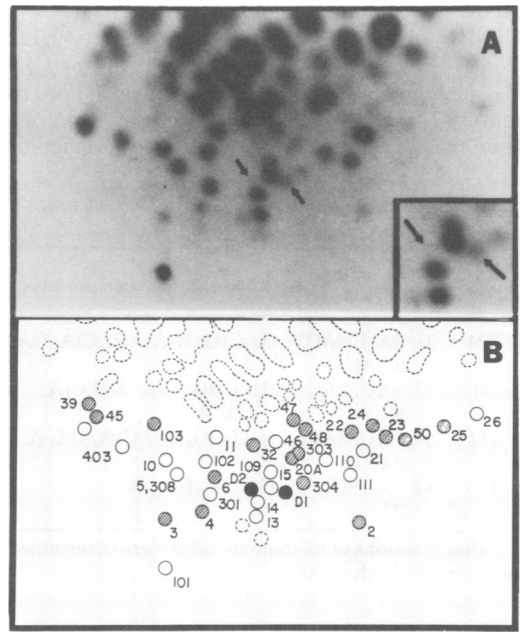

FIG. 4. *Two-dimensional gel electrophoresis of RNase T_1-resistant oligonucleotides of DK2-B_{77}(p8) viral RNA. (A) DK2-B_{77}(p8) 60 to 70S [^{32}P]RNA (1.5 × 10^6 cpm) was digested with RNase T_1 and subjected to two-dimensional gel electrophoresis as described in the text. Exposure was for 2 days at 4°C. Arrows indicate positions of oligonucleotides D1 and D2 observed in DK1-B_{77}(p7) 60 to 70S RNA (see text). (B) Schematic representation of the oligonucleotide fingerprint of DK1-B_{77}(p7). Shaded circles identify oligonucleotides present in reduced intensity. Solid circles identify oligonucleotides indicated by arrows in (A).*

To examine the extent to which defective RNAs had accumulated in B_{77} RSV preparations passaged multiple times in DEF cells, ^{3}H-labeled poly(A)-containing RNA from DK2-B_{77}(p8) virions was mixed with ^{32}P-labeled poly(A)-containing RNA purified from B_{77} virus passaged on CEF (Fig. 5A), B_{77} passaged once on DEF (Fig. 5B), or B_{77} passaged four times on DEF (Fig. 5C) and electrophoresed on denaturing 0.75% agarose gels. A single passage of CK-B_{77} in DEF resulted in substantial amplification of the *td* (2.65-Mdal) RNA relative to the *nd* (3.40-Mdal)

RNA. The molar ratio of *nd* RNA to *td* RNA in CK-B_{77} grown on CEF was approximately 1.0: 0.75 (Fig. 5A), whereas the same ratio for CK-B_{77} passaged one time on DEF was approximately 1:5 (Fig. 5B). CK-B_{77} RSV passaged one time on DEF did not appear to contain significant amounts of the 1.55-Mdal RNA. Analysis of virus passaged four times on DEF revealed that the molar ratio of *nd* RNA to *td* RNA remained fairly constant, but this virus also contained a new RNA, which comigrated with the 1.55-Mdal RNA of DK2-B_{77}(p8) and represented approximately 18% (molar concentration) of the major RNA species. These results suggest that a deleted RNA of 1.55 Mdal becomes amplified after multiple passages on DEF.

TABLE 1. *Compositions of RNase T_1-resistant oligonucleotides*[a]

Oligonucleotide	RNase A digestion products	RNase U2 digestion products
304	G, 3AU, 2AC, 2C, 4U	$(U_3C)G$, U_2A, 2(U,C)A, CA, A
D1	G, 3AU, 2AC, 3C, 4U	$(U_3,C_2)G$, U_2A, 2(U,C)A, CA, A
D2	G, 2AU, 3AC, 3C, 4U	$(U_3,C_2)G$, 3(U,C)A, CA, A
15	G, AU, 9C, 6U	UG, $(U_6,C_{9-10})A$

[a] Compositions of oligonucleotides were determined as described in the text.

RNase T_1-resistant oligonucleotide fingerprint analysis of the major B_{77} RNA species. To determine the extent of the deletions present in each of the viral RNA subunits of duck-passaged B_{77} and to determine which viral RNA subunits contained the new oligonucleotides D1 and D2, ^{32}P-labeled viral RNA was prepared, and the RNA subunits were resolved on 0.75% denaturing agarose gels. Gel fractions containing the individual subunit RNAs were pooled, and the labeled RNA was extracted from the agarose with formamide-phenol and sub-

