

Location of envelope-specific and sarcoma-specific oligonucleotides on RNA of Schmidt-Ruppin Rous sarcoma virus

(deletion mutants of Rous sarcoma virus/mapping RNase-T₁-resistant oligonucleotides/gene order of Rous sarcoma virus)

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ABSTRACT Envelope-specific and sarcoma-specific nucleotide sequences have been located within the 10,000 nucleotides of the RNA of nondefective Schmidt-Ruppin Rous sarcoma virus (nd SR). For this purpose, about 30 RNase-T₁-resistant oligonucleotides were ordered relative to the 3'-poly(A) terminus of the RNA, to construct an oligonucleotide map of the nd SR RNA. A cluster of seven envelope-specific oligonucleotides, identified by their absence from an otherwise very similar oligonucleotide map of an envelope-defective deletion mutant (which lacks the major viral glycoprotein), mapped at a distance of 2800-5000 nucleotides from the poly(A) end of nd SR RNA. A cluster of two sarcoma-specific oligonucleotides, identified by their absence from an otherwise nearly identical oligonucleotide map of a transformation-defective deletion mutant, mapped at a distance of 1000-2000 nucleotides from the poly(A) end of nd SR RNA. The oligonucleotide maps of nd SR and of the two deletion mutants were the same from the poly(A) end up to 650 nucleotides and included one terminal oligonucleotide, termed C, which is found in all avian tumor viruses tested so far. A possible gene order consistent with our data suggests that sarcoma-specific nucleotide sequences map between envelope-specific nucleotide sequences and the poly(A) end of the RNA.

The genome of nondefective (nd) avian sarcoma viruses is thought to be an RNA of about 10,000 nucleotides (1-3) with a poly(A) sequence of about 200 nucleotides as its 3' end (4). The RNA carries four known genetic elements (5): a gene for the internal virion protein or group-specific antigen, termed *gag*; a gene for viral DNA polymerase, termed *pol*; a gene for the viral envelope glycoprotein, termed *env*; and a gene for sarcoma formation, termed here *src*.[‡]

We have recently developed a chemical method to map these genes on the viral RNA. For this purpose 20 to 30 large RNase-T₁-resistant oligonucleotides, which all together represent about 5% of the viral RNA (1, 3), were ordered on the basis of their distance from the 3'-poly(A) end of the RNA to construct an oligonucleotide map (6). A cluster of three sarcoma-specific oligonucleotides, identified by their absence from otherwise identical oligonucleotide maps of transformation-defective (td) deletion mutants, was found to map at a distance of 650-2000 nucleotides from the poly(A) ends of three nd sarcoma virus RNAs (6). Since the size of the sarcoma-specific map segment so identified is about the same as the difference in mass (1200-1500 nucleotides, refs. 8-12)

between nd sarcoma and td viral RNAs, it was concluded that this map segment represented the *src* gene of the nd sarcoma viruses (6).

The present investigation examines the map locations of envelope-specific and sarcoma-specific oligonucleotides of nd Schmidt-Ruppin Rous sarcoma virus (nd SR). This is the only nd virus from which both an envelope-defective, and a td deletion mutant have been isolated, as is necessary for the functional identification of the respective oligonucleotides by our method. The envelope-defective deletion mutant, termed SR N8, lacks the major viral glycoprotein (13, 14) and its RNA is 21% shorter than that of nd SR (15). Two preliminary reports of this work have appeared previously (18, 19).

MATERIALS AND METHODS

Viruses. nd SR clone 85-7, td SR NY 105, both of subgroup A, and SR N8 have been described (14, 15). td SR-A 102 (complete identification td BE 102 SRA) was isolated by end-point dilution (8) from a stock of nd SR clone 85-7, which had been passaged six times without dilution on chick fibroblasts to enrich for td derivatives (9). Fibroblasts infected by the td viruses had a completely normal morphology.

Preparation of Oligonucleotide Maps of Viral RNAs. The method has been described in detail (4, 6). For this purpose viral [³²P]RNA was randomly degraded by alkali. Poly(A)-tagged RNA fragments were selected by binding to oligo(dT)-cellulose, fractionated according to size, and fingerprinted to detect their oligonucleotides (compare legend to Fig. 1). The map position of a given oligonucleotide relative to the poly(A) end of viral RNA was then deduced from the size of the smallest poly(A)-tagged RNA fragment from which it could be isolated.

