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# Mapping of biological functions on RNA of avian tumor viruses: Location of regions required for transformation and determination of host range

(oncornavirus/oligonucleotide map/genetic map/recombination)

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**ABSTRACT** **A map of the large $T_1$ oligonucleotides of the RNA of Prague Rous sarcoma virus, strain B (Pr RSV-B) has recently been established (Coffin and Billeter, submitted for publication). Since the RNA of Rous associated virus, type 1 (RAV-1) lacks many of the large $T_1$ oligonucleotides of Pr RSV-B and contains others not present in the latter, the RNA of recombinants between RAV-1 and Pr RSV-B could be analyzed with regard to the origin of its sequences. Recombinants were selected for transforming capacity (characteristic for Pr RSV-B) and ability to grow on C/B chicken fibroblasts (characteristic for RAV-1). Four out of five recombinants examined had undergone at least two crossovers. The set of Pr RSV-B-specific oligonucleotides present in all recombinants defined an RNA region near the poly(A) segment; this must contain genetic information required for transformation (the *onc* function). All recombinants lost a set of contiguous Pr RSV-B-specific oligonucleotides and concomitantly acquired a set of RAV-1-specific oligonucleotides. These define a region in the middle section of the oligonucleotide map, all or some of which must be required for determining growth capacity on C/B cells (the *env* function).**

Work in several laboratories has shown that oncornavirus RNA can be characterized by the large oligonucleotides resulting from its digestion with $T_1$ RNase and that different strains of avian oncornaviruses yield different and characteristic patterns of large $T_1$ oligonucleotides (1–5). This analytical approach has been used to show that Rous sarcoma virus (RSV) contains two to three identical subunits with a molecular weight of about $3.4 \times 10^6$ (3–8), and to demonstrate that the RNAs of recombinants from a cross between two different strains of avian tumor viruses yield $T_1$ oligonucleotides characteristic for each of the parents (3).

A map of the large $T_1$ oligonucleotides of the RNA of Prague Rous sarcoma virus, subgroup B (Pr RSV-B) has recently been established (Coffin and Billeter, submitted for publication). In this paper we describe a new approach for mapping biological functions on an oncornavirus genome. Crosses are performed between two virus strains that differ in one or more characteristic biological properties as well as in a substantial proportion of their large $T_1$ oligonucleotides. The biological properties of cloned progeny are then correlated with the presence or absence of typical $T_1$ oligonucleotides. If the oligonucleotide map of at least one of the viruses is known, it is possible to locate the selected functions on the genome. Using this approach we have mapped transforming capacity at the 3′-terminal region of Pr RSV-B RNA and host range in the middle section of the genome.

Abbreviations: Pr RSV-B, Prague Rous sarcoma virus, strain B; RAV-1, Rous associated virus, type 1.

## RESULTS

### Preparation and characterization of recombinants between Pr RSV-B and RAV-1

Chicken fibroblast cells (C/O, chf$^-$) (9, 10) were coinfected with Pr RSV-B (subgroup B, a transforming virus) and RAV-1 (Rous-associated virus, type 1, subgroup A, a nontransforming virus), and five presumptive recombinants (transformation positive, capable of growth on C/B cells) were isolated as described in the legend to Fig. 1. [$^{32}$P]RNA of the progeny clones as well as of the parental virus was digested with $T_1$ RNase, and the digest was analyzed by two-dimensional polyacrylamide gel electrophoresis (Fig. 1). The large [$^{32}$P]oligonucleotides of each preparation were characterized as described in Table 1. The parental RNAs yield a set (A) of $T_1$ oligonucleotides common to both strains, as well as sets (B) and (C) characteristic for each parent. Table 1 further lists the presence or absence of $T_1$ oligonucleotides in the five cloned progeny virus. It may be seen that each of the progeny RNAs examined is a recombinant molecule in that it contains some oligonucleotides characteristic for each parent and lacks others.

