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DNA Sequence of the Viral and Cellular *src* Gene of Chickens

II. Comparison of the *src* Genes of Two Strains of Avian Sarcoma Virus and of the Cellular Homolog

TATSUO TAKEYA* AND HIDESABURO HANAFUSA

The Rockefeller University, New York, New York 10021

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The nucleotide sequence of the *src* gene and flanking regions of the Schmidt-Ruppin strain of Rous sarcoma virus (SR-A) was determined. The *src* region of SR-A was very homologous to that of recovered avian sarcoma virus (rASV1441), with only 17 differences among 1,578 nucleotides. The size of the predicted protein was 526 amino acids in both viruses, of which 6 amino acids were different. The differences in nucleotides and amino acids between the two viruses localized within the 5′ two-thirds of the *src* coding region. There were also some differences in the region flanking the 5′ end of *src*. Since rASVs are considered to be recombinants between deletion mutants of SR-A and cellular-*src* (c-*src*) sequences, several segments of c-*src* DNA were also sequenced to understand the molecular basis for the recombination. At 14 of 17 bases where SR-A and rASV1441 differed, rASV1441 had the same sequence as c-*src*. Three of these sequences corresponded to sequences of oligonucleotides which were previously identified in RNAs of nearly all isolates of rASV but which were absent in SR-A RNA. In the 5′-flanking sequences of the *src* gene, c-*src* was more similar to rASV1441 than to SR-A. These results confirm the cellular origin of the *src* sequences of rASVs and provide information about the possible sites of the recombination.

In the accompanying paper (18), we reported the entire nucleotide sequence of a 3.1-kilobase *Eco*RI fragment of one of the recovered avian sarcoma viruses (rASVs), rASV1441, derived from tumors which were induced by infection with transformation-defective (*td*) mutants of the Schmidt-Ruppin strain of Rous sarcoma virus (RSV), subgroup A (SR-A) (5). In this fragment, which contains part of the *env* gene and the entire *src* gene of the virus, the nucleotide sequence of rASV1441 was largely similar to that of SR-A which was reported by Czernilofsky et al. (1). However, we found two significant differences between the two viruses. First, there were some differences in the nucleotide sequences which would result in the alteration of significant parts of the amino acid sequence of the protein products, gp37 and pp60^{src} (18). Second, 24 nucleotides in the region upstream of the *src* coding sequence of rASV1441 were missing in SR-A DNA. Two possibilities were considered to account for these differences: (i) if rASVs were generated by recombination of *td* mutants with cellular *src* (c-*src*), rASVs could have acquired new sequences from c-*src* which are not present in SR-A DNA; or (ii) the differences could be due to the virus source: SR-A used by Czernilofsky et al. (1) could be substantially different from the SR-A used in our laboratory.

To examine these possibilities and to obtain more definitive evidence for the origin of rASVs, we determined the nucleotide sequence of the *src* gene and its flanking regions of our stock of SR-A and compared the results with the corresponding regions of the rASV1441 DNA sequence. We also sequenced several corresponding parts of c-*src* DNA. Comparison of the nucleotide sequences of these three clones provides strong evidence that a significant portion of the rASV1441 *src* sequence was derived from c-*src*.

MATERIALS AND METHODS

Viruses and molecular cloning. Viruses used were rASV1441 and SR-A. Our stock of SR-A will be referred to as SR-A(NY) to distinguish it from the virus used by Czernilofsky et al. (1), which will be referred to as SR-A(SF). rASV1441 is one of the recovered viruses obtained by injecting *td*108 into

chickens. *td*108 is an *src* deletion mutant of SR-A(NY). The isolation, characterization, and preparation of these viruses have been described previously (4, 9, 10, 20). rASV1441 and SR-A(NY) have been molecularly cloned into a vector, λgtWES · B (19). The 3.1-kilobase *Eco*RI fragment, which contains the *src* gene and part of the *env* gene of SR-A(NY), was subcloned into pBR322 (pTT107) (19). DNA sequencing has been carried out on this plasmid.

λRCS3 is a clone that contains an entire c-*src* region (19), which was isolated from a phage library of chicken genome constructed by Dodgson et al. (3).

