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Induction of Tumors and Generation of Recovered Sarcoma Viruses by, and Mapping of Deletions in, Two Molecularly Cloned *src* Deletion Mutants

LU-HAI WANG,* BRIAN EDELSTEIN, AND BRUCE J. MAYER
The Rockefeller University, New York, New York 10021

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*td*108, a transformation-defective (*td*) deletion mutant of the Schmidt-Ruppin strain of Rous sarcoma virus of subgroup A (SR-A), was molecularly cloned. Two isolates of *td* viruses, *td*108-3b and *td*108-4a, obtained by transfection of the molecularly cloned *td*108 DNAs into chicken embryo fibroblasts, were tested for their ability to induce tumors and generate recovered avian sarcoma viruses (rASVs) in chickens. Both *td* viruses were able to induce tumors with a latency and frequency similar to those observed previously with biologically purified *td* mutants of SR-A. rASVs were isolated from most of the tumors examined. The genomic RNAs of those newly obtained rASVs were analyzed by RNase T_1 oligonucleotide fingerprinting. The results showed that they had regained the deleted *src* sequences and contained the same set of marker *src* oligonucleotides as those of rASVs analyzed previously. The *src* oligonucleotides of rASVs are distinguishable from those present in SR-A. We conclude that those rASVs must have been generated by recombination between the molecularly cloned *td* mutants and the c-*src* sequence. The deletions in the *td* mutants were mapped by restriction enzyme analysis and nucleotide sequencing. *td*108-3b was found to contain an internal *src* deletion of 1,416 nucleotides and to retain 57 and 105 nucleotides of the 5′ and 3′ *src* coding sequences, respectively. *td*108-4a contained a *src* deletion of 1,174 nucleotides and retained 180 and 225 nucleotides of the 5′ and 3′ *src* sequences, respectively. Comparison of sequences in the 5′ *src* and its upstream region of *td*108-3b with those of SR-A, rASV1441 (a *td*108-derived rASV analyzed previously), and c-*src* suggested that the 5′ recombination between *td*108 and c-*src* occurred from 7 to 20 nucleotides upstream from the beginning of the *src* coding sequence.

Certain transformation-defective (*td*) mutants of Rous sarcoma virus (RSV) which contain partial deletions of *src* have been shown to be able to induce tumors and generate recovered avian sarcoma viruses (rASVs) after injection into birds (6, 9, 30, 38). Evidence obtained from RNase T_1 oligonucleotide mapping of the *src* sequences of rASVs and the parental sarcoma virus from which the *td* mutants have been derived strongly suggests that the rASVs are generated by recombination between the *td* viral genome and the cellular counterpart, c-*src*, of the RSV *src* sequence (30, 37, 38). This conclusion was strengthened by the peptide analysis of the pp60s encoded by RSV, rASV, and c-*src* sequences (6, 11, 31). Furthermore, comparison of the entire coding sequences of the *src* genes in RSV and rASV as well of as the exons of c-*src* DNA has identified and located the marker nucleotides that differentiate rASV from RSV on the c-*src* DNA (27–29).

An argument has been made that rASVs may have arisen either by mutation of the parental RSV contaminating the stocks of *td* mutants used in the generation of rASVs or by recombination between *td* mutants containing complementary deletions of the *src* sequence (14, 22). These possibilities were considered very unlikely since the *td* viruses used in the experiments of rASV formation were purified by multiple cycles of endpoint dilutions (9) and comparison of nucleotide sequences demonstrated clearly the cellular origin of the regained *src* sequences in a *td*108-derived rASV, rASV1441 (27–29). Nevertheless, to rule out the possibilities raised in the argument completely, we have molecularly cloned *td* viruses of Schmidt-Ruppin RSV, subgroup A (SR-A), and repeated the experiments of injection into chickens. Our results demonstrate that molecularly purified *td* viruses containing partial deletion of *src* are capable of inducing tumors and generating rASVs in chickens with a latency and efficiency similar to those of the biologically purified *td* mutants shown previously (9).

Recently, we have correlated the extent of 3′ *src* sequences retained by various *td* mutants of SR-A with their ability to give rise to rASVs (33). We concluded from that study that retention of the 3′, but most likely not of the 5′, *src* sequence is essential for the generation of rASVs. To identify further the remaining *src* sequences in the *td* viral genomes that are required for the generation of rASVs, we have mapped the precise *src* deletions in the molecularly cloned *td* viral genomes by restriction enzyme and sequencing analyses. Both mutants analyzed were found to have retained portions of both 5′ and 3′ *src* sequences. The *td* virus with a larger *src* deletion still retained 105 nucleotides of the 3′ *src* sequence. Taken together with our recent finding that 5′ *src* sequence is probably not required for the rASV formation, the current results suggest that about 100 nucleotides of the 3′ *src* sequence in the *td* viral genome may be sufficient to mediate the recombination with c-*src* DNA for the generation of rASVs.

MATERIALS AND METHODS

Cells and virus. Chicken embryo fibroblasts (CEF) were prepared and maintained by a published procedure (8). A methylcholanthrene-transformed quail cell line, QT6 (19), was cultured and maintained in a manner similar to CEF.

* Corresponding author.

*td*108, the *td* mutant used in this study, is derived from SR-A and has been shown previously to be able to induce tumors and generate rASVs in both chickens and quails (9, 38).

Isolation of circular viral DNA. The method for isolating circular viral DNA was essentially similar to those published previously (5, 25) with slight differences. QT6 cells were seeded at 5×10^6 cells per 8.5-cm dish. Twelve hours after plating, 50 dishes of QT6 cells were infected with *td*108 at a multiplicity of infection of >3 in the presence of 17 μg of DEAE-dextran per ml. Six hours postinfection, the medium was changed. Forty-four hours after infection, cells were harvested by trypsinization and suspended in a buffer containing 10 mM Tris-hydrochloride (pH 7.4) and 10 mM EDTA at a concentration of 5×10^6 cells per ml. The unintegrated viral DNA was isolated by the method of Hirt (10). Proteinase K, which had been preincubated at 37°C for 1 h, was added to the Hirt supernatant to a concentration of 1 mg/ml and then was incubated at 37°C for 75 min. The DNA solution was deproteinized by four cycles of phenol and chloroform extraction and was concentrated by ethanol precipitation. The DNA pellet was suspended in 10 ml of buffer containing 10 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA. Boiled RNase A was added to the DNA solution to a concentration of 200 μg/ml, and the mixture was incubated at 37°C for 1 h. The solution was further incubated at 37°C for 30 min after the addition of predigested pronase to a concentration of 500 μg/ml. The mixture was deproteinized again by three cycles of phenol and chloroform extraction, and the DNA was concentrated by ethanol precipitation. The circular viral DNA was enriched by extractions with acidic phenol (40). The DNA was suspended in 2.5 ml of buffer containing 1 mM Tris-hydrochloride (pH 8.0) and 1 mM EDTA. The solution was then adjusted to 50 mM sodium acetate (pH 4.0) and 75 mM NaCl. The DNA was extracted for 4 cycles with the phenol which had been extracted and equilibrated with 50 mM sodium acetate (pH 4.0). The DNA in the aqueous phase of final extraction was concentrated by ethanol precipitation after the pH was neutralized with Tris-hydrochloride (pH 8.0). About 900 μg of DNA was recovered after acid phenol extractions.