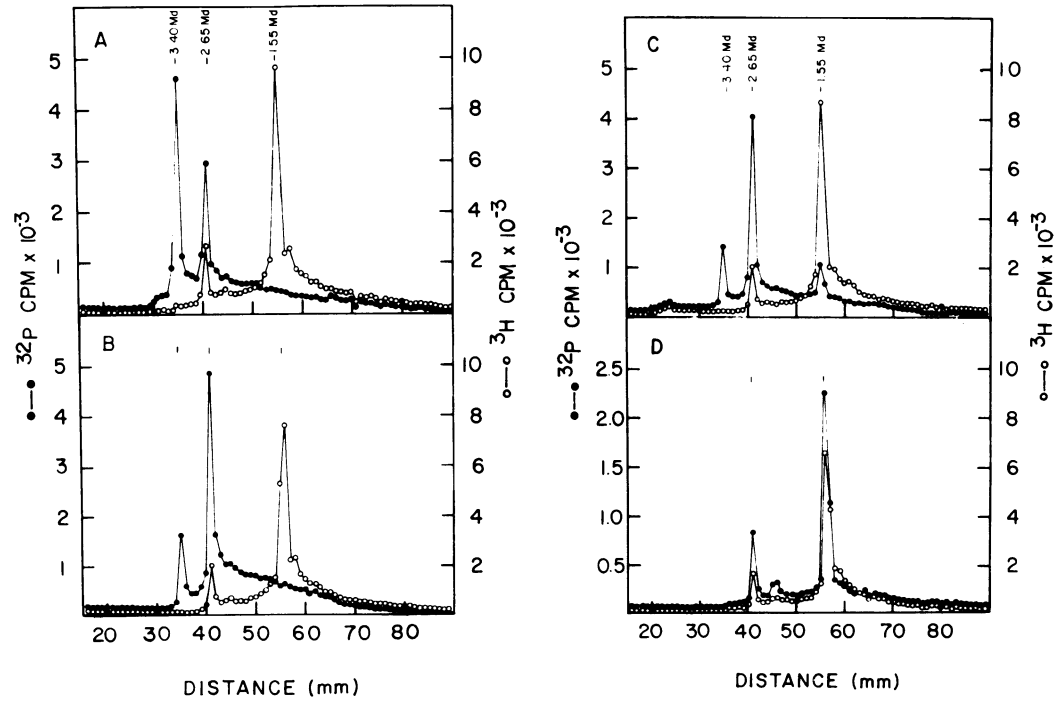

FIG. 5. *Comparison of the RNA subunits of DK1-B_{77}(p7) and DK2-B_{77}(p8) and analysis of the accumulation of defective RNA subunits in DK2-B_{77} virus preparations. Poly(A)-containing [^{3}H]RNA from DK2-B_{77}(p8) was mixed with the poly(A)-containing [^{32}P]RNA purified from DK1-B_{77}(p7), from B_{77} virus passaged in CEF (CK-B_{77}), from B_{77} passaged once in DEF [DK2-B_{77}(p1)], or from B_{77} passaged four times in DEF [DK2-B_{77}(p4)] and electrophoresed on denaturing 0.75% agarose gels containing 5 mM methyl mercuric hydroxide. Electrophoresis was performed at 50 V (2.3 mA/gel) for 5 h. The molecular weights of viral RNA species were estimated relative to the positions of 28 and 18S [^{3}H]rRNA's electrophoresed in parallel. (A) DK2-B_{77}(p8) [^{3}H]RNA (○; 80,000 cpm) mixed with CK-B_{77} [^{32}P]RNA (●; 40,000 cpm). (B) DK2-B_{77}(p8) [^{3}H]RNA (○; 80,000 cpm) mixed with DK2-B_{77}(p1) [^{32}P]RNA (●; 40,000 cpm). (C) DK2-B_{77}(p8) [^{3}H]RNA (○; 80,000 cpm) mixed with DK2-B_{77}(p4) [^{32}P]RNA (●; 40,000 cpm). (D) DK2-B_{77}(p8) [^{3}H]RNA (○; 60,000 cpm) mixed with DK1B_{77}(p1) [^{32}P]RNA (●; 20,000 cpm). Md, Megadaltons.*

jected to RNase T_1-resistant oligonucleotide fingerprint analysis. The 3.40-Mdal RNA of CK-B_{77} (Fig. 6A) contained all of the oligonucleotides found in *nd* B_{77} RNA, whereas the 2.65-Mdal RNA subunit contained significantly reduced amounts of the *src*-specific oligonucleotides (oligonucleotide 6) (Fig. 6B). The fingerprint of the 2.65-Mdal RNA of DK2-B_{77}(p8) was identical to that of the CK-B_{77} *td* subunit (Fig. 6B and C). This species contained no detectable oligonucleotide 6, and the spot which contains oligonucleotides 5 and 308 was reduced in intensity, indicating that it contained a single component (Fig. 6A and C). More importantly, the 2.65-Mdal subunit of DK2-B_{77}(p8) did not contain oligonucleotides D1 and D2.

The oligonucleotide fingerprint of the 1.55-Mdal RNA clearly demonstrates that this RNA contains both the *src* gene deletion characteristic of *td* subunits (oligonucleotides 6 and 5) and the *pol* region deletion spanning oligonucleotides 304 to 4 (Fig. 6F). Oligonucleotides D1 and D2 are present in the 1.55-Mdal RNA (Fig. 6F and inset, solid arrows), although the relative intensities of these oligonucleotides were reduced. The reduced intensity may indicate the presence in the 1.55-Mdal RNA of either D1 or D2, but not both.

The oligonucleotide fingerprint of the 2.25-Mdal RNA is shown in Fig. 6E. Inspection of the fingerprint of this RNA revealed high levels of oligonucleotides 6 and 5, representative of the *src* gene region, and low levels of oligonucleotides 304 to 4. This fingerprint suggests that the majority of the 2.25-Mdal RNA contains all of the *src* gene and is deleted in the polymerase region of the genome. However, the small amount of the 2.25-Mdal RNA precluded a complete analysis.