DNA sequencing. The methods of DNA sequencing and restriction enzyme analysis are described in the accompanying paper (18). The strategy for sequencing of pTT107 was the same as that used for pTT108 (18).

Southern blotting. Southern blotting has been done essentially according to Southern (17), and the details have been described previously (19).

RESULTS AND DISCUSSION

DNA sequencing of the SR-A(NY) *src* gene and its flanking regions. Inserts of two plasmid clones, pTT107 and pTT108, which contain the *Eco*RI 3.1-kilobase fragments of SR-A(NY) and rASV1441, respectively, showed very similar structures by restriction endonuclease analyses (Fig. 1). These inserts contained a part of the *env* gene (gp37) and the entire *src* gene, as described in the accompanying paper (18). However, there were some differences in their digestion patterns, possibly due to base changes (which affect restriction sites), deletion, or substitution. The DNA sequences shown below confirmed that the differences in the restriction patterns were indeed due to these causes (different bands due to base change or deletion are indicated by open

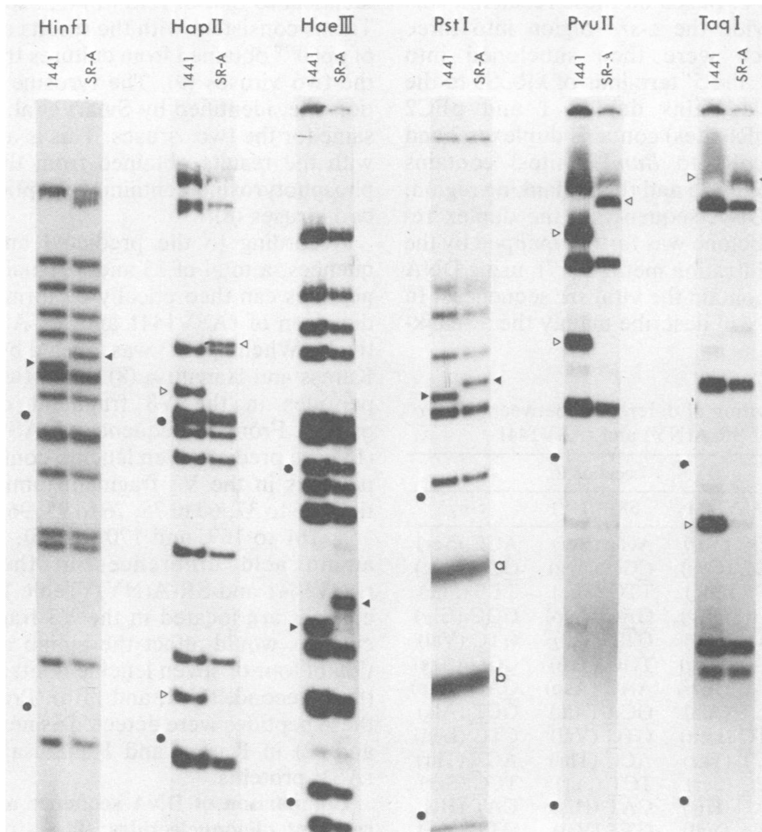

FIG. 1. Comparison of the 3.1-kilobase *Eco*RI fragment of rASV1441 and SR-A(NY) by restriction endonuclease digestion. Subcloned DNA fragments of pTT107 and pTT108 were extracted and digested with the indicated restriction enzyme. DNA fragments were then labeled with either [γ-^{32}P]ATP or [α-^{32}P]cordycepin 5′-triphosphates (18) and fractionated by 5% polyacrylamide gel electrophoresis. Open and closed triangles indicate the fragments which differ due to the base changes and deletions, respectively. Closed circles indicate the positions of two dye markers: xylene cyanol and bromophenol blue (front). When [α-^{32}P]cordycepin 5′-triphosphates was used for 3′-terminal labeling, two unidentified bands (a and b) were observed on the gel. These bands were not stained by ethidium bromide and are not believed to be DNA.

or closed triangles, respectively, in Fig. 1). The region which caused the difference in fragment size was mapped to just upstream of the 5′ end of the *src* coding region.