RESULTS

Maps of RNase-T₁-Resistant Oligonucleotides of nd SR, SR N8, and td SR RNAs. (a) nd SR RNA: The map location of a given oligonucleotide of nd SR was deduced as a first approximation from the length of the smallest size class of poly(A)-tagged [³²P]RNA fragments from which it could be obtained (Fig. 1A-G) (6). To confirm the visual identification of oligonucleotides, their RNase-A-resistant fragments were examined (Table 1, ref. 15). Quantification of all oligonucleotides which first appeared with a given size class of fragments was then used to order these oligonucleotides within the class. The higher the molar yield, the closer an oligonucleotide was placed to the poly(A) end within the map distance covered by this fragment. Two examples are presented in Table 2. The resulting oligonucleotide map of nd SR RNA is shown in Fig. 2. [The numbering of oligonucleotide spots of nd SR is as described previously (15). Some

Abbreviations: nd, nondefective; td, transformation-defective; SR, Schmidt-Ruppin Rous sarcoma virus; SR N8, envelope-defective Schmidt-Ruppin Rous sarcoma virus.

[‡]Because a gene for neoplastic transformation by RNA tumor viruses has so far been defined and characterized only in sarcoma viruses, we refer to it, in agreement with D. Stehelin, R. Guntak, H. E. Varmus, and J. M. Bishop (*J. Mol. Biol.*, in press), by the term *src* instead of the term *onc* (5). The term *onc* would apply to neoplastic transformation by both leukemia and sarcoma viruses.

