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Mapping oligonucleotides of Rous sarcoma virus RNA that segregate with polymerase and group-specific antigen markers in recombinants

[gene order of Rous sarcoma virus/recombinant viruses/DNA nucleotidyltransferase (DNA polymerase) mutants/group-specific antigen variants]

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ABSTRACT **The RNase-T_1-resistant oligonucleotides of two Prague Rous sarcoma viruses with temperature-sensitive (ts) DNA polymerases (DNA nucleotidyltransferases), termed ts LA 337 and 335, of one leukosis virus, RAV-6, and 20 of their recombinant progeny have been mapped relative to the 3′ poly(A) terminus of the viral RNA. The resulting oligonucleotide maps have been correlated with markers of the four known viral genetic elements encoded in the RNA of 10,000 nucleotides. In accord with previous results recombinant RNAs contained (*i*) oligonucleotides characteristic of the *src* gene, coding for sarcoma formation, between the poly(A) end and 2000 nucleotides and (*ii*) oligonucleotides characteristic of the *env* gene, coding for the envelope glycoprotein, between 2500 and 5000 nucleotides from the poly(A) end. (*iii*) A cluster of four oligonucleotides that mapped between 6000 and 8000 nucleotides from the 3′ poly(A) end of each RNA was shared by both parental viruses and all recombinants. Since all other map segments of our recombinants failed to segregate with the ts- or wild-type markers of the parental DNA polymerase gene (*pol*), it was concluded that the ts *pol* lesion maps in this RNA segment. (*iv*) The 5′ segment of each recombinant RNA contained a cluster of four to five oligonucleotides whose parental origin correlated with an electrophoretic marker of one of the parental virion proteins, p27, a major product of the viral *gag* gene. The gene order 5′-*gag-pol-env-src*-poly(A) is consistent with our data.**

Four genetic elements of nondefective (nd) Rous sarcoma virus (RSV) have been defined (1): *gag*, coding for the internal proteins termed group-specific antigens (2, 3); *pol*, coding for the viral DNA polymerase (DNA nucleotidyltransferase); *env*, coding for the envelope glycoprotein; and *src*, coding for sarcoma formation (4). The viral genome is an RNA of 10,000 nucleotides with a poly(A) stretch at the 3′ end (5–7).

Recently a chemical method has been developed to map these genetic elements on the viral RNA (7–9), By this method RNA segments are chemically identified by their large RNase-T_1-resistant oligonucleotides. The location of a given oligonucleotide relative to the poly(A) terminus of the RNA is then deduced from the length of the smallest poly(A)-tagged RNA fragment from which it can be isolated. The resulting order of all large oligonucleotides is termed an oligonucleotide map. The genetic functions of oligonucleotides have been identified by three procedures: (*i*) Oligonucleotides present in nondefective viruses but absent from corresponding deletion mutants have been equated with the genetic function of the wild type that is lacking in the deletion. In this fashion *src* and *env* have been identified and mapped in the order -*env-src*-poly(A) (4, 7–10). (*ii*) Oligonucleotides are identified functionally by correlating with parental gene markers the distribution of parental oligonucleotides in viral recombinants (6, 10). By this method the relative gene order -*pol-env-src*-poly(A) has been obtained (11–13). (*iii*) Another empirical procedure relies on the observation that certain viral oligonucleotides are highly conserved in all avian tumor viruses (8, 12) and if functionally identified can be used to identify gene locations in these viruses.

It is the primary purpose of this paper to identify *gag*- and *pol*-specific oligonucleotides by procedure *ii*. In addition *env* and *src* oligonucleotides are identified by all three procedures. The recombinants used had been selected from crosses between two mutants of Prague RSV of subgroup C, with temperature-sensitive (ts) polymerases, termed tsLA 337 or 335 and leukosis virus RAV-6 (Rous-associated virus) of subgroup B (which lacks a *src* gene) (14). Thus, genetic markers of the *pol*, *env*, and *src* genes of each recombinant are known, while the parental origin of their *gag* gene was unknown. In a previous study with some of these recombinants we have associated *pol* with a RNA segment identified by four oligonucleotides and shared by all parents and recombinant viruses, because all other large recombinant oligonucleotide map segments segregated independently of the *pol* phenotype (13). These four oligonucleotides mapped between 6000 and 8000 nucleotides from the poly(A) end. These findings are confirmed and extended here. Further, we present evidence that a correlation exists between the distribution of parental oligonucleotides near the 5′ end of our recombinant RNAs and a parental electrophoretic marker of the p27 protein. p27 is the major structural protein of the virus and is a product of the *gag* gene (1, 16, 22). This leads us to conclude that the *gag* gene maps near the 5′ end of the RNA.