The DNA sequence of SR-A(NY) was determined for the coding region of the *src* protein, the intercistronic region (between *env* and *src*), and the noncoding region between *src* and the long terminal repeat.

Sequencing of cloned c-*src* DNA. The c-*src* gene was found to be a split gene (14, 15, 19). We found eight regions which were homologous to viral *src* sequences (19). These regions were named duplex 1, 3, 5, and so on, counting from the 5′ terminus as described previously (19). Based on analyses by electron microscopy and restriction endonuclease mapping, we mapped one *Hin*dIII site between duplexes 1 and 3 and one *Bam*HI site after the last duplex. There was a single *Eco*RI site in the c-*src* region, between duplexes 5 and 7. Based on these results, it was possible to divide the c-*src* region into three portions, which were then subcloned into pBR322. pFC1 (the 5′ terminus of λRCS3 to the *Hin*dIII site) contains duplex 1 and pFC2 (*Hin*dIII to *Eco*RI sites) contains duplexes 3 and 5. pRW10 (*Eco*RI to *Bam*HI sites) contains duplexes 7 through 15 and the 3′-flanking region. To carry out DNA sequencing, the duplex region in each subclone was further mapped by the Southern hybridization method (17), using DNA probes which contain the viral *src* sequences. In this paper, we will describe mainly the 5′-flanking region and the regions where rASV1441 and SRA(NY) showed differences. The details of the sequence of c-*src* will be published elsewhere.

TABLE 1. Location of differences between the *src* genes of SR-A(NY) and rASV1441

Position no.[a]	Location in:		
	rASV1441	SR-A(NY)	c-*src*[b]
1,129	AGC (Ser)	AGT (Ser)	AGC (Ser)
1,166	TGC (Cys)	CGC (Arg)	CGC (Arg)
1,273	TTT (Phe)	TTC (Phe)	TTT (Phe)
1,308	GGC (Gly)	GAC (Asp)	GGC (Gly)
1,330	GTC (Val)	GTT (Val)	GTC (Val)
1,403	CGG (Arg)	TGG (Trp)	CGG (Arg)
1,469	GAC (Asp)	AAC (Asn)	GAC (Asp)
1,483	GCT (Ala)	GCA (Ala)	GCT (Ala)
1,490, 1,492	CTC (Leu)	GTG (Val)	CTC (Leu)
1,495	ACT (Thr)	ACC (Thr)	ACT (Thr)
1,594	TCC (Ser)	TCT (Ser)	TCC (Ser)
1,819	CAC (His)	CAT (His)	CAT (His)
2,089	GTA (Val)	GTT (Val)	GTT (Val)
2,092	CAG (Gln)	CAA (Gln)	CAG (Gln)
2,133	ACT (Thr)	ATT (Ile)	ACT (Thr)
2,212	GTC (Val)	GTT (Val)	GTC (Val)

[a] Number indicates the nucleotide position in the DNA sequence of the 3.1-kilobase *Eco*RI fragment of rASV1441 (shown in the accompanying paper [18]).

[b] The nucleotide of the corresponding position of c-*src* is shown for comparison.

Comparison of sequences of SR-A(NY) and rASV1441. In both DNA and protein sequences, the *src* gene of SR-A(NY) was very similar to that of rASV1441 (18). One deletion of 11 nucleotides and one substitution of 4 nucleotides were seen in the 5′-flanking region. Within the *src* coding region, there were 17 single base changes between the two viruses (Table 1). Eleven of 17 were changes in the third base of the codon. The remaining six base changes resulted in changes of amino acids at six positions. These changes in the *src* region localized to the 5′ two-thirds of the gene.

The direct repeats before and after the *src* gene (1, 18) were identical for rASV1441 and SR-A(NY).

The deduced size of SR-A(NY) $pp60^{src}$ was identical to that of rASV1441, 526 amino acids. This is consistent with the results of the analysis of $pp60^{src}$ obtained from cultures transformed by the two viruses (9). The tyrosine phosphorylation site, identified by Smart et al. (16), was the same for the two viruses. This is also consistent with the results obtained from the analysis of phosphotyrosine-containing peptides of these two viruses (8).