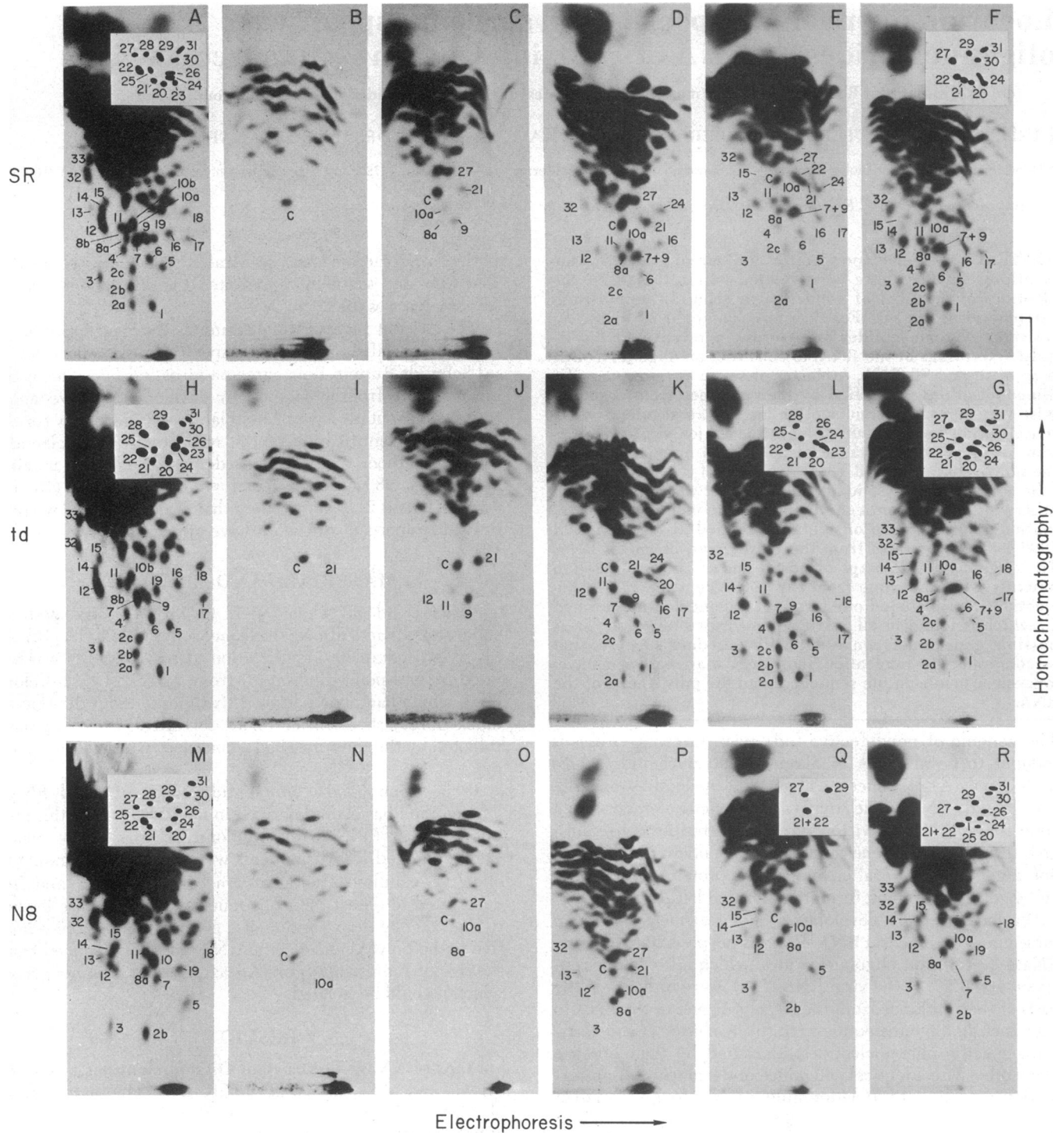


FIG. 1. Fingerprint patterns of RNase-T₁-resistant oligonucleotides of 60-70S viral [³²P]RNAs and of poly(A)-tagged [³²P]RNA fragments of nd SR, td SR, and SR N8. Homomixture b incubated for 24 hr at 60° (6) was used for homochromatography. Homologous spots were given identical numbers in all fingerprint patterns. Some oligonucleotide spots which were still detectable and numbered in the original autoradiographs are no longer visible in the reproductions shown here. The inserts in some panels show tracings and numbers of spots appearing in the center right region of the respective fingerprint pattern. SR: Fingerprints of 60-70S nd SR RNA (A) and poly(A)-tagged RNA fragments of 4-10 S (B), 10-14 S (C), 14-18 S (D), 18-22 S (E), 22-26 S (F), and 26-30 S (G). Approximately 35 × 10⁶ cpm of [³²P]RNA was degraded at pH 11.0 at 50° for 3 min. Sixteen percent of the starting RNA was recovered as poly(A)-tagged fragments after two cycles of binding and elution from oligo(dT)-cellulose (6). These fragments had a broad distribution with a peak at about 21 S after sucrose gradient fractionation (6) done to obtain the size cuts (B-G). td: Fingerprints of 60-70S td SR RNA (H) and of poly(A)-tagged RNA fragments of 4-10 S (I), 10-15 S (J), 15-20 S (K), and 20-28 S (L). About 7 × 10⁶ cpm of 60-70S [³²P]RNA was fragmented for 6 min as described above and approximately 11% was recovered as poly(A)-tagged RNA fragments with an average sedimentation coefficient of 16 S. N8: Fingerprints of 60-70S SR N8 RNA (M) and of poly(A)-tagged RNA fragments of 4-9 S (N), 9-15 S (O), 15-20 S (P), 20-25 S (Q), and 25-30 S (R). About 5 × 10⁶ cpm of 60-70S [³²P]RNA was degraded for 3 min as above and 18% was recovered as poly(A)-containing RNA fragments with an average sedimentation coefficient of 22 S.