Molecular cloning of *td*108 DNA. Ten micrograms of circular *td*108 DNA was digested partially with *Eco*RI. After deproteinization and concentration of the DNA, 1 μg of the *td*108 DNA was ligated to 2 μg of *Eco*RI-cleaved λget · WES · λ B DNA in a 50 μl solution containing 50 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 50 μg of bovine serum albumin per ml, and 1.5 U of DNA ligase. The reaction occurred at 15°C for 2 h. One microgram of the ligated DNA was packaged in vitro into phage particles by the method of Blattner et al. (2). The packaging mix was diluted to 0.4 ml with a buffer containing 10 mM Tris-hydrochloride (pH 7.4), 17 mM NaCl, 1 mM $MgSO_4$, and 0.001% gelatin. Proper samples of the phage solution were titrated on the indicator bacterium, *Escherichia coli* Ed8654 (20). A packaging efficiency of about 10^5 PFU/μg of ligated DNA was obtained. The recombinant phages were screened with [^{32}P]cDNA of *td*108 RNA by the procedure of Benton and Davis (1). About 0.02% of the phage particles contained the *td*108 DNA. Each positive plaque was further purified three times. Among six clones analyzed, two were found to contain only subgenomic DNA, and four appeared to contain the complete genomic DNA of *td*108. The latter four clones, 3b, 4a, 5a, and 7a, were further analyzed. Subcloning of the 3′ *Eco*RI DNA fragments of 3b and 4a *td*108 lambda clones followed the method of Bolivar et al. (3). The *Eco*RI-cleaved plasmid pBR322 DNA was dephosphorylated before ligation to the viral DNA to prevent self-ligation.

Transfection of the molecularly cloned *td*108 DNA and detection of virus production. The *td*108 recombinant DNA was digested partially with *Eco*RI under conditions giving a maximum yield of full-length *td*108 DNA. This was done by using 0.25 U of *Eco*RI per μg of DNA and incubating the reaction mixture at 24°C for 30 min. DNA was transfected into CEF by the calcium phosphate method (7). The transfected cells were transferred at 3-day intervals. After the third transfer, supernatant was taken for the detection of viruses. The production of viruses was detected by assay of the pelletable reverse transcriptase activity (35) and by reinfecting normal CEF and subsequent assay of the reverse transcriptase activity.

Injection of *td* viruses and isolation of rASVs from tumors. Overnight medium from subcultures of the original CEF culture transfected with clone 3b or 4a *td*108 lambda DNA was collected as the *td* virus stocks. Virus stocks of *td*108-3b and *td*108-4a (0.1 ml) were injected into 1-day-old chickens at both wing webs (9). The rASVs were isolated by coculturing the tumor cells and normal CEF by a published method (9). In most cases, culture became confluently transformed 3 to 4 days after cocultivation. Supernatant from the transformed culture was saved as virus stock.

RNA analysis. ^{32}P-labeled viral RNAs were isolated from purified virions (34, 35). The sizes of viral RNAs were analyzed by agarose gel electrophoresis after denaturation of the RNAs with 1 M glyoxal as described previously (18, 36). Genomic sequences of viruses were analyzed by RNase T_1 oligonucleotide fingerprinting as described before (35). For rASVs, in most cases the original coculture was used for isotope labeling and preparation of viral RNAs.

Restriction enzyme analysis of viral DNA. Unlabeled DNA was digested with the appropriate enzyme(s) and analyzed in agarose gel by the procedure of Southern (26) or was labeled with ^{32}P by polynucleotide kinase and then separated in polyacrylamide gels.

DNA sequencing. The standard procedure of Maxam and Gilbert (17) for DNA sequencing was followed, except that a modified method for the cleavage of cytosine residues was used (23).

RESULTS

Molecular cloning of *td*108 DNA. The acid-phenol-purified *td*108 DNA was digested with various restriction enzymes and analyzed by Southern blotting (26). The closed circular DNA was the major species of viral DNA present in the DNA preparation obtained in this way (data not shown). The DNA containing one copy of the long terminal repeat appeared to be more abundant than the DNA containing two long terminal repeats. Neither *Sac*I nor *Sal*I, which each cuts once the DNA of another variant of SR-A, SR-A(SF) (5), cut *td*108 DNA or the DNA of its parental virus, SR-A(NY) (28). Several other restriction enzymes suitable for cleaving lambda and plasmid vector DNAs for cloning cut *td*108 DNA at multiple sites (data not shown). Therefore, we used *td*108 DNA partially digested with *Eco*RI for cloning. Conditions were sought to maximize the yield of full-length viral DNA. We found that the full-length viral DNA was the major product when 1/128 to 1/16 U of *Eco*RI per μg of DNA was used in the digestion. The *td*108 DNA partially digested with *Eco*RI was ligated to *Eco*RI-cleaved λget · WES · λ B DNA and was molecularly cloned. Four clones (3b, 4a, 5a, and 7a) were further analyzed (Fig. 1). Southern blot analysis showed that the *src*-containing *Eco*RI fragment of clone

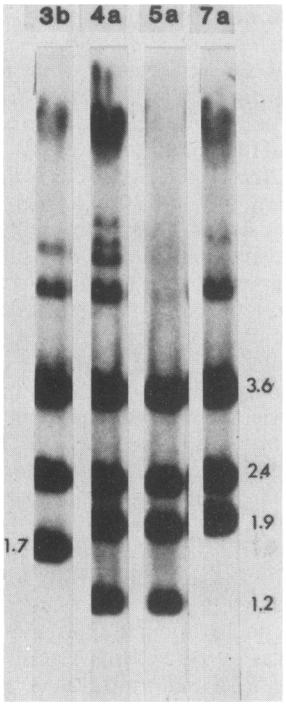

FIG. 1. *Eco*RI restriction pattern of molecularly cloned *td* viral DNAs. Two micrograms of different clones of *td*108 lambda DNAs was digested with *Eco*RI and analyzed as described in the text. DNA fragments running higher than 3.6 kb were the partial digestion products.

