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Evidence for the Common Origin of Viral and Cellular Sequences Involved in Sarcomagenic Transformation

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The *src* genes of six different strains of avian sarcoma virus (ASV) were compared with those of a series of newly isolated sarcoma viruses, termed "recovered avian sarcoma viruses" (rASV's). The rASV's were isolated recently from chicken and quail tumors induced by transformation-defective (td) deletion mutants of Schmidt-Ruppin Rous sarcoma virus. The RNase T_1-resistant oligonucleotide maps were constructed for the RNA genomes of different strains of ASV and td mutants. The *src*-specific sequences, characterized by RNase T_1-resistant oligonucleotides ranging from 9 to 19 nucleotides long, were defined as those mapping between approximately 600 and 2,800 nucleotides from the 3′ polyadenylate end of individual sarcoma viral RNAs, and missing in the corresponding td viral RNAs. Our results revealed that 12 *src*-specific oligonucleotides were highly conserved among several strains of ASV, including the rASV's, whereas certain strains of ASV were found to contain one to three characteristic *src*-specific oligonucleotides. We previously presented evidence supporting the idea that most of the *src*-specific sequences present in rASV RNAs are derived from cellular genetic information. Our present data indicate that the *src* genes of rASV's are closely related to other known ASVs. We conclude that the *src* genes of different strains of ASV and the cellular *sarc* sequences are of common origin, although some divergence has occurred among different viral *src* genes and related cellular sequences.

The genome of avian sarcoma virus (ASV) contains a gene called *src*, which is responsible for sarcoma formation in animals and for transformation of cells in culture (18). The *src* gene has been mapped within the 3′ one-third of the sarcoma viral RNA (6, 25, 55) and contains 1,500 to 2,000 nucleotides (26, 30, 31). The primary product of the *src* gene has recently been shown to be a phosphorylated polypeptide of about 60,000 daltons, termed $pp60^{src}$ (4, 36). The $pp60^{src}$ was found to be associated with a protein kinase activity (8, 13, 27, 32, 40, 43).

The *src* genes of ASVs are considered to have been derived from the normal avian cellular DNA (24, 48–50). This hypothesis has gained support from the following findings. First, by studies of nucleic acid hybridization, chicken and quail cellular DNA and RNA were shown to contain sequences extensively homologous to the *src* gene of ASV (44, 46, 63); second, normal chicken cells contain a phosphoprotein serologically and biochemically related to the $pp60^{src}$ (7, 9, 27, 34, 38); third, sarcoma virus containing an intact *src* gene was generated after injection of chickens and quails with certain transformation-defective (td) deletion mutants of Rous sarcoma virus (RSV) (17, 19, 37, 52, 60, 61).

The sarcoma viruses isolated from tumors in chickens and quails infected with td virus were termed "recovered avian sarcoma viruses" (rASV's) (19, 61). We showed previously that rASV's contain *src* sequences closely related, but not identical, to those of standard ASV, and that the newly acquired *src* sequences are apparently specific to the animal species from which the rASV's are generated (17, 60, 61, 62). The *src* gene and its product, $pp60^{src}$, of rASV's are functionally and biochemically very similar to those of other known ASVs (27).

The *src*-related sequences present in the chicken and quail cellular DNAs are considered to be the most likely candidates for participating in the recombination with td viral genomes to generate rASV's. To compare the *src* sequences present in rASV's with those of various strains of ASV, we used the method of RNase T_1 fingerprinting. This method is very sensitive in detecting minor sequence differences among related RNA species, and is therefore well suited for obtaining information regarding the extent to which the *src* sequences of various strains of ASV are conserved and related to those of rASV's. In this paper we present data indicating that the *src* genes of various strains of ASV and the *src*-related chicken and quail cellular sequences present in rASV are indeed very similar,

though not identical, and therefore likely have been derived from a common origin.

MATERIALS AND METHODS

Cells and viruses. Primary chicken embryo fibroblast (CEF) cultures were prepared from 11-day-old group-specific antigen-negative C/E chicken embryos.

The viruses used in this study and their sources are as follows. Schmidt-Ruppin strain RSV of subgroup A (SR-A) and subgroup B (SR-B) has been maintained in our laboratory and described before (17, 29, 56, 60). SR-B is a recombinant between SR-A and Rous-associated virus-2 (RAV-2), an avian leukosis virus of subgroup B (29, 62). Two sources of Bryan high-titer (BH) strain of RSV were used. One was BH RSV(−) produced from a quail cell line, QCL3, originated by R. Friis, Institute for Virology, Giessen, Germany; this will be termed BH RSV no. 1. BH RSV no. 2 is BH RSV(−), which has been kept in our laboratory for more than 15 years (20). A stock of Fujinami ASV (FuASV) was obtained from B. Wolf, The Rockefeller University, New York, who in turn had obtained the virus from H. Temin, University of Wisconsin, Madison; it was further cloned in our laboratory. Prague RSV of subgroup B (PR-B), described previously (55), was obtained originally from P. Vogt, University of Southern California, Los Angeles. PR-C was prepared by rescue from XC cells after coculture with chicken cells. XC cells were obtained from P. O'Donnell, Sloan-Kettering Institute, New York.

Five different sources of Bratislava 77 (B77) strain ASV were studied. (i) One source of B77 ASV was obtained from H. Temin, who had in turn obtained it from J. Wyke, Imperial Cancer Research Fund, London, England. This stock of B77 ASV was found to be subgroup C by interference assay. (ii) Another source was obtained from V. Smidova, Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Czechoslovakia, as a lyophilized specimen of sarcoma tissue preserved since 1956. A tumor cell suspension was prepared from the sample and inoculated into chickens, and virus was obtained from sarcoma tissues. This stock of B77 ASV was also found to be subgroup C. (iii) A third source of B77 ASV, rescued from B77 ASV-infected rat cells by cocultivation of the rat cells with CEF, was further purified by isolation of foci. The rat cells were obtained from M. Baluda, University of California, Los Angeles, who in turn had received them from J. Svoboda, Czechoslovak Academy of Sciences, Prague. The original B77 ASV stock obtained from the coculture contained about equal proportions of subgroup A and subgroup C transforming viruses. Four clones were isolated from individual foci, and all were identified as subgroup A. One of the isolates was called B77-A no. 1 and used for further studies. (iv) One clone of B77 ASV, named B77-A no. 2 here, was isolated in our laboratory by the formation of colonies on soft agar from a B77 ASV stock, RBA7, and (v) one clone of B77 ASV, named B77-A no. 3, was also isolated by colony formation from another B77 ASV stock, RB77. Both B77-A no. 2 and no. 3 have been characterized as subgroup A by interference assay. Both RBA7 and RB77 ASV stocks were obtained from H. Temin, and both had been passaged through rat cells (H. Temin, personal communication). RBA7 and RB77 ASV stocks were found to contain mostly subgroup A and very small amounts of subgroup C viruses as judged by their efficiency of focus formation on uninfected, RAV-1- and RAV-7-infected CEF (unpublished observation). This is consistent with the recent report by Estis and Temin (14) that a descendant B77 virus from the rat-passaged B77 ASV stock (1) was subgroup A.

rASV-C's are subgroup A sarcoma viruses obtained from chicken tumors induced by certain td mutants of SR-A and have been described previously (17, 19, 27, 60). rASV-Q's are recent isolates of subgroup A sarcoma viruses obtained from quail tumors induced by td108, one of the td mutants of SR-A (61, 62). The term "rASV's" is used here to refer to both rASV-C and rASV-Q. Various td mutants of SR-A and rASV-C were isolated in our laboratory and described recently (28, 60). One clone of td SR-B mutant included in this study, td SR-B 16-8, was isolated and purified by plaque isolation by L. Saxe in our laboratory. td PR-B and td B77-C have also been described (55) and were obtained from P. Duesberg, University of California, Berkeley.