According to the predicted amino acid sequences, a total of 33 and 32 leucine-containing peptides can theoretically be formed by trypsin digestion of rASV1441 and SR-A(NY), respectively. When $pp60^{src}$ was labeled by $[^3H]$leucine, Karess and Hanafusa (8) found two new tryptic peptides in the V3 fragment of rASV1441 $pp60^{src}$. From the sequence of rASV1441 $pp60^{src}$ (18), we predict seven leucine-containing tryptic peptides in the V3 fragment (amino acid positions 16 to 37, 60 t0 75, 76 to 95, 96 to 103, 108 to 152, 161 to 169, and 170 to 175). There are six amino acid differences in the $pp60^{src}$ of rASV1441 and SR-A(NY) (Table 1). Five of six changes are located in the V3 fragment. These changes would affect the amino acid composition of four of seven leucine-containing peptides (first, second, third, and fifth). Probably two of these peptides were detected as new peptides (r1 and r2) in Karess and Hanafusa's analysis of rASV proteins.

Comparison of DNA sequence and RNase T_1-resistant oligonucleotides. The structure of the genomic RNA of various strains of RSV and rASVs has been extensively analyzed by fingerprinting RNase T_1-resistant oligonucleotides (20, 21). So far, 9 *src*-specific oligonucleotides have been identified in SR-A(NY) and 12 spots have been identified in rASV1441 (20, 21). The differences observed between SR-A(NY) and rASV1441 can be divided into two classes: (i)

the appearance of new spots in rASV1441 (spots 15a, 34, and 10c) and (ii) the change in the mobility of a spot due to a base change (spots 8a [SR-A] and 8c [rASV1441]) (20). Sequences which fit the base composition of these oligonucleotides were found and are summarized in Table 2.

To identify the origin of the nucleotide differences between SR-A(NY) and rASV1441, we sequenced the corresponding regions of c-*src* DNA (duplexes 3, 7, 11, and 13). The corresponding nucleotide sequences found in the c-*src* DNA are also summarized in Table 2. The results clearly show the homology between rASV1441 and c-*src*. Spots 15a, 34, and 10c were present in the RNAs of essentially all rASVs but not in SR-A or SR-B (20, 21). Wang et al. (20) found that spot 8c is specific to rASV1441 and is not found in any other rASV. c-*src* contains the nucleotide sequence of spot 8a (Table 2). The base change from T (spot 8a) to C (spot 8c) may have occurred after rASV1441 was generated. Alternatively, the sequence corresponding to 8c may be present in the other allele of the chicken *src* gene.

DNA sequences of the 5'-flanking region of the *src* gene. Since we noticed divergence of DNA sequences in the 5'-flanking regions of SR-A(NY), SR-A(SF), and rASV1441, we compared the nucleotide sequences of the corresponding regions of viral and c-*src* DNA to determine the origin of the differences. pFC1 and pFC2 were used for DNA sequencing analysis of the c-*src* region (Fig. 2). Duplex 1, which is present in pFC1, corresponds to nucleotides 1,029 to 1,111 of rASV1441 (Fig. 2). The remaining 5'-flanking sequence (1,112 to 1,120) and the 5' terminus of the *src* coding region were found in duplex 3 in pFC2. The data obtained by Czernilofsky et al. (1) with SR-A(SF) are also included in Fig. 2 for comparison.

Salient features are as follows. (i) The sequences of SR-A(NY), rASV1441, and c-*src* are very similar to each other. SR-A(SF) has a deletion of about 24 nucleotides (compared with rASV1441) in the area corresponding to the beginning of duplex 1. In the deleted area, however, SR-A(SF) retains the *Pst*I site, which corresponds to the probable slicing acceptor site (18). (ii) The major difference between SR-A(NY) and rASV1441 is the presence of an additional 10 nucleotides in SR-A(NY) at position 1,036 (Fig. 2). This additional 10-nucleotide stretch, which is not present in c-*src*, contains a duplication of 9 nucleotides which just precede this position. (iii) Duplex 1 appears to contain many repeats of short stretches of nucleotides, such as AGCTG, ACGTA, or GTGGCC. (iv) At position 1,061 in Fig. 2, GCTG, present in SR-A(NY) and c-*src*, is changed to ACGTA in rASV1441. (v) It is now believed that RSV was generated through a recombination between the c-*src* gene and a virus such as an avian leukosis virus. The results shown here suggest that this