MATERIALS AND METHODS

All virus strains (13, 14), and procedures for RNA (4, 6, 8, 10, 12, 13) and protein analysis (15, 16) have been described in detail.

RESULTS

The Oligonucleotide Maps of tsLA 337/335, of Leukosis Virus RAV-6, and of 20 tsLA 337/335 × RAV-6 Recombinants. The RNase-T_1-resistant [^{32}P] oligonucleotides of tsLA 337, tsLA 335, RAV-6 (Fig. 1), and 20 tsLA 337/335 × RAV-6 recombinants (some of which are shown in ref. 13) were resolved by two-dimensional electrophoresis–chromatography (termed fingerprinting). Analyses of the RNase A-resistant fragments of the large T_1-resistant oligonucleotides of each virus strain are reported in Table 1. The fingerprint patterns of the parental viruses tsLA337 and tsLA335 were identical (Fig. 1, Table 1). Prague RSV tsLA 337/335-specific oligonucleotides were designated as P-numbers and RAV-6-specific oligonu-

Abbreviations: ts, temperature sensitive; RSV, Rous sarcoma virus; RAV, Rous-associated virus (leukosis).

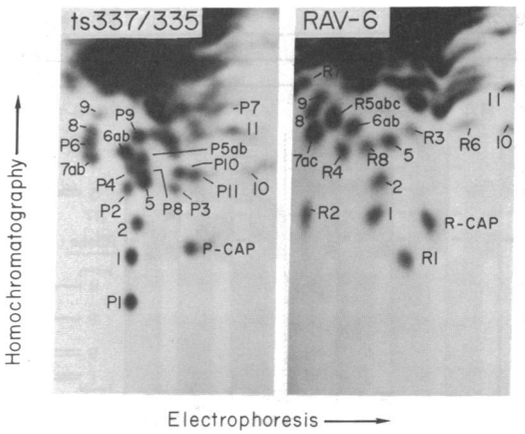

FIG. 1. RNase T_1-resistant oligonucleotides of the 60–70S [^{32}P]RNAs of Prague RSV tsLA 337 (same as 335, not shown) and leukosis virus RAV-6 after electrophoresis and homochromatography (fingerprinting). Preparation of [^{32}P]RNA, its digestion with RNase T_1, fingerprinting, and autoradiography have all been described in detail (4, 7, 8, 10, 12, 13). Fingerprints were derived from RNA which had been alkali fragmented and from which poly(A)-tagged fragments had been removed. The P-, R-, and unlettered numbers identify tsLA 337/335-, RAV-6-, and common oligonucleotides (Table 1, Fig. 2).

cleotides as R-numbers (Table 1, Figs. 1 and 2). Oligonucleotides shared by both viruses were given unlettered numbers. Six of these common oligonucleotides are highly conserved with regard to composition and oligonucleotide map location (Fig. 2) in all avian tumor viruses analyzed to date (12). In all previous analyses the fingerprints of recombinants were found to be mosaics of parental oligonucleotides (6, 10–13). Consequently we have designated recombinant oligonucleotides with the symbols of their parental counterparts (Table 1, Fig. 2). However, recombinant M12 contained one oligonucleotide mapping 3000 nucleotides from the poly(A) end which was not shared with either parent and is not listed in Table 1 and Fig. 2. This oligonucleotide was presumably generated by crossing-over (6).

