Identification of the Viral Sequence Required for the Generation of Recovered Avian Sarcoma Viruses and Characterization of a Series of Replication-Defective Recovered Avian Sarcoma Viruses

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The ability of transformation-defective deletion mutants of Schmidt-Ruppin Rous sarcoma virus to induce tumors and generate recovered sarcoma viruses (rASVs) was correlated with the partial *src* sequences retained in the transformation-defective viral genomes. Since all the transformation-defective viruses that were capable of generating rASVs retained a portion of the 3' *src* sequence, regardless of the extent of the 5' *src* deletion, and those lacking the 3' *src* were unable to generate rASVs, it appears that the 3', but most likely not the 5', *src* sequence retained in the transformation-defective viral genome is essential for rASV formation. However, rASVs derived from a particular mutant, *td109*, which retained a portion of the 3' *src* sequence, but lacked most (if not all) of the 5' *src* sequence, were all found to be defective in replication. Analyses of the genomic sequences of 13 isolates of *td109*-derived rASVs revealed that they contained various deletions in viral envelope (*env*), polymerase (*pol*), and structural protein (*gag*) genes. Ten isolates of rASVs contained *env* deletions. One isolate (rASV3812) contained a deletion of *env* and the 3' half of *pol*, and one isolate (rASV398) contained a deletion of *env* and *pol*. The one with the most extensive deletion (rASV374) had a deletion from the p12-coding sequence through *pol* and *env*. In addition, the 5' *src* region of *td109*-derived rASVs were heterogeneous. Among the 7 isolates analyzed in detail, one isolate of rASV had a small deletion of the 5' *src* sequence, whereas three other isolates contained extra new sequences upstream from *src*. Both *env*⁻ and *env*⁻ *pol*⁻ rASVs were capable of directing the synthesis of precursor and mature *gag* proteins in the infected nonproducer cells. We attribute the deletions in the replication-defective rASVs to the possibility that the 5' recombination site between the *td109* and *c-src* sequence, involved regions of only partial homology due to lack of sufficient 5' *src* sequence in the *td109* genome for homologous recombination. A model of recombination between the viral genome and the *c-src* sequence is proposed to account for the requirement of the 3' *src* sequence and the basis for the generation of deletions in *td109*-derived rASVs.

Certain transformation-defective deletion mutants of Rous sarcoma virus are able to induce tumors and generate sarcoma viruses, called recovered avian sarcoma viruses (rASVs), after injection into birds (5, 10, 20, 28, 38). The rASVs are generated by recombination between transformation-defective viral genomes and the cellular homolog, *c-src*, of the Rous sarcoma virus *src* sequence. This conclusion has been based on (i) the analyses of the genomic RNAs of rASVs, transformation-defective viruses, and Schmidt-Ruppin Rous sarcoma virus subgroup A (SR-A), the parental virus from which transformation-defective viruses have been derived (8, 29, 37, 38); (ii) peptide analyses of the pp60s encoded by SR-A, rASVs, and the *c-src* (5, 15, 29); and (iii) comparison of the complete pp60-coding sequences of rASV1441, SR-A, and *c-src* DNA (24-26). All the rASVs characterized so far are replication competent and appear to have been generated by insertion of the *c-src* sequence into the partially deleted *src* region of the transformation-defective viral genomes at no cost to the replicative sequences. Direct comparison of the *src* sequences of one of the rASVs (rASV1441), SR-A, and *c-src* suggests that the 5' recombination site is immediately upstream from the beginning of the *src* coding sequence, and the 3' recombination site is located within the 3' remaining *src* sequence at least 178 nucleotides from the end of *src* (24, 25). Both 5' and 3' sequences flanking those putative sites of recombination are present in the transformation-defective virus that gave rise to rASV1441 (37, 39; L.-H. Wang, unpublished data).

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To further understand conditions for the recombination between transformation-defective virus and the *c-src* sequence, we have studied various transformation-defective viruses and their ability to generate rASVs. Our results suggest that the 3' *src* sequence, but most likely not the 5' *src* sequence, remaining in the transformation-defective viral genome is essential for the generation of rASVs.

During analyses of rASVs derived from various transformation-defective viruses, we found that the rASVs derived from a particular transformation-defective virus, *td109*, were all defective in replication. The RNA of *td109* retained only a portion of the 3' *src* sequence, but lacked most (if not all) of the 5' *src* sequence. Since *td109* is replication competent, we consider that it is very likely that the defectiveness has resulted from the recombination between *td109* and the *c-src* sequence. Characterization of the RNA genomes of *td109*-derived rASVs revealed that these rASVs contained various lengths of deletion in replicative sequences. Furthermore, unlike the rASVs studied previously, the 5' *src* sequence of *td109*-derived rASVs was rather heterogeneous. This result suggests that the internal deletions and the heterogeneity of the 5' *src* sequence in those rASVs are most likely the result of recombination involving only partial homology at the 5' crossover sites between *td109* and the *c-src* sequence. A preliminary result of this study was reported previously (13).

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEF) were prepared and maintained as described previously (9). All of

the transformation-defective viruses studied here were derived from SR-A. All of them have been described previously (16). An initial account of rASVs obtained from individual tumors induced by transformation-defective mutants was given previously (8, 10).

Isolation of NP clones. Nonproducer (NP) clones of replication-defective rASVs were prepared by isolating transformed colonies of rASV-infected CEF grown in semisolid agar medium containing antiserum against the helper transformation-defective virus (12).