In vitro translation of DK2-B_{77}(p8) virion RNA. Virion RNA has been observed to program cell-free translation systems for the production of both $Pr76^{gag}$ and $Pr180^{gag\text{-}pol}$ (3, 32). To determine whether the 1.55-Mdal RNA observed in DK2-B_{77}(p8) virions can function as an mRNA for synthesis of viral proteins, purified virion RNA was used to program an in vitro protein-synthesizing system. ^{32}P-labeled DK2-B_{77}(p8) poly(A)-containing virion RNA (20 μg) was electrophoresed on 0.75% agarose gels containing 5 mM methyl mercuric hydroxide. RNA was extracted from the regions of the gel containing the 2.65- and 1.55-Mdal RNA species and used to program a rabbit reticulocyte cell-free protein-synthesizing system (Fig. 7). Unfractionated DK2-B_{77}(p8) virion RNA directed the synthesis of $Pr76^{gag}$, trace amounts of $Pr180^{gag\text{-}pol}$, and three additional high-molecular-weight polypeptides with molecular weights of 90,000, 110,000, and 130,000 (Fig. 7, lane 2). Translation of the 2.65-Mdal RNA yielded predominantly $Pr76^{gag}$, although small amounts of the 90,000-, 110,000-, and 130,000-dalton proteins were observed (Fig. 7, lanes 3 through 5). Translation of the 1.55-Mdal RNA resulted in the synthesis of large amounts of the 90,000-, 110,000-, and 130,000-dalton proteins but only small amounts of $Pr76^{gag}$ (Fig. 7, lanes 6 through 8). Whereas the 2.65-Mdal RNA programmed the synthesis of trace amounts of $Pr180^{gag\text{-}pol}$, none of this protein was observed in the reactions programmed by the 1.55-Mdal RNA. From these data, we conclude that the 1.55-Mdal RNA can function as an mRNA in vitro and primarily directs the synthesis of three large polypeptides having molecular weights of 90,000, 110,000, and 130,000. Based on data presented below, it is likely that the 130,000-dalton protein (p130) represents the primary translation product and that the 90,000- and 110,000-dalton proteins are derived by premature termination of translation.

Analysis of virus-specific proteins synthesized in DK2-B_{77}(p8)-infected DEF. To determine whether the 1.55-Mdal RNA functions as an mRNA in cells infected with DK2-B_{77}(p8), DEF infected with either DK2-B_{77}(p8) or CK-B_{77} and CEF infected with CK-B_{77} were incubated for 3 h in media containing [^{35}S]methionine, and the virus-specific proteins were analyzed by immunoprecipitation and polyacrylamide gel electrophoresis. Immunoprecipitation of ^{35}S-labeled proteins from DK2-B_{77}(p8)-infected DEF with antisera directed against the major core protein, p27, revealed the presence of p27-containing polypeptides with molecular weights of 180,000 ($Pr180^{gag\text{-}pol}$), 130,000 (p130), 76,000 ($Pr76^{gag}$), 66,000 ($Pr66^{gag}$), and 27,000 (p27) (Fig. 8, lane 1). However, only four of these proteins ($Pr180^{gag\text{-}pol}$, $Pr76^{gag}$, $Pr66^{gag}$, and p27) were observed in parallel immunoprecipitations of labeled proteins from CK-B_{77}-infected DEF or CK-B_{77}-infected CEF (Fig. 8, lanes 2 and 3). Immunoprecipitation of labeled proteins from DK2-B_{77}(p8)-infected DEF with antisera directed against gp85 specifically precipitated gp85 and p130 but no significant amounts of any other *gag*-related proteins (Fig. 8, lane 4). Immunoprecipitation of labeled proteins from either DEF or CEF infected with CK-B_{77} yielded only gp85 (Fig. 8, lanes 5 and 6). When antisera directed against the $\alpha\beta$ form of viral RNA-dependent DNA polymerase were used, immunoprecipitation of labeled proteins from DK2-B_{77}(p8)-infected DEF precipitated only $Pr180^{gag\text{-}pol}$. No significant amount of p130 was observed (Fig. 8, lane 7). A similar analysis of CK-B_{77}-infected DEF proteins showed less $Pr180^{gag\text{-}pol}$ and a significant amount of the α