In Fig. 2A the large $T_1$ oligonucleotides derived from Pr RSV-B RNA have been aligned in the order determined previously (Coffin and Billeter, submitted for publication) with the oligonucleotides characteristic for Pr RSV-B RNA indicated by large numbers. It was pointed out previously that the mapping procedure was not precise enough to determine the definitive order of all oligonucleotides. As compared to the earlier map, oligonucleotides 1 and 36 have been interchanged, and oligonucleotide 37 has been placed to the right of 23 rather than between 35 and 31, in order to allow a consistent interpretation of the recombination data.

The portions of the progeny RNAs carrying oligonucleotides characteristic for Pr RSV-B RNA are clearly derived from the Pr RSV-B parent. Absence of a set of contiguous Pr RSV-B oligonucleotides indicates the substitution of a segment of the Pr RSV-B genome by one derived from RAV-1 RNA, a deletion, or a combination of the two, such as might result from unequal crossingover [see Duesberg *et al.* (6)]. Deletions alone cannot account for our data, since the loss of Pr RSV-B oligonucleotides was always accompanied by the appearance of RAV-1 oligonucleotides, and (with one exception) oligonucleotides common to both parents never disappeared. The origins of the genome segments, as defined by the above criteria, are indicated in Fig. 2. While RJ-161 can be accounted for by one crossingover, RJ-113, RJ-244, and RJ-233 must have come about by at least two recombina-

Table 1. Some large $T_1$ oligonucleotides of Pr RSV-B RNA and RAV-1 RNA: Presence or absence in recombinants[a]

| Oligonucleotide | | Pancreatic digestion products | Presence in RNA of recombinants[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | RJ-113 | RJ-244 | RJ-123 | RJ-161 | RJ-233 |
| | | *A. $T_1$ oligonucleotides common to Pr RSV-B RNA and RAV-1 RNA* | | | | | |
| 3 | (Pr RSV-B) | $A_2G\ A_2C$ AU 2AC 9C 5U | | | | | |
| 3* | (RAV-1) | $A_2G\ A_2C$ AU 2AC 9C 4U | + | + | + | + | + |
| 4 | (Pr RSV-B) | G $A_3C$ AU AC 8C 5U | | | | | |
| 4* | (RAV-1) | G $A_3C$ AU AC 9C 5U | + | + | + | + | + |
| 10 | (Pr RSV-B) | G $A_2C$ AU 2AC 4C 2U | | | | | |
| 15* | (RAV-1) | G $A_2C$ AU 2-3AC 5-6C 2U | + | + | + | + | + |