Assay of virus production. The ability of the rASV-infected NP cells to produce viral particles was tested by (i) focus formation on CEF (9), (ii) assay of the pelletable reverse transcriptase activity from the culture fluid (34), (iii) labeling of the NP cells with [³H]uridine and isolation of the radiolabeled virus from culture fluid (33, 34), (iv) isolation of virion RNAs from culture medium and analysis of the RNAs by gel electrophoresis followed by RNA blotting and filter hybridization (36), and (v) [³⁵S]methionine labeling and analysis of the virion proteins by gel electrophoresis (6, 15).

Preparation of radioisotope-labeled viral and cellular RNAs. Preparation of ³H-, ¹⁴C-, or ³²P-labeled viral and CEF ribosomal RNAs followed the methods described previously (33, 34, 39). RNAs for size analysis were prepared from viruses harvested at 3- to 5-h intervals, whereas RNAs for oligonucleotide fingerprinting were isolated from virions harvested at 12-h intervals.

Analysis of RNA size. The size of viral RNAs was analyzed by (i) polyacrylamide gel electrophoresis of heat-denatured viral 60 to 70S RNA (4), (ii) sucrose gradient sedimentation (33, 34), and (iii) agarose gel electrophoresis of viral RNAs pretreated with 1 M glyoxal at 50°C (19). Recovery of RNA from preparative polyacrylamide gel was by the published method (3, 36).

Sequence analysis of viral RNAs. RNase T₁-resistant oligonucleotide fingerprinting, mapping, and composition analysis of the oligonucleotides with RNase A have been described previously (34, 35).

Preparation of total poly(A)-containing RNAs from virus-infected cells. Extraction of total cellular RNAs was by the procedures described previously (14, 33). Briefly, cells were disrupted with sodium dodecyl sulfate and digested with proteinase K (14). After deproteinization with phenol and chloroform extractions, the total nucleic acids were concentrated by ethanol precipitation. Polyadenylic acid [poly(A)]-containing RNAs were selected by passing twice through an oligodeoxythymidylic acid-cellulose column (33).

Preparation of gene-specific probes. ³²P-labeled DNA probes were prepared by nick translation (18) of the DNAs specific to various regions of the SR-A genome. The 5' probe spans from the *Eco*RI site within the U3 region of the left-hand long terminal repeat to the *Bam*HI site in the 5' *gag* region with a length of about 500 nucleotides (1, 22); the 5' *gag* probe spans the two *Bam*HI sites within the *gag* region with a length of 1.3 kilobases (kb); and the 3' *gag* probe of 400 nucleotides extends from the second *Bam*HI site in *gag* to the downstream *Eco*RI site. All three probes were prepared from a plasmid clone, pFC3 (obtained from F. Cross), which contains the 5' *Eco*RI fragment of SR-A DNA. The *pol-env* probe represents the 3.8-kb middle *Eco*RI fragment of SR-A DNA. The *src* probe extends from *Hinc*II site to the *Bgl*II site within the *src* region (24), with a length of about 1 kb. This was prepared from the plasmid clone, pTT107 (24), which contains the 3' *Eco*RI DNA fragment of SR-A. Finally the *c* probe of 450 nucleotides represents the *Pvu*II to *Eco*RI DNA fragment containing the U3 region of SR-B DNA and

was isolated from a plasmid clone, pLTR-L6 (obtained from T. Takeya), containing this DNA.

RNA blotting and hybridization. Transfer of the RNAs from agarose gel to nitrocellulose paper was by the procedure of Thomas (27). The conditions for hybridization have been described previously (36).

Protein analysis. Infected cells were labeled with [³⁵S]methionine, and the proteins were extracted and analyzed as described previously (6, 15).

RESULTS

Viral sequence essential for the formation of rASVs. The genomic sequences of various transformation-defective viruses were examined to see whether a correlation exists between the genomic structure and the ability to form rASVs. Three transformation-defective mutants of SR-A, *td103*, *td106*, and *td109*, were analyzed in this study. Other transformation-defective viruses that have been characterized previously (37, 39) were further studied here. The size of the genomic RNAs was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The RNAs of *td106* and *td109* were slightly smaller, whereas the RNA of *td103* was slightly larger than that of Rous-associated virus-2 (RAV-2) (Fig. 1A, G, and H). This result is in agreement with previous analysis (16).

The genomic sequences of the transformation-defective viruses were characterized by fingerprinting of the RNase T₁-resistant oligonucleotides. *td106* RNA contained no *src*-specific oligonucleotides (Fig. 2); *td103* RNA contained *src*-specific oligonucleotides 13 and 35, mapped at the 5' end of *src* (Fig. 3); *td109* RNA retained *src*-specific oligonucleotides 33b, mapped at the 3' end of *src*, and oligonucleotide 27, which, according to the complete SR-A *src* sequence (24), is located at 21 nucleotides downstream from the termination codon of *src* (Fig. 3). As shown previously, *td108* RNA contained both 5' and 3' *src*-specific oligonucleotides (37) (Fig. 2). The presence of 5' and 3' *src*-specific oligonucleotides in *td103* and *td109* RNAs, respectively, is more clearly seen in the fingerprints of poly(A)-containing subgenomic RNAs of the two viruses (Fig. 2E and F). In addition to the absence of 5' *src* oligonucleotides, *td109* RNA lacked oligonucleotide 21 mapped at 217 nucleotides upstream from the initiation codon of *src* (24) (Fig. 3). Besides the *src*-specific spots and oligonucleotide 21, none of the large RNase T₁-resistant oligonucleotides of parental virus, SR-A, was missing in *td109* (Fig. 2). This result and the fact that the genomic RNA of *td109* is smaller than that of RAV-2 despite its retention of the 3' *src* sequence suggest that the 5' boundary of the deletion in *td109* genome extends to at least 217 nucleotides upstream from the AUG codon of *src*. This deletion includes the *src* splicing acceptor site, which is located at 75 nucleotides upstream from the beginning of *src* (23, 24).