TABLE 2. Identification of base sequences corresponding to RNase T_1-resistant oligonucleotides found in viral RNA

Oligonucleotides specific to rASV1441[a]		Corresponding nucleotide sequences in:		
Spot no.	Base composition	rASV1441	SR-A(NY)	c-*src*
15a	6C, G, 2(AC), (AU)	1,112[b] CCCACCACCATG	CCGACCACCATG	CCCACCACCATG
34	4U, 3C, G, (AC), (AAC)	1,309 CTTCAACACTTCTG	ACTTCAACACTTCTG	CTTCAACACTTCTG
10c	3U, 6C, 2(AC), (AU), (AG)	1,482 CTCATTCCCTCACTACAG	CACATTCCGTGACCACAG	CTCATTCCCTCACTACAG
8c	3U, 4C, G, 3(AC), (AAAC)	1,803 CCTACTACTCCAAACACG	CCTACTACTCCAAACATG[c]	CCTACTACTCCAAACATG

[a] The spot number and base compositions are data obtained by Wang et al. (20).
[b] Number indicates the nucleotide position of the DNA sequence of rASV1441 shown in the accompanying paper (18).
[c] Spot 8a in SR-A(NY) RNA has the following base composition: 3U, 4C, G, 2(AC), (AU), (AAAC) (20).

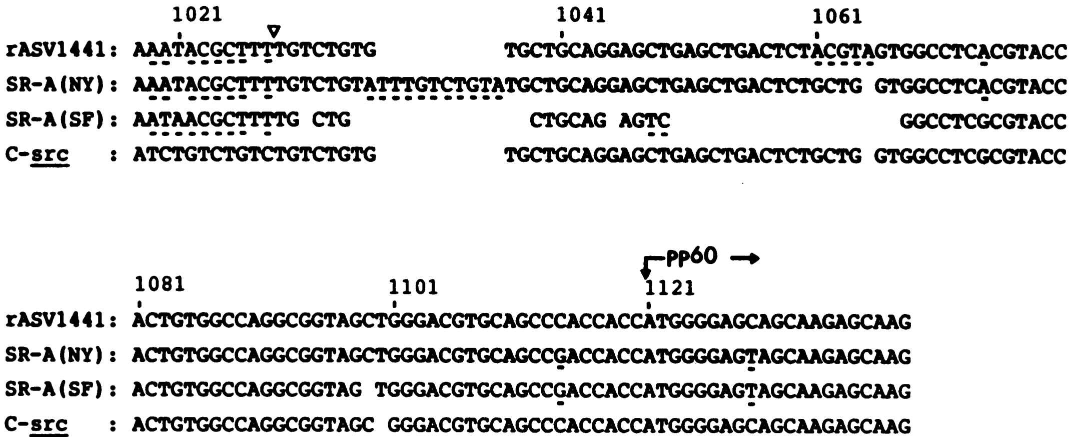

FIG. 2. Comparison of the nucleotide sequence of the 5′ flanking regions of the v-*src* and c-*src* genes. Numbers indicate the position of rASV1441 nucleotides, which were described in the accompanying paper (18). Open triangle indicates the site where divergence between v-*src* and c-*src* starts. Underlines indicate the nucleotides which are different from those of the c-*src* gene.

recombination took place at the 5′ end at positions 1,028 to 1,029. A striking feature is the presence of four TCTGs in a row (1,019 to 1,034) in this region of the c-*src* gene.