| 15 | (Pr RSV-B) | G AU 8C 6U | | | | | |
| 9* | (RAV-1) | G AU 9C 6U | + | + | + | + | + |
| 22 | (Pr RSV-B) | G $A_2U$ 3AU 2C 4U | | | | | |
| 21* | (RAV-1) | G $A_2U$ 3AU 2C 4U | + | + | − | + | + |
| 23 | (Pr RSV-B) | G 2AU 2-3C 5U | | | | | |
| 23* | (RAV-1) | G 3AU 3C 7-8U[c] | + | + | + | + | + |
| 24 | (Pr RSV-B) | G AU AC 2C 5U | | | | | |
| 22* | (RAV-1) | G AU AC 2C 5U | + | + | + | + | + |
| 25 | (Pr RSV-B) | AG AU C 9-10U | | | | | |
| 24* | (RAV-1) | AG AU C 7-8U | + | + | + | + | + |
| | | *B. $T_1$ oligonucleotides specific for Pr RSV-B RNA* | | | | | |
| 1 | | G $3A_4C\ A_2U$ 4AC C 1-2U | + | + | − | − | − |
| 2 | | G $2A_2U\ A_2C$ 2AU AC 7C 6U | − | − | − | − | − |
| 5 | | G $A_3C$ AU 2AC 4C 3U | + | + | + | + | + |
| 6 | (mixture) | G AG 2AU 5AC 13C 8U | + | + | + | + | + |
| 9 | | G $A_2C$ AU 2AC 6C 2-3U | − | − | − | − | − |
| 11 | | $A_4G$ AC 5C 5U | + | + | − | − | + |
| 13 | | G $A_3C$ 3AU 3AC 5C 5U | + | + | + | − | + |
| 14 | | G $A_2U\ A_2C$ 2AU AC 3C 4U | + | + | + | + | + |
| 19 | | G AU 2AC 3C 7U | − | − | − | − | − |
| 20 | | G $A_2U$ AU 2-3C 6-7U | − | − | − | − | − |
| 21 | | G $A_2U$ AC 2C 6U | + | + | + | + | − |
| 26 | | G 3AU 8U | + | + | + | − | − |
| 31 | | $A_3G\ A_3C$ AC 3C 6U | − | − | − | − | − |
| 33 | | G AU 2AC 6C 4-5U | − | − | − | − | − |
| 35 | | G $A_2C$ 5C 2-3U | − | − | − | − | − |
| 36 | | AG $A_2C$ AU 2AC 2C U | + | − | − | − | − |
| 37 | | G 2AC 3-4C U | + | + | − | − | + |
| | | *C. $T_1$ oligonucleotides specific for RAV-1 RNA* | | | | | |
| 1* | | G $A_2C$ 9-10 C 7U | + | + | + | + | + |
| 2* | | AG $A_2U\ A_2C$ 2AU 2AC 3C 4U | + | + | + | + | + |
| 5* | | G $A_2C$ 4AU 4C 7U | − | − | − | + | + |
| 7* | | G AU 3AC 6-7C 3-4U | − | − | − | − | − |
| 11* | | G $A_4U$ 2AU 4C 5U | − | + | + | + | + |
| 12* | | G $A_2U$ AU 5C 8-9U | + | + | + | + | + |
| 13* | | G $A_5C\ A_4C$ 2-3AC C | − | − | + | + | + |
| 14* | | G $A_2C$ AU 2AC 5C U | − | − | + | + | − |
| 16* | | $A_5G\ A_3C$ AC 3-4C 3-4U | + | + | + | + | + |
| 17* | | G 2AU 3AC 4C 3U | − | − | − | + | − |
| 18* | | AG $A_6U\ A_2U$ 2AU 2AC 3-4C 3-4U | − | − | − | − | − |
| 27* | | G 2AU 2-3AC 13-14C 2-3U | − | − | − | − | − |
| 28* | | G $A_5C$ 2-3AC 2C 2-3U | − | − | − | + | + |

