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src Genes of Ten Rous Sarcoma Virus Strains, Including Two Reportedly Transduced from the Cell, Are Completely Allelic; Putative Markers of Transduction Are Not Detected

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The *src* genes of different Rous sarcoma virus (RSV) strains have been reported to be highly conserved by some investigators using RNA-cDNA hybridization, whereas others using oligonucleotide, peptide, and serological analyses have judged *src* genes to be variable in 30 to 50% of the respective markers. Moreover, distinctive *src* oligonucleotides and peptides of so-called recovered RSVs (rRSV's) whose *src* genes were reported to be experimentally transduced from the cell are thought to represent specific markers of host-derived *src* sequences. By contrast, we have pointed out previously that these markers may represent point mutations of parental equivalents. Here we have compared the *src*-specific sequences of eight RSV strains and of two rRSV's to each other and to a molecular clone of the *src*-related chicken locus. Our comparisons are based on RNase T_1-resistant oligonucleotides of RNA hybridized to *src*-specific cDNA, which was prepared by hybridizing RSV cDNA with RNA of isogenic *src* deletion mutants, or to a cloned cellular *src*-related DNA. All of the approximately 20 *src*-oligonucleotides of a given RSV strain were recovered by *src*-specific cDNA's of all other RSV strains or by cellular *src*-related DNA. The number of oligonucleotides varied slightly with the length of the *src* deletion used to prepare *src*-specific cDNA, thus providing a measure for *src* deletion mutants. Our data indicate that the *src* genes of all RSV strains tested, including the two reportedly transduced from the cell, are about 98% conserved and completely allelic with only scattered single nucleotide differences in certain variable regions which are subject to point mutations. Hence, based on the *src* oligonucleotide markers analyzed by us and others, we cannot distinguish between a cellular and viral origin of rRSV's. However, the following are not compatible with a cellular origin of rRSV's. (i) The only putative oligonucleotide marker which is exclusively shared by the two rRSV's studied and which differs from a parental counterpart in a single base was not detectable in cellular *src*-related DNA. (ii) The number of different allelic *src* markers observed by us and others in rRSV's was too large to derive from one or two known cellular *src*-related loci. (iii) The known absence of linkage of the cellular *src*-related locus with other virion sequences was extended to all non-*src* oligonucleotides, including some mapping directly adjacent to *src*. This is difficult to reconcile with the claim that transformation-defective, partial *src* deletion mutants of RSV which contain both, one, or, as we show here, possibly no *src* termini nevertheless transduce at the same frequencies, even though homologous, single or double illegitimate recombinations would be involved. Given (i) our evidence that *src* genes are subject to point mutation under selective conditions similar to those prevailing when rRSV's were generated and (ii) the lack of absolute evidence for the clonal purity of the transformation-defective, partial *src* deletion mutants of RSV used to generate rRSV's, we submit that the *src* genes of rRSV's could have been generated by cross-reactivation of nonoverlapping *src* deletions or mutation of *src* variants possibly present in transformation-defective, partial *src* deletion mutants of RSV. To prove experimental transduction, unambiguous markers need to be identified, or it would be necessary to generate rRSV's with molecularly cloned transformation-defective, partial *src* deletion mutants of RSV. Although our evidence casts doubt on the idea that specific *src* sequences of rRSV's originated by transduction, the close relationship between viral *src* and cellular *src*-related sequences argues that *src* genes originated at one time in

evolution from the cell by events that involved illegitimate recombination and deletion of non-*src* sequences that interrupt the cellular *src* locus.

Ribonucleotide sequences necessary for transformation by Rous sarcoma viruses (RSV) have been defined by subtracting from the RNA of nondefective RSV all sequences found in transformation-defective (td) *src* gene deletion mutants (4, 21, 43) or by correlating sequences that segregate with sarcomagenicity in viral recombinants formed between nondefective and td RSVs (8, 45). These sequences map contiguously as a 1.5-kilobase (kb) RNA sequence near the 3′ end of viral RNA and include the coding sequences of the 60,000-dalton *src* gene product (3, 8, 43).

Although highly conserved in terms of complexity, sequence relationship as measured by RNA-cDNA hybridization (92 to 100%), and chemical composition of some RNase T_1-resistant oligonucleotides (21, 38, 43, 44), the *src*-specific RNA sequences of different RSV strains were diagnosed to contain variable elements. By fingerprinting RNase T_1-resistant oligonucleotides, a method that is sensitive to single base differences, up to 30% of *src*-specific oligonucleotides were found to be variable (43, 45, 49, 50). Consistent with these nucleotide sequence variations, *src* gene products of different viral strains were found to differ in serological determinants (24, 31, 33) and in up to 50% of their tryptic peptides (1, 34). On the basis of these differences some investigators have judged the *src* genes of different RSV strains to be variable (1, 16, 50). Furthermore, distinctive *src* oligonucleotides and *src* peptides were also found in so-called recovered RSVs (rRSV's) (16, 32, 41, 42, 47–50). These are RSVs which were recovered from tumors induced after latent periods of 2 to 3 months by stocks of transformation-defective, partial *src*-deletion mutants of RSV (ptd RSV), which lack over 75% of the parental *src* gene (15, 16, 41). Although these viruses were originally termed rASV (for recovered avian sarcoma viruses), we use here the term rRSV, since other avian sarcoma viruses, i.e., Fujinami sarcoma virus and Y73 sarcoma virus, with transforming genes unrelated to *src* have since been identified (23, 52). Since the rRSV's have complete *src* genes it has been postulated that the 75% fraction of their *src* genes that was absent from ptd RSVs was transduced from cellular *src*-related sequences (15, 16, 41, 42, 47–50). To date, this system is believed to provide the only experimentally reproducible evidence for transduction of cellular transforming genes by retroviruses. Transduction of cellular transforming genes by nontransforming retroviruses has been postulated as the origin of several highly oncogenic retroviruses and, hence, appears relevant to the unknown mechanism by which nontransforming retroviruses cause cancers after long latent periods in animals (8).

To substantiate the view that the *src* genes of rRSV's contain transduced cellular sequence elements, several investigators have argued that the distinctive nonparental markers of rRSV's represent specific sequence elements of cellular origin (16, 41, 42, 47, 48, 50). At variance with this view, we have suggested, in an earlier study comparing T_1 oligonucleotides of some of the same parental and recovered RSVs studied by others (41, 42), the possibility that the *src* genes of rRSV's may have been derived from parental RSVs by point mutations, because the *src* oligonucleotides of two rRSV's appeared either identical or closely related to parental counterparts (32).

To resolve the above discrepancies the nucleotide or amino acid sequences of variable oligonucleotide, peptide, or serological *src* gene markers ought to be compared. As yet, it has not been determined directly whether the distinctive oligonucleotide, peptide, and serological *src* gene markers of different RSV strains and of rRSV's reflect single base differences scattered among allelic *src* regions or unique sequence elements interspersed among conserved sequences.

Here we have compared the *src*-specific RNA sequences of different RSV strains by hybridization with *src*-specific cDNA or cloned cellular *src*-related DNA by using RNase T_1-resistant oligonucleotides as representatives of RNA sequences. We have resolved and compared 20 to 23 *src* oligonucleotides in each of eight RSV strains and in two rRSV's representing about 15% of the *src*-specific RNA. Of these, 11 were conserved in all RSV strains; the remaining oligonucleotides were distinctive or shared by some RSV strains. Allelic sequence counterparts of all distinctive *src* oligonucleotides of different RSV strains were found in others either directly or in the nucleotide sequences of Schmidt-Ruppin (SR) RSV-A (7) or Prague (PR) RSV-C (D. Schwarz, R. Tizard, and W. Gilbert, personal communication) or by competitive hybridization. The same methods also showed that the distinctive *src* oligonucleotides of two rRSV's have allelic counterparts in parental and other RSV strains. Moreover, the only *src* oligonucleotide shared by the two rRSV's that did not have an identical counterpart in parental or other RSVs, was not recovered from an RNase A-, T_1-, and T_2-resistant hybrid formed with DNA of the cellular *src*-related locus.

It is concluded that the *src* genes of eight RSV strains and of two rRSV's are about 98% conserved and completely allelic. Variations among *src*-specific sequences are limited to single nucleotide differences in certain variable regions. Since the same variations were observed among known RSV strains, among clonal variants of the same strain, and among parental and recovered RSVs, and since the only putative transductional oligonucleotide marker of our rRSV's was not found in the cellular *src*-related locus, we submit that the identification of allelic variants of oligonucleotides is not sufficient proof to support the view that the rRSV's carry *src* sequences transduced from the cell. Alternative hypotheses are discussed to explain the origin of rRSV's in animals injected with ptd RSV.

MATERIALS AND METHODS

Viruses and cells. The PR RSV of subgroup B and B77 RSV of subgroup C were the same as those described previously (43). The SR RSV of subgroup A, clone 85-7, and a td *src* deletion mutant of SR RSV-A, termed td 102, have been described (44). A partial *src* deletion of SR RSV-A, 85-7, termed ptd 108, has been characterized previously by us and others (20, 47). The quail cell line producing Bryan RSV(−)3 has also been described (10, 14). A quail line producing a variant Bryan RSV(−), termed RSV(−)16, was the gift of H. Murphy (30). The virus, termed here Bryan RSV(−)1, was obtained from P. K. Vogt and was initially thought to be a stock of Fujinami sarcoma virus (23, 38). Clone 7745 or SR RSV of subgroup D, termed here SR RSV-D1, was the source of the two mutants with a partial *src* deletion, ptd 3 and ptd 14, which were used to generate rRSV 3-3 and rRSV 14-2 and of the complete *src* deletion mutant td SR RSV-D clone 21 (22, 32, 41, 42). In a previous publication we referred to rRSV 3-3 as rASV 3-1 and to rRSV 14-2 as rASV 2-1 (32). The rRSV's were cloned by focus selection to remove excess td rRSV variants (32) before analysis of their *src* genes. A clonal variant of SR RSV-D1, which was derived by cloning SR RSV-D1-transformed rat cells (41) carrying a single provirus and rescuing the virus from a clonal colony of rat cells by fusion with chicken embryo fibroblasts, was termed SR RSV-D2. It was a gift from R. K. Vogt. A td mutant of PR RSV-B was obtained from G. S. Martin.

Viral RNA, cDNA, and a recombinant lambda clone of the *src*-related locus of the chicken. ^{32}P-labeled virus was prepared from the supernatant medium of cells labeled with $^{32}PO_4^{3-}$ (50 mCi/175-cm^2 tissue culture flask) in 20 ml of phosphate-free 199 medium supplemented with 2% calf serum and 1% chicken serum dialyzed against isotonic saline. Labeling was for 12 h. Subsequently, the culture was incubated for an additional 12 h in phosphate-free medium and then for two more 12-h periods with complete medium. Viral RNA (60 to 70S) was prepared as described previously (43).