To further analyze the *src* sequences retained in each transformation-defective viral genome, we isolated total poly(A)-containing RNAs from transformation-defective virus-infected cells and detected the *src* sequence content in viral RNAs by RNA blotting and filter hybridization. For *td109*, RNAs isolated from cells infected with both *td109* and rASV374 were analyzed (Fig. 3). Genomic and two species of spliced mRNAs were detected in *td101*-, *td103*-, *td105*-, and *td108*-infected cells when the 5' probe was used for hybridization (Fig. 3A). The middle and the lower species of RNA in those four transformation-defective viral RNA samples were apparently *env* and truncated *src* mRNAs, respec-

FIG. 1. Polyacrylamide gel electrophoresis of viral RNAs. Isotope-labeled viral 60 to 70S RNAs purified from virions released from infected cells were heat denatured and analyzed in 2.1% sodium dodecyl sulfate-polyacrylamide gels. The RAV-2 genomic RNA and 28S chicken ribosomal RNA were included as internal markers. For rASV157, rASV1702, and rASV382, RNAs obtained from the indicated colony-derived NP clones were used for the analysis. For rASV398 and rASV3812, helper and sarcoma viral RNA mixtures were analyzed.

tively. Only genomic RNA and *env* mRNA were seen in *td106*-, *td107A*-, and *td109*-infected cells. In addition to the three expected species of RNAs, 35S and 22S RNAs were detected in SR-A-infected cells and were apparently derived from the transformation-defective virus present in the SR-A stock used in this experiment. The lack of truncated *src* mRNA from *td109* was expected, as the oligonucleotide mapping suggested that the 5' upstream sequence of *src* was deleted in *td109* RNA. However, lack of truncated *src* mRNAs from *td106* and *td107A* was surprising, since there was no evidence that the 5' *src* flanking sequence was deleted in these two viral RNAs. Direct sequencing of the region containing the *src* mRNA splicing acceptor site is necessary to reveal the basis for the failure to synthesize the truncated *src* mRNAs in *td106*- and *td107A*-infected cells. A 5' *src* probe representing the 5' 332 nucleotides of *src* hybridized to *td103*, *td105*, and *td108* RNAs strongly; it hybridized only weakly with *td101* and even more weakly with *td106* and *td107A* RNAs (Fig. 3B); finally, the 5' *src* probe did not hybridize to a significant level under our conditions with *td109* RNAs despite the fact that it hybridized well with both genomic RNA and *src* mRNA of rASV374 in the same RNA samples (Fig. 3D). This result indicated that most of the 5' 300 nucleotides of the *src* sequence were deleted in *td101*, *td106*, and *td107A* RNAs and probably were completely missing in *td109* RNA. A 3' *src* probe prepared from the *Bgl*I-*Pst*I (45 nucleotides upstream from the UAG codon) DNA fragment (252 base pairs) was also used for the hybridization (Fig. 3C). This probe hybridized strongly to *td101*, *td108*, and *td109* RNAs (Fig. 3C and D); it hybridized weakly, but significantly, to *td105* RNA, but did not hybridize at all to *td103*, *td106*, and *td107A* RNAs (Fig. 3C). Both 5' and 3' *src* probes hybridized well with rASV374 genomic RNA and *src* mRNA (Fig. 3D). The size of the transformation-defective viral genomic RNAs and mRNAs and the pattern of hybridization with 5' and 3' *src* probes were consistent with the result of oligonucleotide mapping.

Figure 4 summarizes the results of current and previous analyses of the genomic sequences of transformation-defec-

tive viruses and their abilities to induce tumors and generate rASVs. The transformation-defective viruses that contained no 3' *src*-specific oligonucleotides were unable to form rASVs even when a portion of the 5' *src* sequence was clearly retained, as in *td103*. By contrast, all of the transformation-defective viruses that retained a portion of the 3' *src* sequence, despite the lack of most (if not all) of the 5' *src* sequence, such as *td109*, were able to give rise to rASVs. We conclude that retention of a portion of the 3' *src* sequence in the transformation-defective viral genome is clearly necessary and probably sufficient to generate rASVs. The reason for the requirement of the 3' *src* sequence is discussed below.

Isolation and biological properties of *td109*-derived rASVs.

Unlike rASVs characterized before (10), all of the rASV isolates obtained from tumors induced by *td109* were defective in replication. The first indication for this came from the fact that about 20 isolates of *td109*-derived rASVs were always low in titer (10^2 to 10^3 focus-forming units per ml compared with an average of 10^6 focus-forming units per ml for rASVs derived from other transformation-defective mutants) and that the transformation spread slowly in the infected cultures (8, 10).

To characterize these *td109*-derived rASVs, cells transformed by 13 independent isolates were cultured, and fully transformed cultures were obtained eventually. The titers of these preparations were around 10^6 focus-forming units per ml. As the first step of characterization, we made cultures of chicken cells infected with about 1,000 focus-forming units of these viruses and then plated them on fresh chicken cells for an infectious center assay. The focus formation by plated cells was virtually abolished when these infected cells were exposed to 5,000 roentgens of X ray before plating. Since this dose of X ray blocks cell division, but does not affect the virus release (11), the result strongly suggested that all of the *td109*-derived rASVs were defective in replication.

