Partial Nucleotide Sequence of Rous Sarcoma Virus-29 Provides Evidence that the Original Rous Sarcoma Virus Was Replication Defective

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Rous sarcoma virus-29 (RSV-29) is the strain of RSV that has the least number of passages beyond its isolation from chicken tumor no. 1 among all current strains of RSV. Biological characterization indicated that it was replication defective. RNA analysis of nonproducer clones of RSV-29-infected chicken embryonic fibroblasts showed the presence of a subgenomic message of 2.6 kilobases containing *src* and a genomic RNA of 7.7 kilobases that contains *gag*, *pol*, and *src*, but not *env*. The *src*-containing *Eco*RI fragment of RSV-29 proviral DNA was molecularly cloned. Sequence analysis of the regions flanking *src* revealed that the *env* gene was completely deleted in RSV-29 and that the sequence across the deletion was exactly the same as the Bryan high-titer strain of RSV. The sequence immediately 3' to *src* in RSV-29 was closely related to that of the Prague strain of RSV. The fact that the strain of RSV which has the minimal number of passages beyond its isolation is replication defective supports the hypothesis of Lerner and Hanafusa (J. Virol. 49:549-556, 1984) that the original RSV is a defective transforming virus. This defective transforming virus is postulated to be the precursor to other defective RSVs like the Bryan high-titer strain and to nondefective RSVs like the Prague strain. The particular clone of RSV-29 that we studied also had a short stretch of sequence duplication at the 3' end of the *pol* gene, which was presumably created by an error of reverse transcription.

In most acutely transforming retroviruses, acquisition of an oncogene from host genomic sequences by a helper virus has resulted in the deletion of one or more genes essential for replication. As a result, these viruses are usually replication defective (11, 17, 24). The common strains of Rous sarcoma virus (RSV), Schmidt-Ruppin (SR-RSV) (22) and Prague (PR-RSV) (12), are anomalous in that they contain an oncogene and yet preserve all the genes for replication (34). However, the Bryan high-titer strain of RSV (BH-RSV) has been found to lack the *env* gene, rendering the virus replication defective (4, 8, 21). BH-RSV could have been a deletion variant derived from nondefective RSV. However, the possibility that BH-RSV arose by an independent transduction of *c-src* by a nondefective helper virus could not be ruled out. Sequence analysis of molecularly cloned BH-RSV proviral DNA has defined the exact nature of this deletion. The DNA sequence has also revealed extensive homology between the sequence from the 3' end of *src* to the long terminal repeat in BH-RSV and the corresponding sequence at the 3' end of a helper virus, Rous-associated virus 2 (RAV-2). This observation suggested the steps by which recombination between the helper virus and the 3' end of *c-src* message produced the 3' end of BH-RSV (1, 13).

RSV-29 is a variant of RSV obtained from a stock of Rous chicken tumor no. 1 that was dried and stored since 1929. In 1963, one ampoule of this dried tumor was suspended in buffer and injected into chickens. Extracts of the tumors that developed contained viable tumorigenic virus, which was called RSV-29 (26).

We found that RSV-29 is also replication defective. We therefore decided to examine RSV-29 for the nature of its defectiveness and for the structure of its 3' end to define its

relationship to other strains of RSV and possibly to refine our views regarding the putative recombinations that took place between the helper virus and the genomic *c-src* sequences to give rise to the RSVs.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEF) were from *gs⁻chf⁻* embryos (SPAFAS, Inc., Norwich, Conn.) and were grown before and after transformation in Scherer medium as described previously (7), but the 1:1 mixture of Ham F-10 and Dulbecco modified Eagle medium or minimal essential medium were also used in some experiments. RSV-29, provided by R. M. Dougherty, is only two tissue culture passages beyond a chicken tumor induced with a dried preparation stored by W. J. Purdy in 1929 (26).

Cloning of RSV-29-infected chicken cells. A secondary culture of CEF was infected with RSV-29 and its helper virus at high virus dilution (multiplicity of infection of 10^{-4} to 10^{-5} focus-forming units per cell). After 5 h, the infected cells were trypsinized and plated out at a density of 10^6 cells per 100-mm dish and overlaid with minimal essential medium containing 0.36% agar. Individual colonies were picked after 14 days of incubation and were cultured separately on a feeder layer of uninfected CEF. Clones of cells that were morphologically transformed but did not produce focus-forming virus were superinfected with helper virus RAV-2. Clones that consistently produced infectious focus-forming virus on superinfection with RAV-2 but that did not do so without helper virus were designated nonproducer (NP) clones and were used for studies on viral RNA. Proviral DNA was prepared from NP clone 1.2.