For preparation of cDNA, virions were purified by equilibrium density gradient centrifugation in 20 to 60% sucrose gradients (23, 43). Typical reactions contained 2 to 3 units of absorbancy at 260 nm per ml of purified virus (measured in neutral solution containing 0.2% sodium dodecyl sulfate), 20 mM Tris (pH 8.5), 60 mM KCl, 8 mM $MgCl_2$, 2 mM deoxynucleoside triphosphates and sufficient [^{3}H]TTP or [^{3}H]dCTP to give a final specific activity of 20 to 50 cpm/ng of [^{3}H]cDNA, 0.025% (wt/vol) nonionic detergent Nonidet P-40, 20 μg of actinomycin D per ml, and 70 mM dithiothreitol. Reactions were incubated with gentle shaking at 40°C for 16 to 20 h. Subsequently, EDTA was added to 20 mM, the solution was extracted twice with phenol, and the nucleic acids were precipitated with 2.5 volumes of ethanol. After 2 h at −20°C the nucleic acids were pelleted and incubated overnight at room temperature in 0.3 ml of 0.3 N NaOH, and after neutralization with 2 N acetic acid the solution was chromatographed on a Bio-Gel (Bio-Rad Laboratories) P100 column (1 by 15 cm) in 0.01 M NaCl–0.01 M Tris (pH 7.4)–0.1% sodium dodecyl sulfate. The [^{3}H]cDNA eluting in the void volume was recovered by ethanol precipitation directly or after an additional phenol extraction. The yield was 0.3 to 4 μg of cDNA per unit of absorbancy at 260 nm of virus.

The recombinant lambda phage λCS3 containing the *src*-related locus of the chicken was a gift of G. Cooper. It was propagated as described previously (35).

Nucleic acid hybridization and fingerprinting of T_1 oligonucleotides of RNA-cDNA hybrids. *src*-specific cDNA was prepared by incubating 1 to 2 μg of cDNA with 10 to 20 μg of td RSV RNA in 10 μl of 50% formamide containing 0.45 M NaCl–0.045 M sodium citrate–20 mM sodium phosphate buffer (pH 7) for 3 to 12 h at 40°C. For competitive hybridizations between *src*-specific RNAs, *src*-specific cDNA was hybridized under the same conditions with a 10-fold excess of RSV RNA. Subsequently about 0.25 μg of RSV [^{32}P]RNA (4×10^6 cpm/μg) was added in 10 μl of the above solution, and incubation was continued for an additional 3 h. The solution was diluted 10-fold with 0.15 M NaCl–0.015 M sodium citrate and then incubated at 40°C with 10 U of RNase T_1 for 50 min. The hybrid was separated from digested RNA by chromatography on the Bio-Gel column described above, except that the column was developed with buffer containing 0.1 M NaCl. After ethanol precipitation the hybrid was dissolved in 100 μl of 5 mM NaCl–5 mM Tris (pH 7.4)–0.5 mM EDTA; after heat dissociation for 1 min and subsequent quenching at 0°C, the [^{32}P]RNA was digested with 8 U of RNase T_1 for 30 min at 40°C. The solution was then fingerprinted by electrophoresis at pH 2.5 followed by homochromatography as described previously (12).

RESULTS

Identification and comparison of the *src*-specific oligonucleotides of eight different RSV strains by hybridization with *src*-specific cDNA. (i) Strategy. To determine the extent of relatedness among the *src* genes of different RSV strains, the *src*-specific sequences of different RSVs were compared by RNA-cDNA hybridization and fingerprinting T_1 oligonucleotides of hybridized RNA. This method

distinguishes unambiguously between sequences which may be identical or differ from each other only in single base substitutions and hence form T_1-resistant hybrids and sequences with substantial nonhomologies which would not form T_1-resistant hybrids (5, 12, 23, 28). To detect a maximum of unique *src* oligonucleotides, the background of viral non-*src* oligonucleotides was omitted by fingerprinting only *src*-specific RSV RNA that was hybridized by *src*-specific cDNA. The *src*-specific cDNA was prepared by annealing cDNA of nondefective RSV with a 10-fold excess by weight of RNA from the corresponding isogenic *src* deletion mutants. The *src*-specific cDNA was then hybridized to 60 to 70S [^{32}P]-RNAs of different RSVs, and the resulting RNase T_1-resistant hybrids were fingerprinted as described above.

One possible problem with this method is that the *src* genes of different RSVs may not be completely allelic. In this case a hypothetical unique *src* sequence element of a given RSV strain would not be hybridized by *src*-specific cDNA from another. Another problem is that the complexity of the *src*-specific cDNA would vary with the extent of the *src* deletion mutant used to prepare the probe.

(ii) *src*-specific cDNA's of different RSV strains are allelic. To test for the possibility that *src* genes of different RSV strains might not be allelic, the *src* oligonucleotides of PR RSV-B recovered with two different *src*-specific cDNA's, one prepared from PR RSV-B cDNA and td PR RSV-B RNA and the other prepared from SR RSV-A cDNA and td SR RSV-A 102 RNA, were compared. It is shown in Fig. 1A and B that the same oligonucleotides were obtained. We conclude that the *src* gene of PR RSV does not contain unique *src* sequences detectable as T_1 oligonucleotides that are not allelic with SR RSV. (The only difference was that the PR RSV cDNA hybrid contained the 5'-terminal cap oligonucleotide of PR RSV RNA [cap in Fig. 1B]. It is likely that this oligonucleotide was not sufficiently outcompeted by td SR RSV RNA due to a probable excess of strong-stop cDNA that spans the sequence between the 5' end and the tRNA primer located near the 5' end of RSV RNA [17].)

The possibility that the *src* genes of PR and SR RSV may each contain small unique sequences only detectable as very small oligonucleotides not resolved as unique species by our method was then tested by competitive hybridization. In this experiment *src*-specific cDNA of SR RSV-D1 was first hybridized with PR RSV-B RNA to anneal all related *src* sequences (see above). The resulting hybrid was then incubated under annealing conditions with SR RSV-D1 [^{32}P]RNA. As is shown in Fig. 1C, no oligonucleotides were recovered, except polyadenylic acid [poly(A)], which due to its size and resistance to RNase T_1 copurified with RNA-cDNA hybrids in our Bio-Gel column. We conclude that the *src* genes of PR and SR RSV are completely allelic and do not contain unique sequence elements.

Judging from their autoradiographic densities we note that not all *src* oligonucleotides were recovered at equimolar ratios by our method (Fig. 1, 2, and 4). This was particularly true when the same *src*-specific RNA was analyzed with different *src*-specific cDNA's (Fig. 1 and 4). This is probably due to experimental shortcomings of our method, such as nonequimolar distribution of *src* cDNA segments, the preferential loss of some not completely base-paired oligonucleotides from RNA-cDNA hybrids upon digestion with RNase T_1 (5, 12), and technical difficulties in transferring RNA digests from the electrophoretic dimension to two separate DEAE thin-layer plates (12). (An example of nonequimolar distribution of cDNA is described below.)

Fingerprints of the *src*-specific RNAs of the six additional RSV strains, including SR RSV-A, SR RSV-D2, three replication-defective variants of Bryan RSV(−) [i.e., RSV(−)3, 16, and 1], and RSV B77, all obtained with *src*-specific cDNA prepared with PR RSV-B cDNA and td PR RSV-B RNA are shown in Fig. 2. The defectiveness of the three RSV(−) variants was verified by measuring electrophoretically the sizes of the viral RNAs, which, in agreement with previous analyses (10, 30, 44), were 8, 8.5, and 8 kb for RSV(−)3, 16, and 1, respectively (Table 1). The size of all nondefective RSVs and rRSV was 10 kb, again in agreement with previous analyses (8, 32, 43, 44). The compositions of all large *src* oligonucleotides (numbered as in Fig. 2) in terms of RNase A-resistant sequences are listed in Table 1. About 20 to 23 unique *src* oligonucleotides were resolved in each RSV RNA which together represented 200 to 250 (or 15%) of the about 1,600 nucleotides of *src*-specific RNA.

On the basis of their distribution in other viral strains the 20 to 23 *src* oligonucleotides of all RSV strains analyzed by us fell into three classes: a completely conserved class of 11 *src* oligonucleotides, numbered 1 to 11, that all RSV strains analyzed had in common; a second class of about 12 relatively conserved *src* oligonucleotides that each RSV strain shared with 1 to 9 of the 10 strains compared (which include two rRSV's); and a third class of about one to three *src* oligonucleotides that were distinctive for a given RSV strain. To determine whether distinctive and relatively conserved *src* oligonucle-