Oligonucleotide maps of tsLA 337/335 and RAV-6 (Fig. 2) were derived by determining, from discrete sizes of poly(A)-tagged RNA fragments, the distance between each oligonucleotide and the poly(A) end of the viral RNA (not shown). Fingerprint patterns of these poly(A)-tagged fragments have been published (13). Oligonucleotides shared by both parental viruses are connected by single horizontal lines and oligonucleotides highly conserved among all avian tumor viruses are connected by double lines. Oligonucleotide maps of twenty tsLA 337/335 × RAV-6 recombinants are shown on the same figure. The oligonucleotide maps of ten recombinants (V1–V5, M2, M4, M6, M10, M12) were derived directly from fingerprints of poly(A)-tagged RNA fragments (some are shown in ref. 13). The oligonucleotide maps of the remaining recombinants (M1, M3, M5, M7–9, M11, M13–15) were drawn by assigning for each recombinant oligonucleotide a map location which is equivalent to its parental counterpart. This is in accord with previous evidence that rearrangements and permutations do not occur during recombination and that all avian tumor viruses share highly conserved oligonucleotides at equivalent map locations (8, 12, 13). The presence of a parental oligonucleotide is indicated by + and the absence by − in three different columns at the appropriate map location for each oligonucleotide of a recombinant (Fig. 2). The right column records tsLA 337/335-specific oligonucleotides (P-numbers), the middle column records oligonucleotides shared by both parents (unlettered numbers), and the left column records RAV-6-specific oligonucleotides (R-numbers). The stippled area connecting

Table 1. RNase-T_1-resistant oligonucleotides of Prague RSV-C tsLA 337/335, RAV-6, and tsLA 337/335 × RAV-6 recombinants

ts337/335*	RAV-6*	RNase A digestion products†
C	C	G,(AC),(AU),(AAU),(AAAC)
1	1	5U,9C,2(AC),(AU),(AAC),(AAG)
2	2	5U,7C,G,(AC),(AU),(AAAC)
5	5	5U,6C,(AAG),(AAAAN)
6ab	6ab	4U,5C,2G,4(AC),2(AU),(AAC),(AAAAAN)
7b		U,2C,G,(AC),(AU),(AAAAAAN)
7a	7a	5C,2(AC),(AU),(AG),(AAC)
	7c	6C,2(AC),(AU),(AG),(AAU),(AAAAAAN)
8	8	6C,3(AC),(AAG)
9	9	3C,(AU),(AG),(AAAAAAAN)
10	10	6U,2C,G,4(AU),(AAU)
11	11	9U,6C,G,(AU)
P1		7U,8C,G,3(AC),(AAC)
P2		3U,7C,G,3(AC),(AU)
P3		4U,3C,G,(AC),2(AU),(AAC),(AAU)
P4		4U,5C,G,2(AC),(AU),(AAAC)
P5ab		6U,8C,G,4(AC),2(AU),(AG)
P6		U,6C,G,3(AC),(AAAN)
P7		5U,3C,G,(AU),(AAU)
P8		4U,3C,(AC),(AAG),(AAAAN)
P9		4U,4C,(AC),(AAAAG)
P10		5U,3C,2(AC),4(AU),(AG)
P11		5U,3C,G,(AC),2(AU),(AAAAN)
P-CAP‡		4U,4C,3(AC),3(AU),CAP
	R1	5U,8C,G,(AC),2(AU),(AAC),2(AAU)
	R2	2C,G,2(AC),2(AAAAN)
	R3	3U,3C,(AG),(AAU),(AAAU)
	R4	2U,7C,G,2(AC),(AU),(AAC)
	R5abc	5U,12C,3G,6(AC),2(AU),(AAC),2(AAAN)
	R6	6U,3C,G,2-3(AU),(AAAAN)
	R7	3C,2(AAC),(AAG)
	R8	5-6U,6C,(AAG),(AAAAN)
	R-CAP‡	4U,6C,3(AC),2(AU),CAP

* Numbers, capital letters, or number-capital letter combinations refer to oligonucleotides of tsLA 337/335, RAV-6 (Fig. 1), or tsLA 337/335 × RAV-6 recombinants (not shown here) (see also *text*). Oligonucleotide spots containing more than one G residue are identified by lower-case letters to indicate the number of unresolved or partially resolved oligonucleotides overlapping in such a spot.