Preparation of DNA probes. Viral DNA probes (see Fig. 1B) were prepared from the following viral DNA plasmid clones: pSRA-2, which contains the entire SR-RSV-A (SF) genome (3); pFC3, which contains the 2.5-kilobase-pair

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(kbp) *EcoRI* DNA fragment of SR-RSV-A (NY) containing the 5' region of *gag* (obtained from F. Cross); pLTR-L6, which contains the *PvuII*-to-*EcoRI* DNA fragment of the SR-RSV-B U3 region (obtained from T. Takeya); and pTT107, which contains the 3.1-kbp *EcoRI* fragment of SR-RSV-A (NY) containing the *env-src* region (31). DNA fragments representing defined regions of the viral genome were prepared by digestion of the above plasmid DNAs with appropriate restriction enzymes followed by agarose gel electrophoresis and electroelution. The purified viral DNA fragments were labeled with [32 P]dCTP by nick translation (14).

Viral RNA analysis. Total poly(A)-containing RNAs were prepared from two to four 10-cm dishes of RSV-29-infected CEF according to the procedures described previously (33). Portions (5 to 10 μ g) of each poly(A)⁺ RNA preparation were used for analysis by agarose gel electrophoresis, Northern blotting, and hybridization with various viral DNA probes (Fig. 1). RNase T₁ resistant oligonucleotide fingerprinting has been described previously (32).

Molecular cloning of RSV-29 proviral DNA fragment. Total cellular DNA was extracted from RSV-29 clone 1.2 as described previously (25) and was digested to completion with an excess of *EcoRI*. A fraction of the digested DNA was electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and probed with the 0.8-kilobase (kb) *PvuII* fragment of pTT107. This fragment contained the 3' third of

FIG. 1. (A) Portions (10 μ g) of poly(A)-containing RNA from nonproducing (n) and producing (p) clones of CEF infected with RSV-29 alone and a mixture of RSV-29 and RSV-29-associated virus, respectively, were analyzed by Northern blotting and hybridization with nick-translated probes indicated below the lanes. (B) Probes used for the Northern analysis. SR-A, Representation of the SR-RSV-A genome. The arrow indicates the splice acceptor site for the *src* message. IC, Intercistronic region of RSV. The probes used are indicated by lines below the corresponding segments of the SR-RSV-A genome. A + below a probe indicates that it hybridizes to viral RNA; a - indicates no hybridization. Enzymes: R, *EcoRI*; Ba, *BamHI*; K, *KpnI*; X, *XhoI*; S, *SalI*; N, *NcoI*; A, *AccI*; H, *HincII*; Bg, *BglI*; P, *PvuII*. The hatched box indicates the minimum extent of the deletion that would explain the results. The putative genomic and subgenomic RNAs from RSV-29 are shown at the bottom.

FIG. 2. (A) Southern hybridization of total cellular DNA from SR-RSV- and RSV-29-infected CEF. The probe used is shown in panel B. (B) Size of *src*-containing *EcoRI* fragment expected from a nondefective RSV (SR-RSV or PR-RSV) and from a defective RSV with a deletion of *env* (BH-RSV or RSV-29). The sizes of the *EcoRI* fragments are indicated in kb below the figures. Enzymes: R, *EcoRI*; H, *HindIII*; K, *KpnI*.

the *src* gene of SR-RSV-A (NY) and hybridized with a 5.0-kbp *EcoRI* fragment of RSV-29 proviral DNA (Fig. 2). The rest of the digested DNA was fractionated under identical conditions with appropriate molecular weight markers, and the 5.0-kbp DNA fragment was recovered by electroelution. This DNA was cloned into lambda gt WES, and the recombinant plaques were screened by hybridization with the same 0.8-kb *PvuII* fragment of pTT107 described above. One of the positive recombinants was purified by three rounds of plaque purification, and the 5-kbp *src*-containing *EcoRI* fragment was subcloned into the *EcoRI* site of pBR322 to construct plasmid pR29. All procedures used were essentially as described previously (14).