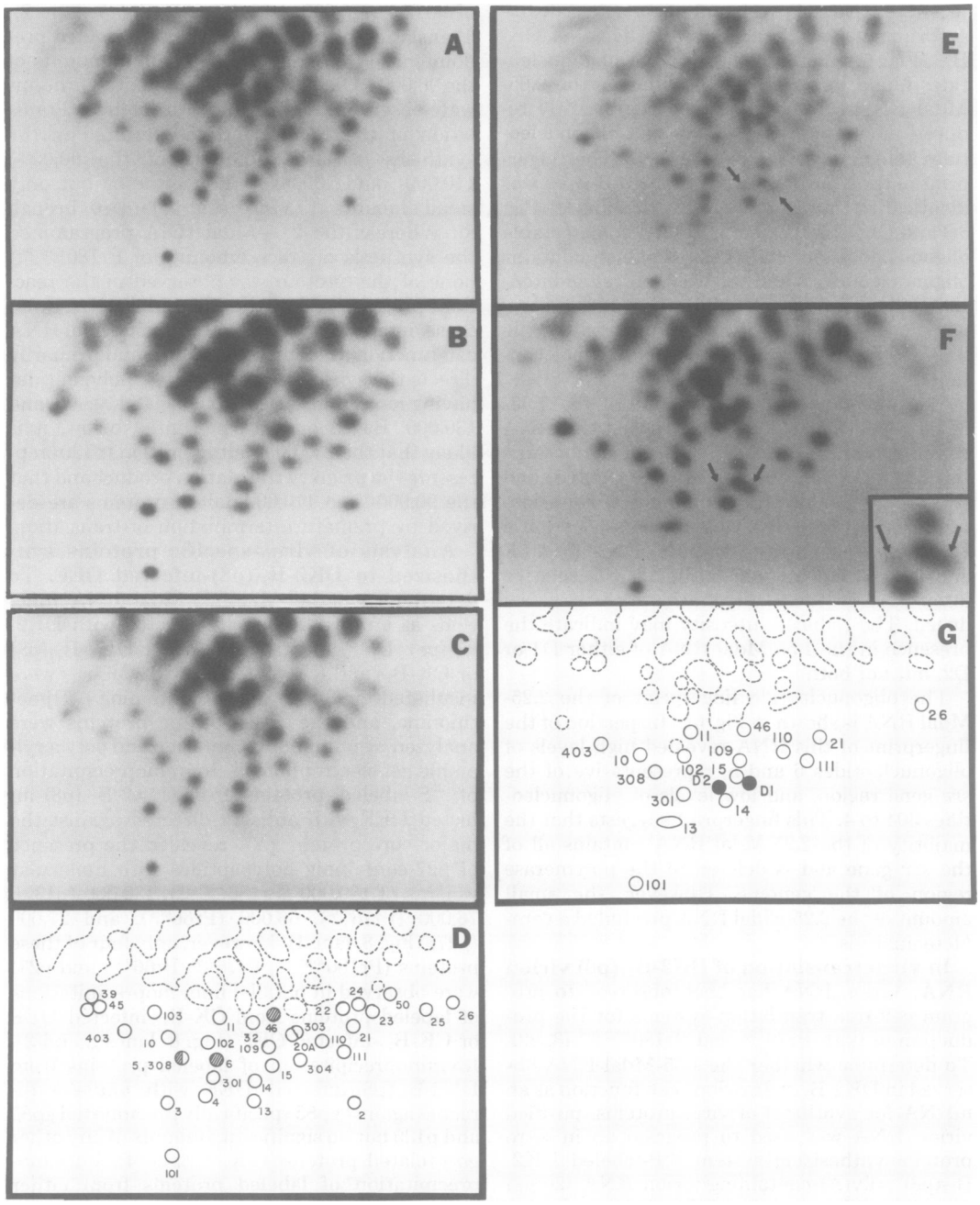

FIG. 6. *RNase T_1-resistant oligonucleotide fingerprint analysis of the major B_{77} RNA species. Poly(A)-containing [^{32}P]RNA was isolated from CK-B_{77} and DK2-B_{77}(p8) and electrophoresed on denaturing 0.75% agarose gels. After electrophoresis gels were sliced into 1-mm fractions and counted for Cerenkov radiation. One to three gel slices corresponding to each of the major RNA species present in either CK-B_{77} or DK2-B_{77}(p8) were pooled, and the [^{32}P]RNA was recovered by formamide-phenol extraction as described in the text. Recoveries of RNA ranged from 63 to 82%. [^{32}P]RNA was digested with RNase T_1 and subjected to two-dimensional gel electrophoresis as described in the text. Autoradiography was for 6 to 16 days at 4°C, using DuPont Lightning Plus intensifying screens. (A) RNase T_1 fingerprint of the 3.40-Mdal [^{32}P]RNA subunit (106,000 cpm) of CK-B_{77} RNA. (B) RNase T_1 fingerprint of the 2.65-Mdal [^{32}P]RNA subunit (125,000 cpm) of CK-B_{77} RNA. (C) RNase T_1 fingerprint of the 2.65-Mdal [^{32}P]RNA subunit (51,000 cpm) of DK2-B_{77}(p8) RNA.*

subunit of reverse transcriptase (Fig. 8, lane 8). The significance of the 76,000-dalton protein observed in this track is not understood. Analysis of CK-B_{77}-infected CEF proteins revealed only the presence of the α subunit of reverse transcriptase. Little or no $Pr180^{gag\text{-}pol}$ is evident (Fig. 8, lane 9).

The data of Fig. 8 demonstrate that DEF infected with DK2-B_{77}(p8) contain a unique viral protein of approximately 130,000 daltons not present in chicken or duck cells infected with CK-B_{77} RSV. p130 contains peptide sequences which render it cross-reactive with antisera directed against *gag* and *env* polypeptides but not with antisera directed against reverse transcriptase. Therefore, the synthesis of p130 appears to be the result of a *gag-env* gene fusion. Furthermore, the difference between the molecular weight of p130 and the total molecular weight of the polypeptides derived from the *gag*, *pol*, and *env* genes (about 250,000) indicates that such fusion could result from a deletion of about 3,300 nucleotides spanning the polymerase gene region. This is further supported by the observation (Fig. 7) that the 1.55-Mdal RNA (containing a deletion of the *pol* region) directs the synthesis of p130.