Seven isolates of *td109*-derived rASVs were chosen for further studies. Single foci were isolated from cultures infected with each of these viruses, and the production of infectious virus, polymerase-containing particles, and viri-

FIG. 2. Fingerprint patterns of RNase T_1 -resistant oligonucleotides of genomic and subgenomic viral RNAs. ^{32}P -labeled viral RNAs were completely digested with RNase T_1 , and the digestion products were analyzed by two-dimensional separation. (A through D) Fingerprints of viral 60 to 70S RNAs. Samples in A and B were total RNAs after in vitro degradation of 60 to 70S RNA and removal of poly(A)-containing RNAs. The fingerprint patterns of total 60 to 70S RNA before and after removal of poly(A)-containing RNAs differ only in the poly(A) spot. (E through H) Subgenomic poly(A)-containing RNAs of 15 to 20S (E), 12 to 15S (F and G), and 18 to 23S (H) were prepared by alkaline degradation of viral 60 to 70S RNAs followed by selection and fractionation of the poly(A)-containing RNAs as described previously (33, 34). Oligonucleotides indicated by open triangles are *src*-specific spots of SR-A RNA remained in the transformation-defective viral genomes. The open circle in panel B indicates the position of oligonucleotide 21, which is missing in the *td109* RNA fingerprint. Numbering of oligonucleotides follows those of SR-A RNA published previously (35, 37–39), except the four new oligonucleotides, 51 to 54.

ons labeled with either $[^3\text{H}]$ leucine or $[^3\text{H}]$ uridine was examined. None of the seven isolates produced infectious viruses. Four of the seven rASV isolates tested (157, 1702, 382, and 542) produced virions containing reverse transcriptase, two (3812 and 398) produced reverse transcriptase-negative virions, and one (374) produced no virions. All of the NP foci produced 10^5 to 10^6 focus-forming units of rASV per ml upon superinfection with Rous-associated virus-1 helper virus.

Size of the genomic RNAs of *td109*-derived rASVs. The size of the genomic RNAs of *td109*-derived rASVs was analyzed by gel electrophoresis and sucrose gradient sedimentation. The results of typical isolates of rASVs are shown in Fig. 1 and 5. In the case of rASV157, rASV1702, and rASV382, virion 60 to 70S RNAs from individual focus-derived NP cultures were analyzed. The RNA genomes were significantly smaller than that of RAV-2, with the exception of rASV382 (Fig. 1). The size of the genomic RNAs was identical among several clones of NP cells for each isolate of rASV (data not shown). The RNA of rASV1702 was slightly smaller than that of rASV157 (Fig. 5 and other data not shown). For rASV3812 and rASV398, the virion RNAs were prepared from producer cultures infected with both helper

and sarcoma viruses. In each case two RNA species were detected. The larger species migrating closely with RAV-2 RNA corresponds to the helper *td109* RNA, and the smaller species migrating between RAV-2 and 28S RNA is apparently the sarcoma viral genomic RNA (Fig. 1). The smaller RNA species recovered from preparative polyacrylamide gels similar to those shown in Fig. 1E and F were further analyzed by sucrose gradient sedimentation (Fig. 6) and agarose gel electrophoresis (Fig. 5A). rASV398 and rASV3812 RNAs sedimented as 30 and 32S, respectively. The size difference of the two sarcoma viral RNAs is most clearly seen in the agarose gels shown in Fig. 5A.

In addition to the virion RNAs, we have also analyzed the intracellular virus-specific RNAs prepared from rASV-infected NP clones. The *src* DNA probe detected two RNA species from each rASV-infected NP clone, the genomic RNA and the spliced subgenomic *src* mRNA (Fig. 5B). The genomic RNA of rASV374 was the smallest among all of the *td109*-derived rASVs analyzed. The size of the genomic RNA of each rASV was determined from its mobility in the agarose gel with *Hind*III-digested lambda DNA as markers. The results of analysis of the size of the rASV genomic

FIG. 3. Analysis of the remaining *src* sequence in transformation-defective viral RNAs. Total poly(A)-containing RNAs were isolated from virus-infected CEF. About 6 μ g of RNA from each sample was subjected to agarose gel electrophoresis, RNA blotting, and filter hybridization (see the text). RNAs similarly obtained from SR-A-infected cells were analyzed in parallel for molecular weight markers. For *td109*, RNAs isolated from CEF infected with a *td109*-rASV374 mixture were used for the analysis (D). Two lanes in D represent two independent RNA samples. The 5' probe is described in the text. The 5' *src* probe was prepared by nick translation of the *NcoI-HincII* *src* DNA fragment isolated from pTT107 and represented the 5' 332 nucleotides of the *src*. The 3' *src* probe was prepared from *BglI-PstI* fragment (252 base pairs) and spanned from 297 to 45 nucleotides upstream from the *src* termination codon. In general, 3×10^6 to 5×10^6 cpm of the probe was used for hybridization with the 9- by 15-cm filter in 6 ml of solution at 37°C for 48 h (36). The specific activity of the probes ranged from 5×10^7 to 3×10^8 cpm/ μ g of DNA.

RNAs (see Fig. 9) represent average values of several gels similar to that shown in Fig. 5.