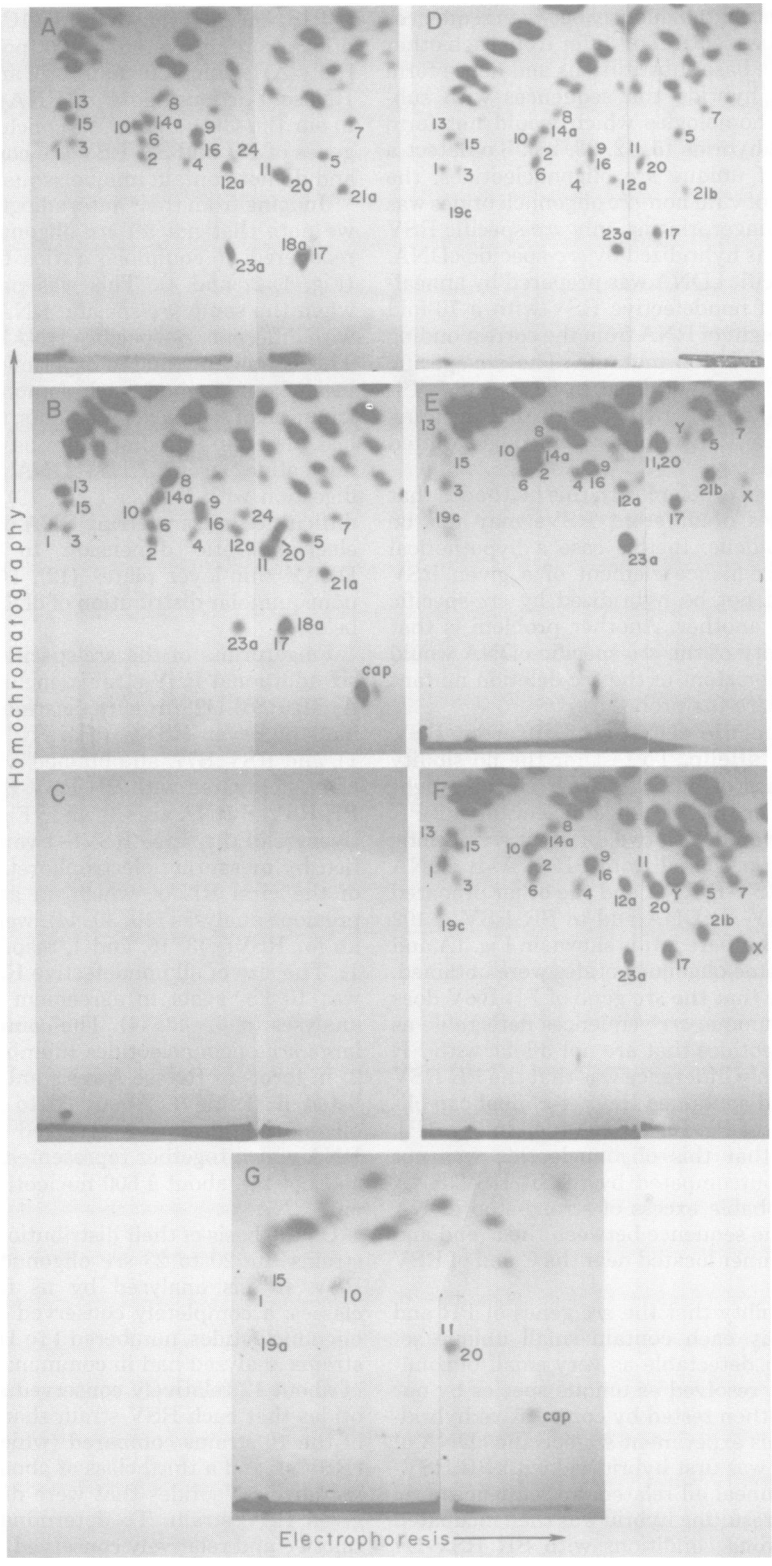
A
13
15
1
3
10
8
14a
6
2
9
16
4
24
12a
11
20
5
7
21a
18a
23a
17
D
13
15
1
3
10
8
14a
2
6
4
9
16
11
12a
20
5
7
21b
19c
23a
17
B
13
15
1
3
10
8
14a
6
2
9
16
4
24
12a
11
20
5
7
21a
18a
23a
17
cap
E
13
15
1
3
19c
10
8
14a
6
2
4
9
16
11,20
12a
Y
5
7
21b
X
17
23a
C
F
13
15
1
3
19c
10
8
14a
6
2
4
9
16
11
12a
20
Y
5
7
21b
X
17
23a
G
15
1
10
19a
11
20
cap
Homochromatography
Electrophoresis

FIG. 1.

otides of different RSV strains reflect allelic variants of others, as suggested by the fact that they were obtained with the same *src*-specific cDNA probe, the following criteria were applied: (i) the presence of one must be mutually exclusive for the other because their map locations have to be the same; (ii) their complexity must be high enough to insure uniqueness; and (iii) sequences must be close enough to explain differences in terms of one or two (in larger oligonucleotides; c.f. Table 1, no. 23a, b, and c) point mutations.

Mapping of *src* oligonucleotides was accomplished by two methods. One method located *src* oligonucleotides of PR RSV-B relative to the 3′ poly(A) coordinate of viral RNA by fingerprinting RNA from T_1-resistant hybrids formed between poly(A)-tagged PR RSV-B RNA fragments of discrete size classes (43) and *src*-specific cDNA of PR RSV-B (Fig. 3). The other method matched *src* oligonucleotide sequences with the known nucleotide sequence of cloned proviral DNA of SR RSV-A (7) or of PR RSV-C (Schwarz et al., personal communication). The oligonucleotide maps obtained by both methods were in complete agreement (Fig. 3). The position of each *src* oligonucleotide detected in the sequence of SR RSV-A or PR RSV-C DNA is recorded in Table 1 using the nucleotide position numbers in the 5′ to 3′ order of the SR RSV DNA sequence reported by Czernilofsky et al. (7) and is depicted in Fig. 3.

Together with the compositional analyses, the oligonucleotide maps allowed unambiguous identification of allelic pairs among nonidentical *src* oligonucleotides of different RSV strains (Table 1). Once identified, allelic variants of a given oligonucleotide sequence were given the same numbers and were distinguished alphabetically in Fig. 1, 2, and 4 and Table 1.

(iii) Complexity of *src*-specific cDNA varies with the *src* deletion mutant used to prepare the probe. It was noted that the number of *src* oligonucleotides of a given RSV strain varied slightly with some, but not all, *src* deletion mutants used to prepare *src*-specific cDNA. For instance, the *src* oligonucleotide patterns of PR RSV-B obtained with *src*-specific cDNA prepared with PR RSV-B cDNA and td PR RSV-B RNA or SR-RSV-A cDNA and td SR RSV-A 102 RNA were the same, indicating that these deletions were essentially colinear (Fig. 1A and B). The *src* deletion mutant td SR RSV-D clone 21 presents an example of a deletion that extends in the 5′ direction beyond the limits set by td SR RSV-A 102 and td PR RSV-B. Hence, SR RSV-D1 RNA hybridized by *src*-specific cDNA formed with SR RSV-D1 or rRSV 14-2 cDNA and td SR RSV-D21 RNA included oligonucleotides x and y (Table 1), which are not found in hybrids formed with the *src*-deletion mutant td PR RSV-B (Fig. 1 D, E, and F, 3, and 4). The recovery of these oligonucleotides varied with the cDNA used. For example, little of x and y was recovered with cDNA of SR RSV-D1, but much more was recovered with cDNA of rRSV's 14-2 and 3-3 (cf. Fig. 1 and 4). This reflects the presence of an excess of spontaneously generated td rRSV's present in our rRSV stocks, as determined by gel electrophoresis of viral RNAs (data not shown) (32). By contrast to td SR RSV-D21 these td rRSV's lacked only *src* sequences and not sequences outside of the 5′ *src* gene border (data not shown). Hence, a mixture of rRSV and td rRSV cDNA's hybridized with td SR RSV-D21 RNA would contain an excess of unhybridized cDNA of the non-*src* region that is deleted in td SR RSV-D21. This cDNA would hybridize RNA represented by oligonucleotides x and y (Fig. 3).

By contrast td SR RSV-A 108 represents an *src* deletion mutant with residual *src* oligonucleotides (47, 48). The residual *src* oligonucleotides of td 108 were isolated from a hybrid formed

FIG. 1. *Fingerprint patterns of RNase T_1-resistant oligonucleotides of src-specific RSV [^{32}P]RNA. ^{32}P-labeled 50 to 70S RSV RNAs were hybridized with src-specific cDNA's prepared from RSV cDNA's and RNAs of corresponding isogenic td RSV. The RNase T_1-resistant hybrids were denatured and subjected to fingerprinting at pH 2.5 (12) as described in the text. Because homochromatography was on two separate commercial DEAE thin-layer plates to enhance resolution (12), there is a splice mark in the middle of each fingerprint. A, src oligonucleotides of PR RSV-B recovered from src-specific cDNA's prepared from PR RSV-B cDNA and td PR RSV-B RNA. B, src oligonucleotides of PR RSV-B recovered from src-specific cDNA prepared from SR RSV-A cDNA and td SR RSV-A RNA. C, Competitive hybridization between SR RSV RSV-D1 [^{32}P]RNA and unlabeled PR RSV-B RNA with src-specific cDNA prepared from SR RSV-D1 cDNA and td SR RSV-D21 RNA; only poly(A), which is resistant to RNase T_1 digestion, is found on the fingerprint; detailed experimental procedures are described in the text. D, src oligonucleotides of SR RSV-D1 recovered from src-specific cDNA prepared from PR RSV cDNA and td PR RSV-B RNA. E, src oligonucleotides of SR RSV-D1 protected by src-specific cDNA prepared from SR RSV-D1 cDNA and td SR RSV-D21 RNA; oligonucleotides x and y map outside the src gene as illustrated in Fig. 3. F, src oligonucleotides of SR RSV-D1 protected by src-specific cDNA prepared from rRSV 14-2 cDNA and td SR RSV-D21 RNA. G, Residual src oligonucleotides of td SR RSV-A 108 recovered from src-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B RNA.*

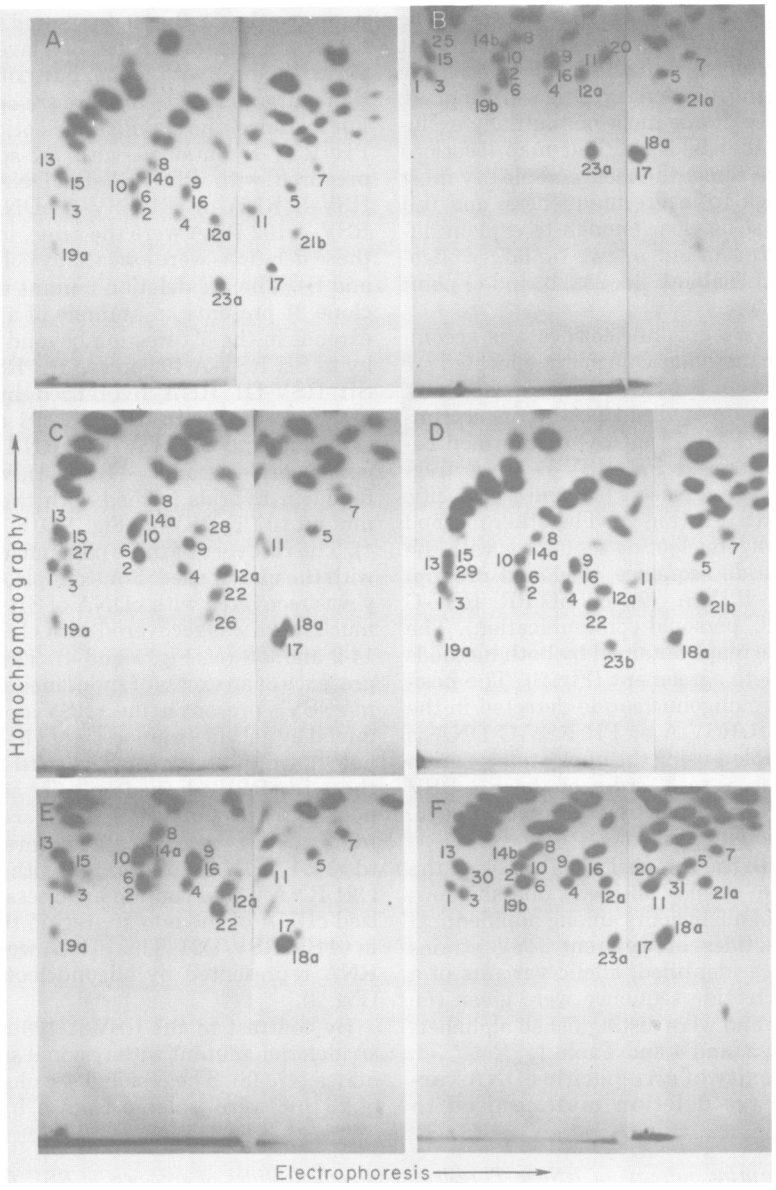

FIG. 2. *Fingerprint patterns of src oligonucleotides of six strains of RSV. ^{32}P-labeled 60 to 70S RSV RNAs were hybridized with src-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B RNA. The procedures were as described for Fig. 1. src-specific RNAs of the following viruses were fingerprinted: A, SR RSV-A; B, SR RSV-D2; C, Bryan RSV(−)3; D, Bryan RSV(−)16; E, Bryan RSV(−)1; and F, B77 RSV.*

between td 108 [^{32}P]RNA and *src*-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B RNA (Fig. 1G).