† RNase-A-resistant fragments of RNase-T_1-resistant oligonucleotides were determined as described (6, 8). Residue symbols in parentheses indicate nucleotide sequences.

‡ This oligonucleotide was shown to contain m^7GpppG^mpCp, the 5′-terminal sequence of tumor virus RNA (17, 18).

recombinant oligonucleotides over two columns is an interpretation of our data. We assume that a given recombinant map segment was inherited from tsLA 337/335 if the stippled area connects + symbols of the middle and right column and that segments were derived from RAV-6 if + symbols of the middle and left column are connected. Switches would indicate a minimum number of cross-over points ranging between 2 and 5.

Comparing oligonucleotide map segments with the known genetic functions of the parents and their recombinants, we make the following seven deductions.

The Map Locations of *src*, *env*, and *pol* in Recombinants. (*i*) All recombinant RNAs shared between poly(A) and nucleotide 2000 three to four oligonucleotides (P3, P4, P5ab) with

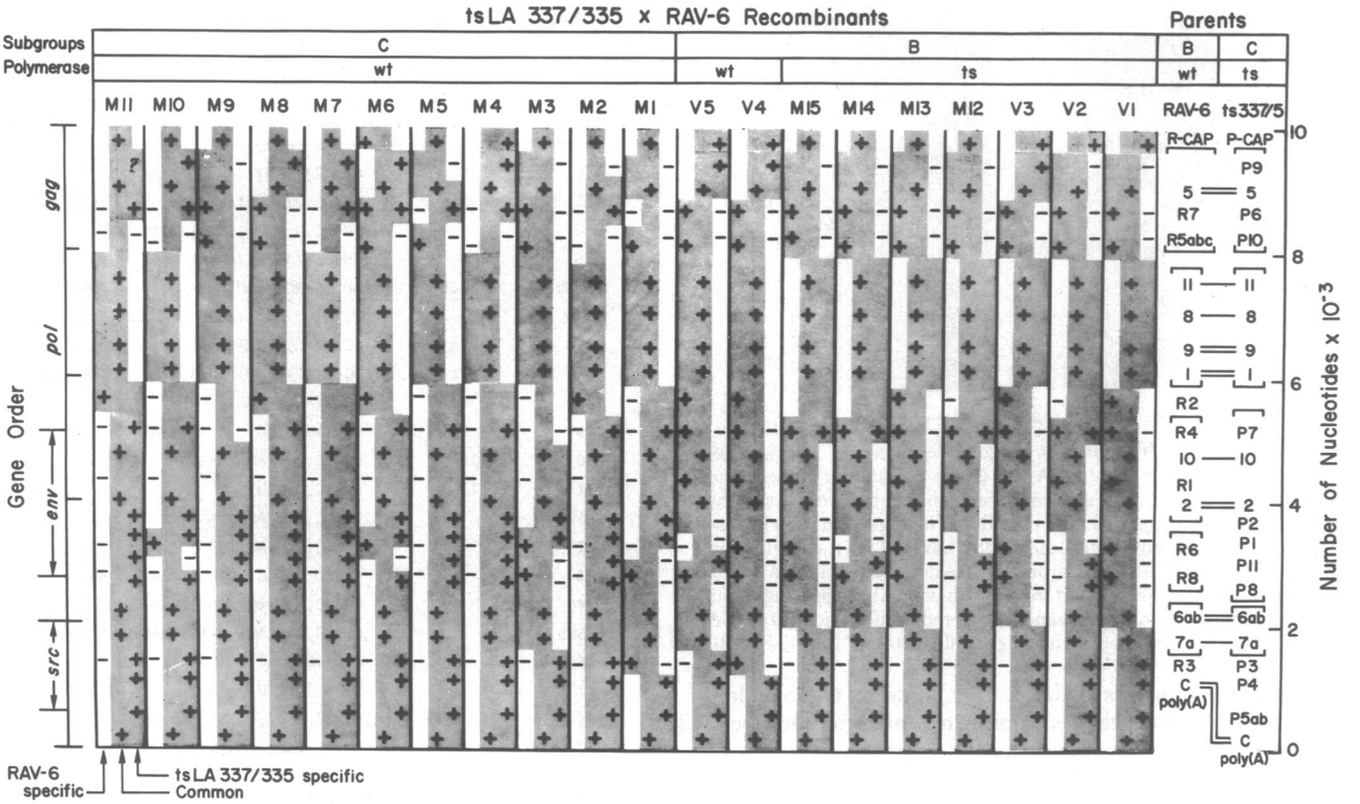

FIG. 2. Oligonucleotide maps of tsLA 337/335, RAV-6, and 20 tsLA 337/335 × RAV-6 recombinants. RNase-T_1-resistant oligonucleotides are numbered as described for Fig. 1 and Table 1 and have been ordered linearly on the basis of their distance from the 3′ poly(A) end of the viral RNA (4, 7, 8, 12, 13). The right ordinate shows the size of viral RNA in nucleotides (5, 6). The relative location of oligonucleotides within brackets is uncertain. The oligonucleotide map of RAV-6 is 15% shorter than that of tsLA 337/335 because RAV-6 lacks the *src* gene (see *text*). Horizontal lines connect oligonucleotides shared by both viruses at equivalent map locations. Double lines connect oligonucleotides highly conserved among avian tumor viruses (8–12). The left ordinate indicates the most probable gene order of avian tumor virus RNA derived from these and other data (*text* and refs. 7–13).

The oligonucleotide maps of the 20 recombinant RNAs show, in three different columns, the presence (+) or the absence (−) of a given parental oligonucleotide at the map location equivalent to its parental counterpart. The right column records tsLA 337/335-specific oligonucleotides, the left column RAV-6-specific oligonucleotides, and the middle column oligonucleotides shared with both parents. The stippled area connects all adjacent recombinant oligonucleotides between two columns as they might have been inherited from RAV-6 (left and middle column) or from tsLA 337/335 (right and middle). CAP oligonucleotides whose parental origins are not known are denoted by + and a stippled area in the middle column only.