Restriction mapping. pR29 was digested with single and multiple restriction enzymes, and the DNA fragments were fractionated by electrophoresis through agarose or acrylamide gels and stained with ethidium bromide. The map was confirmed by partial digestion of 32 P-end-labeled DNA with appropriate restriction enzymes.

DNA sequencing. The *KpnI*-to-*NcoI* fragment measuring 300 base pairs (bp) on the 5' side of *src* was subcloned from pR29 into replicative forms of M13mp18 and M13mp19 between the *KpnI* and *HincII* sites. The 450-bp *PstI*-to-*EcoRI* fragment on the 3' side of *src* was subcloned between the *PstI* and *EcoRI* sites of the M13mp8 and M13mp9 replicative forms. The recombinant plasmids were used to transform *Escherichia coli* JM101, which was plated on YT agar (16) containing isopropylthiogalactoside and dibromodichloro-indolylgalactoside. White plaques were screened for the presence of inserts by gel electrophoresis analysis of the size of phage DNA from culture supernatants (16).

One recombinant virus of each type was selected and sequenced by the dideoxy method (20) with both [32 P]dATP and [35 S]dATP (New England Nuclear Corp., Boston, Mass.). The products of the sequencing reactions were fractionated on 8 and 5% polyacrylamide sequencing gels in buffer consisting of 50 mM Tris base, 50 mM boric acid, and 2 mM disodium EDTA. Each fragment was sequenced at least twice on either strand.

The DNA sequences were analyzed with the computer programs in the Sequence Analysis Package designed by the Biomathematics Computation Laboratory of the University of California, San Francisco.

RESULTS

Defectiveness of RSV-29. In the early 1960s, Simons found that RSV-29 is replication defective (P. J. Simons, personal communication). To confirm the defectiveness of the virus, CEF infected with high dilutions (multiplicity of infection of 10^{-5}) of the initial RSV-29 stock were plated in soft agar for the isolation of colonies. About 70% of the isolated colonies, which originally did not produce transforming virus, released high titers of transforming virus upon superinfection with helper viruses (RAV-1 or RAV-2). By the interference assay with avian leukosis virus of known subgroups (6, 34), we found that the original RSV-29 stock is of subgroup A. Since the virus appears to be defective in the *env* gene (see below), this subgroup specificity represents that of a helper virus associated with the original stock of RSV-29.

Analysis of RSV-29 RNAs. The total poly(A)-containing RNAs obtained from RSV-29-infected NP clones and the clone doubly infected with both RSV-29 and RSV-29-associated virus were analyzed. Representative results of one NP clone and one producer clone are shown in Fig. 1A. The NP clones contained two species of RSV-29-specific RNAs, measuring 7.7 and 2.6 kb, detectable by several probes. The doubly infected producer clones contained an additional 2.8-kb species of RNA. The 7.7-kb RNA is most likely the genomic RNA, and the 2.6-kb RNA is the spliced *src* mRNA of RSV-29. The producer clone also contained the helper viral genomic RNA that could not be separated from the 7.7-kb RSV-29 genomic RNA but that was revealed by hybridization with probes derived from *env* and the intergenic region between *env* and *src* of the SR-RSV-A genome. The 2.8-kb RNA appeared to be the RSV-29-associated virus *env* mRNA. Neither the 7.7-kb nor the 2.6-kb RNA of RSV-29 hybridized with probes derived from *env* and intergenic regions. The result of the hybridization of RSV-29 RNAs with various SR-RSV-A-derived DNA probes is summarized in Fig. 1B. Since probes 5, 6, and 7 (Fig. 1B) failed to hybridize to RSV-29 genomic RNA while all other probes did, it is concluded that the RSV-29 genome contains a deletion in the *env* region. The 5' boundary of the deletion is located between the *Bam*HI and *Xho*I sites, since the genomic RNA hybridized to probe 4. The 3' boundary is located between the *Acc*I site and the splice acceptor site for *src* mRNA (position -76 from the initiation codon of *src*), because probe 8 hybridized with the 7.7-kb genomic RNA and with the 2.6-kb mRNA. There was no evidence of deletions in the other replicative genes or in the *src* gene.