By using both the oligonucleotide map of PR RSV-B (Fig. 3) and the known *src* sequences of SR RSV-A (7) and PR RSV-C, the RSV oligonucleotides recovered by cDNA probes formed with some but not other *src*-deletions were located at the 5′ or 3′ boundary of the *src*-specific sequence (Fig. 3). Hence, fingerprinting *src* oligonucleotides of known RSVs with cDNA probes formed with different *src* deletion mutants proved to be a reliable method to define the boundaries of the *src* deletion in a given td RSV virus (Fig. 3). The result also confirmed earlier analyses which indicated that the dele-

TABLE 1. *Composition of T_1 oligonucleotides in the src gene region*

No.[b]	Oligonucleotide		Oligonucleotide present in strain[a]:									
	Composition	Map position[c]	PR RSV-B	SR RSV-A	SR RSV-D1	SR RSV-D2	Bryan RSV(−)3	Bryan RSV(−)16	Bryan RSV(−)1	B77 RSV	rRSV 14-2	rRSV 3-3
1	3C,G,3AC	69-78	+	+	+	+	+	+	+	+	+	+
2	U,2C,G,AC,2A$_2$C	334-345	+	+	+	+	+	+	+	+	+	+
3	4U,G,A$_4$C	502-511	+	+	+	+	+	+	+	+	+	+
4	U,5C,AC,AU,AG	395-406	+	+	+	+	+	+	+	+	+	+
5	4U,4C,G,2AC	247-259	+	+	+	+	+	+	+	+	+	+
6	U,5C,G,A$_3$C	491-501	+	+	+	+	+	+	+	+	+	+
7	4U,2C,G,A$_2$C	514-523	+	+	+	+	+	+	+	+	+	+
8	2C,AU,A$_3$G	889-896	+	+	+	+	+	+	+	+	+	+
9	U,4C,G,AU,A$_2$C	1,178-1,188	+	+	+	+	+	+	+	+	+	+
10	4C,AU,AC,AG	1,393-1,402	+	+	+	+	+	+	+	+	+	+
11	2U,3C,AC,AU,A$_2$G	1,324-1,335	+	+	+	+	+	+	+	+	+	+
12a	U,5C,G,AC,2AU	1,016-1,018	+	+	+	+	+	+	+	+	+	
12b	U,4C,G,AC,2A U											+
13	3C,2AC,AG	874-882	+	+	+		+	+	+	+	+	+
14a	U,C,2AC,A$_2$G	607-616	+	+	+		+	+	+		+	+
14b	U,C,2AC,AG					+				+		
15	2C,2AC,A$_2$G	1,375-1,383	+	+	+	+	+	+	+		+	+
16	2U,4C,AC,AU,AG	424-435	+	+	+	+		+	+	+	+	+
17	4U,5C,G,3AC,AU	639-656	+	+	+	+	+		+	+	+	+
18a	4U,6C,2AC,AU,AG	364-382	+			+	+	+	+	+	+	
18b	4U,6C,G,2AC,AU											+
19a	4C,AC,A$_2$C,A$_2$G	96-107		+			+	+	+		+	+
19b	4C,G,AC,AU,A$_2$C					+				+		
19c	4C,G,2AC,A$_2$C				+							
20	2U,5C,AU,A$_2$G	1,279-1,290	+	+	+	+				+	+	+
21a	4U,3C,G,AC,A$_2$C		+			+				+	+	+
21b	4U,3C,G,2AC,A$_2$C	184-198		+	+			+				
22	2U,3C,2AC,A$_2$G						+	+	+			
23a	3U,4C,G,2AC,AU,A$_3$C	692-709	+	+	+	+				+		
23b	3U,6C,G,3AC,AU							+				
23c	3U,4C,G,3AC,A$_3$C										+	+
24	4U,5C,AC,A$_2$G		+									
25	4C,AG,A$_2$C					+						
26	2U,3C,G,AC,A$_2$C						+					
27	2C,G,4AC						+					
28	U,3C,G,AC,AU						+					
29	4C,AC,AG,								+			
30	4C,A$_2$C,A$_2$G										+	
31	3U,2C,AC,A$_2$G										+	
x	4U,3C,G,AC,2AU,A$_2$C	−(184-200)										
y	2U,C,AC,AU,A$_2$G	−(162-171)										

[a] RNA sizes, estimated from their electrophoretic mobilities in polyacrylamide gels, were as follows: PR RSV-B, 10 kb (this study); SR RSV-A, 10 kb (8, 44); SR RSV-D1, 10 kb (32); SR RSV-D2, 10 kb (this study); Bryan RSV(−)3, 8.0 kb (this study; 9, 30); Bryan RSV(−)16, 8.5 kb (this study); Bryan RSV(−)1, 8.0 kb (this study); B77 RSV, 10 kb (8, 43); rRSV 14-2 (this study; 32); and rRSV 3-3, 10 kb (this study; 32).

[b] Numbers refer to those shown in Fig. 1, 3, and 4. Allelic variants of a given oligonucleotide are distinguished alphabetically.

[c] Map positions are those of proviral DNA sequence of SR RSV-A described by Czernilofsky et al. (7).

tions of different td RSV isolates lack different amounts of *src* sequences (13, 20, 22).

The *src*-specific RNA sequences of two rRSV's and of parental SR RSV-D1 are completely allelic and differ by a few single base variations. To determine whether the *src* genes of two rRSV's derived from SR RSV-D1 via ptd RSVs might contain specific sequence elements acquired by transduction from the cell, we compared the *src* genes of rRSV 14-2 and 3-3 to that of parental SR RSV-D1 and to those of other RSVs. rRSV 14-2 contained four *src* oligonucleotides, no. 18a, 19a, 21a, and 23c, not shared with the parental SR RSV-D1 (Fig. 4; Table 1). SR RSV-D1 contained three *src* oligonucleotides, no. 19c, 21b, and 23a, not shared with rRSV 14-2 (Fig. 2; Table 1). It was possible to demonstrate that all differences that set apart rRSV 14-2 from SR RSV-D1 appear to represent single base changes. For example, although an allelic counterpart of rRSV 14-2 oligonucleotide no. 18a has not been detected in parental SR RSV-D1 in the form of a large, unique T_1 oligonucleotide, a parental sequence equivalent was indicated because SR RSV-D1 RNA was able to outcompete all rRSV 14-2 *src* sequences upon

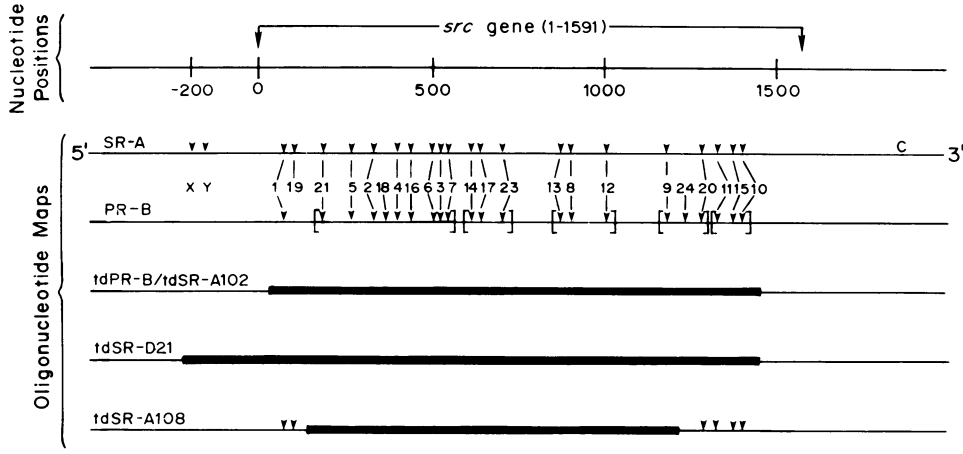

FIG. 3. *Synopsis of the nucleotide positions of the src gene sequence of proviral SR RSV-A DNA as described by Czernilofsky et al. (7), of the oligonucleotide maps of the src-specific RNA sequences of PR RSV-B and SR RSV-A, and of the src deletion map of four transformation-defective RSV mutants. The src oligonucleotide map of SR RSV-A was derived by matching oligonucleotide sequences with the known sequence of SR RSV-A (7) and of PR RSV-C (Schwarz et al., personal communication) and the oligonucleotide map of PR RSV-B. The src oligonucleotide map of PR RSV-B was obtained by hybridizing different size classes of poly(A)-tagged RNA fragments with src-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B. Oligonucleotides whose relative order within a cluster could not be determined by this method are in brackets. Oligonucleotide numbers are shown in Fig. 1, 2, and 4 and Table 1. Definition of the deletions of td RSV RNAs was by fingerprinting that part of PR or SR RSV RNA that was hybridized by src-specific cDNA's prepared with the respective td RSV RNAs as described in the text. The deletion map of the td SR RSV-A 108 was independently derived by fingerprinting the remaining src oligonucleotides recovered from src-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B RNA (Fig. 1G).*