Table 1. RNase-T₁-resistant oligonucleotides of nd SR, td SR, and SR N8

Spot no.*†	RNase A digestion products†	Spot no.*†	RNase A digestion products†
①	8U,5C,2(AC),2(AU),(AG),(AAC),(AAU)	14	U,5C,3(AC),(AAG)
2a	6U,7C,G,3(AC),(AAC)	15	6C,(AU),(AG),(AAAN)
2b	4U,8C,2(AC),(AU),(AAC),(AAG)	16	5U,4C,G,2(AU),(AAAN)
2c	5U,9C,G,(AC),(AU),(AAAC)	17	8U,2C,G,4(AU),(AAU)
3	C,G,2(AC),(AAAU),(AAAAAN)	18	7U,6C,G,(AU)
④	3U,6C,G,3(AC),(AU)	19	5U,3C,2(AC),3(AU),(AG)
5	6U,6C,G,2(AC),4(AU)	20	SR: 2U,C,G,3(AU),(AAU),(AAAAAN)
⑥	5U,6C,G,2(AC),(AU),2(AAU)	20	td: 2U,G,2(AU),(AAU),(AAAC)
7	SR: 12U,14C,2(AC),(AU),(AAG),(AAAC), 2(AAAU),(AAAG)	20	N8: 2U,C,G,2(AU),(AAAC)
7	td: 9U,11C,2(AC),(AU),2(AAG),(AAAC), (AAAN)	21	3U,2C,G,(AC),(AU),(AAC)
7	N8: 5U,5C,(AAG),(AAAU)	22	8U,9C,2G,3(AC),2(AU),(AAU)
8a‡	5U,6C,G,2(AC),(AU),(AAAC)	23	4U,3C,G,2(AU),(AAU)
8b‡	SR: 5U,9C,G,2(AC),0.5-1(AU)	24	6U,3C,2G,2(AC),3(AU),2(AAU)
8b‡	td: 6U,10C,G,2(AC)	25	4U,5C,G,(AC),(AU)
8b‡	N8: Not done	26	SR: 2U,C,G,2(AU),(AAU)
⑨	5U,9C,G,(AC),4(AU),(AG),(AAC),(AAU)	26	td: 3U,C,G,2(AU),(AAU)
10a‡	3U,5C,G,2(AC),1-2(AU)	26	N8: 3U,C,G,(AC),2(AU),(AAU)
10b‡	3U,5C,G,3(AC),2-3(AU)	27	2U,C,(AAG),(AAAN)
11	SR: 3U,5C,G,2(AC),(AU),(AAC)	28	4U,2C,G,(AU),(AAAU)
11	td: 4U,6C,G,2(AC),(AU),(AAC)	29	SR: 8U,4C,2G,2(AC),2(AU),(AAC),(AAU)
11	N8: 3U,6C,G,2(AC),(AU)	29	td: 8U,4C,2G,2(AC),2(AU),1-2(AAU)
12	U,16C,G,7(AC),2(AU),2(AG),(AAC), (AAU),(AAAU),(AAAAAN)	30	5U,3C,G,2(AU)
13	4C,(AC),(AAC),(AAG)	31	5U,2C,2(AU),(AG)
		32	9C,G,5(AC),(AG)
		33	7C,4(AC),2(AG),2(AAC)
		C	G,(AC),(AU),(AAU),(AAAC)

* Numbers refer to oligonucleotide spots shown in Fig. 1. Numbers of spots not found in SR N8 are in circles and of spots not found in td SR are in squares. The difference between the composition of spot 1 reported here and that reported previously (15) is due to a previous typographical error.

† If homologous spots of nd SR, td SR, and SR N8 had the same composition, it is listed only once; if differences were found separate compositions are listed. Spots 21-25 and 28-33 of SR N8 were not analyzed.

‡ Compositions of 8a and 10a of nd SR and SR N8 were obtained from poly(A)-tagged fragments shown in Fig. 1D and P, respectively. The composition of spot 8b of nd SR was either determined directly from the fingerprint of 60-70S RNA where it was partially separated from the neighboring spots or estimated by subtracting 8a from 8a,b when the two overlapped. The composition of spot 10b of nd SR was estimated by subtracting 10a from 10a,b (Fig. 1A). The composition of 10b of SR N8 was estimated from fingerprints not shown here in which spot 10 was partially separated from 8b and contained only 10a and 10b. Spots 8b and 10b of td SR were analyzed directly from fingerprints of 60-70S RNA (Fig. 1H).

spots which contained multiple oligonucleotides previously were resolved here and assigned letter designations, e.g., 2a, 2b, and 2c (Fig. 1A, Table 1).]

In cases where an autoradiographic spot of 60-70S viral RNA consisted of two or more overlapping oligonucleotides under the conditions used here, compositional analyses of that spot from different poly(A)-tagged RNA fragments were performed as an approach to determine the map location of each of the mixed oligonucleotides. For example, an autoradiographic spot in the fingerprint position of oligonucleotides 7 and 9 had a composition of 4U, 5C, 0.5 (A-C), 2.5 (A-U), (A-G), (A-A-C), (A-A-U) (in Fig. 1C), 7U, 7C, (A-C), 4 (A-U), (A-G), (A-A-C), (A-A-U), 0.8 (A-A-G), 0.6 (A-A-N) (in Fig. 1D) and 6U, 10C, G, 1.5 (A-C), 4 (A-U), (A-G), (A-A-C), (A-A-U), (A-A-G), 0.8 (A-A-A-N) (in Fig. 1E). This indicates that oligonucleotide 9 appeared first and then was mixed with increasing amounts of oligonucleotide 7 when bigger fragments were analyzed (compare Table 1). Similarly, compositional analyses of spot 12 from different fragments indicated that more oligonucleotide components appeared when the size of the poly(A)-fragments increased (Table 1). Spot 10 of 60-70S nd SR RNA (Fig. 1A) appeared to consist in some experiments of two and in others of three overlapping oligonucleotides (8b, 10a, and 10b, Table 1). One of these, 10a, mapped very close to the poly(A) end