Preparation of ^{32}P-labeled viral RNAs. The procedures were essentially as described previously (54, 55, 58) with minor modifications. The virus-producing culture was preincubated in phosphate-free medium 199 (55) for 3 to 5 h; at the time of labeling, the medium was replaced with fresh phosphate-free medium, and 0.5 to 2 mCi of [^{32}P]phosphate per ml (Amersham Corp., Arlington Heights, Ill.) was added. Initial labeling was for 12 h; subsequently, F10 medium supplemented with 10% tryptose phosphate broth, 5% calf serum, 1% chicken serum, 1% dimethyl sulfoxide, and 1 μg of amphotericin B per ml was added, and virus was harvested at 12-h intervals for 2 to 3 days. The labeled virus was purified from the medium by direct pelleting at 10,500 rpm for 12 h in a Sorvall GSA rotor at 4°C. For cultures producing relatively small amounts of virus, the ^{32}P-labeled virus was further purified by 20 to 60% linear sucrose gradient equilibrium sedimentation.

The RNA was extracted from the pelleted or gradient-purified virus in a solution containing 0.01 M Tris-hydrochloride (pH 7.2), 10 mM NaCl, 5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 0.5 mg of self-digested proteinase K per ml (EM Laboratories, Inc., E. Merck, Elmsford, N. Y.), and 20 μg of carrier yeast RNA per ml at 37°C for 1 h. The digestion mixture was then extracted with buffered phenol containing 1% SDS and 1% β-mercaptoethanol. The aqueous phase was adjusted to 50 mM NaCl and precipitated with 2.5 volumes of 95% ethanol. The labeled viral 60 to 70S RNA was purified by sucrose gradient sedimentation. Purified ^{32}P-labeled viral 60 to 70S RNA was used for analyses either directly or after further purification by passing through an oligodeoxythymidine [oligo(dT)$_{\leq 10}$]-cellulose (Collaborative Research, Inc., Waltham, Mass.) column. The latter procedure was routinely used for those virus-infected cultures that yielded only relatively small amounts of viral 60 to 70S RNA.

Preparation of ^{32}P-labeled poly(A)-containing viral subgenomic RNA fragments. The details of the procedures have been described (55). Briefly, ^{32}P-

labeled 60 to 70S viral RNA was randomly nicked with 50 mM Na_2CO_3 (pH 11) at 50°C for various times. Polyadenylate [poly(A)]-containing RNAs were selected by two cycles of binding to and eluting from an oligo(dT)-cellulose column. They were then separated according to size by sucrose gradient sedimentation. RNAs were pooled into consecutive fractions from the gradient such that the difference in S value between the smallest and the largest RNA species within each pool was about 3 to 5. For mapping oligonucleotides within the *src* region, each size class of poly(A)-containing RNA fragments was subjected to one more cycle of oligo(dT)-cellulose binding and sucrose gradient sedimentation. Each pool of RNA fragments sedimented in the second sucrose gradient at a position corresponding to the average S value of the pool from the first sucrose gradient (data not shown). Only peak fractions of a given size class of poly(A) fragments were pooled to minimize overlapping with the next size class of RNA.

RNase T_1 fingerprinting and oligonucleotide mapping. The ^{32}P-labeled viral RNA was exhaustively digested with RNase T_1, and the digestion mixture was separated by combined electrophoresis and homochromatography, a two-dimensional process called "fingerprinting," as described previously (55). The location of a given large RNase T_1-resistant oligonucleotide relative to the 3′ poly(A) end of the viral RNA was estimated from the size of the smallest poly(A)-tagged RNA fragments from which the oligonucleotide could be recovered according to a method called "oligonucleotide mapping" (55). Partial sequence analysis of RNase T_1-resistant oligonucleotides with RNase A has been described (54, 55, 58).

Identification of the *src*-specific sequences. For nondefective ASVs studied here, except PR-C and rASV-Q, the *src*-specific sequences of each sarcoma virus were identified as sequences deleted in the corresponding td mutant and were associated with 10 to 21S poly(A)-tagged subgenomic RNA fragments of the sarcoma virus, since *src* has been mapped within 700 and 2,700 nucleotides from the 3′ poly(A) end of the sarcoma viral RNA (6, 25, 26, 31, 55, 56). For envelope-defective sarcoma viruses, such as BH RSV (20) and FuASV (T. Hanafusa, unpublished observation), *src* sequences were defined as the sequences mapping within the domain corresponding to the *src* region of other sarcoma viruses, since no td mutants from these sarcoma viruses were available for comparison.

RESULTS

src-specific sequences of SR RSV and rASV. Figure 1 shows the fingerprint patterns of unfragmented 60 to 70S viral RNAs and 10 to 21S poly(A)-tagged subgenomic viral RNAs of SR-B, td SR-B, rASV-C, and rASV-Q. The *src* sequences of rASV's have been analyzed and described previously (60, 61); therefore, only one representative clone each of rASV-C and rASV-Q is presented here for comparison with other ASVs. SR-B was isolated from a cross between SR-A and RAV-2 by selecting for a subgroup B transforming virus (29). Results of mapping and partial sequence analysis of the RNase T_1-resistant oligonucleotides of SR-B, SR-A, and RAV-2 RNAs revealed that SR-B used in this study had inherited the *c* region, *env*, the 3′ portion of *pol*, and the 5′ portion of *gag* from RAV-2 and the rest of the genome from SR-A (Fig. 1 and other data not shown). The RNA of SR-B contained an identical set of 10 oligonucleotides (Fig. 1A) previously identified as *src* specific in SR-A (60, 61). All of these oligonucleotides were absent in td SR-B RNA (Fig. 1B,F) and mapped between 600 and 2,700 nucleotides from the 3′ poly(A) end of the SR-B RNA, since they could be recovered from 10 to 21S poly(A)-tagged viral RNA fragments (Fig. 1E and other data not shown). All of the 10 *src*-specific oligonucleotides of SR-B were present in rASV-C RNA, and all but one (oligonucleotide 10a) were present in rASV-Q RNA. We showed previously that rASV-C RNA contained oligonucleotides 10c, 15a, and 34 and that rASV-Q RNA contained oligonucleotides 10c, 34, Q1, and Q2 not present in SR-A (60, 61); these new oligonucleotides were also absent in SR-B RNA (Fig. 1). All of these new oligonucleotides of rASV-C and rASV-Q were mapped within the *src* region of the respective genome (60, 61) and segregated with the transforming function, since they were missing in the RNA of td mutants derived from rASV-C (60). Among the new *src*-specific oligonucleotides of rASV's, oligonucleotides 10c and 34 were also present in several other strains of ASV (see below); by contrast, oligonucleotide 15a seemed to be present only in rASV-C (60), oligonucleotide Q2 was unique to rASV-Q, and oligonucleotide Q1 was present only in rASV-Q and FuASV (see below).