3b was about 200 nucleotides smaller than the corresponding fragments of clones 4a, 5a, and 7a (Fig. 1), of which the 3′ *Eco*RI fragments were identical in size to that of parental *td*108 DNA (see below). Clone 3b was apparently derived from a *td*108 DNA molecule with further deletions. It is not clear at which step this deletion occurred. In addition, clones 4a and 5a contained an additional 1.2-kilobase (kb) DNA fragment upon *Eco*RI cleavage. The structure of the clones is shown in Fig. 2. Hybridization of the *Eco*RI fragments of clone 4a with DNA probes specific to various regions of the SR-A genome (data not shown) suggested that the 1.2-kb DNA fragment represented a viral DNA molecule containing an extensive internal deletion and retaining only extreme 5′ and 3′ genomic sequences. This deleted viral DNA was apparently ligated in tandem to a full-length *td*108 DNA molecule during the ligation reaction between viral and lambda DNAs.

The 1.2-kb fragment was absent from the viral genome of *td*108-4a obtained by transfection into CEF of clone 4a DNA partially digested with *Eco*RI (see below). *Eco*RI digestion of the *td*108-4a-infected CEF DNA produced three fragments identical in size to those of the parental virus, *td*108 (Fig. 3). It is not surprising that the 1.2-kb fragment was lost in the viral genome after transfection of the *td*108 lambda clone 4a DNA since that sequence could be freed from the rest of the viral genome after *Eco*RI digestion before transfection (see Fig. 2 for the structure of clone 4a). Alternatively, the 1.2-kb DNA might still be linked to the other viral sequence after partial *Eco*RI digestion, but since it would be located outside the complete unit of nonpermuted viral genome resulting from the transfection, it might not be transcribed or linked physically to the viral genome during transcription.

Interestingly, the largest *Eco*RI restriction DNA fragment of SR-A(NY) and all the *td* mutants derived from it was about 200 nucleotides smaller than the corresponding fragment of Prague RSV, subgroup A (PR-A), PR-C, and B77 (Fig. 3 and other data not shown). However, SR-A(NY) and all the *td* mutants derived from it replicate as efficiently as the other avian sarcoma viruses.

Isolation of the molecularly cloned *td*108 viruses and tumor induction by these viruses. Lambda *td*108 recombinant DNAs partially digested by *Eco*RI were used to transfect CEF. All four clones were able to infect CEF and produce infectious virions. The *td* viruses obtained by transfection of recombinant clones 3b, 4a, 5a, and 7a were called *td*108-3b, *td*108-4a, *td*108-5a, and *td*108-7a, respectively. The *td*108-3b and *td*108-4a stocks obtained from the supernatant of transfected cultures were injected into 1-day-old chickens. Results of such an experiment are shown in Table 1. One or two chickens of each group died within 2 weeks of injection without obvious reason. Most of the chickens surviving the initial injection of *td*108-3b and *td*108-4a developed solid tumors 6 to 8 weeks after infection. A small number of *td* mutant-infected chickens did not develop solid tumors but instead contracted lymphoid leukosis 4 months after the infection. None of the chickens from the control group developed either solid tumors or lymphoid leukosis. As observed before (9), solid tumors appeared in various tissues and organs distant from the sites of virus injection. A total of 21 suspected tumors were found in nine birds and were

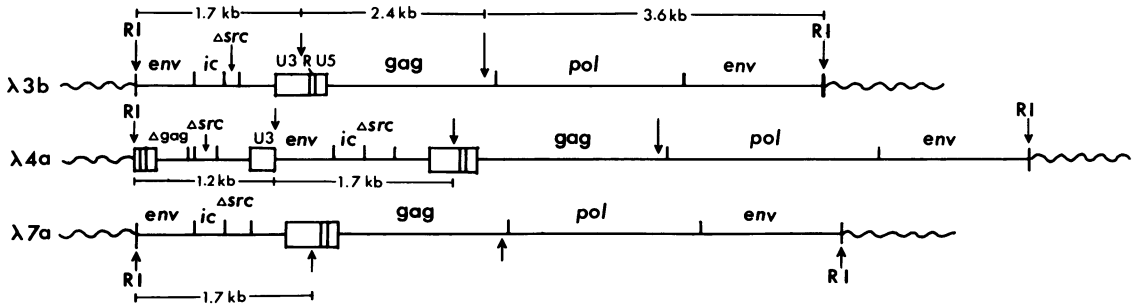

FIG. 2. Structure of the *td*108 lambda recombinant DNA clones. The structures of the recombinant DNA clones were deduced by partial digestion of each clone with *Eco*RI and detection of the restriction DNA fragments with probes specific to various regions of the viral genome (data not shown). Vertical arrows indicate the *Eco*RI sites. Wavy lines designate lambda DNA arms. The ic region represents the intercistronic sequence between *env* and *src*. The structure of clone 5a, which is not shown here, is identical to that of clone 4a. The sizes of the *gag*- and *pol-env*-containing *Eco*RI fragments for each clone are identical.

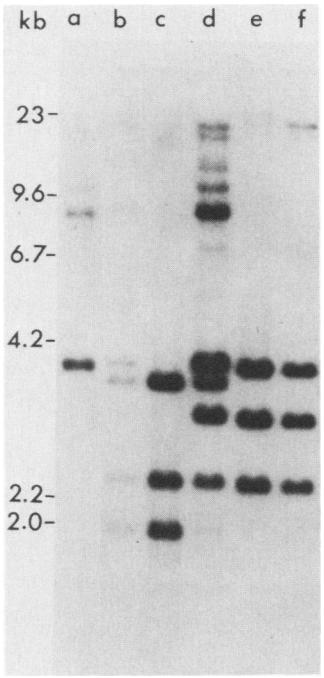

FIG. 3. *Eco*RI digestion of the integrated proviral DNAs. Five micrograms of DNA from each sample was digested completely with *Eco*RI and analyzed. A probe representing the entire SR-A DNA in a SR-A plasmid clone, pSR2 (5), was used for hybridization as follows: Lane a, uninfected CEF DNA; lane b, *td*108-4a-infected CEF DNA; lane c, unintegrated *td*108 DNA enriched in circular forms; lane d, SR-A(NY)-infected CEF DNA; lane e, a wild-type PR-A-infected quail cell clone, QPA4 (16); lane f, a wild-type B77 ASV-infected quail cell clone, QB77-8 (16). Unlabeled *Hin*dIII-digested lambda DNA was used as molecular weight markers.

removed for virus isolation. rASVs were isolated from 16 tumors. Out of 10 samples subjected to histopathological examination, 8 were diagnosed as fibrosarcomas. Two kidney tumors were diagnosed as carcinomas, and rASVs were isolated from these also.