5′ Boundary of the polymerase region deletion. The generation of the new oligonucleotides (D1 and D2) observed in the 2.25- and 1.55-Mdal RNAs (Fig. 6E and F) could occur either by the acquisition of new nonviral sequences or by the breaking and rejoining of viral sequences as a consequence of a deletion event. Comparison of the partial sequence data of oligonucleotides D1 and D2 with oligonucleotides deleted in the *pol* gene region indicated that these new oligonucleotides might be related to each other and to an oligonucleotide, oligonucleotide 304, which maps toward the 5′ end of the deleted region (Table 1). To examine the sequence relationship of D1, D2, and 304, the primary nucleotide sequences of these oligonucleotides were determined. DK2-B_{77}(p8) 60 to 70S RNA (labeled at a low specific activity with ^{32}P) was digested to completion with RNase T_1, and the oligonucleotides were resolved by two-dimensional gel electrophoresis. Oligonucleotides D1, D2, and 304 were excised from the gel, eluted, and labeled at their 5′ termini with [γ-^{32}P]ATP. Each labeled oligonucleotide was repurified by gel electrophoresis and analyzed by the direct sequencing procedures of Donis-Keller et al. (10). The resulting sequence ladders for oligonucleotides D1, D2, and 304 are shown in Fig. 9A, B, and C, respectively.

The primary sequences of oligonucleotides D1, D2, and 304 (Fig. 10) are further supported by the complete RNase A and U2 digestion products listed for each oligonucleotide in Table 1. To rule out the possibility that any of the cleavages observed in D1 and D2 were due to contamination of these oligonucleotides with oligonucleotide 15 (which migrates between D1 and D2 in the second dimension), a similar analysis was performed on this oligonucleotide. A comparison of the sequence ladders of oligonucleotides D1, D2, and 15 showed that no cleavages unique for oligonucleotide 15 are present in the sequence ladders of D1 or D2 (data not shown).

The primary sequences of oligonucleotides D1 and 304 (Fig. 10) show identity at 16 of 18 positions. The positions of nonhomology reside at the 3′ terminus of oligonucleotide 304, suggesting that oligonucleotide D1 is derived from oligonucleotide 304 by deletion of viral sequences at a point within oligonucleotide 304. The reduced intensity of oligonucleotide D1 in Fig. 6F suggests that all 1.55-Mdal RNA species contain this oligonucleotide and that some may contain oligonucleotide D2 instead. Oligonucleotide D2 may also be derived from oligonucleotide 304 by a similar deletion of viral sequences. The extensive sequence homology among oligonucleotides D1, D2, and 304 supports this suggestion. The location of oligonucleotide 304 near the 5′ end of the deleted sequences is, therefore, consistent with the hypothesis that this oligonucleotide defines the 5′ terminus of the *pol* region deletion (see below).

DISCUSSION

The genetic compositions of two independently derived preparations of B_{77} RSV were analyzed after each was passaged seven or more times on DEF. RNase T_1-resistant oligonucleotide fingerprint analysis of virion RNA from both preparations of duck-passaged B_{77} revealed that approximately 75% of the RNAs contain a

(D) Schematic of (A). Shaded circles identify oligonucleotides present in the RNase T_1 fingerprint of the 3.40-Mdal RNA and absent in the RNase T_1 fingerprints of the 2.65-Mdal RNAs. (E) RNase T_1 fingerprint of the 2.25-Mdal [^{32}P]RNA subunit (35,700 cpm) of DK2-B_{77}(p8) RNA. Arrows identify oligonucleotides D1 and D2. (F) RNase T_1 fingerprint of the 1.55-Mdal [^{32}P]RNA subunit (167,000 cpm) of DK2-B_{77}(p8) RNA. Arrows identify oligonucleotides as in (E). (G) Schematic of (F). Numbers identify oligonucleotides characterized in Fig. 1. Solid circles identify oligonucleotides identified by arrows in (E). Dashed circles identify minor oligonucleotides present only in this particular preparation of DK2-B_{77}(p8).