The relative order of *src*-specific oligonucleotides of each viral RNA was derived from the analysis of poly(A)-tagged RNA fragments of various lengths (Fig. 1E–H and other data not shown). The *src*-specific oligonucleotides recovered from poly(A)-containing RNA fragments were analyzed for their composition of RNase A-resistant fragments (Table 1). Oligonucleotides derived from different viral RNAs but having identical map locations and compositions of RNase A-resistant fragments were considered homologous oligonucleotides and given the same numbers. The highly conserved *src*-specific oligonucleotides of different strains of ASV have been designated according to the numbering used for SR-A and rASV's (11, 56, 60).

src-specific sequences of PR-B and PR-C. PR-B and PR-C RNAs had an identical set of 10 *src*-specific oligonucleotides (Fig. 2). The RNA of a td mutant of PR-B lacked all 10

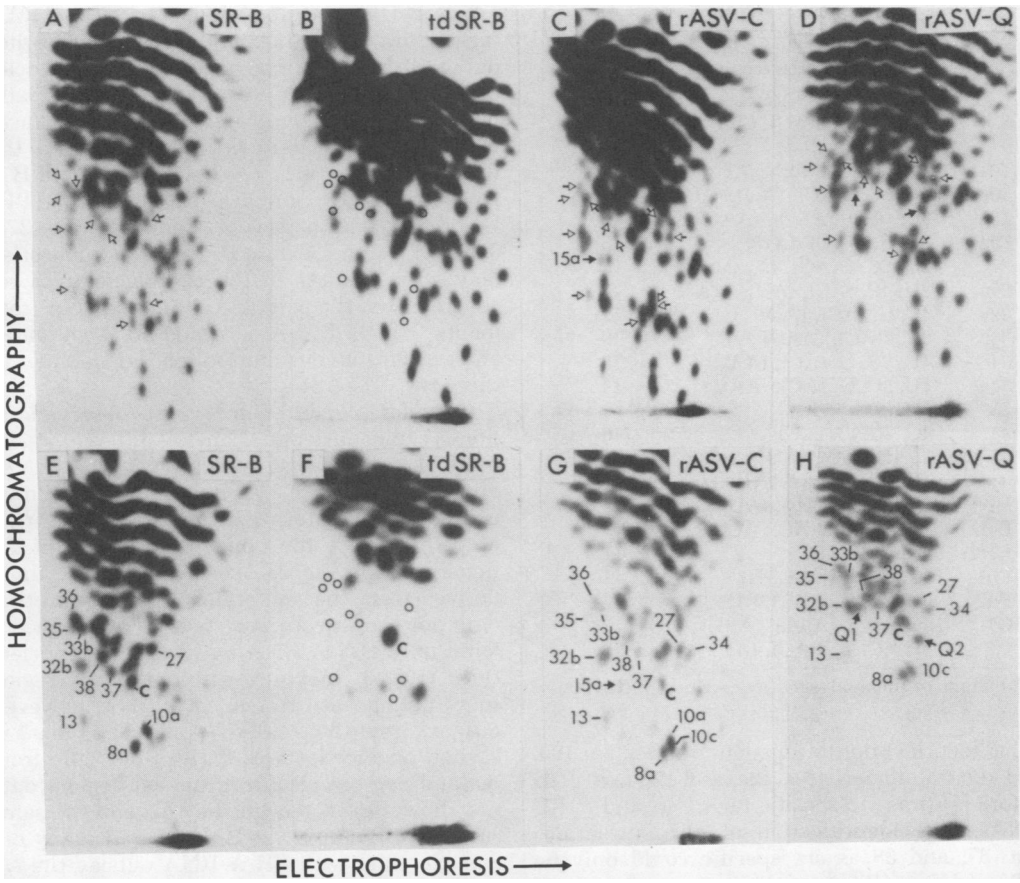

FIG. 1. *Fingerprint patterns of RNase T_1-resistant oligonucleotides of SR-B, td SR-B, rASV-C, and rASV-Q [^{32}P]RNAs. ^{32}P-labeled viral 60 to 70S RNAs or poly(A)-containing RNA fragments were digested exhaustively with RNase T_1, and the digestion products were separated by combined electrophoresis and homochromatography. Some of the viral RNA analyzed (A) was obtained after alkaline degradation of 60 to 70S RNA and removal of poly(A)-containing RNA. The fingerprint patterns of total poly(A)-containing and non-poly(A)-containing RNAs for a given virus, however, are identical except for the poly(A) spot. (A–D) Viral 60 to 70S RNAs; (E, G, H) 18 to 21S poly(A)-containing RNA fragments prepared from alkaline degradation of viral 60 to 70S RNAs; (F) 10 to 21S poly(A)-tagged RNAs with average size about 14S. Oligonucleotide spots indicated by open arrows are the highly conserved src-specific oligonucleotides common to various strains of ASV. Spots indicated by solid arrows are src-specific oligonucleotides unique to certain strains of ASV. Open circles in panels B and F indicate the corresponding chromatographic positions of the src-specific oligonucleotides missing in the td SR-B RNA. The lower molar yields of certain oligonucleotides, such as 13, 15a, and 35, have been observed repeatedly in the fingerprints of total viral 60 to 70S or subgenomic RNAs. This is also true for newly cloned sarcoma virus. The real reason for this phenomenon is not clear. However, the low recovery has been often seen with cytidine-rich oligonucleotides. This may be due to partial susceptibility of these oligonucleotides to the digestion of RNase T_1 preparation used or due to partial degradation of these oligonucleotides during the process of fingerprinting because of the presence of relatively fragile bonds in these nucleotide sequences. As a result, mapping of these oligonucleotides may have been biased by the low recovery due to the reason mentioned above.*

oligonucleotides. Eight of the *src*-specific oligonucleotides of PR-B and PR-C were also present in SR-A RNA. PR RSV RNAs contained oligonucleotide 10c not present in SR-A RNA; conversely, SR-A RNA contained oligonucleotides 13 and 35 lacking in PR-B and PR-C RNAs. Oligonucleotide 27a of PR RSV had a map location within the *src* region similar to that of oligonucleotide 27 of SR-A (see Fig. 5), but had a different composition of RNase A-resistant fragments compared with the latter spot (Table 1). These results confirm and extend our pre-