Analysis of *td*108 and rASV RNAs. The sizes of the genomic RNAs of parental *td*108, *td*108-3b, *td*108-4a, and several isolates of rASV derived from the latter two molecularly cloned *td* viruses were analyzed by agarose gel electrophoresis. The original cocultures of tumor cells and normal CEF or rASV-infected CEF cultures were labeled with ^{32}P, and the viral RNAs were isolated from the labeled viruses for size and sequence analyses. rASV RNAs consisted both of sarcoma viral RNA equivalent in size to the SR-A 39S RNA and of the parental *td* viral RNA (Fig. 4). The RNA of *td*108-4a appeared to be identical to that of parental *td*108, whereas *td*108-3b RNA was slightly smaller than either *td*108 or *td*108-4a RNA. This is consistent with the size difference detected in the 3′ *Eco*RI DNA fragments of the two clones (Fig. 1). The SR-A RNA consisted of both the 39S sarcoma viral RNA component and the 35S *td* viral RNA component apparently derived from the spontaneous *td* virus segregant present in the SR-A stock.

The genomic sequences of the three molecularly cloned *td* viruses, *td*108-3b, -4a, and -7a, as well as the 16 isolates of rASVs derived from clones 3b and 4a, were analyzed by RNase T_1 oligonucleotide fingerprinting. Figure 5 shows the result of such analysis. Table 2 summarizes the composition of *src*-specific oligonucleotides in each viral RNA. It has been shown previously that *td*108 RNA retains two 5′ *src*-specific oligonucleotides of SR-A, spots 13 and 35 (37), which map at 111 and 143 nucleotides, respectively, downstream from the beginning of *src* (27, 28). In addition, it retains the 3′ oligonucleotides 33b and 27 (37), which map at 205 nucleotides upstream and at 21 nucleotides downstream, respectively, from the UAG codon of *src* (27, 28). Oligonucleotide compositions of *td*108-4a, *td*108-7a, and parental *td*108 RNAs are identical. The RNA of *td*108-3b contained oligonucleotide 27 but lacked oligonucleotides 13, 33b, and 35. Except for the *src* oligonucleotides, *td*108-3b had a fingerprint pattern identical to that of parental *td*108. The presence or absence of those *src*-specific oligonucleotides in each *td* viral RNA was more clearly seen when 10 to 15S polyadenylic acid-containing RNAs of the *td* viruses were analyzed (data not shown). Figure 5 shows the typical fingerprint patterns represented by three isolates of rASV, rASV2158, derived from *td*108-3b, and rASV2168 and

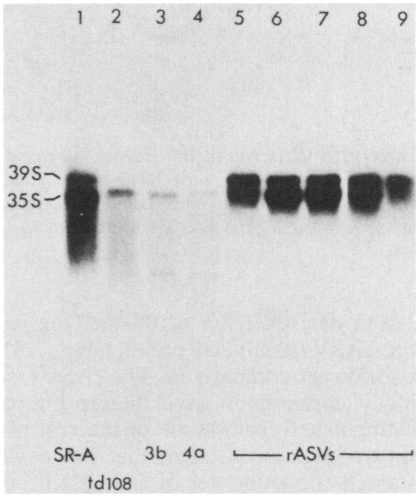

FIG. 4. Analysis of the genomic RNA size of molecularly cloned *td* viruses and rASVs derived from them. CEF infected with various viruses was labeled with ^{32}P, and the labeled viral RNAs were isolated from purified virions. For rASVs, original cocultures of the tumor cells and CEF were used for the labeling and RNA preparation. The purified ^{32}P-labeled viral RNAs were denatured with 1 M glyoxal at 50°C and analyzed in 1% agarose gels. After electrophoresis, the gel was dried under vacuum onto a layer each of Whatman DE81 and 3MM paper and subjected to exposures. Lanes 5, 6, and 8 are *td*108-3b-derived rASVs, 2158, 2160, and 2163 respectively; lanes 7 and 9 are *td*108-4a-derived rASVs, 2168 and 2172, respectively. The faint bands migrating faster than the genomic RNAs in lanes 2, 3, and 4 were apparently the 28S rRNA contaminants. The SR-A stock used in this experiment apparently contained a significant level of *td* virus as reflected by the presence of 35S RNA (lane 1).

TABLE 1. Tumor induction in chickens by molecularly cloned *td* viruses

td clone	No. of chickens			
	Injected	Dead	With solid tumors	With lymphoid leukosis
*td*108-3b	7	1 (1–2 wk)	4 (6–8.5 wk)	2 (16–18 wk)
*td*108-4a	8	2 (1–2 wk)	5 (6–7 wk)	1 (16–19 wk)
Control	7	2 (1–2 wk)	0	0