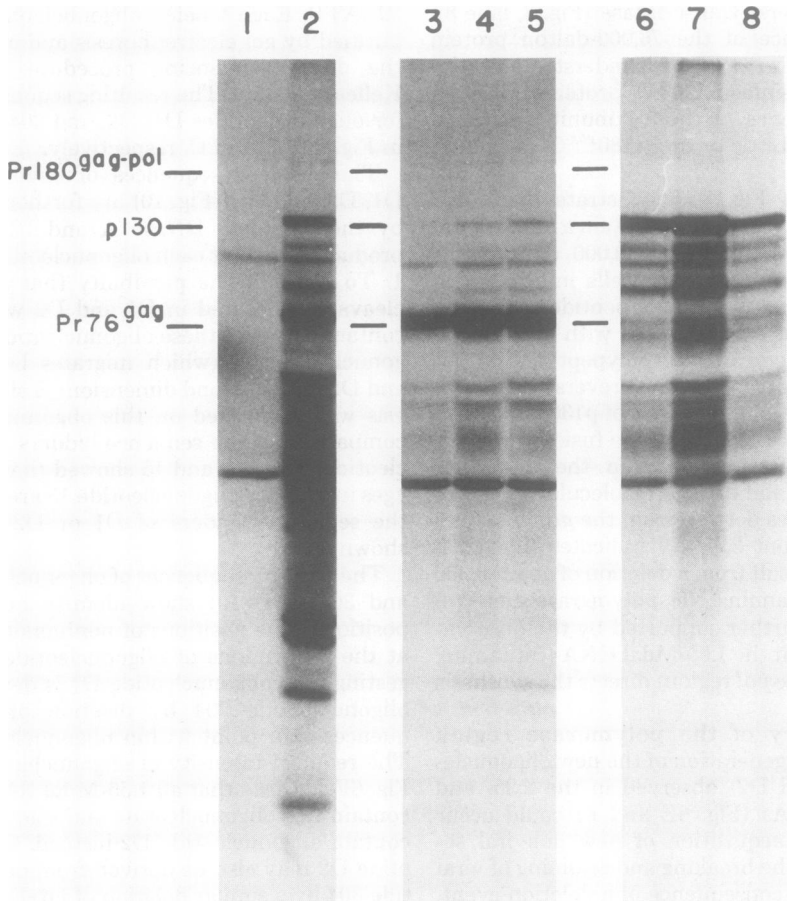

FIG. 7. *In vitro translation of DK2-B_{77}(p8) viral RNA. Poly(A)-containing DK2-B_{77}(p8) [^{32}P]RNA (20 μg; 20,000 cpm/μg) was electrophoresed on 0.75% agarose gels containing 5 mM methyl mercuric hydroxide. The gel slices containing the 2.65- and 1.55-Mdal RNA species were pooled, and the RNA was recovered as described in the text. The purified RNA was redissolved in 22 μl of a rabbit reticulocyte cell-free lysate containing 5.5 μCi (4 pmol) of [^{35}S]methionine. After 2 h of incubation at 30°C, protein synthesis was terminated, and the proteins synthesized were analyzed by SDS-polyacrylamide gel electrophoresis. The RNAs used to program each reaction were as follows: lane 1, no RNA; lane 2, 3 μg of poly(A)-containing DK2-B_{77} RNA, unfractionated; lanes 3 through 5, RNAs recovered from fractions containing the 2.65-Mdal RNA species (170, 265, and 253 ng, respectively); lanes 6 through 8, RNAs recovered from fractions containing the 1.55-Mdal RNA species (545, 885, and 730 ng, respectively). Molecular weights were assigned based on electrophoretic mobilities relative to the following molecular weight standards: Escherichia coli RNA polymerase, β and β′ chains, 155,000 and 165,000, respectively; rabbit muscle phosphorylase, 92,500; bovine serum albumin, 66,000; human immunoglobulin G, heavy chain, 54,000; ovalbumin, 42,000; human immunoglobulin G, light chain, 22,000; and cytochrome c, 12,000.*

deletion which spans oligonucleotides 304 to 4 and encompasses all of the B_{77} polymerase gene. More than 90% of the RNAs also contain a deletion which spans the *src*-specific oligonucleotides 6 and 5 and is identical to the deletion observed in *td* B_{77}. Virion RNA from duck-passaged B_{77} also contains two oligonucleotides (D1 and D2) not observed in the RNA of B_{77} virus grown on CEF. Analysis of the virion RNA of duck-passaged B_{77} by denaturing agarose gel electrophoresis revealed four major subunits with molecular weights of 3.40×10^6, 2.65×10^6, 2.25×10^6, and 1.55×10^6. Although the 3.40- and 2.65-Mdal RNA species comigrated with the *nd* and *td* RNAs of B_{77} propagated on CEF, no counterparts to the 2.25- and 1.55-Mdal RNAs were observed in the RNA of B_{77} grown on CEF. Oligonucleotide fingerprint analysis of these RNA species revealed that the 2.65-Mdal RNA contains the *src*-specific deletion and that the