Genomic sequences of *td109*-derived rASVs. The genomic RNAs of various isolates of *td109*-derived rASVs were analyzed by fingerprinting of RNase T₁-resistant oligonucleotides. Figure 7 shows the results of such analyses of several

representative isolates of rASV. One of the replication competent rASVs studied previously, rASV145, which was derived from *td108* (37), is included here for comparison. Again, the RNAs of rASV157, rASV1702, and rASV382 used for this analysis were isolated from the viral particles produced by the sarcoma virus-infected NP cells, genomic

FIG. 4. Deletion maps of transformation-defective viral genomes and their abilities to generate rASVs. The top scale represents the 3' *EcoRI* DNA fragment of SR-A. The termination sites of gp37 and the initiation and termination sites of *src* are indicated by the corresponding codons. The *c* region is the sequence between *src* and the 3' end of the SR-A genome as described previously (32, 34). The numbers above the scale represent the large RNase T₁-resistant oligonucleotides located within the 3' region of the SR-A genome (37, 39), and their precise locations based on the nucleotide sequence are indicated under the scale by the numbers of nucleotides downstream or upstream from the beginning of *src*. The nucleotide numbers correspond to the 3' G residues of individual oligonucleotides. The extent of *src* deletion in each transformation-defective viral genome has been estimated from oligonucleotide mapping (37, 39; data not shown) and nucleic acid hybridization (8; data not shown) and is indicated by the open box. The hatched area of the box denotes the region within which the 5' boundary of the deletion lies, but the precise location has not been determined. The ability to form rASV has been shown previously in various transformation-defective viruses (10).

FIG. 5. Agarose gel electrophoresis of rASV RNAs. (A) Preparations of rASV398 and rASV3812 RNAs similar to those described in Fig. 6 were analyzed. The 32 P-labeled viral and marker RNAs were denatured with 1 M glyoxal and electrophoresed in 1% agarose gel (19). After separation, the gel was dried under vacuum on a layer each of Whatman DE81 and 3MM papers and then subjected to exposure. Lanes a, rASV398; b, rASV3812. (B) Total poly(A)-containing RNAs from sarcoma virus-infected NP cells were separated as in A. The RNAs were transferred from the agarose gel to a sheet of nitrocellulose paper (27). The 1-kb *HincII*-*BglI* fragment of *src* DNA (see the text) was used as a probe for hybridization with the RNA filter paper. The far left and right lanes show *HindIII*-digested, 32 P-labeled lambda DNAs analyzed in the same gel as molecular weight markers. Lanes a, SR N8, an *env* deletion mutant derived from SR-A (35); b, rASV157; c, rASV1702; d, rASV382; e, rASV3812; f, rASV398; g, rASV374. The right-hand two lanes represent part of the same gel, since longer exposure time was needed to detect the rASV374 RNAs; the photograph was cut and spliced to the rest, which had a shorter exposure.

RNAs of rASV3812 and rASV398 were isolated from the mixture of helper and sarcoma viral RNAs by preparative polyacrylamide gel similar to those shown in Fig. 1E and F, and rASV374 RNA was analyzed as a mixture with the *td109* RNA. The RNAs of *td109*-derived rASVs contained the same set of *src*-specific oligonucleotides, including those not present in SR-A (Fig. 7, spots 8c, 10c, 15a, and 34), as the rASVs derived from other transformation-defective viruses (37, 38). Like rASVs studied before, *td109*-derived rASVs showed variations with respect to *src*-specific oligonucleotides 8c and 15a (Fig. 7). Some rASVs contained spot 8a in place of 8c, and some rASVs did not have spot 15a. The corresponding sequence of oligonucleotide 8c, CCUACUACUCAAACACG, in SR-A and *c-src* DNA involves a C to U change of the penultimate C residue, which is the third position for the His codon (24–26). The sequence of spot 15a, CCCACCACCAUG, is identical to that present in *c-src* DNA, and the corresponding sequence in SR-A involves a C to G transversion of the third C residue, which is outside the *src* coding sequence (24–26). The variations in these two oligonucleotides among rASVs may be due to polymorphism in chicken *c-src* DNA. Alternatively, these sequences may represent hot spots for mutation. rASV1702 lacked two 5' *src* oligonucleotides, 13, and 35, whereas rASV382 con-

tained four new oligonucleotides, 51, 52, 53, and 54, not present in either *td109* or SR-A. The composition of the RNase A-resistant fragments of those spots is shown in Table 1. Analysis of the poly(A)-containing subgenomic RNA fragments of rASV382 revealed that oligonucleotides 51 to 54 were derived from the region immediately upstream from the *src* (Fig. 2G and H).

As expected, all of the *td109*-derived rASVs lacked oligonucleotide 21, which was deleted in the *td109* genome. The RNAs of rASV157 and rASV1702 lacked all of the *env*-specific oligonucleotides defined previously (35). The RNA of rASV382 deleted all but one *env* oligonucleotide, spot 17, which was mapped at the 5' end of *env* in SR-A RNA (35) and appeared to be located immediately upstream from the four new oligonucleotides in the rASV382 RNA genome (Fig. 2H). The RNA of rASV3812 lacked oligonucleotides of *env* and 3' *pol* regions; however, it contained two (spots 51 and 52) of the four new oligonucleotides detected in rASV382 RNA. The RNA of rASV398 lacked oligonucleotides in the entire *env* and *pol* regions. The oligonucleotide composition of the rASV374 genome is not clear since it was analyzed as a mixture with *td109* RNA; however, it contained at least one (spot 51) of the four new oligonucleotides.

To map the deletion in the rASV374 genome, we prepared the total poly(A)-containing RNAs from rASV374 and helper virus-infected cells and analyzed the sequence content of

FIG. 6. Sucrose gradient sedimentation of rASV398 and rASV3812 RNAs. Genomic RNAs of rASV398 (A) and rASV3812 (B) recovered from preparative polyacrylamide gels similar to those shown in Fig. 1E and F were used in this experiment. Each sarcoma viral RNA and markers, including 35S RAV-2 RNA, 18 and 28S rRNAs, and 4S tRNA, were sedimented in a 10 to 25% linear sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.2), 0.1 M NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. Sedimentation was performed in an SW40 rotor at 40,000 rpm for 4.5 h at 22°C.