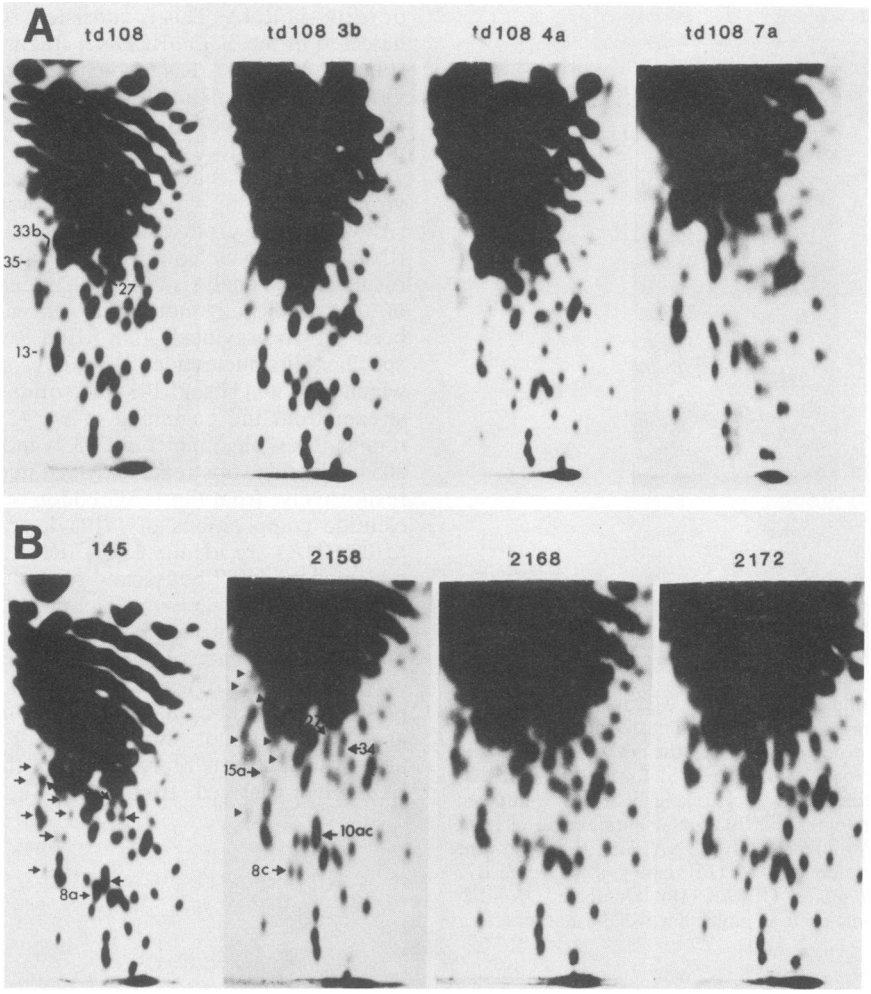

FIG. 5. Fingerprint patterns of the RNase T_1-resistant oligonucleotides of *td* virus (A) and rASV (B) RNAs. ^{32}P-labeled viral 60 to 70S RNA was digested completely with RNase T_1, and the digestion products were separated by electrophoresis (from left to right) and homochromatography (from bottom to top). Oligonucleotide spots indicated by arrows are *src* specific. Spots 8c, 10c, 15a, and 34 are those not present in SR-A. rASV2158 was derived from *td*108-3b, and rASV2168 and -2172 were derived from *td*108-4a.

rASV2172, both derived from *td*108-4a. One isolate of the *td*108-derived rASVs analyzed previously, rASV145, is included here for direct comparison. The rASVs isolated from the two kidney carcinomas gave fingerprint patterns that were not distinguished from those of the rest of the rASVs (data not shown). It can be seen that the newly obtained rASVs contained the same set of *src*-specific oligonucleotides, including those not present in SR-A (Fig. 5 and Table 2, spots 8c, 10c, 15a, and 34), as the rASVs derived from other *td* viruses (37, 38). Like rASVs analyzed before (37, 38), current rASVs also showed variation with respect to *src*-specific oligonucleotides 8c and 15a, which are located at 682 nucleotides downstream and 9 nucleotides upstream, respectively, from the beginning of *src* (27). Both spots 8c and 15a are present in the c-*src* sequence (29). Of the seven isolates of *td*108-3b-derived rASVs analyzed, three contained spot 15a, and the rest did not; six isolates contained spot 8c, and one isolate contained spot 8a in place of 8c. Only one of the nine *td*108-4a-derived rASVs analyzed had spot 15a; five isolates contained spot 8c, and four isolates contained spot 8a. Spot 8c differs from spot 8a in SR-A by a single C-to-U base change (27, 37). Spot 15a differs from the corresponding sequence in SR-A by a single C-to-G base change (27). The reason for the variation of these two oligonucleotides is not clear. It may be due to polymorphism of chicken c-*src* alleles or due to hot spots for mutation. There is no apparent linkage between spots 8a and 15a or between spots 8c and 15a among the rASVs currently or previously analyzed. Analysis of 12 isolates of current rASVs derived from four chickens showed that individual tumor-derived rASVs from a single chicken always give the identical composition of oligonucleotides, with one possible exception concerning spot 15a (data not shown).

Mapping the deletions in *td*108-3b and *td*108-4a. To define the deletions in the molecularly cloned *td* viruses further, the 3′ *Eco*RI DNA fragments of *td*108-lambda clones 3b and 4a were subcloned in plasmid pBR322 and analyzed. The 1.7-kb DNA of clone 3b and the 1.2- and 1.9-kb DNAs of clone 4a were digested with *Hin*fI and *Eco*RII and were compared with 3.1-kb *src*-containing *Eco*RI fragment of SR-A DNA (Fig. 6). *Eco*RII digestion of the 1.7- and 1.9-kb DNA fragments produced a subset of fragments seen in SR-A DNA. In addition, it generated from each DNA a fragment (Fig. 6, arrows) not seen in the SR-A DNA digests. These

TABLE 2. Composition of *src*-specific oligonucleotides in *td* virus and rASV RNAs

Viral RNA	Presence of RNAse T_1 oligonucleotide[a]:													
	15a	13	35	34	10c	37	32b	10a	8a	8c	38	36	33b	27
*td*108	−	+	+	−	−	−	−	−	−	−	−	−	+	+
*td*108-3b	−	−	−	−	−	−	−	−	−	−	−	−	−	+
*td*108-4a	−	+	+	−	−	−	−	−	−	−	−	−	+	+
*td*108-7a	−	+	+	−	−	−	−	−	−	−	−	−	+	+
rASV145	+	+	+	+	+	+	+	+	+	−	+	+	+	+
rASV2158	+	+	+	+	+	+	+	+	−	+	+	+	+	+
rASV2168	−	+	+	+	+	+	+	+	+	−	+	+	+	+
rASV2172	−	+	+	+	+	+	+	+	−	+	+	+	+	+

[a] The order of spots from left to right is the 5′-to-3′ order of these oligonucleotides in rASV RNA (27, 37). All except spots 15a and 27 are derived from the coding region of *src*. Spot 15a spans nucleotides −9 to +3 of *src*, and spot 27 spans 9 to 19 nucleotides from the termination codon of *src* (27). Spots 8a and 8c are isomers of the same stretch of *src* sequence and differ from each other by only one base change (see text).

new fragments were apparently generated as a result of the deletion in each *td* virus clone. *Hin*fI digestion showed that the 1.9-kb DNA fragment lacked the fragments of 580, 292, 194 (see below), 144, 48, and 32 base pairs (bp) (Fig. 6). The 1.7-kb fragment lacked these fragments and also lacked fragments of 207, 173, 147, and 92 bp. The 1.2-kb DNA, which appeared to be an extensively deleted proviral DNA (see above), generated only the fragments of 236, 207, 173, 147, and 119 bp corresponding to those of SR-A. The other fragments derived from the 1.2-kb fragments were not present in the 3.1-kb SR-A DNA and apparently were derived from another genomic region of the deleted provirus DNA.

The DNA fragments running at positions corresponding to 292 and 200 bp from *Hin*fI-digested 1.7-kb DNA were named 1.7 Ha and 1.7 Hb, respectively, and were further analyzed. Similarly, the 207- and 194-bp fragments derived from 1.9-kb DNA were named 1.9 Ha and 1.9 Hb, respectively. These fragments were initially considered to be the possible locations of the deletions. To identify the DNA fragments containing the deletion, the ^{32}P-end-labeled 1.7 Ha, 1.7 Hb, 1.9 Ha, and 1.9 Hb fragments were subjected to digestion with various enzymes known to cut the SR-A DNA within the corresponding regions of each fragment. The restriction enzyme digestion revealed that the 1.7 Ha and 1.9 Ha fragments contained the deletions (data not shown). The size of all the restriction fragments generated outside the deletion agrees completely with the expected size of the fragments generated from the SR-A 3′ *Eco*RI DNA fragment. From the known positions of various restriction enzyme sites on the SR-A 3.1-kb DNA (27), a detailed deletion map of clones 3b and 4a and the respective *Hin*fI fragment containing the *src* deletions was constructed (Fig. 7). The major deletion in clone 3b was located between the *Eco*RII and *Ava*II sites (Fig. 7). The major deletion in clone 4a was located between the *Hae*III and *Dde*I sites (Fig. 7).