[a] $^{32}P$-Labeled RNA was digested with $T_1$ RNase, and the products were separated by two-dimensional polyacrylamide gel electrophoresis as described in the legend to Fig. 1. The large $T_1$ oligonucleotides listed were extracted and digested with pancreatic RNase; the digestion products were identified. In the case of the parental RNAs and the recombinants RJ-161 and RJ-244 the pancreatic digestion products were quantitated; in the other cases their relative proportion was estimated visually from the radioautograph. The molar content of C and U is accurate to only about ± 20%.

[b] The absence of an oligonucleotide was determined by inspection and/or by the pancreatic RNase analysis of the oligonucleotides present in the area.

[c] Visual estimation of the radioautograph is consistent with 2 AU at the most.

tional events. RJ-123 is somewhat more difficult to interpret. The right-hand third of the RNA is derived from Pr RSV-B, the middle section from RAV-1, while the left-hand third contains the Pr RSV-B-specific oligonucleotide 13 almost at the terminus but lacks oligonucleotide 11 at its very end. Formally, this finding could be explained by three crossovers, but it is also possible that oligonucleotide 11 was lost because of a point mutation or a terminal deletion. On the

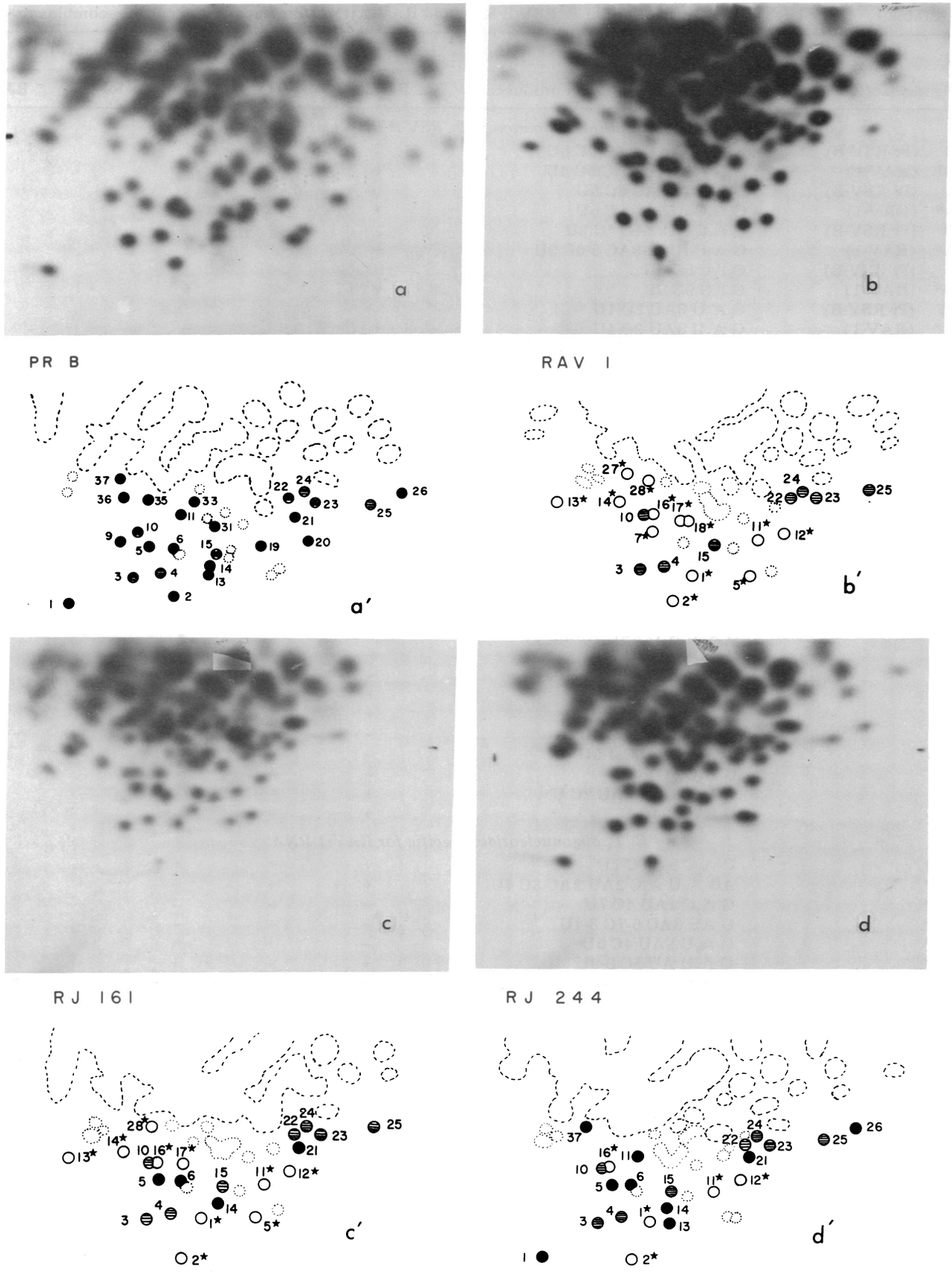


FIG. 1. (*Legend appears at top of following page.*)