(Fig. 2). It could be identified unambiguously as a distinct oligonucleotide when small RNA fragments of nd SR (or SR N8, see below) were analyzed (Fig. 1D, E and P, Q; Table 1). This was not the case with 8b and 10b, whose approximate compositions (Table 1) and map locations were derived indirectly. These were absent from all poly(A)-tagged RNA fragments of nd SR (or SR N8 and td SR, see below) analyzed here but were present in 60-70S RNA (Fig. 1A, H, and M). They were also recovered from poly(A)-tagged intact 30-40S RNA, although frequently at lower yields than most other oligonucleotides (not shown). This is consistent with their locations close to the 5' end of the viral RNA (Fig. 2) and with the observation that the RNA extracted from purified virus is partially degraded, causing preferential loss of oligonucleotides (1, 3) at the 5' end when poly(A)-containing species are selected.

(b) SR N8 RNA: In accord with a previous study (15) all large oligonucleotides of SR N8 RNA were found to have homologous counterparts in nd SR RNA (Figs. 1M and A; Table 1). Homologous oligonucleotides of SR N8 and nd SR or td SR (see below) were given identical numbers. SR N8 RNA lacked eight oligonucleotides which were present in the RNA of nd SR. An oligonucleotide map of SR N8 RNA was derived from the fingerprints of poly(A)-tagged RNA fragments shown in Fig. 1M-R. Comparison of the oligonu-

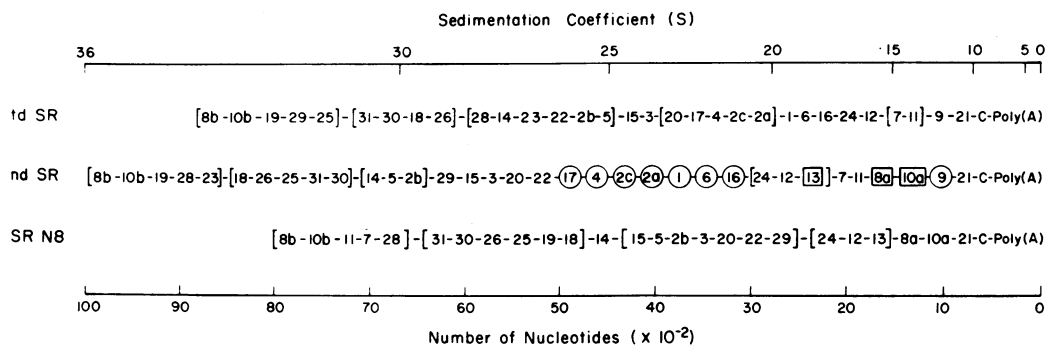


FIG. 2. Oligonucleotide maps of viral 30–40S RNAs. RNase- T_1 -resistant oligonucleotides of nd SR, td SR, and SR N8, numbered as in the fingerprints shown in Fig. 1, were ordered linearly on the basis of their distance from the 3'-poly(A) end (see *text* and ref. 6). Those oligonucleotides which could not be mapped accurately are put in brackets. The top scale represents the size of viral RNA or RNA fragments used to map oligonucleotides in S units and the bottom scale represents size in numbers of nucleotides. The S units were converted into molecular weight units by Spirin's formula [molecular weight = $1550 \cdot S^{2.1}$ (17)]. The number of nucleotides was calculated by dividing the molecular weight of a RNA fragment by 320, the average molecular weight of a nucleotide in Rous sarcoma virus RNA (3). Oligonucleotides of nd SR absent from SR N8 are in circles and those absent from td SR are in squares (see Table 1).

cleotide maps of SR N8 and nd SR shows (Fig. 2) that most oligonucleotides shared by SR N8 and nd SR also had approximately the same order on the respective RNA relative to the poly(A) end. Seven out of eight nd SR-specific oligonucleotides (circled in Fig. 2 and Table 1) were clustered together in a map segment about 2800–5000 nucleotides away from the poly(A) end. One other nd-SR-specific oligonucleotide (no. 9) mapped near the poly(A) end of the RNA. Two oligonucleotide spots, possibly shared by nd SR and SR N8 (nos. 7 and 11), mapped together near the poly(A) end of nd SR RNA but at the opposite end of SR N8 RNA (Fig. 2). However, since the compositions of these oligonucleotides differed in the two viruses (Table 1) further analysis would be required to determine the significance of this difference.