hybridization with rRSV 14-2 *src*-specific cDNA (Fig. 4; see below). Further, an allelic sequence equivalent of rRSV 14-2 oligonucleotide no. 18a with internal Gs was directly identified in the *src* sequence of SR RSV-A (position 364 through 382 in Czernilofsky's sequence, Table 1); an equivalent without internal Gs was found in the PR RSV-C sequence at nucleotide positions 7487 to 7505 (Schwarz et al., personal communication). (The *src* gene of PR RSV-C extends from positions 7126 to 8703 in the complete sequence of 9302 nucleotides.) The internal Gs would make this sequence undetectable as a unique, large, T_1-resistant oligonucleotide in SR RSV-A. The same must be true for its equivalent in SR RSV-D1. Moreover, oligonucleotide no. 18a is present in SR RSV-D2, a clonal variant of SR RSV-D1 (see below and Fig. 2). Oligonucleotide no. 19a of rRSV 14-2 is allelic with oligonucleotide no. 19c in SR RSV-D1, from which it differs by a change from AGG to AAG. Oligonucleotide no. 19a is also found in the closely related SR RSV-A (Table 1). Oligonucleotide 21a of rRSV 14-2 is allelic with oligonucleotide no. 21b in SR RSV-D1, which is also found in SR RSV-A (positions 184 through 198, Table 1). Oligonucleotide no. 21a of rRSV 14-2 differs from oligonucleotide no. 21b of SR RSV-D1 or SR RSV-A in an AC-to-AG change in position 184 through 186 (Table 1). Oligonucleotide no. 21a was also present in SR RSV-D2, a cloned variant of SR RSV-D1 (see below and Fig. 2). rRSV 14-2 oligonucleotide no. 23c is allelic with SR RSV-D1 oligonucleotide no. 23a, because the presence of these oligonucleotides is mutually exclusive, and they differ from each other by a single base between AC and AU. Hence, we demonstrated that each of three distinctive oligonucleotides that set apart rRSV 14-2 and the parental SR RSV-D1 has an allelic counterpart in the others, i.e., rRSV 14-2 oligonucleotides no. 19a, 21a, and 23c correspond to SR RSV-D1 oligonucleotides no. 19c, 21b, and 23a. The distinctive rRSV 14-2 oligonucleotide no. 18a has an allelic sequence equivalent in SR RSV-D1 and a direct counterpart in SR RSV-D2, a clonal variant of SR RSV-D1. In addition, most of these oligonucleotides have identical or homologous T_1 oligonucleotide counterparts in other RSV strains (Table 1). The distinctive, nonparental *src* oligonucleotides of rRSV 3-3, no. 12b, 18b, 19a, 21a, and 23c, also had allelic counterparts in SR RSV-D1, rRSV 14-2, or other RSV strains as is shown in Table 1 and explained above for the allelic equivalent of no. 18a and 18b in SR RSV-D1.

Nevertheless, the above experiments did not

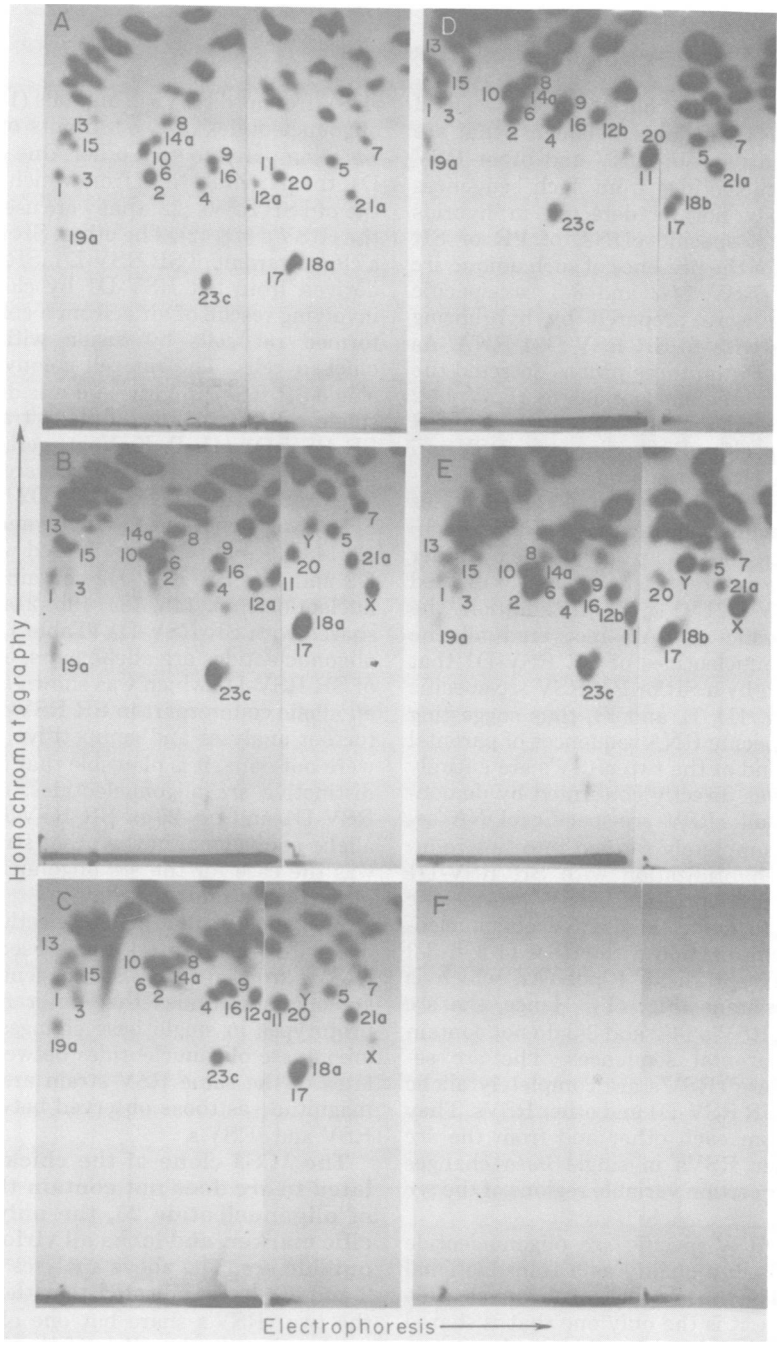

FIG. 4. *Fingerprint patterns of RNase T_1-resistant oligonucleotides of src-specific rRSV [^{32}P]RNA. A, src oligonucleotides of rRSV 14-2 recovered from src-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B RNA. B, src oligonucleotides of rRSV 14-2 recovered from src-specific cDNA prepared from rRSV 14-2 cDNA and td SR RSV-D21 RNA. Two additional oligonucleotides, x and y, are found that are not present in panel A. These are identical to x and y of SR RSV-D1 in Fig. 1E and have been shown to map outside src (Fig. 3). C, src oligonucleotides of rRSV 14-2 recovered from src-specific cDNA prepared from SR RSV-D1 cDNA and td SR RSV-D21. The fingerprint pattern is exactly the same as in panel B. D, src oligonucleotides of rRSV 3-3 recovered from src-specific cDNA prepared from PR RSV-B cDNA and td PR RSV-B RNA. Several rRSV 3-3 oligonucleotides are different from those of rRSV 14-2 (panel A). E, src oligonucleotides of rRSV 3-3 recovered from src-specific cDNA prepared from rRSV 3-3 cDNA and td SR RSV-D21 RNA. Oligonucleotides x and y are also found and are identical to those in panel B. F, Competitive hybridization between rRSV 3-3 [^{32}P]RNA and unlabeled SR RSV-D1 RNA with src-specific cDNA prepared from rRSV 3-3 cDNA and td SR RSV-D21 RNA. None of the distinctive src oligonucleotides was recovered, except poly(A), which is located at the origin of the chromatographic dimension on DEAE-cellulose.*

exclude the possibility that the rRSV's could contain unique *src*-sequence elements that are not allelic with parental RSV and other RSV strains. Oligonucleotides from such sequences would obviously not be detected in hybrids formed with *src*-specific cDNA of PR or SR RSV. To test for the presence of such unique *src* sequences in rRSV 14-2 and 3-3, *src*-specific cDNA probes were prepared by hybridizing rRSV cDNA's with td SR RSV-D21 RNA. As can be seen in Fig. 4, these probes detected the same rRSV *src* oligonucleotides in rRSV 14-2 and 3-3 as those detected by PR RSV-B or SR RSV-D1 *src*-specific cDNA. Since the rRSV *src*-specific cDNA's were prepared with td SR RSV-D21 RNA they also picked up oligonucleotides x and y. As has been shown above (Fig. 1 and 3), these oligonucleotides map outside the 5′ border of the *src* gene and represent sequences deleted in td SR RSV-D21 (Fig. 3). In addition, the rRSV *src*-specific cDNA's recovered all the same *src* oligonucleotides of SR RSV-D1 that were recovered by a SR or PR RSV *src*-specific cDNA (cf. Fig. 1D, E, and F), thus suggesting that the *src*-specific RNA sequences of parental SR RSV-D1 and of the two rRSV's are entirely allelic. This was directly confirmed by demonstrating that all rRSV *src*-specific cDNA sequences are completely formed into heteroduplexes by prehybridization with SR RSV-D1 RNA. Such prehybridized rRSV *src*-specific cDNA failed to detect any rRSV oligonucleotides upon hybridization with rRSV 14-2 or 3-3 [^{32}P]RNAs, except the [^{32}P]poly(A), which is RNase T_1 resistant (Fig. 4F). Hence, the *src* sequences of rRSV's 14-2 and 3-3 do not contain unique, nonparental sequences. The *src* sequences of these rRSV's are completely allelic with those of SR RSV-D1 and other RSVs. They only differ from each other and from the *src* genes of other RSVs in single base changes scattered over certain variable regions of the *src* gene.

The only rRSV-specific *src* oligonucleotide that could possibly qualify as a transductional marker would be the 23c allele of oligonucleotide no. 23, because it is the only one that is shared by both rRSV's and lacks an identical counterpart in parental and other RSVs.

***src*-specific sequences of SR RSV-D1 and of a clonal variant termed SR RSV-D2 differ from each other in single base variations like those that distinguish SR RSV-D1 and rRSV's 14-2 and 3-3.** To assess the likelihood that the single base differences between the *src* oligonucleotides of rRSV's 14-2 and 3-3 and of the parental prototype SR RSV-D1 may be the result of mutations arising under the strong selective conditions used to generate rRSV from ptd RSVs in animals (15, 41), the *src* oligonucleotides of two variants of SR RSV-D were compared to each other. One was SR RSV-D1, the parental strain from which were derived the ptd SR RSVs (22) that were used to generate the rRSV's (41, 42). The other, SR RSV-D2, was a clonal variant of SR RSV-D1. SR RSV-D2 was derived from SR RSV-D1 by clonal selection involving rescue of virus from a colony of transformed rat cells by fusion with susceptible chicken cells. The rat cell colony contained a single SR RSV provirus and was derived from a single cell of a culture initially transformed by SR RSV-D1 (41; P. K. Vogt, personal communication). Comparisons of the *src* oligonucleotides of SR RSV-D1 and RSV-D2 indicated that SR RSV-D1 contained four oligonucleotides, no. 13, 14a, 19c, and 21b, not shared with SR RSV-D2 and that SR RSV-D2 contained five oligonucleotides, no. 14b, 18a, 19b, 21a, and 25, not shared with SR RSV-D1 (Table 1). All lettered oligonucleotides are allelic pairs, except no. 18a of SR RSV-D2 which was shown above to have an allelic counterpart in SR RSV-D1. Although further analyses and competitive hybridization were not done, it is plausible that the remaining distinctive *src* oligonucleotides, no. 13 of SR RSV-D1 and no. 25 of SR RSV-D2, also have allelic equivalents in the other strain since this was the case for the *src* oligonucleotides of all other RSV strains tested.