The *td* viruses are mutants of SR-A(NY) with a deletion within the *src* region but retaining the 5′-flanking region (21). One possible explanation for the presence of a substantial difference between SR-A(NY) and rASV1441 in this 5′-flanking region is that, to generate rASV, at least three recombination events between *td* virus and c-*src* took place within this region: one between nucleotide 1,028 and the short insertion (1,036), one between this insertion (1,036) and the short substitution (1,061 to 1,065) present in rASV1441, and one between nucleotide 1,099 and spot 15a. The substitution of the sequence ACGTA (position 1,061 in Fig. 2) may have taken place on rASV1441 DNA during the process of rearrangement of this region (it is interesting to see that the same sequence is present at position 1,073).

Preliminary data from the analysis of DNA from tumors which were initially produced in chickens as a result of the injection of *td* mutants showed that often viral DNA in tumors had an unusual size distribution; many tumors contain smaller-sized DNAs, and the deletions seem to be in the 5′-flanking region of the *src* gene (M. Nadel, D. H. Spector, and H. Hanafusa, unpublished data). In fact, DNA of the tumor from which rASV1441 was obtained had a 5′-flanking region larger than the standard RSV or rASV1441 itself. These results also suggest that the 5′-flanking region is involved in recombination. The variation in the size of viral DNA in these tumors suggests that more than one step of the recombination might be needed to generate rASVs.

It is not immediately clear why a noncoding region (duplex 1) was incorporated into the viral genome when RSV was originally generated. Possibly the attachment of this region was essential for the expression of the *src* coding region since it provided a site for slicing (18).

Origin of rASV sequence. Although we have not determined the exact extent of the deletion within the *td*108 genome, fingerprinting analysis showed that *td*108 retains RNase T_1-resistant oligonucleotides 35 and 20, which map at nucleotide positions 1,255 to 1,263 and 2,390 to 2,401 in the rASV1441 sequence (12, 18, 20). The distance between these two positions, therefore, constitutes the maximum length of the deletion in *td*108. The deleted area in *td*108 corresponds well to the region of base changes between rASV1441 and SR-A(NY) in the 5′ two-thirds of their *src* genes.

The sequence of rASV1441 matches that of c-*src* in the regions corresponding to the three T_1 oligonucleotide changes that distinguish SR-A(NY) and rASV1441. Furthermore, DNA sequencing of the c-*src* gene revealed that the sequence of rASV1441 was identical to that of c-*src* at 14 of 17 positions where base changes were found between rASV1441 and SR-A(NY) (Table 1). Two of these sites, positions 2,092 and 2,212, correspond to *Pvu*II and *Taq*I sites in c-*src* present in rASV1441 but absent in SR-A(NY). At the remaining three positions where rASV1441 and SR-A(NY) differed, c-*src* shared the same bases with SR-A(NY). At these three positions rASV1441 may have a point mutation or contain an allele of the c-*src* gene as discussed

above. One of these three positions is the sequence represented by oligonucleotide 8a in SR-A(NY).

Tryptic peptide analysis of phosphorylated pp60^{src} showed that the phosphotyrosine-containing peptides of rASV1441 and SR-A(NY) migrated to the same position whereas that of c-*src* (pp60$^{c\text{-}src}$) migrated to a different position (8). Therefore, the 3'-end region of the *src* gene of rASV1441, probably including the tyrosine phosphorylation site, may have originated from SR-A(NY). This has been discussed in the accompanying paper (18). These results are summarized schematically in Fig. 3.

In the 5'-flanking region, c-*src* is also more similar to rASV1441 than to SR-A(NY). The possible involvement of this region in recombination was discussed above. These results, therefore, suggest that the recombination between *td*108 and c-*src* took place at the 5'-flanking region and at a position in the *src* gene between the *Taq*I site (at nucleotide 2,212) and the phosphotyrosine site (nucleotide 2,366).

Wang et al. (20, 21) have shown that oligonucleotides 15a, 34, and 10c are present in nearly all rASVs examined. All of these sequences are now found in c-*src* and in rASV1441 but not in SR-A(NY). These findings, together with the observation that c-*src* shares homology with rASV1441 but not with SR-A(NY) at 14 of 17 scattered positions within the *src* region, lend strong support to the idea that rASVs acquired a significant portion of the *src* sequence from c-*src*.