the tsLA 337/335 parent. Since all recombinants analyzed here were selected for the *src* gene of tsLA 337/335 and since RAV-6 lacks a *src* gene [leukosis viruses lack *src* (4, 7–9, 19)] it follows that the *src* gene for these recombinants resides in this map segment. This is consistent with previous analyses, which have mapped the *src* gene of Prague RSV-C, the wild-type of tsLA 337/335, in this position (7), and additional results, which have indicated that the *src* oligonucleotides are highly conserved among all avian sarcoma viruses (8, 12, 13). In addition all recombinants shared with both of their parents and all other avian tumor viruses oligonucleotide C at the poly(A) end. (*ii*) Three conserved oligonucleotides (6ab, 7a) were found between 1800 and 2500 nucleotides in all recombinants and both parents. These oligonucleotides could belong to any viral gene except the *src* gene, which is absent from RAV-6. However, this map segment is probably too small to represent *gag*, which codes for a protein of 76,000 daltons (16), corresponding to 2300 nucleotides, or *pol*, which codes for a protein of 100,000 daltons (20, 21), corresponding to 3000 nucleotides. Hence it is not likely to be an integral part of these genes (see below). Instead it may be a conserved part of the *env* gene which maps near *src* between 2500 and 5000 nucleotides (4, 8, 11, 12, 13) or it may represent a yet undefined genetic function of the virus. (*iii*) The map segments between 2500 and 5000 nucleotides are known to be part of the *env* gene (4, 8, 11–13). As expected (4, 12), all recombinants selected for subgroup B *env* gene from RAV-6 had RAV-6-specific oligonucleotides and all recombinants selected for the subgroup C *env* from tsLA 337/335 had tsLA 337/335-specific oligonucleotides in this segment (Fig. 2). Moreover, both parents and all recombinants shared the highly conserved *env*-specific oligonucleotide (no. 2) in this map segment. (*iv*) Between 5000 and 6000 nucleotides recombinants either contained or lacked oligonucleotide R2 or P7 irrespective of their selected biological markers. Since these oligonucleotides did not segregate with a known genetic marker their function cannot be determined. (*v*) All recombinants shared with both parents a map segment between 6000 and 8000 nucleotides defined by four conserved oligonucleotides. Two of these are highly conserved among all avian tumor viruses (12). Since no tsLA 337/335-specific oligonucleotide map segment was found that segregated with all ts *pol* recombinants and no RAV-6 map segment was found that segregated with all wild-type *pol* recombinants (see below), we concluded that the ts *pol* lesion of LA 337/335 is associated with the RNA segment identified by these conserved oligonucleotides.