(c) td SR RNA: The RNA of deletion mutant td SR was found to be about 15% smaller (size class *b*, 7–12) than that of the nd parent, nd SR (not shown here, ref. 15). This difference is the same as that between other nd and corresponding td viruses (4, 6–12). Comparisons of the fingerprint patterns of td SR and nd SR RNAs indicate that all oligonucleotides found in td SR RNA had homologous counterparts

(same number) in nd SR RNA (Fig. 1A, H, and Table 1). Three oligonucleotides (nos. 8a, 10a, and 13, Fig. 1A; Table 1; Fig. 2) present in the RNA of nd SR (and SR N8) were missing in the RNA of td SR. An oligonucleotide map of td SR (Fig. 2) was derived from the fingerprints of poly(A)-tagged RNA fragments shown in Fig. 1H–L. Two independent isolates of td SR [NY 105 (15) and BE 102] were indistinguishable with regard to all results described here.

A comparison of the oligonucleotide maps of the td SR and nd SR (or SR N8) shows that (i) all oligonucleotides common to nd SR and td SR had approximately the same map locations on the respective viral RNAs and (ii) two of the three oligonucleotides found in nd SR (or SR N8) but not in td SR (8a and 10a) mapped together in nd SR (or SR N8) RNA at a distance of 1000 to 2000 nucleotides from the poly(A) end (Fig. 2). Oligonucleotide 13 was separated from oligonucleotides 8a and 10a in nd SR RNA by oligonucleotides common to nd SR and td SR (nos. 11 and 7). The mapping of spot 13 was complicated because, as noted previously (7, 15), it often appeared at low relative molar yield. Preliminary evidence suggests that oligonucleotide 27 of nd SR (Fig. 1A and M; Table 1) may also be absent from td SR.

Common Heteropolymeric Sequences Adjacent to the Poly(A) Terminus of nd SR, td SR, and SR N8 RNAs. The fingerprint patterns of poly(A)-tagged RNA fragments of up to 600 nucleotides (about 10 S) indicated that each viral variant contained an oligonucleotide, termed C (Table 1), which mapped very close to poly(A) (Fig. 1B, I, and N; Fig. 2). This oligonucleotide has been found in all avian tumor virus RNAs investigated so far (6). All other small oligonucleotides of these fragments formed a very similar, if not identical, pattern (Fig. 1B, I, and N). This pattern appeared strain-specific because it was different from those of similar RNA fragments of other virus strains studied previously (6).

Table 2. Relative molar yields of unique RNase- T_1 -resistant oligonucleotides from 14 to 18S and 18 to 22S poly(A)-tagged fragments of nd SR RNA*

Spot no.†	14–18S	18–22S	Spot no.†	14–18S	18–22S
1	6.7	11.4	11	23.9	19.0
2a	5.6	9.7	13	12.6	24.5
2c	4.6	10.4	15	np‡	8.5
3	np‡	12.6	16	16.7	21.7
4	5.4	10.8	17	1.4	6.5
6	10.3	12.9	21	42.3	43.2
8a	37.6	25.2	C	91.6	54.2
10a	39.9	27.4			

* Relative molar yields of oligonucleotides are expressed as cpm per nucleotide, obtained by dividing the total radioactivity of a given oligonucleotide by the number of its constituent mononucleotides (Table 1). Total radioactivity of each unique oligonucleotide from a fingerprint was determined as described (1, 3, 6, 15). The RNA fragments are those described in Fig. 1D and E. The resulting preliminary sequence of the 14–18S fragment is C, 21, 10a, 8a, 11, 16, 13, 6, 1, 2a, 4, 2c, 17, and that of the 18–22S fragment is C, 21, 10a, 8a, 13, 16, 11, 6, 1, 3, 4, 2c, 2a, 15, and 17.

† Numbers are the same as in Fig. 1 and Table 1.

‡ “np” means not yet present.