Restriction enzyme digestion showed that the 1.7 Hb fragment was related to the 207-bp *Hin*fI fragment of SR-A DNA and contained a deletion of about 10 nucleotides between *Hae*III and the 3′ end of the 1.7 Hb fragment (Fig. 7). Similarly, the 1.9 Hb fragment was shown to be equivalent to the 207-bp *Hin*fI fragment of SR-A and contained two small deletions of about 5 and 10 nucleotides (Fig. 7).

Nucleotide sequences of the restriction fragments containing the deletions. Having identified the restriction fragments of *td*108-3b and *td*108-4a DNAs that harbored the *src* deletions, we then proceeded to sequence those DNA fragments to determine the precise boundaries of the deletions (Fig. 8). The sequence of the 1.7 Hb *Hae*III-*Hin*fI fragment revealed that it contained a 10-bp deletion in comparison with that of SR-A(NY) between positions −83 and −84 from the AUG codon of *src* (Fig. 8A). This result confirmed the restriction enzyme analysis of the 1.7 Hb fragment. The sequence in this region of *td*108-3b was identical to that of rASV1441 determined previously and differed from that of SR-A(NY) by only the 10-bp deletion and an A-to-G base change at position −84 (28; Fig. 8A). Next, we sequenced the 1.7 Ha

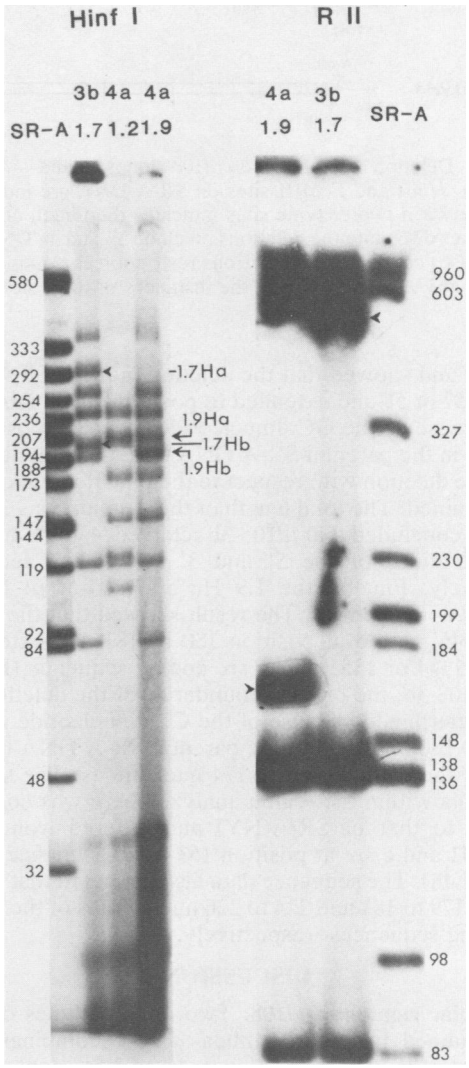

FIG. 6. *Hin*fI and *Eco*RI digestion of SR-A and *td* viral DNAs. The 3′ *Eco*RI DNA fragments of SR-A and *td*108 lambda clones (subcloned in plasmid pBR322) were digested completely with either *Hin*fI or *Eco*RII. The digested DNAs were labeled at the 5′ ends with ^{32}P and separated on 5% polyacrylamide gels (acrylamide:bisacrylamide = 19:1). The size of each restriction fragment of SR-A DNA is indicated by the number of base pairs according to the known *Eco*RII and *Hin*fI sites. The mobility of the SR-A DNAs was slightly faster than that of *td* viral DNAs in the *Hin*fI experiment shown here, probably due to different amounts of salt present in the samples. Such an effect was not seen in other experiments (data not shown).

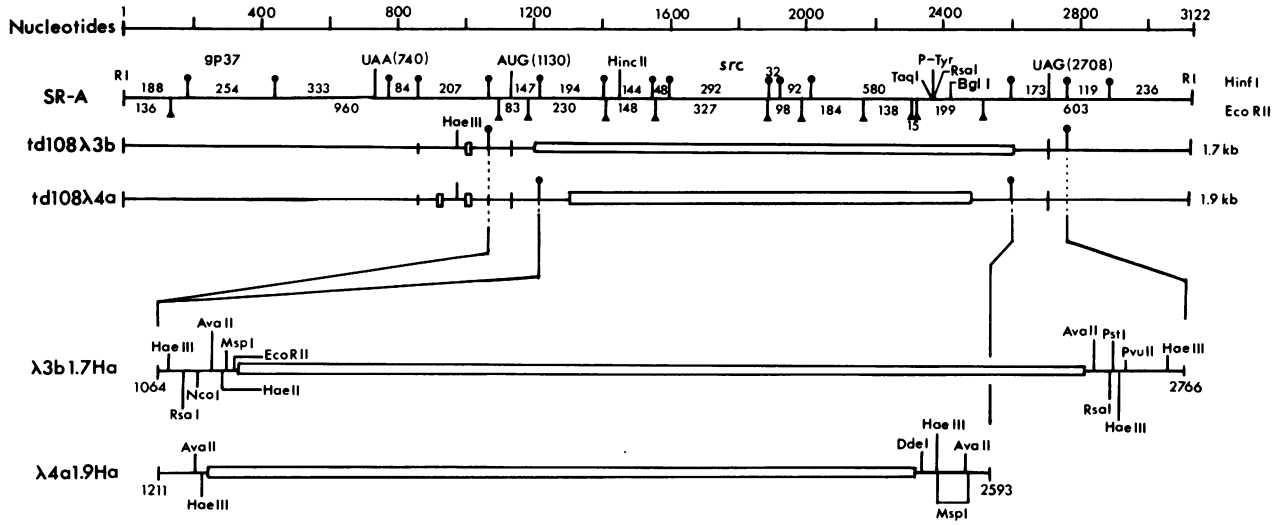

FIG. 7. Deletion map of lambda *td*108 clones 3b and 4a DNA. The 3′ *Eco*RI (RI) DNA fragments of SR-A DNA and clone 3b and 4a DNAs are shown. *Hin*fI and *Eco*RII sites on SR-A DNA are indicated, respectively, above and below the line representing the SR-A DNA. The number between two enzyme sites indicates the length of the restriction fragments in bp based on the known sequence of SR-A (27). The empty boxes designate the deletions on clone 3b and 4a DNA. The detailed restriction maps of 1.7 Ha and 1.9 Ha fragments were constructed from analyses conducted with various restriction enzymes shown on the map. The numbers in parentheses after the initiation and termination codons of gp37 and *src* indicate the distances of those codons from the left-hand *Eco*RI site by number of nucleotides (27).