FIG. 7. Fingerprint patterns of RNase T_1 -resistant oligonucleotides of rASV RNAs. Conditions for the analysis are as described in Fig. 2. For rASV157, rASV1702, and rASV382, ^{32}P -labeled viral 60 to 70S RNAs prepared from NP clones (clone number indicated by the number after hyphane) were analyzed. For rASV398 and rASV3812, the smaller sarcoma viral RNA species recovered from polyacrylamide gels similar to those shown in Fig. 1E and F were used for the analysis. For rASV374, mixed helper and sarcoma viral RNAs were analyzed. The fingerprint of one of the *td108*-derived rASVs, rASV145, is included here for comparison. Oligonucleotides indicated by filled triangles in panels A to G and blackened in panel H are *src* spots specific to rASVs in comparison with SR-A. Oligonucleotides indicated by open triangles and shaded are *src* spots common to rASVs and SR-A (37–39). The sketch in panel H depicts the fingerprint pattern of rASV145 RNA. The numbering of spots is as described previously (35, 37–39).

virus-specific RNAs by using probes specific to various regions of the genome. Both 5' and *c* probes detected four viral RNA species corresponding to helper virus 35S genomic RNA and 22S *env* mRNA and rASV374 4.4-kb genomic RNA and 21S *src* mRNA (Fig. 8). The *src* probe detected only the latter two RNA species. The rASV374 genomic RNA was not hybridizable with *pol*, *env*, and 3' *gag* probes, but could be detected with a 5' *gag* probe. This result indicates that the deletion in rASV374 genome extends beyond the *Bam*HI site in the p12 region (22). From the size of its genome and the known sequences of *src* and *c* regions

in SR-A (24), it can be deduced that rASV374 retains about 2.1 kb of 5'-terminal sequences of *td109* RNA.

Figure 9 summarizes the result of the analysis of the genomic RNAs of *td109*-derived rASVs. Of 13 independent isolates of rASVs that have been analyzed, 10 appeared to contain *env* deletion similar to rASV157, the remaining 3 isolates, rASV3812, rASV398, and rASV374, had more extensive deletions (Fig. 9). All but rASV374-infected NP cells were capable of producing viral particles. The ability to produce viral particles was checked by more than one criterion described above. The failure of rASV374-infected NP clones to produce detectable virions was concluded from assays of infectivity, reverse transcriptase activity, viral RNA-containing particles and [^{35}S]methionine-labeled viral particles. The inability of rASV374-infected cells to release detectable levels of virions is most likely due to the deletion of the p15 sequence, which is required for the processing of *gag* proteins (2, 30, 31). The result of the analyses of the sequence content in *td109*-derived rASVs is consistent with the analyses of the size of their genomic RNAs and *src* mRNAs. The rASV157 RNA was slightly smaller than that of SR N8 (Fig. 6B), which is an *env* deletion mutant of SR-A, probably due to the deletion of about 200 nucleotides of intergenic sequence between *env* and *src*. Both genomic

TABLE 1. RNase T_1 -resistant oligonucleotides specific to *td109*-derived rASVs

Spot no.	RNase A-resistant fragments ^a
51	6U, 7C, G, AC, 3AU, AAU
52	4U, 6C, G, AU, AAAU
53	3U, 6C, G, AC, AU
54	5U, 2C, AC, 2AU, AG

^a Oligonucleotides were recovered from the fingerprints of rASV382, rASV3812, and rASV374 (Fig. 2, Fig. 7, and data not shown). They were analyzed by RNase A digestion as described previously (34).

FIG. 8. Sequence content of rASV374 RNAs. Two independent samples of poly(A)-containing RNAs prepared from rASV374 and *td109* helper virus-infected CEF (same preparations shown in Fig. 3D) were analyzed by RNA blotting and hybridization. The RNA filter (same filter used in the experiments shown in Fig. 3D) was hybridized sequentially with the DNA probe indicated after eluting the probe of the previous cycle of hybridization.

RNA and *src* mRNA of rASV1702 were smaller than those of rASV157 (Fig. 6B), due to the deletion of the 5' *src* sequence. The genomic RNA and *src* mRNA of rASV382 were larger than those of rASV157 (Fig. 6B), probably due to the retention of residual 5' *env* sequence represented by oligonucleotide 17 and the presence of the extra sequences represented by the four new oligonucleotides. This indicated that those extra sequences must be located within the 3' 3 kb (the size of the *src* mRNA) of the rASV382 genome and supported the result of oligonucleotide mapping. It also indicated that splicing of the 5' leader sequence occurs upstream from those extra sequences.