Other explanations for the origin of the *src* sequences of rASVs have been proposed (12). One of them attributes the generation of the rASVs to the possible presence of minute amounts of contaminating sarcoma virus(es) in *td* virus preparations. This is highly unlikely because individual rASVs derived from a single *td* mutant, *td*109, have different genetic structures (6, 8), and viral DNAs present in individual tumors produced by single *td* mutants also vary in size (Nadel et al., unpublished data). These results indicate that processes leading to the formation of rASV are different in each case.

Another possibility is that one or more minor component of *td* virus, containing the partial *src* sequence absent in the major component of *td* virus, is present as a contaminant(s) in the *td* virus preparations injected into chickens (12). These minor *td* virus components of minor sarcoma virus contaminants are considered to recombine with the major *td* virus component to give rise to fully transforming virus. Results presented in this paper indicate that this is also unlikely. Oligonucleotide analyses have shown that the *td* viruses injected in our experiments contain *src* sequences homologous to SR-

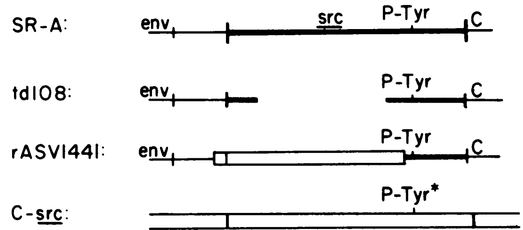

FIG. 3. Genome structures of *td*108, SR-A(NY), rASV1441, and c-*src*. The derivation of the *src* gene and its flanking regions in each virus is shown. Solid line indicates SR-A(NY) origin, and open bar indicates the cellular origin. P-Tyr indicates the site of phosphotyrosine. The structure of P-Tyr of pp60$^{c\text{-}src}$ is different from that of pp60$^{v\text{-}src}$ (8, 16) and is shown as P-Tyr*.

A(NY). For example, neither SR-A(NY) nor any of our *td* viruses have oligonucleotide 15a, although many of them retain sequences corresponding to this region (21). To generate rASVs, these minor virus components must contain sequences represented by oligonucleotides 15a, 34, and 10c, which are not present in the parental SR-A(NY).

One of the bases for the hypothesis (12) that the sequences acquired by rASVs were of viral rather than cellular origin was the observation that oligonucleotides 34 and 10c in rASVs were also found in RNAs of other strains of RSV (12, 21). We previously interpreted this as evidence that the *src* sequences of RSV originated from c-*src* and then diverged in different strains (21). The results presented here are consistent with this interpretation.

The significance of rASV formation in chickens injected with *src* deletion mutants is that this provides evidence that cellular *src* sequences can function as a part of the viral genome. Recently, Neel et al. (B. G. Neel, L.-H. Wang, B. Mathey-Prevot, T. Hanafusa, H. Hanafusa, and W. S. Hayward, Proc. Natl. Acad. Sci. U.S.A., in press) recovered an avian sarcoma virus, 16L, from sarcomas formed in chickens infected with avian leukosis virus. The genome of 16L virus contains the *fps* gene, the transforming gene of Fujinami sarcoma virus (7, 11), flanked by the 5' and 3' sequences of injected avian leukosis virus. Thus, this is the first example of isolation of a sarcoma virus from an animal in which an oncogene-negative retrovirus was introduced. This can be considered an extreme example of the formation of rASV.

Direct demonstration of the function of cellular *onc* genes came from experiments of Oskarsson et al. (13) and DeFeo et al. (2). They have shown that cellular genes homologous to mammalian sarcoma virus-transforming genes are capable of causing cell transformation when

introduced into cells together with a viral long terminal repeat containing the promoter for transcription. We have not obtained such direct proof that c-*src* itself is oncogenic. Our attempts to transform cells by transfecting with c-*src* DNA ligated to a viral long terminal repeat have been unsuccessful. Thus, the formation of rASV was the only evidence available to date to indicate that a significant part of c-*src* is functionally equivalent to viral *src*. Investigations are in progress to examine differences between c-*src* and viral *src* to determine whether any region is critical for biological function as a transforming gene.

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