TABLE 1. *src-specific RNase T_1-resistant oligonucleotides of ASVs*

Spot no.	RNase A digestion products[a]	No. of nucleotides
8a	3U, 5C, G, 2(AC), (AU), (AAAC)	19
10a	4U, 5C, G, 2(AC), (AU)	16
10c	4U, 5C, 2(AC), (AU), (AG)	17
13	4C, (AC), (AAC), (AAG)	12
15a	6C, G, 2(AC), (AU)	13
27	2U, 2C, (AAG), (A_{4-5}N)	12–13
27a	2U, 4C, (AC), (AU), (AAG)	13
32b	4C, G, (AC), (A_4N)	12
33b	3C, 2(AC), (AAG)	10
34	4U, 3C, G, (AC), (AAC)	13
34a	4U, 3C, G, (AC), (AAU)	13
35	5C, (AC), (AG)	9
36	4C, 2(AC), (AG)	10
37	U, 4C, (AC), (AU), (AG)	11
38	4C, G, (AC), (AAAU)	11
B1	U, 4C, G, 2(AC), (AU)	12
BH1	4U, 3C, G, 2(AC), (AAC)	15
BH2	5C, G, (A_4N)	11
Fu1	3C, G, (AC), (A_{4-5}N)	11–12
Fu2	3U, 3C, G, 2(AC), (AAC)	14
Q1	2C, (AC), (AAC), (A_4G)	12
Q2	4U, 5C, G, 2(AC), (AU)	16

[a] Details of the analysis are described in the text.

vious identification of oligonucleotides 8a, 10a, and 10c (numbered as 9, 12a, and 12c for PR-B before [55]) as *src* specific for PR-B and PR-C RNAs. The identification of oligonucleotides 27a, 37, and 38 as *src* specific could only be achieved from the partial sequence analysis of the oligonucleotides recovered from the small poly(A)-containing RNA fragments ranging from 15 to 21S, because these spots contained an isomer(s) derived from the sequences outside the *src* region of the RNA. Direct comparison of the unfragmented sarcoma and td viral RNAs, therefore, would not allow immediate identification of these oligonucleotides as *src* specific (Fig. 2A,B).

src-specific sequences of B77 ASV. To clarify the identity of B77 ASV, we analyzed B77 ASVs from five different sources. Two were identified as B77-C, and three were B77-A derived from transformed rat cells. The RNA of the B77-C obtained from two different sources gave the identical fingerprint patterns (Fig. 3A) as that of B77-C studied before (55). Therefore, in later discussions in this study, the virus will be referred to as B77-C without reference to the source. On the contrary, the RNAs of the B77-A ASVs obtained from the rat cells gave distinctive patterns. However, most of the differences found among different isolates appeared to be in the region outside the *src* gene. The RNA of all B77 ASVs analyzed shared nine oligonucleotides (Fig. 3A–D) associated with 10 to 21S poly(A)-tagged viral RNA fragments (Fig. 3F and other data not shown). Among the shared oligonucleotides, three spots, 8a, 10a, and 10c, have already been identified as *src* oligonucleotides specific to B77-C ASV studied previously (55). None of the nine oligonucleotides was present in the td B77 RNA (Fig. 3B,E). Similar to the situation in PR-B and td PR-B, the lack of oligonucleotides 27a, 32b, 33b, 37, and 38 in td B77 RNA became clear only after analysis of the oligonucleotides recovered from the poly(A)-containing RNA fragments (Fig. 3). B77-A no. 2 and no. 3 contained an *src*-specific oligonucleotide B1 unique to these two B77 ASVs.

Considerable differences were observed for non-*src* oligonucleotides among different stocks of B77. Nevertheless, all the B77 ASV RNAs analyzed, except B77-A no. 1, had a similar characteristic pattern of oligonucleotides (for example, compare the upper right region of the fingerprints in Fig. 3A, B, and C), except those derived from the *env* region (Fig. 3 and other data not shown). As expected, B77-C RNA differed from B77-A RNA by several oligonucleotides (Fig. 3) mapping within the *env* region (data not shown). B77-A no. 1 RNA, however, differed from those of B77-A no. 2 and no. 3 in several oligonucleotides derived not only from *pol* and *gag* but also from the *env* region (data not shown) even though they were of the same subgroup. B77-A no. 1 RNA shared many oligonucleotides with SR-A RNA outside the *src* region (compare B77-A no. 1 in Fig. 3D with rASV-C or rASV-Q, which had sequences identical to those of SR-A outside the *src* region). It seems likely that B77-A no. 1 was derived by recombination between B77 ASV and an SR-A-related subgroup A virus. Apart from the oligonucleotides shared with B77-C ASVs, B77-A no. 2 and no. 3 RNAs contained several oligonucleotides, mapping within the 5′ two-thirds of the genome, unique to these viruses (Fig. 3C and other data not shown). B77-A no. 2 and no. 3 were apparently generated by recombination between B77-ASV and a subgroup A virus different from the one that gave rise to B77-A no. 1 (see above). Furthermore, the RNA of B77-C contained a 5′-terminal cap oligonucleotide named class I (58), whereas RNAs of B77-A viruses had a class IV (58) cap oligonucleotide (Fig. 3).

src-specific sequences of BH RSV and FuASV. Figure 4 shows fingerprint patterns of BH RSV no. 1, BH RSV no. 2, and FuASV RNAs. The three sarcoma viruses shared 10 oligonucleotides (Fig. 4A–C) that mapped within the segment of the genome corresponding to the *src* region of the nondefective ASV. This is consistent with the previous report that FuASV