Oligonucleotides near the 5′ End of Recombinant RNAs Segregate with an Electrophoretic Marker of p27, a Major *gag* Gene Product. (*vi*) The 5′-terminal map segment, between 8000 and 10,000 nucleotides but not including the 5′-terminal CAP oligonucleotide (see below), was defined in some recom-

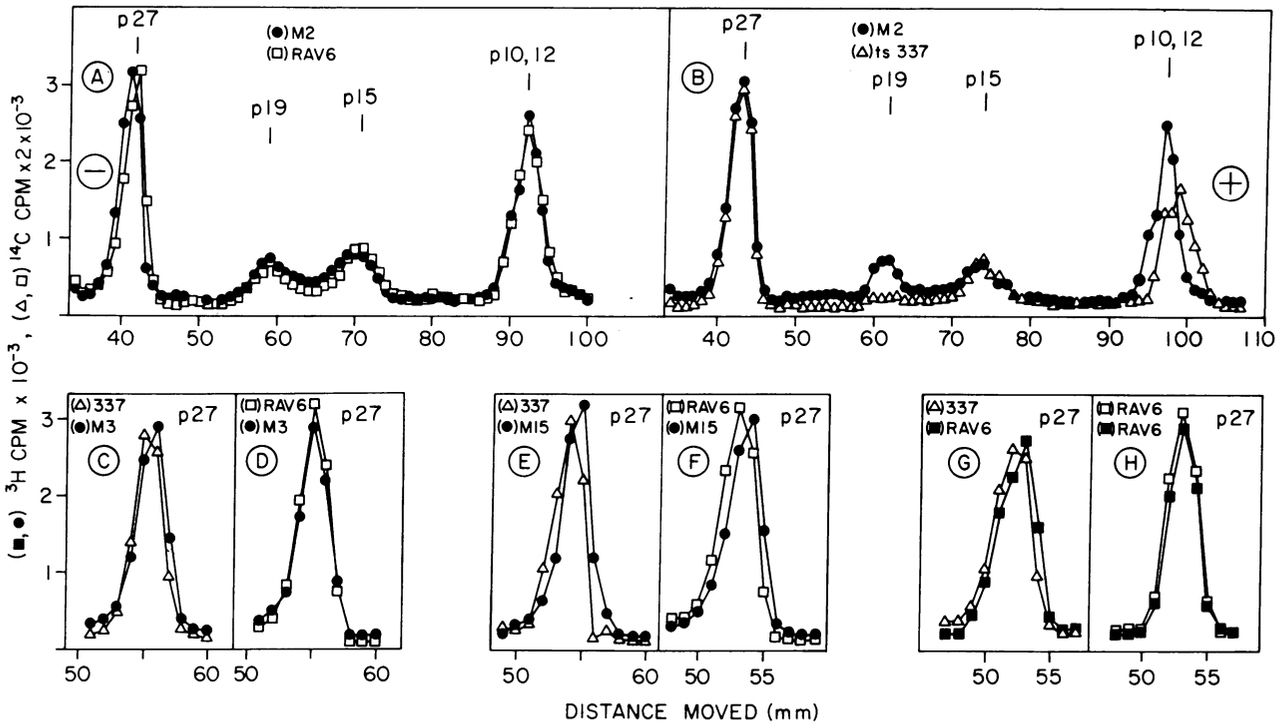

FIG. 3. Polyacrylamide gel electrophoresis of the *gag* proteins of tsLA 337, RAV-6, and tsLA 337 × RAV-6 recombinants M2, M3, and M15. Preparation of radioactive viral proteins and the conditions for gel electrophoresis were as described (15) except that 17.5% polyacrylamide gels of 13 cm length were used. Electrophoresis was for 30–34 hr at 8–10 V/cm. Simultaneous electrophoresis of amino-acid-labeled M2 virus with RAV-6 (A), and with tsLA 337 (B). Designation of proteins p27, p19, p15, p12, p10 followed the convention of August *et al.* (22). Only the lower segments from 40 to 100 mm from the top of the gel are shown. (C) and (D) show the p27 proteins of M3 with those of tsLA 337 and RAV-6 after migration into a segment from 50 to 60 mm from the top of the gel. (E) and (F) show the p27 proteins of M15 with those of tsLA 337 and of RAV-6. A control experiment comparing the p27 of tsLA 337 and RAV-6 is shown in (G) and one comparing different preparations of RAV-6 is shown in (H).

binants (V1, V2, M3, M9, M12–15) by RAV-6-specific oligonucleotides R7, R5abc, in others (M2, M4, M7, M10, M11) by tsLA 337/335 oligonucleotides P6 and P9, and in still others (V3, V4, V5, M5, M6, M8) by a combination of RAV-6 and tsLA 337/335 oligonucleotides (Fig. 2). The presence of three RAV-6 and two tsLA 337/335 oligonucleotides in recombinant M6 could be due to unequal crossing-over (10) or could signal a mixture of two viruses. Since the oligonucleotides of this map segment segregated independently of the three known viral gene markers of our recombinants, it would appear by exclusion that *gag* might be associated with this map segment. To test this directly we have examined the *gag* gene products of tsLA 337/335 and RAV-6, e.g., proteins p27, p19, p15, and p10, p12 (14) for possible electrophoretic markers. It is shown in Fig. 3 that the major *gag* gene product, p27, of RAV-6 had a slightly higher electrophoretic mobility than its counterpart in tsLA 337/335 (Fig. 3G and H). These two p27 products also differed in two out of 19 tryptic peptides (unpublished) resolved by cation exchange chromatography (24). Further, it was found that a correlation exists in recombinants between the distribution of this electrophoretic marker of p27 and the parental origin of their oligonucleotides near the 5′ end of the RNA. Out of 12 recombinants that contained besides the CAP oligonucleotide only oligonucleotides from one parent in the 5′-RNA segment, seven (V1, V2, M1, M3, M9, M12, M13) had RAV-6 oligonucleotides and a RAV-6 p27 (see M3, Fig. 3C and D; others not shown) and three recombinants (M2, M7, M10) had tsLA 337 oligonucleotides and a tsLA 337 p27 (see M2, Fig. 3A and B; others not shown). Some other recombinants that contained both RAV-6 and tsLA 337 oligonucleotides (V5, M6, M8) or only tsLA 337 oligonucleotides (M11) or only RAV-6 oligonucleotides (M15) near the 5′ end contained p27 proteins that migrated unlike those of either parent, e.g., M15 (Fig. 3E and F). M4, which had tsLA 337 oligonucleotides in the 5′ RNA segment, had a p27 migrating almost like that of tsLA 337, yet differing from it in several tryptic peptides (not shown). We conclude that p27 maps near the 5′ end of the RNA. The finding of recombinant p27 products with an electrophoretic mobility unlike that of either parent could be the result of crossing-over. This was directly demonstrated in the 5′ segment of the V5 and M6 oligonucleotide maps (Fig. 2) and may have occurred in M15 and M4 without involving oligonucleotides detected by our method.

The p15 proteins were indistinguishable in all viruses tested. The pattern of p19 and p10,12 proteins of RAV-6 differed from the patterns of tsLA 337 (Fig. 3A and B) in that p19 of tsLA 337 was missing or present at low concentration in some preparations, and p10,12 was shifted to faster migrating protein components. Sixteen of 17 recombinants analyzed had p19 and p10,12 patterns like that of RAV-6. One recombinant (M12) had the p19 and p10,12 pattern of tsLA 337. These electrophoretic properties could not be correlated with any oligonucleotide markers (Fig. 2).

The 5′-Terminal Oligonucleotides. (*vii*) The 5′-terminal oligonucleotides, termed CAP (17, 18), differed slightly in RAV-6 and tsLA 337 (Table 1, Fig. 2). Because this difference was not noticed in early experiments (13) their parental origin was not determined in all recombinants (Fig. 2). These oligonucleotides are probably not translated into protein, because they lack an initiation triplet (18) and therefore are not expected to represent viral genes.