DISCUSSION

Map Location of Envelope-Specific Sequences in nd SR. The experiments reported here indicate that the seven out of eight oligonucleotides that are present in nd SR but absent from SR N8 cluster together in a segment of the nd SR RNA that maps about 2800–5000 nucleotides away from the poly(A) end. It would appear that these seven oligonucleotides define an nd SR RNA segment of about 2200 nucleotides that was deleted from nd SR to generate the enve-

lope-defective SR N8. However, our experiments cannot determine whether this RNA segment is the mRNA for the envelope glycoprotein or plays an indirect role in its synthesis. Nevertheless, the simplest interpretation of our results is that this RNA segment is part or all of the *env* gene of nd SR RNA (18, 19).

Assignment of this map segment for envelope-specific oligonucleotides is consistent with independent results obtained by mapping envelope-specific oligonucleotides of viral recombinants selected for a cross between the *env* and the *src* genes of their parents (1, 3). Each of these recombinants shared oligonucleotides with the parent that had donated the envelope glycoprotein, in the map segment identified here as envelope-specific (18, 20).

nd SR also differs from SR N8 in a few other oligonucleotides (nos. 9, 7, and 11, see above, and Fig. 2). These differences may be mutational or recombinational changes which might have affected each virus after SR N8 was generated from nd SR. Such minor differences should not invalidate functional identification of nd-virus-specific oligonucleotides, by comparison with deletion mutants, as long as the oligonucleotide maps of the deletion mutant and the respective nd-virus share enough homologous oligonucleotides to demonstrate colinearity of all their common genetic elements. This is clearly the case for nd SR and SR N8 and was shown in other cases previously (6).

Sarcoma-Specific Sequences. Two adjacently mapping oligonucleotides (8a and 10a) of nd SR (and also SR N8) RNA are thought to be sarcoma-specific because they are absent from td SR. This is consistent with their compositions and map locations, which are similar to those of sarcoma-specific oligonucleotides of Rous sarcoma viruses Prague B, C, and B77 (4, 6). [The difference is that 10a is a single oligonucleotide, whereas the analogous spot of Prague B, C, and B77 contains two oligonucleotides (6)]. We are uncertain whether oligonucleotide 13 of nd SR is sarcoma-specific, although it is absent from td SR, because unambiguous identification of a specific sequence by our method requires at least two adjacently mapping oligonucleotides. A single distinct oligonucleotide mapping between oligonucleotides shared by two RNAs may reflect a point mutation rather than a deletion. Further work analyzing the RNAs of other td deletion mutants of nd SR, as well as of nd recombinants between nd SR and leukosis viruses and their corresponding td derivatives, is necessary to identify more definitely sarcoma-specific sequences of nd SR.

Relative Location of Envelope, Sarcoma-Specific, and Other Sequences on nd SR RNA. From the data described here and previously (4, 6, 15, 18, 19) the following partial map emerges for nd SR beginning at the poly(A) coordinate in nucleotides ($\times 10^{-2}$): 0–2, poly(A); 2–6.5, a short heteropolymeric sequence common to nd SR, SR N8, and td SR, which includes an avian tumor virus group-specific element identified by oligonucleotide C (4, 6); 6.5–20, sarcoma-specific sequences of about 1400 nucleotides (7, 10–12); 20–28, a map segment shared by nd SR, SR N8, and td SR which is identified by oligonucleotides 12, 24, and (except for td SR) 13 (Fig. 2); 28–50, envelope-specific sequences; 50–100, presumably *pol* and *gag* genes (6).

We cannot yet determine from our data whether sarcoma-specific and envelope-specific sequences are directly adjacent or separated by other sequences. This is because the map segment 20–28 could represent residual envelope-specific RNA sequences not deleted in SR N8 or could be se-

quences with other functions. Further mapping of other deletion mutants and of the viral recombinants mentioned above is necessary to answer these questions.

Generation of Deletion Mutants. Two mechanisms have been discussed to explain the generation of deletion mutants (6, 15): (i) deletions occur by late initiation or early termination during transcription of viral RNA to proviral DNA or of DNA to viral RNA; (ii) deletions occur by recombinational events at the level of proviral DNA (16). The observations that the oligonucleotide maps of both deletion mutants described here show deletions from within the RNA, rather than from the ends as expected from transcriptional deletions, would argue for the second mechanism. A proviral DNA intermediate (16) could by formation and subsequent elimination of loops delete any sequences from the genome with equal probability.

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