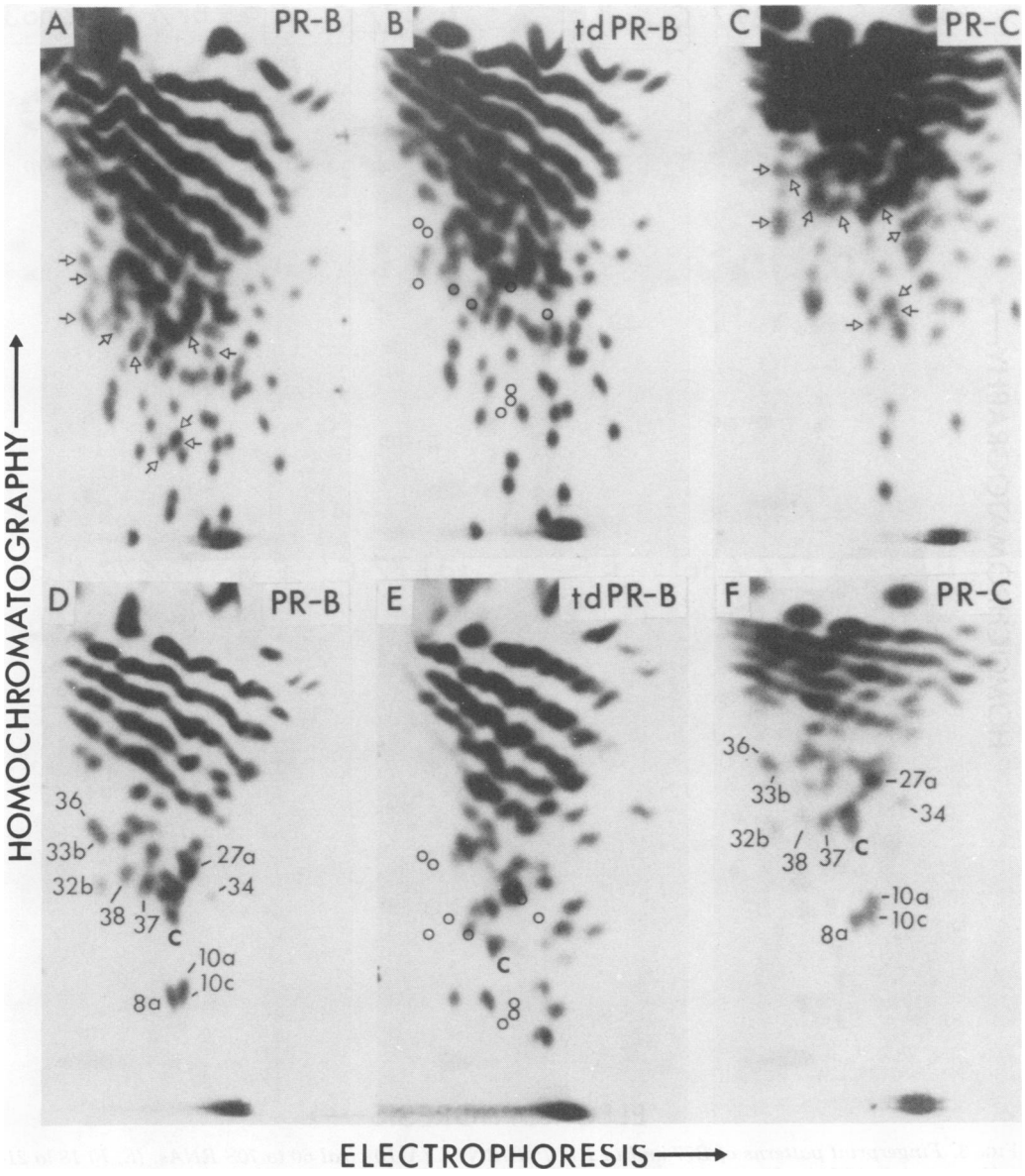

FIG. 2. *Fingerprint patterns of PR-B, td PR-B, and PR-C [^{32}P]RNAs. (A-C) Viral 60 to 70S RNAs; (D-F) 18 to 21S poly(A)-containing viral RNA fragments. Designation of open arrows is the same as in Fig. 1. Open circles in panels B and E indicate the positions where PR-B src-specific oligonucleotides, which are missing in td PR-B RNA, would have been located.*

shared 90% of its *src* sequences with those of other ASVs (45, 51). In addition, BH RSV no. 1, BH RSV no. 2, and FuASV RNAs contained one, two, and three *src*-specific oligonucleotides characteristic of each virus, respectively (Fig. 4A-C).

Both BH RSV (20) and FuASV (Hanafusa, unpublished observation) have been shown to be defective in viral envelope glycoprotein. This was confirmed by the findings that both BH RSV (11, 33, 41) and FuASV (data not shown) RNAs had an electrophoretic mobility identical with that of avian leukosis virus RNA when analyzed by SDS-polyacrylamide gel electrophoresis. In addition, they contained fewer large RNase T_1-resistant oligonucleotides than did nondefective sarcoma viruses (Fig. 1-4) and contained no *env*-specific oligonucleotides of non-