Viral protein synthesis in *td109* and *td109*-derived rASV-infected cells. The ability of the CEF infected with *td109* or *td109*-derived rASVs to direct the synthesis of viral proteins was examined. There were no differences in the profiles of viral proteins in *td108*- and *td109*-infected cells (Fig. 10). This is consistent with the biological properties and the result of oligonucleotide mapping of *td109*. The NP cells infected with rASV157, rASV398, or rASV3812 were all

capable of synthesizing the precursor Pr76 and the mature *gag* proteins (Fig. 10). The relative amount of immunoprecipitable p19 in the experiment with rASVs was lower compared with that of *td108* and *td109*. However, different preparations of antiserum were used in the two experiments. A similar result with respect to p19, i.e., a relatively lower amount compared with other *gag* proteins, was obtained from *td109*-infected cells when they were analyzed in parallel with the rASV-infected cells (data not shown). This observation is most likely due to the deficiency of p19-recognizing antibodies in the serum used in the later experiments. A small amount of the precursor Pr180 could also be detected in *td108*-, *td109*-, and rASV157-infected cells, but not in rASV398- or rASV3812-infected cells. Instead, a protein species corresponding to about 140 kilodaltons was present in rASV3812-infected cells. It is possible that this polypeptide represents the truncated Pr180 that would be predicted from the oligonucleotide mapping. However, we do not have direct evidence to support this hypothesis. Our preliminary analysis of the viral proteins in rASV374-infected NP cells showed that neither Pr76 nor mature *gag* proteins could be detected; instead, a polypeptide of about 59 kilodaltons was precipitable with serum against disrupted virions (data not shown). This polypeptide may represent the truncated Pr76. The abilities of rASVs to direct the synthesis of viral proteins in the infected cells are in good agreement with the locations of deletions obtained by oligonucleotide mapping.

DISCUSSION

The data presented above indicate that the 3' *src* sequence, but probably not the 5' *src* sequence, remaining in the transformation-defective viral genome is essential for the generation of rASVs. However, all of the rASVs derived from *td109*, which lacks most, if not all, of the 5' *src* sequence, contain deletions of various length. Figure 11 depicts a model of recombination between the transformation-defective viral genome and the *c-src* sequence that can account for those observations. In this model, we assume that recombination occurs between the homologous *src* sequences remaining in the transformation defective viral genome and the corresponding regions of *c-src*. The recombination may occur between provirus or unintegrated viral DNA and *c-src* DNA. Recombination between integrated provirus and the *c-src* DNA would not be restricted to the initial stages of infection and could occur in subsequent genera-

FIG. 9. Deletion maps and biological properties of *td109*-derived rASVs. The top line represents the genome of SR-A. The open boxes depict the deletion of viral sequences, and the blackened boxes designate the sequences derived from the *c-src* DNA by recombination. The locations of oligonucleotides 17 and 21 and the four new oligonucleotides are indicated. The order of four new spots is not certain.

FIG. 10. Protein synthesis in virus-infected cells. The uninfected (lane a) and virus-infected CEF (lanes b to i) were labeled with [35 S]methionine for 4 h. Total cellular proteins were extracted, precipitated with either preimmune serum (lanes d, f, and h) or sera against disrupted virions (lanes b, c, e, g, and i), and analyzed in 5 to 15% gradient sodium dodecyl sulfate-polyacrylamide gels. The positions of molecular mass markers are indicated by the numbers in kilodaltons at far left and right lanes of the panels. The upper band of the p19 doublet has an apparent molecular mass of about 24 kilodaltons. This polypeptide was shown to contain most of the p19 sequence (21). Viral proteins p12 and p15 were not separated under our gel condition. Lanes: b, *td108*; c, *td109*; d and e, rASV157; f and g, rASV398; h and i, rASV3812. All rASV-infected cells analyzed were NP clones.

tions of the infected cells. It is also possible that recombination happens between the DNAs synthesized from the viral genome and *c-src* mRNA packaged in the virion. At present we do not have data to distinguish those possibilities. Figure 11A illustrates the situation where a portion of both 5' and 3' *src* sequences remain in the transformation-defective viral genome. Recombination via those homologous sequences will result in the insertion of the *c-src* sequence without the loss of viral genetic information. Figure 11B depicts the situation of *td109*, where few 5' *src* sequences are available for base pairing with the *c-src*. Instead, the 5' region of *c-src* recombines with certain upstream sequences of the viral genome. Such recombination will result in the loss of viral sequences downstream from the 5' site of recombination. For example, recombination between sites a and a' or between sites b and b' would result in deletion of *env* or deletion of *env* and *pol*, respectively. Furthermore, more than one site on *c-src* DNA may participate in such recombination involving probably only partial homology. The only selection forces are preservation of the sarcomagenic function of the integrated *c-src* sequence and rescuability of the final viral recombinant genome. The model can explain the observed deletions and the heterogeneity in the 5' *src* region of *td109*-derived rASVs. Figure 11C illustrates the situation where either none of the *src* sequences or only the 5' *src* sequence, but not the 3' *src* sequence, remains in the viral genome. Recombination between the 3' *c-src* and the *c* region in the latter case, i.e., *td103*, would have generated rASVs. However, the *c* region in SR-A has only about 450 nucleotides (24) and contains essential sequences for replication and the expression of the viral genome (22); the deletion of such sequences as a result of recombination would produce a recombinant unable to replicate or express itself. It is also likely that there are no suitable sequences within such a

small region that are able to form minimum base-pairing with the 3' *c-src* sequence for recombination to take place. This can explain the failure of those transformation-defective viruses lacking the 3' *src* sequences to generate rASVs. Enrietto et al. also showed that a portion of the 3' *src* sequence was retained in the transformation-defective mutants that were

FIG. 11. Model of recombination between the transformation-defective viral genome and chicken *c-src* sequence. The straight horizontal lines represent transformation-defective viral genomes, and the open boxes indicate the deletions. Curving lines represent the *c-src* sequences. The short vertical lines connecting the transformation-defective viral genome and the *c-src* sequence depict the homology between these two sequences. The short blackened boxes in B designate the possible sites of recombination on the *td109* genome and *c-src* sequence. Details are described in the text.

able to generate rASVs (5). One of the transformation-defective mutants, *td1621*, studied by Enrietto et al. contained a deletion extending beyond the *src* mRNA splicing acceptor site, but retained almost all of the 3' half of the *src* sequence. This mutant was unable to induce tumors and generate rASVs. Another mutant, *td4511*, was also unable to form rASVs despite the fact that it retained both 5' and 3' *src* sequences in its genome. These observations are in conflict with our results with *td109* and other transformation-defective viruses. The reason for these differences is not clear at the present time.