The 32 P-labeled RNAs from cells infected with RSV-29 and its helper virus were also analyzed by RNase T₁-resistant oligonucleotide fingerprinting. The patterns of both viral RNAs, except for the *src*- and *env*-specific oligonucleotides, superimposed (data not shown). The pattern of the non-*src* oligonucleotides is different from those of several strains of helper viruses originally isolated from BH-RSV (RAV-1, RAV-2, RAV-3, and RAV-7).

Molecular cloning of a 5-kbp *pol*-*src* fragment of RSV-29. When digested with *Eco*RI, the RSV-29 proviral DNA gave a 5-kbp fragment that hybridized with a probe from the *src* gene (Fig. 2). These data suggest that the genomic structure of RSV-29 is similar to that of BH-RSV (13) in which the *Eco*RI sites in *gag* and U3 are preserved but the site in *env* is lost. This 5-kbp fragment was prepared from CEF transformed clonally with RSV-29, molecularly cloned into lambda phage vector, and then subcloned into pBR322.

Restriction site mapping of pR29. The restriction map of the *Eco*RI fragment of RSV-29 is presented and compared

with those of PR-RSV-C and BH-RSV (Fig. 3). In the *pol* gene, the map of RSV-29 is the same as that of PR-RSV with two exceptions: (i) an extra *Pvu*II site is present near the 3' end of *pol* which is absent in PR-RSV, SR-RSV, and BH-RSV, and (ii) a *Bam*HI site at the 3' end of *pol* that is present in PR-RSV but is absent in RSV-29. BH-RSV also lacks this *Bam*HI site. In the *src* gene, the restriction maps of PR-RSV and RSV-29 are similar except for the absence of one *Bgl*II and two *Bal*I sites in RSV-29. None of the restriction sites located in the *env* gene of PR-RSV (23) were found in RSV-29. Furthermore, the restriction fragments bridging across the putative deletion in pR29, e.g., the *Nco*I 1.2-kbp fragment and the *Bal*I 1.3-kbp fragment, comigrated on electrophoresis with the corresponding fragments from pBH-beta (13), suggesting that the sizes of the deletions in the viruses RSV-29 and BH-RSV are very similar.

***pol*-*src* junction in RSV-29.** To study the deletion in detail, the 300-bp *Kpn*I-*Nco*I fragment of pR29 was cloned into M13 phage vectors in both orientations and sequenced. The sequence from the *Kpn*I site in *pol* to the *Nco*I site at the initiation codon of *src* is presented in Fig. 4A.

The sequence across the deletion of *env* is exactly the same as in BH-RSV (Fig. 4B). Following the termination codon of *pol* (as defined in PR-RSV-C) are 6 nucleotides that are also present in PR-RSV-C, and immediately 3' to this are the 91 nucleotides preceding the initiation codon of *src* in BH, PR-C, and SR-A strains of RSV, as well as in recovered avian sarcoma virus 1441 and *c-src* (13, 23, 28, 29).

As shown in Fig. 4A and discussed below (see Fig. 7), the RSV-29 clone we studied has a mutation in the 3' end of *pol*. It has 13 bases replacing 2 bases at the same position in the sequence of PR-RSV (23) which would result in a frame shift in the carboxy-terminal part of the *pol* protein, replacing the last 28 amino acids of the PR-RSV *pol* protein with 43 different amino acids. The new reading frame is terminated by an opal termination codon at -61 relative to the initiation codon of *src*.

3' end of RSV-29. The 450-bp fragment from the *Pst*I site

FIG. 3. Restriction map of RSV-29 proviral DNA compared with PR-RSV-C (PR-C) (23) and BH-RSV (13) between the *Eco*RI site in *gag* and the *Eco*RI site in U3. A + or - sign indicates whether the site was present or absent in BH-RSV and RSV-29. A dotted line marking an enzyme site indicates a site present in BH-RSV or RSV-29 which is absent in PR-RSV. Where two restriction enzyme sites cannot be resolved in the scale of this diagram, they are indicated in a vertical array at the same site on the PR-RSV-C map; this array is the same for the BH-RSV and RSV-29 maps. Enzymes: R, *Eco*RI; Ps, *Pst*I; H, *Hind*III; Hp, *Hpa*I; Xb, *Xba*I; Ba, *Bam*HI; BI, *Bal*I; N, *Nco*I; A, *Acc*I; Bg, *Bgl*II; X, *Xho*I; P, *Pvu*II; B, *Bgl*II; K, *Kpn*I. Δ indicates that *Pst*I sites were not determined for BH-RSV (13).