We conclude that clonal selection under conditions as stringent as those used to generate rRSV's from ptd RSV stocks generates variant *src* oligonucleotides that differ from parental prototypes in single base changes. Differences among *src* oligonucleotides between clonal isolates of the same RSV strain are of the same magnitude as those observed between parental RSV and rRSV's.

The λCS3 clone of the chicken locus related to *src* does not contain the 23c allele of oligonucleotide 23, the only rRSV-specific marker, and lacks all virion sequences outside *src*. The above analyses of rRSV's 14-2 and 3-3 have indicated that the *src* genes of the two rRSV's share but one oligonucleotide marker, i.e., the 23c allele of oligonucleotide no. 23, that was not found in parental RSV or any other RSVs (Table 1). Other RSVs contained either the no. 23a or 23b allele or undetected variants of this sequence (Table 1). The same oligonucleotide no. 23c was also detected previously in these rRSV's (32, 41) and in two of seven rRSV's described by Wang et al. (47).

On this basis it has been argued that this oligonucleotide is a marker of novel, cell-derived *src* sequences (41, 47). To test this possibility directly we have asked whether the 23c allele

can be detected in the *src*-related locus of the chicken, recently cloned in lambda phage by Shalloway et al. (35). For this purpose rRSV 14-2 RNA was hybridized to DNA of the λCS3 clone of the *src*-related locus. After treatment of the reaction mixture with RNase T_1 or with RNases A, T_1, and T_2, the resistant hybrids were isolated by chromatography, and the RNA of hybrid was fingerprinted as described above. The T_1-resistant hybrid indeed contained all of the rRSV 14-2 *src* oligonucleotides identified above (Table 1), including no. 23c (Fig. 5A). By contrast an RNase A-, T_1-, and T_2-resistant hybrid formed with λCS3 lacked only oligonucleotide no. 23c (Fig. 5B), whereas an RNase A-, T_1-, and T_2-resistant hybrid formed with rRSV 14-2 *src*-specific cDNA contained oligonucleotide no. 23c (Fig. 5C).

Since the λCS3 DNA hybridized all *src* oligonucleotides of rRSV 14-2, we confirm and extend the conclusion reached by Shalloway et al. (35) that the complexity of the *src*-related sequences of the chicken locus and of viral *src* are about the same. Further, it is concluded that the 23c allele of *src* oligonucleotide no. 23 is not present in the chicken *src*-locus λCS3. The absence of 23c in the λCS3-rRSV 14-2 hybrid is particularly convincing, since all other rRSV 14-

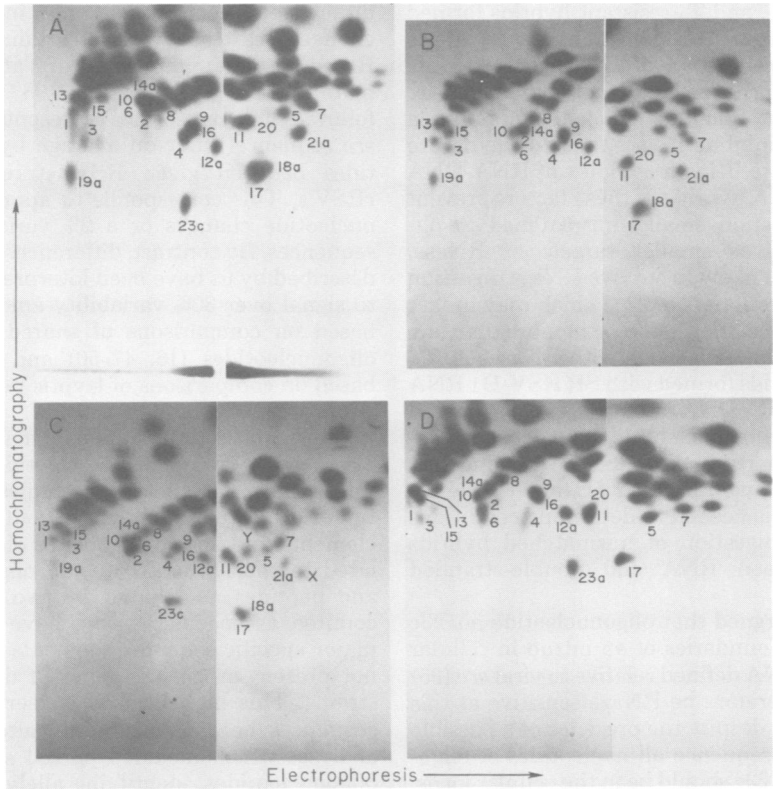

FIG. 5. *T_1 oligonucleotides of rRSV 14-2 RNA recovered from RNase T_1-resistant (A) and RNase A-, T_1-, and T_2-resistant hybrids (B) formed with DNA of the cellular src-related locus of the chicken cloned in lambda phage (λCS3). Two control experiments show rRSV 14-2 src oligonucleotides recovered from an RNase A-, T_1-, and T_2-resistant hybrid formed with rRSV 14-2 src-specific cDNA (C) and SR RSV-D1 src oligonucleotides recovered from an RNase A-, T_1-, and T_2-resistant hybrid formed with PR RSV-B src-specific cDNA (D). The λCS3 hybrids were formed by incubating 0.5 μg of rRSV 14-2 [^{32}P]RNA (2 × 10^6 cpm) with 20 to 40 μg of λCS3 DNA containing 0.6 to 1.2 μg of src-related DNA which had been degraded to <1,000 nucleotide fragments by incubation in 0.3 N NaOH at 95 to 100°C for 10 to 15 min, in 10 to 20 μl of 70% formamide containing 0.3 M NaCl–0.03 M sodium citrate–20 mM sodium phosphate (pH 7) at 40°C overnight. Digestion of the resulting hybrids was at 0.1 to 0.2 μg of nucleic acid per μl in 0.3 M NaCl–0.03 M sodium citrate (pH 7) at 40°C for 45 min either with RNase T_1 at 50 U/ml or with RNase A at 50 μg/ml, RNase T_1 at 50 U/ml, and RNase T2 at 10 to 25 U/ml. After digestion the solution was extracted once with phenol-chloroform, and the hybrid was isolated by chromatography on Bio-Gel P100 equilibrated with 0.25 M NaCl and otherwise as described in the text. The RSV RNA-cDNA hybrids were prepared by the same method, except that RNase digestion was at <0.1 μg of nucleic acid per μl.*

2 *src* oligonucleotides of the hybrid survived the RNase treatment. Nevertheless, it does not appear plausible that, except for 23c, the cellular locus would contain all rRSV 14-2 specific alleles of the variable *src* oligonucleotides listed in Table 1. This may reflect incomplete digestion of the smaller *src* oligonucleotides in possibly mismatched regions of rRSV RNA-λCS3 DNA hybrids. This is suggested because under the same conditions of digestion we recovered with λCS3 DNA the 21b allele of SR RSV-D1 (data not shown) and recovered the 21a allele of rRSV 14-2, and because in this (Fig. 5B) and in other similar experiments some *src* oligonucleotides were present at less than equimolar ratios in RNase A-, T_1-, and T_2-resistant hybrids formed with λCS3. Incomplete digestion may be due to the high concentration of DNA, including six non-*src* sequences that interrupt the chicken *src* locus (35) and flanking chicken and lambda DNA, which inhibits RNase, and to the nature of the substrate being a complex of RNA-DNA and DNA-DNA hybrids. If these factors prevent complete digestion, smaller mismatched *src* oligonucleotides, as smaller targets of RNase, would be more likely to survive RNase digestion than the largest one, no. 23c, which may in fact differ from its cellular homolog in more than one base. By contrast, digestion with RNases A, T_1, and T_2 of hybrids formed with SR RSV-D1 RNA and *src*-specific cDNA of PR RSV-B eliminated all rRSV *src* oligonucleotides that lacked identical counterparts in PR RSV-B (oligonucleotides no. 19c and 21b; Fig. 5D and Table 1). Further work is needed to determine conditions of complete digestion of mismatched hybrids formed between RNA and double-stranded DNA.

It may be argued that oligonucleotide no. 23c includes the boundaries of an intron in cellular *src*-related DNA defined relative to viral *src* (35) and would therefore be RNase sensitive at this site. However, despite the presence of a possible intron in this sequence all nucleotides of oligonucleotide no. 23c should be in the cellular locus, if it were its transductional origin, and therefore should have been base paired in the hybrid. Such a hybrid would remain RNase resistant (C. A. Dekker, personal communication).

With regard to the hypothesis that the cellular *src*-related locus is transducible by ptd RSVs, it is important to point out that not a single non-*src* oligonucleotide of rRSV 14-2 (Fig. 5A and B) or of several other RSVs tested (data not shown) was hybridized by λCS3 DNA. Not even the oligonucleotides x and y, which map immediately adjacent to the 5′ end of *src* in viral RNA (Fig. 2) were recovered, although the λCS3 clone contains about 10 kb of chicken DNA mapping at the 5′ end of the *src*-related locus and with some uncertainty about 0.5 to 1 kb of chicken DNA flanking its 3′ end (35; G. Cooper, personal communication). Hence, *src* transduction by ptd RSVs lacking one or both *src* termini (16, 47; see below) would require illegitimate recombination.

DISCUSSION

Conserved and variable *src* gene elements. The results described here and the identification of highly conserved *src* oligonucleotides (43, 49) and RNA-cDNA hybridizations published previously (38) indicate that the *src*-specific RNA sequences of different RSV strains are highly conserved and completely allelic and differ from each other only in single base changes scattered within certain *src* regions. Based on data summarized in Table 1, we estimate that *src* genes are about 98% conserved as follows. Oligonucleotides representing 15% of the *src* sequence differ on average by five nucleotides between given RSV strains, including rRSV's. This corresponds to approximately 30 nucleotide changes or a 2% variability in *src* sequences. By contrast, differences such as those described by us have been interpreted by others to signal over 30% variability among *src* genes based on comparisons of shared and specific oligonucleotides (16, 47–50) and of over 50% based on comparisons of tryptic peptides of p60 *src* proteins (1, 34, 42).

There are two possible explanations for this discrepancy. (i) The oligonucleotide and peptide fingerprinting in the above studies did not distinguish between nonidentical, allelic sequence elements and specific sequence elements unrelated to each other. Thus, all oligonucleotides and peptides not shared by two viruses were counted as specific. As we have shown here, major specific sequence elements are probably not present in the *src* genes of different RSV strains. This high level of conservation of *src* sequences became clear only after secondary analyses which included partial sequencing of oligonucleotides, identifying allelic pairs of oligonucleotides on oligonucleotide maps derived by fingerprinting poly(A)-tagged RNA fragments as well as by identifying their position in the nucleotide sequence of viral DNA, and by competitive hybridization of *src*-specific RNAs from different RSV strains with *src*-specific cDNA. Hence, counting all nonidentical oligonucleotides of two *src* genes without distinguishing allelic and nonallelic variants must lead to an overestimate of sequence diversity. Since the amino acid sequences of viral *src* peptides and proteins were not analyzed, most, if not all, *src* peptides reported to be distinctive (1, 33, 34, 42) as well as the distinctive serological markers (24,

31, 33) of different *src* proteins probably also represent allelic variants of highly conserved sequences.