FIG. 1. *(on preceding page)* Two-dimensional polyacrylamide gel electrophoresis of $T_1$ oligonucleotides from the RNA of Pr RSV-B, RAV-1, and two recombinants between Pr RSV-B and RAV-1. Chick fibroblasts (Chf$^-$, C/O, obtained from R. Friis), grown as described earlier (18), were infected with Pr RSV-B clone 3 (Coffin and Billeter, submitted for publication; about 370 focus-forming units per 50-mm dish) and RAV-1 (original stock obtained from H. Temin, Madison, Wisc.; 0.2 ml of culture medium from infected cells per 50-mm dish; titer, $>5 \times 10^3$/ml). After 4 days the cells were subcultured, and after a further 4 days the medium was harvested, diluted, and used to generate foci on C/BDE$^-$ cells (obtained from R. Friis) under agar [about 70 foci per 50-mm dish, as described (19)]. Thirty of these foci were extracted, and a virus stock solution was prepared from each focus on C/BDE$^-$ cells. While all of the preparations transformed C/O cells, only 15 transformed C/BDE$^-$ cells. Five of the stock solutions were again plated under focus assay conditions on C/BDE$^-$ cells; a subclone was selected from each of the clones and used to prepare virus stock solutions. $^{32}$P-Labeled virus RNA was prepared (4), and two-dimensional polyacrylamide gel electrophoresis of the RNase $T_1$ digest was carried out as described earlier (4). (a) Pr RSV-B RNA; (b) RAV-1-RNA; (c) RJ-161; (d) RJ-244. (a'–d') Tracings of a–d. Hatched spots, oligonucleotides common to Pr RSV-B and RAV-1 RNA; light spots and black spots, oligonucleotides specific for RAV-1 and Pr RSV-B RNA, respectively. Dotted, oligonucleotides not considered in the present analyses because they were not mapped.

other hand, if the map order of 11 and 13 is reversed, the data can be simply explained by two crossovers. The absence of oligonucleotide 22, which is common to both parents, may be ascribed to a point mutation or a deletion (which might be caused by unequal crossingover occurring in this region).

It should be noted that an uneven number of crossovers within a region containing only large $T_1$ oligonucleotides common to both parents can be detected but not localized, while even numbers of crossovers within such a region remain undetected.

### Mapping of transforming capacity and subgroup specificity

Fig. 2 shows that the only $T_1$ oligonucleotides characteristic for Pr RSV-B RNA present in *all* of the transforming recombinants are 5, 6†, and 14, which are adjacent to each other and situated at the 3'-terminal segment of the genome. Since RAV-1 cannot transform chicken fibroblasts, we conclude that genetic information required for transformation is located in this region. The results do not exclude the possibility that other parts of the genome, common to RAV-1 and Pr RSV-B, are also required for transformation, nor do they allow the conclusion that the entire region defined by oligonucleotides 5, 6, and 14 is required for this function.

The acquisition of growth capacity on C/B cells, a feature of subgroup A specificity, is correlated with the absence of the Pr RSV-B-specific oligonucleotides 9, 33, 2, 19, 35, 31, and 20, which are situated in an RNA region located somewhat to the right of the middle of the oligonucleotide map, and the presence of RAV-1-specific oligonucleotides 1*, 2*, 12*, and 16*. We have not yet fully characterized our recombinants in regard to their growth properties on other cell types and can therefore not exclude the possibility of partial heterozygosity in regard to subgroup specificity [see Wyke *et al.* (11)].

Finally, it should be noted that our experiment permits us to roughly map the RAV-1 oligonucleotides (see Fig. 2). The large $T_1$ oligonucleotides common to both RAV-1 and Pr RSV-B RNA have been written in the same order as in Pr RSV-B RNA on the assumption that the RNAs are partly homologous. While there is no direct evidence to support this assignment, it leads to a self-consistent interpretation of the recombination data. Since in the recombinant RJ-113 the RAV-1-specific oligonucleotides 1*, 2*, 12*, and 16* are present when the Pr RSV-B-specific nucleotides 9, 33, 2, 19, 35, 31, and 20 are missing, we conclude that 1*, 2*, 12*, and 16* are located within the region defined by the missing Pr RSV-B nucleotides, namely, in the middle section of the oligonucleotide map. Further deductions of this nature lead to the partial map order for RAV-1 shown in Fig. 2.