[A]

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KpnI
----- 10      20      30      40      50      60
GGTACCCTCT CGAAAAGTTA AACCGGACAT CACCCAAAAG GATGAGGTGA CTAAGAAAGA

70      80      90      100     110     120
TGAGGCGAGC CCTCTTTTTC CAGGCATTTC TGATTGGATA CCCAGGGAGA AACCGCCAGC
          |
          env
          splice acc
          pol
          mutation

130     140     150     160     170     180
AACAAAGCAAG AAGGACTCCA GGGAGAAACC GCCAGCAACA AGCAAGAAAG ACCCGAGAGAA

190     200     210     220     230     240
GACACCCCTTG CTGCCAAGCA GAGTTAAATTA TATTGTCTGT GTGCTGCAGG AGCTGAGCTG
          |          |          |          |          |
          pol      Δenv      src      splice acc      pol
          stop      in RSV-29      splice acc      stop
          PR-C

250     260     270     280     290     300
ACTCTGCTGG TGGCCTCGCG TACCACTGTG GCCAGGCGGT AGCTGGGACA GTGCAGCCGA

start
src
|309
CCACCATGG
-----
NcoI

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[B]

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PR-C      src      R29
pol      spl      pol
stop      acc      stop
---      |      ---
RSV-29    GAAGACACCCTTGCTGCCAACGAGAGTTAATTATATGTCTGTGTGCTGCAGGAGCTGAGCTGACTC
BH-RSV    *****A*****
PR (pol)   *****C**AT*A*TGCTGTCCT
PR (src)   *TAG*CTA*****A*****
          10/15

src
start
---
RSV-29    TGCTGGTGGCCTCGCGTACCACTGTGGCCAGGCGGTAGCTGGGACAGTCCAGCCGACCACCATGG
BH-RSV    *****A*****
PR (src)   *****-*****

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[C]

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PR-C      v-src      src
pol      -91      splice
stop      |      acc
---      |      |
RSV-29    CGAGAGTTAATTATATGTCTGTGT      GCTGCAGGAGCTGAGCTGAC
PR (pol)   *****C**AT*A*TGCTGTC**CTCTTT*GTGAGGTTA
c-src     TCTGACACCCATCTG**C*GTC**CTGTGT*****
          12/19

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FIG. 4. (A) DNA sequence of 309-bp *KpnI*-*NcoI* fragment of RSV-29. The pol mutation refers to 13 bases in the *pol* gene of RSV-29 which replaced 2 bases in the same position in PR-RSV-C and is discussed in detail in the legend to Fig. 7. (B) RSV-29 sequence from the end of *pol* to the *NcoI* site compared with that of BH-RSV and with the sequence of PR-RSV *pol* [PR(pol)] and that of PR-RSV from 104 bases upstream from the initiation codon of *src* [PR(src)]. The box shows a putative recombination point in a nondefective RSV that could generate the deletion in RSV-29 or BH-RSV. (C) Sequence of RSV-29 across the deletion of *env* compared with that of PR(pol) (see above) and the region upstream of *c-src* (*c-src*). The box shows another recombination point between the 3' end of *pol* and the region upstream of *c-src* that would produce a defective virus like RSV-29. In panels B and C, a * indicates the same nucleotide as RSV-29, and a - indicates a nucleotide that is present in RSV-29 but absent in the other sequences.

at the 3' end of *src* to the *EcoRI* site in U3 was cloned into M13mp8 and mp9 vectors and sequenced (Fig. 5A). There is extensive homology between PR-RSV-C (23) and RSV-29 in the regions designated as F3 (30), F1A, F1B, F1D (13), PPT, and U3 (Fig. 5B). F1C is interrupted in PR-RSV-C by F2 sequences. In contrast, the F1C of RSV-29 is uninterrupted and homologous to that of SR-RSV-A (13, 28).

The 3' end of the RSV-29 *src* gene also contains the

38-nucleotide sequence that is presumably derived from the chicken *c-src* locus 1 kb downstream from the *c-src* termination codon, called *c-src'* (30). It also contains box P (Fig. 5A), which has been hypothesized to be involved in the recombination between *c-src* and *c-src'* (Fig. 6), and box Q, which may have been involved in the recombination between helper virus (RAV-2) and *c-src'* sequences to generate the 3' end of BH-RSV (13) (Fig. 6).