(ii) A second reason for the overestimate of specific *src* sequences in some previous studies is that the materials analyzed included *src* as well as non-*src* elements. For example, *src* oligonucleotides were identified on fingerprints of total viral RNA or of poly(A)-tagged fragments of RNA which include adjacent 3'-terminal and also 5'-terminal non-*src* elements (16, 42, 47–50). Thus, some non-*src* oligonucleotides have been counted as *src* oligonucleotides in these studies. For instance, several large, presumably rRSV-specific *src* oligonucleotides identified in poly(A)-tagged RNA fragments in one previous study (42) were not detectable by us studying *src* specific oligonucleotides of the same viral RNAs by fingerprinting a T_1-resistant hybrid formed with *src*-specific cDNA. These rRSV oligonucleotides were possibly from the *env* region (see comparisons below). Another study considered an oligonucleotide, termed no. 27 (in references 16 and 47), as a 3'-terminal *src* oligonucleotide. However, this oligonucleotide probably does not belong to the coding sequence of *src* since it was not discovered by our cDNA probes and λCS3 DNA and since it is located in the proviral DNA sequence of SR RSV-A outside the 3' border of *src* at positions 1,602 through 1,612 (7). Likewise, since *src* peptide analyses were carried out with *src* proteins translated in vitro from viral RNA, other non-*src* proteins may have been present, as suggested by the electrophoretic heterogeneity of the starting materials (42) and by the presence of specific peptides that are absent from authentic *src* proteins isolated from infected cells (33, 42). Such contaminants could include products of premature chain termination of other viral proteins also encoded by viral RNA (29).

Are the nonparental *src* oligonucleotides of rRSVs transduced from cellular *src*-related sequences? (i) Lack of evidence for unique rRSV-specific *src* oligonucleotides. Only scattered single base variations were observed between *src* sequences of parental RSV and two rRSV's. Hence, the *src* genes of the two rRSV's studied by us lack identifiable markers other than scattered single-base differences to demonstrate their putative transductional origin. This proves correct the view considered by us in a previous study that distinctive oligonucleotides of these two rRSV's are allelic with their parental counterparts and hence may be derived from parental *src* genes (see below) rather than representing unique sequence elements derived from the cell (32). By contrast, other investigators have considered distinctive *src* oligonucleotides and peptides of the rRSV's studied here (41, 42) and of other rRSV's (16, 41, 42, 47–50) as specific or novel (41, 42) sequences and have held the view that these are specific markers of *src* sequences transduced from the cell.

Here we present a critical comparison of our results with those described previously to determine whether the distinctive *src* oligonucleotides of rRSV's described by others may indeed represent unambiguous markers of cellular *src*-related sequences that could not have been derived from parental *src* genes by point mutations. The *src*-specific oligonucleotides of rRSV's 14-2 and 3-3 (Table 1) include the nonparental *src* oligonucleotide no. 23c detected previously in rRSV 14-2 and termed no. 11 by Robins and Duesberg (32) or no. 5c by Vigne et al. (41, 42). We have shown here that this oligonucleotide is allelic with the oligonucleotide no. 23a of the parental SR RSV-D1. Three other *src*-specific oligonucleotides of rRSV's were described by Vigne et al. (41, 42); two of these, no. 5a and 5d, are mixtures and hence cannot be unambiguously compared with ours. A third one, no. 5b, was found in rRSV 14-2. It is probably the same as our parental SR RSV-D1 oligonucleotide no. 23a since it has the same chromatographic properties as no. 23a, although it is reported to have an A_2C rather than an A_3C element as does our no. 23a oligonucleotide. Since the rRSV 14-2 stock studied by Vigne et al. (41, 42) was reported to contain no. 5c as well as its probable parental equivalent, no. 5b, it is possible that the rRSV 14-2 stock studied by Vigne et al. contained parental SR RSV-D1 or that the *src* genes of a part of the rRSV population in this stock had mutated back to the parental phenotype. Three other large oligonucleotides of rRSV 14-2 and 3-3 described by Vigne et al. (42), no. 1b, c, and d, were not discovered in the *src*-specific RNA of the same viruses by our methods; neither are they detectable in the sequences of Czernilofsky et al. (7) and of Schwarz et al. (personal communication). Probably, these oligonucleotides derive from neighboring, *env*-related sequences, a possibility acknowledged by Vigne et al. (42).

The *src* oligonucleotides identified by us also include five of the six nonparental *src* oligonucleotides found in rRSV's described by Wang et al. (47, 48). Their oligonucleotides no. 8c, 10c, 34 (47), and Q2 (48) are the same as our oligonucleotides no. 23c, 18a, 21a, and 17, respectively (Table 1). Three of these, no. 17, 18a, and 21a, have been detected by us in a number of known RSV strains and are consequently not likely to be unambiguous markers of cellular *src*-related sequences. Moreover, no. 17, considered to be a

marker of a quail-specific *src* sequence and hence termed Q2 by Wang et al. (48), was present in our SR RSV-A strain, in our rRSV 14-2 with a *src* sequence presumably recovered from chicken (42), and in our SR RSV-D1 and in other RSV strains (Table 1). Further Q2 appears at least allelic if not identical with a parental counterpart, no. 10a (48), since the presence of these oligonucleotides in quail-recovered and parental RSVs is mutually exclusive (48). Oligonucleotide no. 18a was shown to have an allelic sequence counterpart in DNA sequence positions 364 through 382 of SR RSV-A, the parental virus from which the rRSV's of Hanafusa et al. (15, 16) are derived. In SR RSV-A this sequence carries internal Gs and therefore does not appear as a large oligonucleotide in T_1 digests of SR RSV-A RNA, but it does appear in our SR RSV-D2, in PR RSV-B, and Bryan RSV(−)1, 3, and 16 (Table 1). Oligonucleotide no. 21a is present in our PR RSV-B, SR RSV-D2, and B77. It differs from oligonucleotide no. 21b of SR RSV-A, or SR RSV-D1 and RSV(−)16, in an AC-to-AG change (Table 1). No. 23c is the only oligonucleotide that we (Table 1; 32) and Wang et al. (47) have found exclusively in rRSV's, but not in all rRSV's (47, 48). However, this oligonucleotide, too, has an allelic equivalent in SR RSV-A and SR RSV-D1 and other RSV strains, no. 23a and 23b (Table 1). Moreover, we have shown that the rRSV-specific allele of this oligonucleotide does not have an identical counterpart in the cloned *src*-related chicken locus studied here. Therefore, it cannot be considered a marker for transduction.

A fifth rRSV-specific *src* oligonucleotide described by Wang et al. (47, 49), no. 15a, appears to be the allelic equivalent of our highly conserved *src* oligonucleotide no. 12a (Table 1), if we assume an AU-to-AC and a U-to-C change. (The latter difference might well be a technical artifact characteristic of our common methods of analyzing RNase A-resistant fragments by DEAE-paper electrophoresis.) This is even more plausible since Wang et al. (47) have analyzed essentially the same SR RSV-A strain as we did, yet we did not find their no. 15a and they did not find our no. 12a. There is only one other *src* oligonucleotide described by Wang et al., no. Q1, which is thought to be rRSV specific because it was isolated from a virus recovered from a quail tumor (48), which has not been discovered in any RSV strains analyzed by us or in the RSV sequences of Czernilofsky et al. (7) and of Schwarz et al. (personal communication), assuming no more than a single base difference. However, since it was later acknowledged by Wang et al. (49, 50) that this oligonucleotide is also present in an RSV stock that had been thought to be Fujinami sarcoma virus and was propagated in chicken cells, it is likely that even this oligonucleotide is not a specific marker of a cellular *src*-related sequence.

We conclude that all nonparental *src* oligonucleotides of rRSV's analyzed by us and most, if not all, of those analyzed by others are allelic with *src* oligonucleotides of parental RSVs with only scattered single nucleotide differences. Moreover, transduction could not be validated as the origin of the only presumably transduced rRSV-specific *src* marker detected here, oligonucleotide no. 23c, because it lacked an identical counterpart in the cloned cellular *src* locus studied by us. Therefore, we submit that the distinctive oligonucleotides associated with rRSV's are not valid markers of transduction since they may be derived from parental *src* genes by point mutations.

(ii) The number of presumably transduced, *src*-specific oligonucleotide markers of rRSV's exceeds that of known cellular *src*-related loci. Even if one considers nonparental *src* oligonucleotides differing from parental allelic equivalents in single base changes as sufficient markers of cellular *src*-related sequences, the transductional origin of these markers is not readily reconciled with the pattern of their appearance in rRSV's. If the *src* genes of rRSV are indeed derived (from the cell) via ptd RSV lacking over 75% of *src* (16, 20, 22, 32, 47), and the cell contains only one or, at most, a few copies of *src*-related sequences per haploid genome (18, 35, 37), it would follow that all or most rRSV's share at least 75% of the cellular *src*-specific markers. Since the two rRSV's analyzed by us each contain different sets of allelic variants of the same *src* oligonucleotides (i.e., rRSV 14-2 contains no. 12a and 18a, whereas rRSV 3-3 contains no. 12b and 18b [Table 1]), each of these viruses must have recovered its *src* gene from a different cellular locus (if the distinctive oligonucleotides were indeed markers of cellular *src*-related sequences). Others have described further *src* oligonucleotides not shared by all of their rRSV's or by those analyzed here, i.e., no. 8a, 8c, 15a (47, 48), and 5b (41, 42) (which are allelic with our oligonucleotides no. 23, 23, 12, and 23, respectively; see above and Table 1), implying the existence of additional distinct cellular *src*-related loci. Since the number of cellular *src* loci necessary to explain the transductional origin of all nonparental *src* oligonucleotides of rRSV's appears larger than the number of known cellular *src* loci, it would follow that the nonparental *src* oligonucleotides of rRSV's reflect either variable or polymorphic cellular *src*-related sequences and hence could not serve as

reliable markers for transduction or else that these oligonucleotides are not transduced from the cell.