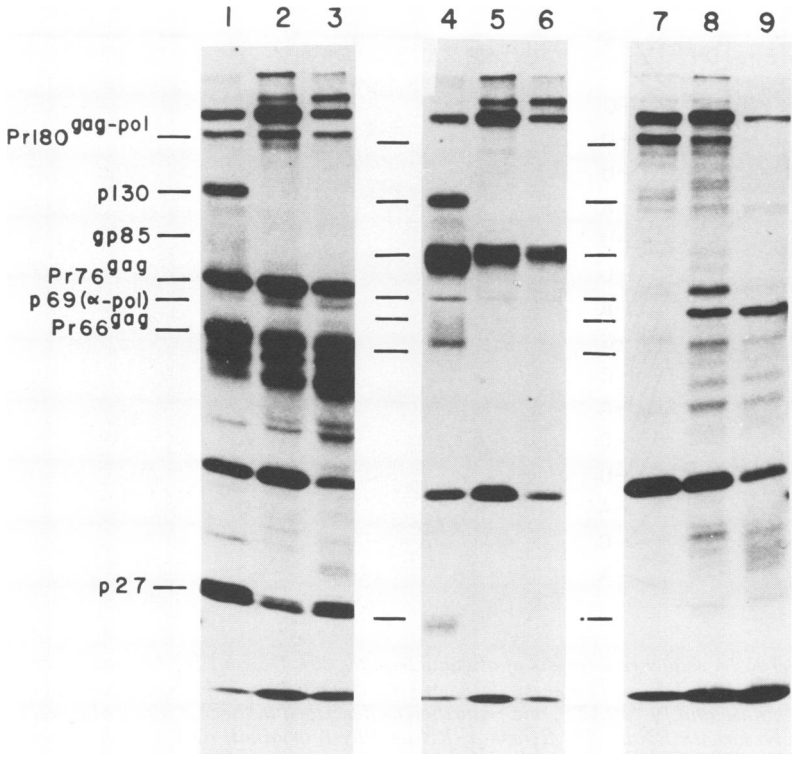

FIG. 8. *Immunoprecipitation of viral proteins from [^{35}S]methionine-labeled DK2-B_{77}(p8)-infected DEF. [^{35}S]methionine-labeled cell lysates were prepared from DK2-B_{77}(p8)-infected DEF (lanes 1, 4, and 7), CK-B_{77}-infected DEF (lanes 2, 5, and 8), and CK-B_{77}-infected CEF (lanes 3, 6, and 9). Virus-specific proteins in each lysate were immunoprecipitated with antisera directed against viral proteins p27 (lanes 1 through 3), gp85 (lanes 4 through 6), or αβ DNA polymerase (lanes 7 through 9) and analyzed by SDS-polyacrylamide gel electrophoresis. Molecular weights were assigned as described in the legend to Fig. 7.*

2.25-Mdal RNA contains the polymerase region deletion; both of these deletions were observed in the 1.55-Mdal RNA, which was the major RNA subunit species detected in duck-passaged B_{77}. The new oligonucleotides D1 and D2, observed in the duck-passaged virus, were present in the 2.25- and 1.55-Mdal RNA species, but not in the 2.65-Mdal RNA. Comparison of the nucleotide sequences of oligonucleotides D1, D2, and 304 (an oligonucleotide deleted in the defective RNA) suggested that oligonucleotides D1 and D2 are derived from oligonucleotide 304. Oligonucleotide 304, therefore, likely defines the 5′ terminus of the sequences lost as a consequence of the polymerase region deletion.

The deletion in the 2.25- and 1.55-Mdal RNA species which spans oligonucleotides 304 to 4 (Fig. 1) encompasses sequences derived from all three replicative genes (*gag, pol,* and *env*). A number of oligonucleotides located within the region deleted in duck-passaged B_{77} have been mapped to the *env* and *pol* genes. Analysis of the recombinant progeny obtained from mixed infections of CEF with an *nd* avian sarcoma virus and Rous-associated virus of different subgroups has indicated that the sequences defined by oligonucleotides 109 to 111 (Fig. 2) are involved in determining viral subgroup specificity (18, 19). Although oligonucleotide 4, which is present in all avian leukosis = sarcoma viruses, was originally mapped to the 3′ side of oligonucleotide 109 (19), the mapping of oligonucleotide 4, but not oligonucleotide 109, to the region deleted in the 1.55-Mdal RNA (Fig. 2) suggests that the order of these oligonucleotides could be reversed. The absence of oligonucleotide 4 in the NY8 variant of Schmidt-Ruppin RSV, subgroup A (39), indicates that this oligonucleotide is not contained within the *pol* gene, since the NY8 variant of RSV contains a functional polymerase gene (20). In addition, a 28S mRNA species believed to direct the synthesis of the viral glycoproteins (17, 42) has been shown to contain oligonucleotide 4 (28). Therefore, oligonucleotide 4 is contained within the *env* gene. Based on arguments reviewed by Coffin et al. (8), the