fragment and showed that the deletion in *td*108-3b started at position 57 or 58 and extended to position 1473 or 1474 (Fig. 8B). Because of the GC dinucleotide repeat at positions 57 and 1473 in the parental SR-A DNA (27), the exact breakage site of the deletion with respect to the dinculeotide could not be determined. The total length of the deletion was 1,416 bp. It can be concluded that *td*108-3b retained 56 to 58 and 104 to 106 nucleotides of the 5′ and 3′ *src* coding sequences, respectively. Finally, the 1.9 Ha 5′ *Hin*fI-*Msp*I fragment (Fig. 7) was sequenced. The result showed that the deletion in *td*108-4a started at position 180 or 181 and extended to position 1354 or 1355 of the *src* coding sequence (Fig. 8C). As in *td*108-3b, the precise boundaries of the deletion could not be determined because of the CT dinucleotide repeat at positions 180 and 1354 in the parental SR-A DNA (27). The length of the deletion was 1,174 nucleotides. The sequence of *td*108-4a within the region analyzed here was completely identical to that of SR-A(NY) and differed from that of rASV1441 and c-*src* at position 153 (C-to-T change) (27–29; current data). The sequence data also indicated that *td*108-4a retained 179 to 181 and 224 to 226 nucleotides of the 5′ and 3′ *src* coding sequences, respectively.

DISCUSSION

Molecular cloning of *td*108. Two unusual types of clones were obtained from the lambda-*td*108 recombinants. The first type, represented by clones 4a and 5a, contained the additional 1.2-kb viral DNA fragment linked to a copy of complete genomic DNA. Formation of such a chimeric recombinant DNA molecule was quite possible under our conditions, which favored intermolecular ligation. However, the source of the 1.2-kb DNA was not clear. It might have existed in our *td*108 DNA preparation used for the cloning. The acid-phenol method could not separate low-molecular-weight DNAs from the circular DNAs (40). However, the 1.2-kb DNA was not detected in the DNA blot of the *td*108 DNA (data not shown). The second unusual type of clone, clone 3b, had a *src* deletion larger than that of parental *td*108. Similarly, we did not detect the 1.7-kb DNA band of clone 3b in the *Eco*RI digest of the original *td*108 DNA (data not shown). However, we could not rule out the possibility that both 1.2-kb DNA and *td*108-3b DNA existed at low quantity in our *td*108 DNA preparation. They could be derived from *td*108 after passages of the virus stock in culture. Alternatively, those DNAs with further deletions could result from cloning by an unknown mechanism.

Origin of rASVs. Our results demonstrate that the two *td* viruses derived from molecularly cloned viral DNAs were able to induce tumors and generate rASVs after injection of the viruses into chickens. The latency (about 7 weeks) and the efficiency (about 70%) were similar to those observed previously with biologically purified *td* viruses (9). The current data confirm our previous studies and those of others showing that rASVs are generated by recombination between *td* viruses and the c-*src* sequence based on studies of the viral genomic and c-*src* sequences and the pp60s encoded by these sequences (6, 9, 27–31, 37, 38). It was argued previously that the minor differences observed in *src* nucleotide sequences and tryptic peptides of pp60s among SR-A, rASVs, and c-*src* could not allow one to assign unambiguously the origin of the *src* sequences in rASVs (14, 22). Our current results with molecularly cloned *td* viruses rule out unequivocally the possibilities raised previously that rASVs might have emerged from contaminants in the *td* virus stocks or might have been generated by recombination between two *td* viruses containing complementary deletions of *src* (14, 22).

Sequence essential for the generation of rASVs. We have shown in a recent study that all the rASVs derived from a particular *td* mutant, *td*109, contain deletions of various lengths in the *env*, *pol*, and *gag* genes; in addition, some rASVs show heterogeneity, with either deletion or extra sequences, at the 5′ region of the regained *src* sequences (33). The genome of *td*109 was shown to have deletions of most, if not all, of the 5′ *src* and its immediate upstream region, including the splice acceptor site for *src* mRNA. We