Further, if recombination between viral and cellular *src*-related sequences was as common as suggested by the hypothesis that ptd RSVs transduced cellular *src* sequences at predictable frequency (15, 16, 41), it would be expected that the *src* genes of different RSV strains would have converged upon passage in animals or cell culture to assume the oligonucleotide pattern typical of cellular *src*-related sequences. The fact that this was not observed (Table 1) again suggests either that the *src* genes of exogenous viruses do not readily recombine with the cellular relatives or that the cellular *src*-related sequences show the same degree of variability as viral *src* genes, in which case the variable allelic oligonucleotide markers described here and elsewhere could not be valid markers of transduced cellular *src*-related sequences.

(iii) The question of clonal purity of the ptd *src* deletion mutants used to generate rRSV. In principle two methods could be used to substantiate the claim that rRSV's contain *src* sequences transduced from the cell. (i) If unique rRSV *src* oligonucleotides could be found that are not in other RSVs a single cellular sequence of origin might be identified. We have not been able to find such markers in the rRSV's analyzed here, nor is there unambiguous evidence for such markers in rRSV's described by others. (ii) Alternatively, unambiguous proof of transduction of cellular information could be obtained if a pure partial *src*-deletion mutant was inoculated into an animal from which an rRSV with a complete *src* gene was recovered. This alone would substantiate the claim that *src*-related sequences were transduced from the cell. Although the predominant viral species in the ptd RSV stocks used to generate rRSV's lacked over 75% of the viral *src* genes (20, 22), it cannot be excluded that minor components carrying the remaining *src* sequences were also present in these ptd RSV stocks since these viruses were not molecularly cloned. This is suggested because long-term persistence of stable heterozygotes has been described previously in retroviruses subjected to extensive biological cloning (25, 27) even under highly selective conditions nonpermissive for the host range of one of two viral components (40; Duesberg and Vogt, unpublished data). Heterogeneity has been biochemically demonstrated by fingerprinting T_1 oligonucleotides of the ptd SR RSV-D14 used to generate the rRSV 14-2 (32), although it had been biologically cloned by plaque purification (22, 41, 42). A similar heterogeneity was biochemically detectable in ptd SR RSV-A 107 despite biological cloning by endpoint dilution (20). Likewise, the cloned rRSV 14-2 stock described by Vigne et al. (42) was heterogeneous, because it contained two allelic variants of the same oligonucleotide (5b and 5c; see above). Thus, unambiguous evidence for neither marker rescue nor the clonal purity of the ptd RSV stocks used to generate rRSV is available to substantiate the claims that rRSV's contain transduced cellular *src*-related sequences.

(iv) Cellular versus viral origin of *src* genes of rRSV's. On the basis of our results, that the *src* genes of rRSV's and of parental rRSV's are completely allelic with only scattered single base variations, and that the complexities of *src*-related sequences of the cell and of viral *src* are about the same, we cannot distinguish between two possible origins of the *src* genes of rRSV's: transduction of cellular sequences, very similar to viral *src* genes, or recovery by cross-reactivation of parental deletions or back-mutations of parental variants, whose presence in stocks of ptd RSVs has not been excluded.

In the following we compare the merits of the two alternatives. Although *src* transduction is formally possible, since the complexity of *src*-related sequences of the cell and of viral *src* are about the same, it appears genetically very complicated for the following reasons. Viruses lacking one or both *src* termini would have to rely on illegitimate recombination to transduce *src*, since the cellular locus is not flanked by endogenous viral sequences other than *src* (18, 19, 35). We have shown here at a higher level of resolution that the cloned cellular *src*-related locus studied by us lacks any detectable viral non-*src* oligonucleotides, including x and y, which are immediately adjacent to the 5′ end of *src*.

Several lines of evidence cannot be reconciled with the transduction hypothesis. As pointed out above the only rRSV-specific oligonucleotide marker identified by us and others studying the same rRSV's (41, 42), the no. 23c allele of *src* oligonucleotide no. 23, is not present in a molecularly cloned, *src*-related locus of chickens. Also, we pointed out that the apparent discrepancy between the number of nonparental, presumably transduced *src*-markers of rRSV's described by us and others (over five) and the number of cellular *src*-related loci (one or at most two) argues against the transductional origin of these markers.

In addition it is difficult to reconcile available data on the genetic structure of ptd RSVs with their known frequencies and presumed mechanism of transduction. It has been postulated that residual, terminal *src* sequences of ptd RSVs are necessary for transduction (16, 41, 42, 47). These were located at the 5′ end of *src* in ptd RSVs

studied by one group (22, 41) and at the 3′ end in ptd RSVs studied by the other group (16, 47). Specifically, it was postulated by Hanafusa et al. (16) and Wang et al. (47) that a putative *src* oligonucleotide marker from the 3′ end of *src*, oligonucleotide no. 27, is required because all ptd RSVs studied by these investigators share this oligonucleotide but vary in other residual *src* oligonucleotides. However, as we point out above this oligonucleotide was not detected by our *src*-specific cDNA and λCS3 DNA hybrids. Instead, it was detected outside the 3′ border of *src* in the adjacent *c* region at positions 1,602 through 1,612 of the sequence of Czernilofsky et al. (7) (Fig. 3). Its presence in all ptd RSVs described by Hanafusa et al. might reflect the essential role of the *c* region in replicating viruses (43). In view of this, it would appear that three classes of ptd RSVs transduce *src* at the same predictable frequencies of >50% within 2 months of inoculation of viruses into the animals (15, 16); those which lack verifiable oligonucleotide markers of either *src* terminus like td SR RSV-A 105 (16, 47, 50), those which lack the 5′ terminus but contain markers of the 3′ terminus of *src* like td SR RSV-A 101 and 109 (16, 47, 50), and those which retain both *src* termini like td SR RSV-A 108 (Fig. 2) (16, 47, 48, 50). It appears surprising that each of these classes of deletions is said to transduce at the same frequency (15, 16), because double illegitimate recombination would be necessary for *src* deletions with no residual *src* termini, single illegitimate recombination would be necessary for *src* deletions with one residual *src* terminus, and homologous, asymmetric recombination would suffice for transduction by *src*-deletions with both *src* termini.

Finally, the six intervening sequences of the cellular *src* locus would have to be eliminated to make the cellular *src* DNA colinear with viral *src* (35). Since the cellular *src*-related mRNA is about twice the size of viral *src* mRNA (51) and hence may not be colinear with *src*, it cannot be assumed that regular splicing removes all introns of the cellular *src*-related locus. There is also no evidence that the cellular *src*-related protein (34) functions like the viral p60 *src* protein.

Experimentally reproducible *src* transduction by ptd RSV with a 3′ *src* terminus is particularly incompatible with the total lack of evidence for *src* recombination between *env*-defective Bryan RSV(−) and its helper virus or between Moloney leukemia and sarcoma virus, which has a genetic structure similar to RSV(−) (8, 25). Since both RSV(−) (10, 26, 44) and Moloney sarcoma virus (25) share sequences adjacent to the 3′ boundary of *src* with their respective helper viruses, they should each be able to form nondefective sarcoma viruses by one homologous recombination near the 3′ end of *src* and one illegitimate recombination at 5′ end of *src*. Yet there is no evidence for the formation of nondefective RSV (26) or Moloney sarcoma virus (25) despite extensive passage of these viruses in animals and cell culture (39a). This type of recombination should, in fact, be more frequent than that reported between ptd RSV and cellular *src*-related sequences, because two viruses are more likely to interact with each other than are a virus and a cellular gene, and because no sequences of nonhomology need to be removed from viral *src*.

In view of our results, alternate hypotheses, some of which we proposed previously (8, 32), are tenable to explain the phenomenon of generating rRSV's from ptd RSV. For example, rRSV may have been generated by cross-reactivation among nonoverlapping *src* deletions involving asymmetric recombination (13, 26) or by mutations of nontransforming variants with complete *src* genes which may be present in the ptd RSV stocks inoculated into animals. (Indeed, a nontransforming variant of rRSV, td 113, with a complete *src* gene has been observed by Wang et al. [47].) Such components might be present as minor components in stocks of ptd RSVs and may therefore escape biochemical detection which is usually limited to a range of 2 orders of magnitude, whereas biological assays may detect minor components present at concentrations of less than 10^{-6}. The presence of biochemically detectable contaminants in some biologically cloned stocks of ptd RSVs has been documented above. The single base differences observed between the *src* oligonucleotides of rRSV and parental RSVs could be the products of these processes or could reflect spontaneous mutations in variable *src* regions which arose during the selection process that led to the formation of tumorigenic rRSV in the animal.

The occurrence of spontaneous mutations in *src* oligonucleotides upon clonal selection under conditions as stringent as those that led to the formation of rRSV from ptd RSV was shown here for SR RSV-D2, a clonal variant of SR RSV-D1. The single nucleotide differences observed among the *src* genes of the six other laboratory strains of RSV examined here may also reflect point mutations arisen upon clonal selections. Single base changes also occur regularly in oligonucleotide sequences of other avian tumor viral genes after passage of viruses under selective conditions (6, 9, 11, 40) and have been observed previously (32) and here in comparisons between rRSV's 3-3 and 14-2 and parental SR RSV-D1 (data not shown). Furthermore, single base changes set apart known allelic oligonucleotide sequences of many related avian

tumor viruses (5, 6, 46; compare 36 and 39). Likewise, spontaneous mutations of oligonucleotides were recently observed upon clonal passage of influenza virus (2).

The failure of some ptd RSVs (15, 16) and of all complete *src* deletions of RSV to generate rRSV's may then simply reflect their clonal purity. Since deletion of the *src* gene has been shown to proceed via partially deleted intermediates (6), stocks of ptd RSVs are more likely to be heterogeneous, i.e., containing intermediates with nonoverlapping *src* deletions that could regenerate *src* by cross-reactivation, than the terminal products of this process, i.e. td RSVs with complete *src* deletions.

Hence, to prove transduction of cellular *src*-related sequences, it would be necessary to find definitive markers or to reproduce the phenomenon with molecularly cloned ptd RSVs.

Origin of RSV. Although our evidence casts doubt on the idea that the distinctive oligonucleotides of the *src* genes of rRSV's originated by experimental transduction of a cellular sequence, the close relationship between viral *src* and cellular *src*-related sequences argues that *src* genes of RSV originated from the cell at one time in evolution. However, the generation of RSV from cellular *src*-related sequences and a retrovirus without *src* must be a rare and genetically complicated event, because it would require illegitimate recombination and deletion of non-*src* sequences that interrupt the *src*-related sequence of the cell.

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ADDENDUM IN PROOF

A recent analysis of the *src* peptides of recovered RSVs by R. Karess and H. Hanafusa (Cell **24**:155–164, 1981) indicates that of about 20 rRSV *src* peptides resolved, 17 are shared with parental RSV, 1 to 2 are specific, and 2 resemble peptides of a cellular protein serologically related to the viral *src* protein. Although these results are in good agreement with the oligonucleotide analysis described herein, they were interpreted as evidence for the cellular origin of rRSV *src* genes.

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