† What is designated as oligonucleotide 6 from Pr RSV-B RNA is a mixture of two oligonucleotides (Coffin and Billeter, submitted for publication).

## DISCUSSION

The complete absence of a number of typical RAV-1 and Pr RSV-B oligonucleotides from all progeny clones selected as recombinants argues against contamination by parental virus, so that there is no reason to ascribe the host range properties to phenotypic mixing. The large proportion of double crossovers found in our small sample may be the consequence of extensive recombination (11, 12), but it should also be considered that proviral DNA passes through a circular state (13, 14) and that recombination between DNA circles can intrinsically give rise to a high yield of double recombinants (15, 16). The fact that in three out of five recombinational events at the right-hand side of the genome crossingover took place at apparently the same position may indicate a "hot-spot" for recombination. This may be due to a relatively rare, short region, subject to recombination in both parental RNAs (for example, a region of homology within an otherwise unrelated sequence) and/or reflect the result of selection for viable progeny, since recombination within cistrons may not always lead to functional proteins and, for instance, recombination in an intercistronic region may be the least detrimental.

As stated in *Results*, we have modified the $T_1$ oligonucleotide map of Pr RSV-B RNA of Coffin and Billeter by two transpositions because otherwise four or more crossovers would have been required to explain the $T_1$ oligonucleotide composition of several progeny clones. Considerable refinement of the $T_1$ oligonucleotide maps should become possible as more data on recombination, in particular between two mapped genomes, are obtained.

Correlation of the biological properties of the recombinants with their $T_1$ oligonucleotide map allowed us to establish a coarse genetic map, on which transforming capacity has been located close to the poly(A)-carrying terminus and host range somewhat to the right of the middle. The location of the transforming function is in good agreement with the previous observation that transformation defective variants of Pr RSV-B lack oligonucleotides 6 and 5 (Coffin and Billeter, submitted for publication). Lai *et al.* (1) have described three different strains of RSV in which one or two $T_1$ oligonucleotides are missing in transformation defective variants. They attribute this to a deletion in the 3'-terminal region (17). The localization of the host range marker in the middle of the genome is supported by our finding (unpublished results) that the RNA of Prague Rous sarcoma viruses of subgroups B, C, and A differ in regard to their large $T_1$ oligonucleotides only in the middle region defined by Pr RSV-B oligonucleotides 37 through 20. The assignment also agrees with the finding that rd-SR-N8, a Rous sarcoma virus