Certain features of the model can be tested experimentally. One of them is the site of initial recombination. Although extensive sequence homology is not involved in the recombination predicted in Fig. 11B, certain short stretches of homology are likely to be required for the formation of the recombination intermediate. We have compared the 5' *c-src* (26) and Rous sarcoma virus PR-C DNA sequences (22) to search for the possible sites of recombination. A significant homology was found between the beginning of *env*, AGGGAACCTTTG (nucleotides no. 5,269 to no. 5,280 in the PR-C genome [22]) and the sequence slightly upstream from the exon 1 of *c-src* DNA (26), AAGGAACCTCTG (T. Takeya and H. Hanafusa, unpublished data). Recombination involving these sites would generate *env*⁻ rASVs similar to rASV157. Homology involving fewer bases than the site at the beginning of *env* were also found at the beginning of *pol*, within *pol*, and in the 3' region of *gag*. Recombination at these sites could produce various deletions corresponding to those seen in the *td109*-derived rASVs. Most of the *td109*-derived rASVs contain *env* deletions. We suspect that the sequence homology described above is the basis for this favored site of recombination. The sequence flanking the 5' recombination sites in *td109*-derived rASV genomes can be analyzed and compared with the corresponding regions on *td109* genome and *c-src* DNA to locate the sites of recombination. Sequence comparison of those regions should serve as a direct test for the type of recombination proposed for the formation of *td109*-derived rASVs.

The second feature of the model is the suggestion of multiple sites of recombination on *c-src* DNA. This is proposed based on the observation that certain *td109*-derived rASVs, such as rASV1702, have a deleted 5' *src* coding sequence, whereas others, such as rASV382 and rASV3812, contain new sequences upstream from the 5' end of *src*. We assume that the presence of extra sequences or the 5' *src* deletion is the result of recombination at a site further upstream or downstream from the beginning of the *c-src* coding sequence. This can be checked by sequence analysis of those regions in rASV382 and rASV3812. Furthermore, if these sequences are indeed part of the 5' *c-src* DNA sequence, probes can be prepared to see whether they are also present in the *c-src* mRNA. This will provide a test as to whether *c-src* mRNA is involved in the recombination processes generating rASVs.

Third, we propose that the requirement of the 3' *src* sequence in the transformation-defective viral genome for the generation of rASVs is to provide the 3' homology for the recombination. Comparison of *c-src* and SR-A *src* sequences revealed that the 3'-terminal 36 nucleotides of SR-A *src* coding sequence are different from the corresponding region of *c-src* (26). Obviously, no base pairing between *v-src* and *c-src* could be formed via this terminal sequence to promote the recombination. Therefore, to have homologous recombination, the transformation-defective viral genome must retain an additional sequence upstream from this terminal region.

The minimum length of sequence required, beyond the 3'-terminal 36 nucleotides, can be defined by the ability of that sequence to form a stable intermediate for the recombination. This can be tested by examining the ability of rASV formation from transformation-defective viruses containing the defined length of the 3' *src* sequence. Alternatively, the requirement for the 3' *src* sequence may reflect a functional difference in this region of *v-src* and *c-src*. The last 36 nucleotides of *v-src* differ greatly from those of *c-src* (26). It is possible that retention of this region from *v-src* is important for oncogenic potential.

The latent period for tumor induction by various partial *src* deletion mutant is in general 4 to 8 weeks (5, 10, 28, 38; our unpublished data). Possible reasons for the requirement of a long latent period are as follows. (i) Recombination between the transformation-defective viral genome and *c-src* described above is apparently a rare event. (ii) Most of the induced tumors were found at sites distant from the site of injection; spreading of the injected virus to various tissues and organs requires certain period of time. (iii) It is difficult to detect tumors at the very early stage, in particular the tumors located internally; the time of the tumor detection usually represents a relatively late stage of the tumor development. (iv) Further mutations of the *c-src* sequence after recombination may be required for the oncogenic ability. Since *td109* induces tumors and generates rASVs with a latency and efficiency similar to those of other transformation-defective viruses, it can be inferred that sequence homology at either the 5' or the 3' *src* region is sufficient to form the initial recombination complex at an equivalent rate.

The 5' *src* deletion in rASV1702 is particularly interesting. Although a smaller *src*-encoded protein, corresponding to the deletion described above, was found in the infected cells (15), the truncated *src* protein is fully capable of inducing transformation of CEF in culture. However, the *in vivo* oncogenicity of rASV1702 is greatly reduced (15, 17). It has been proposed that the reduced oncogenicity is due to decreased membrane-binding ability of the *src* protein because of the deletion of N-terminal amino acids (17) that may affect the addition of fatty acid and membrane association (7). Although no deletion or insertion of sequences at the 5' *src* of rASV157 was detected by oligonucleotide mapping, it was previously found that rASV157 coded for a slightly larger *src* protein of 62 kilodaltons (15), which also had a decreased membrane-binding ability (17). Like rASV1702, rASV157 transforms CEF in culture efficiently, but has a decreased, although not as low as rASV1702, *in vivo* oncogenicity (15, 17). Sequence analysis of the 5' *src* regions of rASV1702 and rASV157 genomes is necessary to reveal the basis for these observations.

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