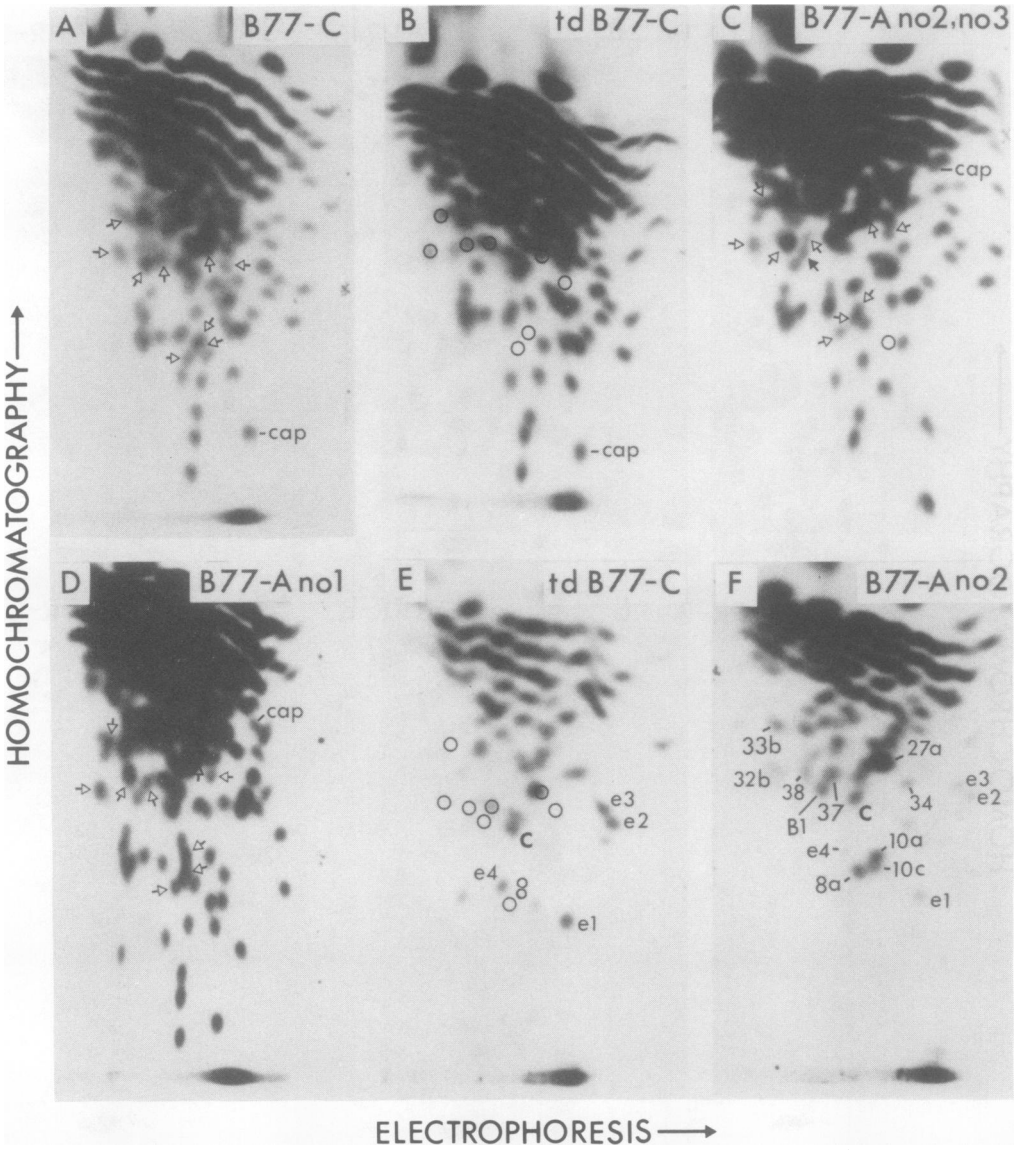

FIG. 3. *Fingerprint patterns of B77 and td B77 [^{32}P]RNAs. (A–D) viral 60 to 70S RNAs; (E, F) 18 to 21S poly(A)-containing RNAs. Designations of open and solid arrows are as in Fig. 1. B77-A no. 3 RNA contains an oligonucleotide absent in B77-A no. 2 RNA; this oligonucleotide is indicated by the open circle in the fingerprint pattern of B77-A no. 2 shown in panel C. Open circles in panels B and E indicate the corresponding chromatographic positions of the src-specific oligonucleotides missing in the td B77 RNA. The numberings e1, e2, e3, and e4 in panels E and F indicate the env-specific oligonucleotides shared by B77 ASV and td B77 RNAs. The cap-containing oligonucleotide is designated as "cap" in panels A to D.*

defective sarcoma or leukosis viruses analyzed so far (12, 25, 56, 57, 59). FuASV RNA differed from RNA of BH RSV no. 1 and no. 2 by only seven and four oligonucleotides, respectively, out of about 30 large RNase T_1-resistant oligonucleotides (Fig. 4A–C), even though FuASV (16) and RSV (39) were independently isolated at almost the same time. The authenticity of FuASV analyzed here is not certain. It is possible that this stock of FuASV is actually a variant of BH RSV and has been misnamed. Nevertheless, since its fingerprints were different from those of two stocks of BH RSV studied, we decided to follow the original name of the stock as it was given to us. We recently have analyzed an independent stock of FuASV which we believe to be

the authentic Fujinami sarcoma virus isolated 66 years ago. Our new results revealed that Fujinami virus contained a transforming gene unrelated to *src* of other ASVs and seemed to represent a new class of avian sarcoma virus (T. Hanafusa, L.-H. Wang, S. Anderson, R. K. Karess, W. S. Hayward, and H. Hanafusa, Proc. Natl. Acad. Sci. U.S.A., in press). It was also somewhat surprising that the RNAs of BH RSV no. 1 and no. 2 differed from each other by four oligonucleotides in addition to the variation within the *src* region. The variation probably reflects the extensive cloning of this defective RSV in each laboratory since its isolation (5, 35) and its frequent association with various types of helper virus. Recombination between BH RSV and different helper viruses could generate the diversity in sequences outside the *src* region. The difference between BH RSV no. 1 and no. 2 in their *src* sequences could have been gener-

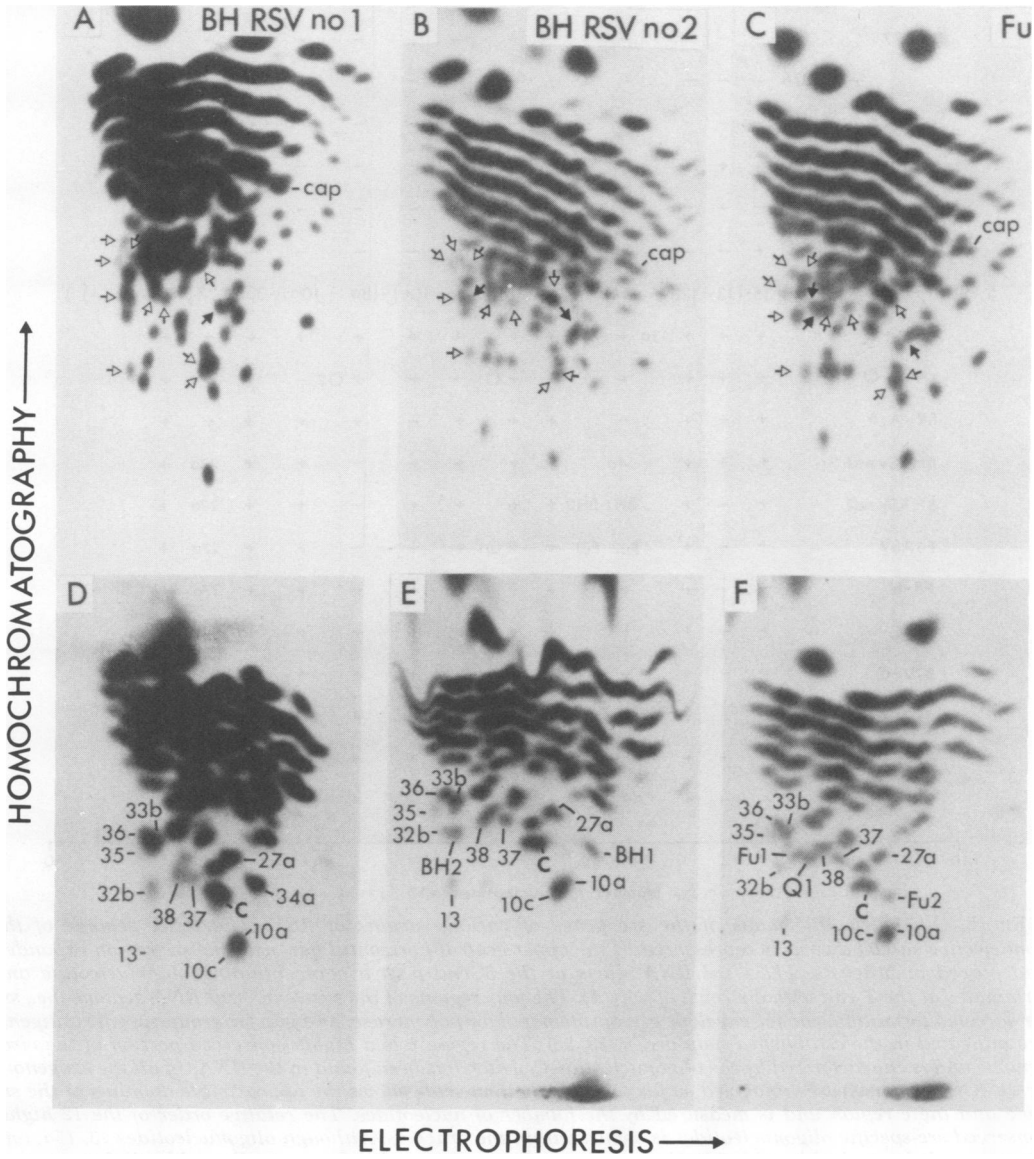

FIG. 4. *Fingerprint patterns of BH RSV and FuASV [^{32}P]RNA. (A-C) 60 to 70S RNAs; (D-F) 18 to 21S poly(A)-containing RNAs of corresponding viruses shown in panels A through C. Designations of open and solid arrows are the same as in Fig. 1. The cap-containing oligonucleotides of BH RSV and FuASV RNAs are indicated in panels A through C.*