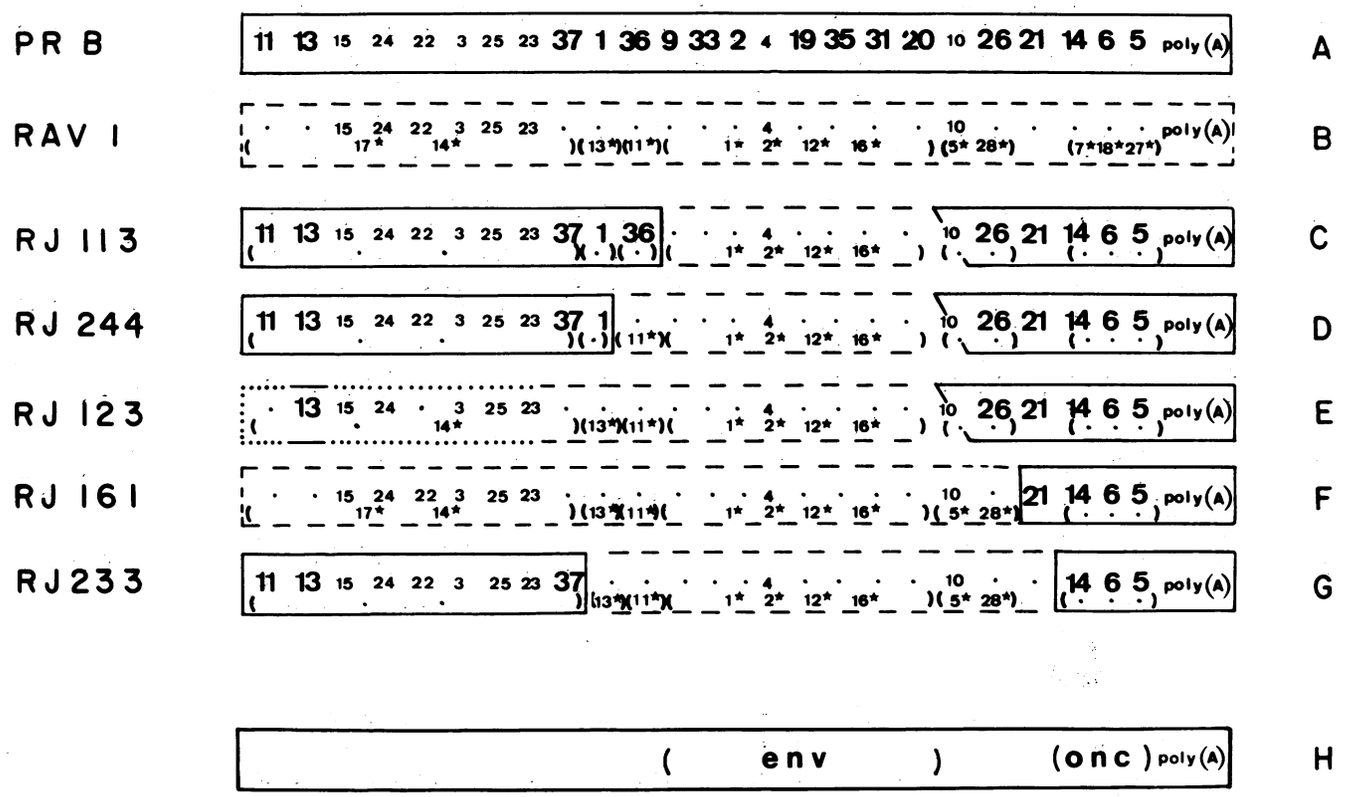


FIG. 2. Oligonucleotide maps of the RNA of Pr RSV-B, RAV-1, and five recombinants between Pr RSV-B and RAV-1. Maps indicate order of oligonucleotides and not relative distances, since the markers are not expected to be distributed evenly over the RNA.(A) Pr RSV-B RNA, as determined by Coffin and Billeter (submitted for publication), however, with two modifications (see *text*). (B) RAV-1: top part of map, oligonucleotides common to Pr RSV-B and RAV-1 (the order is assumed to be the same as in Pr RSV-B RNA); bottom part, oligonucleotides specific for RAV-1. The order of the oligonucleotides within parentheses is unknown; the blocks have been ordered as described in the *text*. (C–G) Recombinant clones (described in the legend to Fig. 1). (C) RJ-113; (D) RJ-244; (E) RJ-123; (F) RJ-161; (G) RJ-233. Small numbers, oligonucleotides common to Pr RSV-B and RAV-1 [using Pr RSV-B numbering system (see Table 1)]; large numbers, oligonucleotides specific for Pr RSV-B; starred numbers, oligonucleotides specific for RAV-1. Continuous lines, Pr RSV-B derived regions; dashed lines, RAV-1 derived regions; dotted lines, undetermined. (H) Regions *within* which genetic information for host range (growth on C/B cells) designated *env* and transformation capacity (*onc*) is located, relative to the oligonucleotide map.

lacking the large envelope glycoprotein, contains a deletion in the middle section of its RNA (L. H. Wang, P. Duesberg, S. Kawai, and H. Hanafusa, personal communication).

It should be possible to locate further biological functions by the method outlined above, using mutations in reverse transcriptase and in the internal proteins. Our mapping procedure does not require mutants with substantial changes, such as deletions, in their RNA, but depends only on the availability of two virus strains that differ in biological properties, contain different sets of characteristic oligonucleotides, and are capable of undergoing recombination at a substantial number of different sites, conditions that can be met without too much difficulty.

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