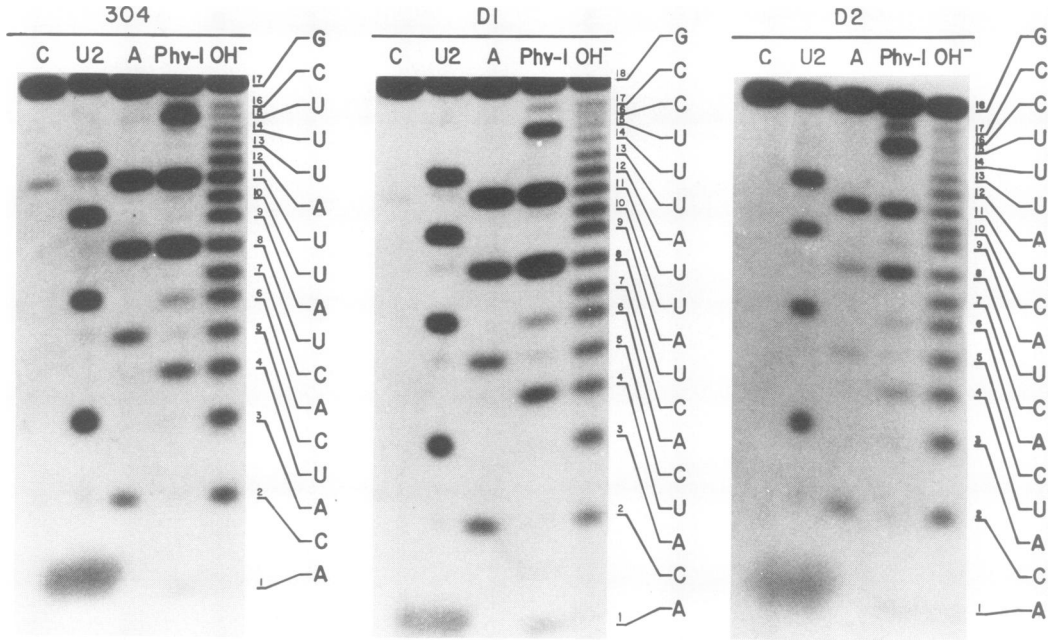

FIG. 9. *Direct RNA sequence analysis of oligonucleotides 304, D1, and D2. Oligonucleotides 304, D1, and D2 were isolated from an RNase T_1 digest of 20 μg of DK2-B_{77}(p8) 60 to 70S RNA, labeled at their 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP, and repurified as described in the text. Each oligonucleotide was then partially hydrolyzed with RNase U2, RNase A, RNase Phy-1, or alkali (OH^-) as described in the text and analyzed by electrophoresis on a 20% polyacrylamide gel. Lanes marked C show the electrophoretic profile of the untreated oligonucleotide. Each reaction contained 1.6 × 10^6 to 2.2 × 10^4 cpm of ^{32}P-labeled oligonucleotide. Autoradiography was for 48 h at −20°C, using DuPont Lightning Plus intensifying screens.*

Oligonucleotide	1	5	10	15	
304	5′ — A C A U C A C U A U U A U U U C G				— 3′
D1	A C A U C A C U A U U A U U U C C G				
D2	A C A U C A C U A C U A U U U C C G				
15	C U C C U C C U C C U C C U U A U G				

FIG. 10. *Nucleotide sequences of oligonucleotides 304, D1, D2, and 15.*

3′ boundary of the *pol* gene has been assigned to include oligonucleotide 32, and 5′ and 3′ boundaries of the *env* gene have been set to include oligonucleotides 4 and 10, respectively. The *pol* gene must extend leftward at least through oligonucleotide 3 since mapping of recombinants has localized the temperature-sensitive polymerase lesion of *ts*LA337-Prague strain of RSV, subgroup C (25), to a position located between oligonucleotides 24 and 3 (18, 19). The 5′ boundary of the *gag* gene is probably located between oligonucleotides 403 and 301 (Fig. 2) because oligonucleotides 13 and 403 have been identified as part of the leader sequence in subgenomic mRNA species (21, 28, 42).

The inclusion of *gag* gene sequences in the *pol* region deletion is indicated by the analysis of viral proteins synthesized in vivo and in vitro. Detection of a new viral protein, p130, which contains *gag*- and *env*- but not *pol*-specific antigenic determinants, suggests that the termination codon of the *gag* gene and all of the *pol* gene sequences have been lost as a result of the deletion, yielding a fused *gag-env* gene. The in vitro synthesis of p130 in reticulocyte lysates programmed with purified 1.55-Mdal RNA indicates that p130 synthesis in vivo does not result from a low level of suppression of the *gag* termination codon. This conclusion is further supported by a comparison of the amino acid sequences predicted from the primary sequence of oligonucleotide 304 and the amino acid sequence of the p15 polypeptide of avian myeloblastosis virus strain BAI-A (R. Sauer, personal communication). One of the reading frames of oligonucleotide 304 predicts the hexapeptide sequence Asp-Ile-Thr-Ile-Ile-Ser (Table 2). The substitution of a C residue for the A at position

TABLE 2. *Predicted amino acid sequence of oligonucleotide 304*

Polypeptide	Amino acid sequence with the following nucleotide sequence:					
	(G)AC	AUC	ACU	AUU	AUU	UCG
Oligonucleotide 304 (predicted)	Asp	Ile	Thr	Ile	Ile	Ser
Avian myeloblastosis virus residues 41 through 46[a]	Asp	Leu	Thr	Ile	Ile	Ser

[a] See text.

3 in the sequence of oligonucleotide 304 would result in a Leu residue instead of an Ile residue at position 42 (Table 2). Such single-base changes are common in avian sarcoma-leukosis viruses (8, 19, 33, 34). Since oligonucleotide 304 probably defines the 5′ terminus of the deleted sequences, the deletion observed in duck-passaged B_{77} includes the carboxy-terminal 80 to 85 amino acids (about 250 nucleotides) of $Pr76^{gag}$.

The amount of *env*-specific sequences included in the *pol* region deletion can only be approximated. Since about 67,000 daltons of p130 is *gag* related, the remainder of p130 (63,000 daltons) must be encoded by the *env* gene. Assuming that the polypeptide backbone of the viral glycoprotein complex has a molecular weight of 62,000 to 70,000 (9, 14, 23, 29), as much as 200 nucleotides of the *env* gene could be contained within the *pol* region deletion.

The mechanism(s) responsible for the generation of deletions such as those observed in the 1.55-Mdal RNA of DK-B_{77} RSV is unknown. Formation of such deletion mutants could arise by loss of sequences from the integrated provirus by chromosomal rearrangements, loss of sequences by asymmetric viral recombination, or the packaging and replication of aberrantly processed (spliced) RNA. Although such deletion mutants are not apparent in stocks of B_{77} RSV grown on chicken cells, we also cannot rule out their presence in these stocks and their subsequent amplification in duck embryo cells. Clearly, additional studies are needed to define the origin of these deletion mutants.

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