ated via point mutations during passage of each virus. Similar to all the other exogenous avian tumor viral RNAs analyzed so far, BH RSV and FuASV RNAs contained oligonucleotide C (55) very close to the 3′ terminus (Fig. 4D–F). The cap oligonucleotide of BH RSV and FuASV belongs to class IV, the same class as SR-A and B77-A ASV (Fig. 4A–C; 58).

Comparison of the *src*-specific sequences of different strains of ASV. Figure 5 summarizes the results of our sequence analysis of ASVs and their td mutants studied here and earlier (60, 61). The compositions of the RNase A-resistant fragments of *src*-specific oligonucleotides present in different strains of ASV are listed in Table 1. The length of the oligonucleo-

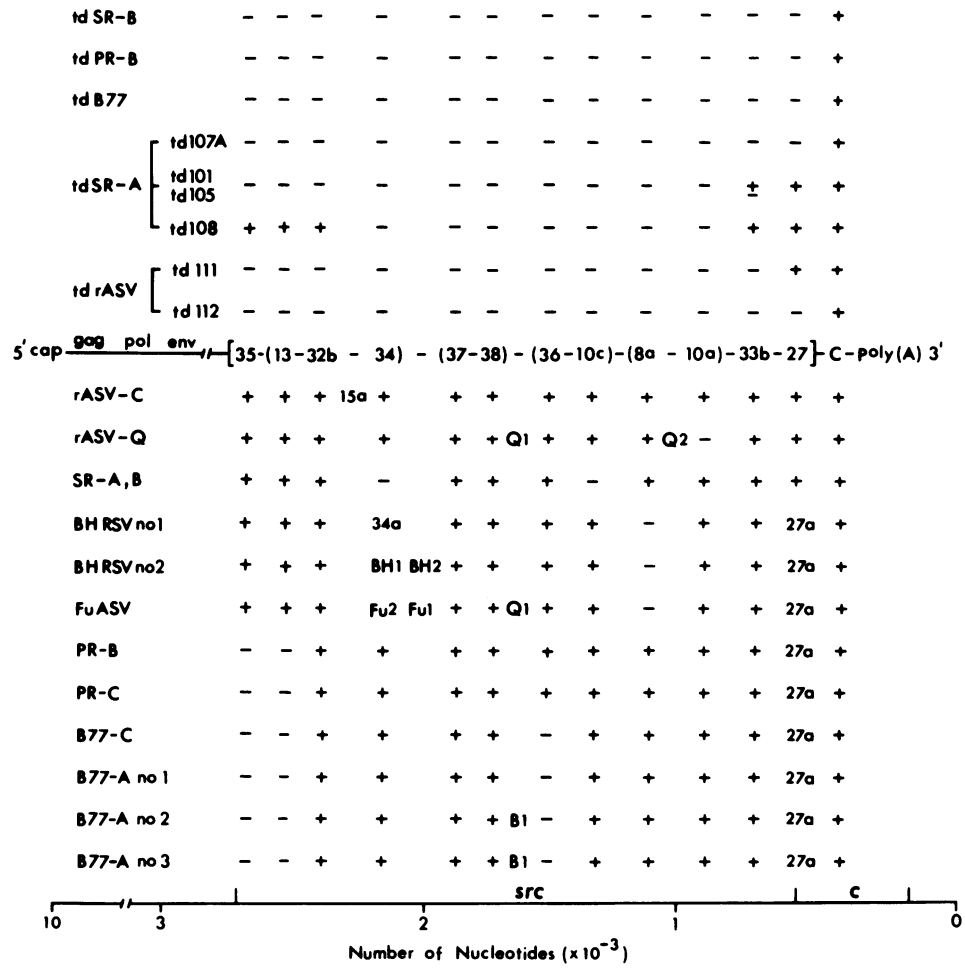

FIG. 5. *Oligonucleotide maps of the src genes of various strains of ASV. The RNA genome of the nondefective sarcoma virus is represented by the upper central horizontal bar, with the src region expanded and placed in brackets. The viral RNA starts at the 5′ end with a cap-containing oligonucleotide and terminates at the 3′ end with a stretch of poly(A). The four regions of the sarcoma viral RNA genome (i.e., src for sarcoma formation, env for envelope glycoprotein, pol for polymerase, and gag for group-specific antigens) are indicated in the established map order (12, 53). The region c is a highly conserved portion of the viral genome and is characterized by the oligonucleotide C, which has been found in the RNAs of all the exogenous avian RNA tumor viruses analyzed so far (53). The bottom scale shows the approximate domains of the src gene and the c region and is measured by the number of nucleotides. The relative order of the 12 highly conserved src-specific oligonucleotides is shown within the brackets. Although oligonucleotides 13, 15a, and 35 are clearly located within the 5′ half of src, their precise mapping has been complicated by the low recovery of these oligonucleotides in the fingerprints (see Fig. 1). Therefore, their map locations are considered tentative. The relative order of oligonucleotides within parentheses is also uncertain. The + and − symbols above and under each oligonucleotide designate the presence or absence of that oligonucleotide in the given viral RNA. Oligonucleotide 33b is present in td101 RNA, but absent in td105 RNA.*

tides analyzed ranged from 9 to 19 nucleotides. Most of these appeared only once within a given sarcoma virus genome, since they were absent in the corresponding td viral RNA. The smaller *src*-specific oligonucleotides, such as 27a, 32b, 33b, 37, and 38, were apparently isomers of or identical to certain oligonucleotides outside the *src* region in some sarcoma viral RNAs (see above).