DISCUSSION

Our results provide direct evidence that p27, the major *gag* gene product, maps near the 5′ end of the viral RNA and

suggest that the four known genes of RSV have the order *gag-pol-env-src*-poly(A). This map is consistent with biochemical and genetic evidence described previously (4, 7, 8, 11–13). Our identification of the *gag* gene as a map segment of about 2000 nucleotides near the 5′ end of the RNA, which includes strain-specific as well as conserved oligonucleotides, is consistent with the known characteristics of its protein product. The *gag*-gene proteins contain serologically and biochemically conserved as well as strain-specific elements (2, 3). The primary *gag* gene product is a precursor protein of 76,000 daltons (16), corresponding to 2300 nucleotides. This protein is also the only one that is effectively translated *in vitro* from viral RNA (23). This is an independent argument for the 5′ location of the *gag* gene, since eukaryotic RNAs are thought to have only a single active initiation site for translation near their 5′ end (24).

It is difficult to correlate the oligonucleotides in each 5′ cluster with specific *gag* proteins, because the oligonucleotides analyzed here represent < 5% of the RNA (6, 10) and would not be expected to include correlates for every viral protein. It is possible that oligonucleotide spot R5abc, which covaried with all RAV-6-specific p27 proteins, and P6, which covaried with all tsLA-337-specific p27 proteins, is p27 specific. However, because these oligonucleotides were also found in recombinants (V5, M6, M11, M15) with p27 proteins that migrated like neither parental counterpart, their identification remains uncertain.

There is still some uncertainty about the identification of the *pol* gene, because a viral strain-specific *pol* oligonucleotide has not yet been identified. Thus the proposed association of *pol* with a map segment identified by four conserved oligonucleotides between 6000 and 8000 nucleotides from the poly(A) end is indirect, based on the exclusion of other map segments, which did not segregate with a ts *pol* marker. The association of the *pol* gene with four conserved or highly conserved oligonucleotides is also consistent with the notion that the polymerases of different viral strains are serologically (25, 26) and biochemically (20, 21) closely related. It may be argued that a map segment between *env* and *src* and characterized by oligonucleotides 6ab and 7a, which is also conserved in all virus strains tested here, may be *pol* specific. However, this has been considered unlikely (see above) because it appears too small to code for the viral polymerase.

Mechanism of Recombination. RNA tumor viruses are thought to recombine by crossing-over, presumably at the level of proviral DNA (6, 10). Little is known about the detailed mechanism. The oligonucleotide maps of our recombinants suggest a minimum of two to five cross-over points (Fig. 2). (Cross-overs that do not involve RNase-T_1-resistant oligonucleotides would not be detected by our method.) Thus far, our data do not suggest that RNA segments exist that are especially recombination prone. However, some peculiarities were noted; all 20 recombinants lacked the oligonucleotide P10 of tsLA 337/335. Sixteen out of 17 recombinants had a p19 quantitatively (p19 was not or barely detected in tsLA 337; see above) and a p10,12 qualitatively typical of RAV-6. This is particularly puzzling in the cases of M2, M7, and M10, which contain a tsLA 337-specific p27 but RAV-6-like p19 and p10,12 and in the case of M12, where the opposite distribution was observed. Covariation of the *gag* proteins would have been expected, since they derive from a common precursor (16), but these inconsistencies can be explained by intragenic crossing-over. A linkage between *gag* and *src* has been observed in genetic experiments (27, 28) reviewed previously (13). Such a linkage is not suggested by biochemical data reported here and previously (12), although 15 out of the 20 recombinants studied here appear to contain sarcoma parental CAP-oligonucleotides (preliminary observation). The apparent discrepancy may reflect a technical bias. Recombinants studied biochemically must be from biochemically distinguishable parents, whereas recombinants studied genetically may be from biochemically indistinguishable, congenic parents differing only in conditional lesions. The linkage patterns of *gag* and *src* may be different in recombination between biochemically distinct and congenic parents.

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