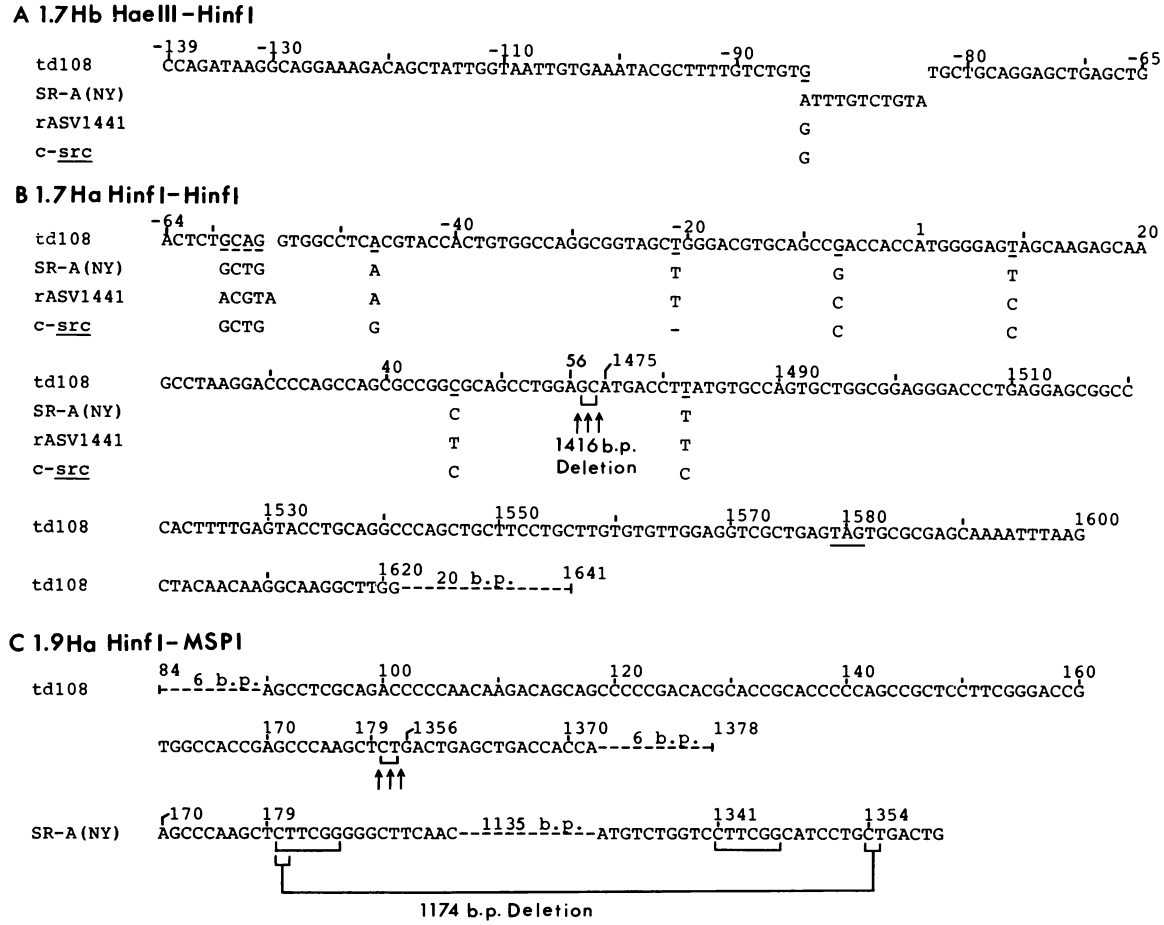

FIG. 8. Nucleotide sequences of *td* viral DNA restriction fragments containing the deletions. Sequences were obtained by the sequencing method of Maxam and Gilbert (17). The numbering starts at the first nucleotide the *src* coding sequence. The sequences of the 3′ 20 nucleotides of the 1.7 Ha fragment and the 5′ and 3′ 6 nucleotides of the 1.9 Ha *Hin*fI-*Msp*I fragment were not determined. The nucleotides of divergence among *td*108, SR-A, rASV1441, and c-*src* are underlined, and the differences are shown. Sequences of SR-A, rASV1441, and c-*src* are taken from published data (27, 29). The vertical arrows indicate the possible sites of breakage that generated the deletions.

hypothesized that deletions and heterogeneity at the 5′ *src* region of those rASVs resulted from recombination between the *td*109 genome sequence and the c-*src* sequence at multiple sites involving only partial homology because of the lack of the 5′ *src* region in *td*109 (33). None of the 16 isolates of *td*108-3b- and *td*108-4a-derived rASVs analyzed here contain either deletions in replicative sequences or heterogeneity at the 5′ *src* region. This is consistent with the hypothesis since both *td* viruses retain a portion of the 5′ *src* sequence and have no deletions in the upstream region. In the same recent study (33), we concluded that retaining certain 3′ *src* sequences, but most likely not the 5′ *src* sequences, was essential for the generation of rASV, although lack of the 5′ *src* sequence and its upstream sequence in *td*109 was most likely responsible for the deletions in the rASVs derived from it. Since *td*108-3b retains only 104 to 106 nucleotides of the 3′ *src* sequence, it implies that 68 to 70 nucleotides of the 3′ *src* sequence are sufficient to mediate the homologous recombination in the 3′ region of the *td* viral genome and the c-*src* sequence since the homology between SR-A *src* and c-*src* ends at 37 nucleotides before the termination codon (29). It is also possible that the 3′ terminal sequence of v-*src* differentiates its transforming potential from that of c-*src* and that retaining this region in *td* mutants is, therefore, essential for the generation of rASVs.

Recombination sites between *td*108 and c-*src* in the generation of rASV1441. Sequence analyses of SR-A, rASV1441, and c-*src* have located several differences among them (27–29). The sequence differences at the 5′ *src* and its upstream flanking regions among *td*108, SR-A, rASV1441, and c-*src* are shown in Fig. 8. The sequences of *td*108 and SR-A are identical except for a T-to-A transversion at position −57, an A-to-G change at position −84, and a 10-bp insertion next to it in SR-A (Fig. 8). It is not clear whether the 10-bp (TTTGTCTGTA) tandem repeat is present in the original SR-A genome or unique to this particular SR-A clone. Comparison of the sequences among *td*108, rASV1441, and c-*src* in this region showed that sequences upstream from position −20 were identical between *td*108 and rASV1441, except for the unique 5-bp changes of rASV1441 at position −56. By contrast, rASV1441 and c-*src* sequences were identical at positions −7 and 10 and differed from those of *td*108. These data suggest that the 5′ recombination site between *td*108 and c-*src* in the generation of rASV1441 is located between positions −7 and −20. This is one of the possible recombination sites suggested previously based on

the sequence comparison of SR-A(NY), rASV1441, and c-*src* (28). The nucleotide at position 46 of the rASV1441 *src* sequence differs from that in *td*108, SR-A, and c-*src* and is most likely due to mutation after recombination. Comparison of the 3′ *src* sequences of SR-A, rASV1441, and c-*src* reveals that nucleotides at positions 1400, 1405, 1421, and 1482 are identical in SR-A and rASV1441 but different in c-*src* (28, 29). This implies that the *src* sequences from position 1400 and the downstream region were apparently derived originally from SR-A. The current sequence data of the two *td*108 variants show that *td*108-4a retains the 3′ *src* sequence downstream from position 1354 and is most likely the parental virus of rASV1441. It can also be concluded that the 3′ recombination site between the *td*108 genome and c-*src* is located between positions 1354 and 1400 of the *src* sequence. The precise crossing-over site could not be determined because the sequences within that region are identical in SR-A, rASV1441, and c-*src* (28, 29).

Generation of *td* mutants. Nondefective avian sarcoma virus segregates *td* deletion mutants at a high rate upon passage of the virus in cell culture (13, 15, 32). Sequence data of SR-A and PR-C RSV reveal that *src* is flanked by 100 to 120 nucleotides of sequences with about 80% homology (4, 24, 27). It has been proposed that those directly repeated sequences may play a role in the generation of *td* viruses (4). Deletion mediated by those directly repeated sequences most likely would result in the loss of entire *src* sequence, but this cannot account for the generation of partial *src* deletion mutants from SR-A and PR-C (12, 33, 39). A recent study by Omer et al. shows that deletions in three *td* mutants are flanked by small directly repeated sequences outside the *src* coding region (21). Neither of the deletions in the two *td* mutants analyzed here was flanked by directly repeated sequences. However, a 6-bp direct repeat was found at the neighborhood of the 5′ and 3′ deletion boundaries of *td*108-4a (Fig. 8C). The significance of this direct repeat in the generation of the *td*108-4a deletion is not clear. It is likely that an alternative mechanism to that mediated by the repeated sequences is responsible for the generation of certain *td* mutants, especially those that retain partial *src* sequences.

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