The overall result of our analysis indicates that 12 *src*-specific oligonucleotides are highly conserved among different strains of ASV. Oligonucleotides 32b, 33b, 37, and 27 (or 27a) were present in all sarcoma viruses analyzed, and oligonucleotides 10a and 10c were present in all except one virus. Oligonucleotides 8a and 34 were present in 9, whereas oligonucleotide 36 was present in 8 of 12 isolates of sarcoma viruses studied here. Oligonucleotides 13 and 35 were present in 6 of the 12 sarcoma viruses. In addition to the variations within the 12 highly conserved *src*-specific oligonucleotides, we also found that certain strains of ASV contained one to three *src*-specific oligonucleotides unique to that virus. None of the 12 *src*-specific oligonucleotides was present in td PR-B or td B77, but one to five of these oligonucleotides were retained in some td mutants of SR-A and rASV-C (Fig. 5).

DISCUSSION

We have compared the *src* genes of different strains of ASV with *src* sequences which have been shown to be derived from chicken and quail cells, namely, those of rASV-C and rASV-Q (60, 61). The results indicate that the great majority of the *src*-specific oligonucleotides of ASV and rASV RNAs are conserved. Some *src*-specific oligonucleotides previously found to be new to rASV's when compared with SR-A RNA (60, 61) are widely present in other strains of ASV. These data suggest that *src* genes of different strains of ASV and the cellular *sarc* sequences present in rASV's (60, 61) are of common origin and may have originated from a single set of sequences.

Several approaches have been used to study the sequences of the *src* genes of ASVs and of related cellular DNA and RNA sequences. Those approaches include nucleic acid hybridization (21, 44, 45, 63), electron microscope heteroduplex mapping (26, 31), and RNase T_1 oligonucleotide fingerprinting (6, 25, 55). Comparison of the *src*-specific sequences of different strains of ASV by nucleic acid hybridization indicate that *src* genes of ASVs analyzed so far have more than 80% sequence homology among one another (21, 45, 51). Heteroduplex mapping studies have yielded similar results, although only a few derivatives of RSV have been compared (26, 31). Interpretation of the results of hybridization study relies on the representativeness of the complementary DNA transcribed from the *src* region of the RNA template, conditions used for the hybridization, and the method used to detect the hybrid. Heteroduplex mapping could not detect stretches of mismatching smaller than 100 nucleotides (10). Neither method can detect minor sequence diversities among closely related RNA species, although both methods are effective in measuring the gross extent of homology. Oligonucleotide fingerprinting is more sensitive in detecting minor sequence differences such as single base changes within a RNase T_1-resistant oligonucleotide. Although the total large RNase T_1-resistant oligonucleotides represent only 5 to 10% of the viral genomic RNA sequence, statistically such oligonucleotides are distributed rather evenly throughout the RNA (53); thus the pattern of conservation and diversity indicated in the oligonucleotide maps should reflect rather accurately the relationship of different RNA species. The sensitivity of this method has been best illustrated in the analysis of avian tumor viral RNAs. For example, by hybridization, the *env* genes of different strains or subgroups of avian RNA tumor viruses share more than 70% of their sequences (21–23, 47, 51), but a quite distinctive set of oligonucleotides is generated from this region of the genome for each virus (12, 55–57, 59). Even the highly conserved *c* region displays a characteristic oligonucleotide pattern for each strain of the avian tumor virus, despite the fact that all of the exogenous strains share the oligonucleotide termed "C" (53, 55). Since it is already known from hybridization studies that the *src* genes of ASVs and the cellular *sarc* sequences are highly related (44, 46, 63), a more sensitive method of sequence study may detect the minor differences among them and thus provide some insight into the origin of the *src* genes of different strains of ASV. For the reasons discussed above, we chose the method of oligonucleotide fingerprinting to analyze the viral and cellular transforming sequences. Our overall results indicate that by using the method with this degree of sensitivity, we could detect only minor sequence variations among the *src*-specific oligonucleotides of different strains of ASV and newly isolated rASV-C and rASV-Q. In view of the postulated mechanism (17, 19, 60, 61) for the generation of rASV's and the striking similarity of rASV *src* sequences to those of other known ASVs, it is tempting to speculate that all of the ASVs have been generated by recombination between certain leukosis viruses and the cellular *sarc* sequences. An increasing body of evidence has been accumulated to suggest that murine

sarcoma viruses have been generated by a similar process (15, 42).

The constraint of variation in *src* genes among different strains of ASV most likely reflects the necessity of maintaining a functional *src* gene, the property for which these ASVs have been selected. Recombination between sarcoma virus and cellular *sarc* could also explain the convergence of viral *src* sequences. The presence of an almost identical copy of *src* sequences in chicken and quail cells, reflected in the *src* sequences of rASV-C and rASV-Q, implies that such sequences probably code for a function essential for the normal cell. The expression of the cellular *sarc* must be under a strict regulation (44, 63) since uncontrolled expression of such sequences could possibly lead to sarcomagenic transformation.

Although some *src*-specific oligonucleotides were found to be characteristic of certain ASV RNAs, most of the variations in oligonucleotides may be interpreted as a result of single base changes. For example, oligonucleotide 34a in BH RSV no. 1 RNA appeared to result from a "C"-to-"U" transition of oligonucleotide 34; oligonucleotide Q2 in rASV-Q RNA is apparently an isomer of an oligonucleotide, 10a, present in all other ASV RNAs analyzed. In addition, oligonucleotides BH1 and Fu2 may be considered as homologous sequences of oligonucleotide 34, as is the case with oligonucleotides 27 and 27a. This leaves only oligonucleotides 15a, BH2, Fu1, and B1 unique to RNAs of rASV-C, BH RSV no. 2, FuASV, and B77-A (no. 2 and no. 3), respectively. The variations in *src* sequences are consistent with the findings that different strains of ASV induce characteristic focus morphology of transformed cells (18) and have different serological properties for their primary protein products, pp60's (3, 32, 43). Our data also agree with the recent report that pp60's of different strains of ASV contain common as well as characteristic tryptic peptides (2).

Although SR-A, SR-B, PR-B, PR-C, and BH RSV are considered as derivatives of the original RSV isolated by Rous (39), it is not entirely clear whether they have been derived from a single tumor or from independent tumors. The fact that the RNAs of PR, SR, and BH RSV all form a distinctive fingerprint pattern suggests that they may actually be independent isolates of ASV obtained from different tumors. Alternatively, they could be independent clones of the original RSV, which was a mixture of several strains of virus. Recombination with associated leukosis virus or accumulation of mutations during passage and cloning of these viruses in each laboratory could also account for the observed sequence diversity. In view of the generation of rASV, a possibility that RSV rarely recombines with cellular sequences also cannot be excluded. Whatever the cause of the divergence, all these derivatives of Rous virus did not characterize them as a unique group when compared with an independent isolate of ASV, B77 ASV. We have found that B77 ASVs obtained from different sources share a characteristic set of oligonucleotides as well as a set of rather well conserved *src* sequences. All of these B77 ASVs can be considered derivatives of the original B77 ASV. This demonstrates the effectiveness of oligonucleotide fingerprinting for the classification of closely related viruses.

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