FIG. 5. (A) Sequence of the *Pst*I-*Eco*RI fragment of RSV-29. F3 (30) and F1A-D (13) are previously defined segments of the sequences flanking *src* in RSV. dr refers to the limits of the direct repeat flanking *src* in SR-RSV and PR-RSV as originally defined by Schwartz et al. (23). Boxes P and Q are hypothetical points of recombination required to generate the 3' end of v-*src* (13; legend to Fig. 6). (B) Comparison of 3' ends of RSV-29 and PR-RSV-C (PR-C). A * indicates a nucleotide that is present in RSV-29, and a - in a given sequence indicates a base that is absent in that sequence but present in the other sequence. A series of ~ indicates where PR-RSV-C has acquired an F2 (13) sequence.

DISCUSSION

Deletion of *env*. DNA sequence data confirmed the conclusion from RNA analysis that RSV-29 contains a deletion of the entire *env* gene. The deletion involves exactly the same sequences as in BH-RSV (13). As discussed by Lerner

and Hanafusa (13), this *pol*-*src* junction could be formed as a result of recombination between the helper virus and c-*src* using homologous sequences existing downstream from *pol* in PR-RSV-C (sequences of *pol* and its neighboring regions of helper viruses have not been determined, but they are probably similar to those of PR-RSV-C) and upstream of

FIG. 6. Model for the generation of the 3' end of *v-src* (12). Boxes P and Q are putative recombination points between *c-src*, *c-src'* (30) and 3' end of helper virus. The lengths of the relevant regions in number of nucleotides are indicated. Recombination 1 probably took place during reverse transcription. Recombination 2 could have happened by a rearrangement at the *c-src* locus, by aberrant processing of a helper virus-*c-src* hybrid RNA, or by an error in reverse transcription.

c-src in the genome of chickens (Fig. 4C). Such a recombination would cause a deletion of the entire *env* sequence and produce a replication-defective virus. Alternatively, similar homologous sequences existing downstream from *pol* and upstream from *v-src* within the PR-RSV-C genome could also be utilized to cause the *env* deletion in the nondefective virus (Fig. 4B). Thus, as with BH-RSV, analysis of the sequence across the deletion of *env* fails to distinguish whether the deletion occurred during the generation of sarcoma virus in chicken tumor no. 1 or occurred secondarily in an originally nondefective PR-RSV-C-like virus.

The exact homologies in the deletion in BH-RSV and RSV-29 suggest that these two viruses have descended from a common progenitor virus and that the differences in their 3' sequences might have arisen in BH-RSV by recombination with helper viruses during subsequent passages (see below). The fact that a strain of RSV with the least number of passages beyond chicken tumor no. 1 has deleted its *env*

gene and is therefore replication defective suggests that the original isolate of RSV was also replication defective. This view is supported by the observations of Hagino-Yamagishi et al. (5), who found that two independently isolated avian sarcoma viruses (S1 and S2), obtained by inoculating chickens with avian lymphoid leukemia virus, contained *src* and were replication defective. This implies that *src*, like other oncogenes, can be transduced into avian lymphoid leukemia virus to form a replication-defective transforming retrovirus. Furthermore, while replication-defective mutants generated from nondefective virus have been selected by isolating single colonies (10, 15), it is difficult to explain how a pure stock of a defective virus (e.g., RSV-29 and BH-RSV) could be established by passage of nondefective virus in chickens in which defective viruses do not appear to have any selective advantage over their nondefective counterparts. Taking all these arguments into consideration, we propose that, like all other transforming retroviruses, RSV was originally isolated as a replication-defective virus, and that the commonly studied replication-competent strains have been generated during subsequent recombinations with helper virus.

Mutation in *pol*. The discovery of the 13 nucleotides in RSV-29 substituting for 2 nucleotides in *pol* (as compared with PR-RSV-C [23] and BH-RSV [M. Sudol and H. Hanafusa, unpublished data]) is interesting. We do not know whether the mutation and the resulting missense translation are peculiar to the particular NP clone that was sequenced or are present in all clones of RSV-29. It would be interesting to study the functional and structural properties of the *pol* gene product in this clone. On the basis of the findings of Hippenmeyer and Grandgenett (9), the mutation in this domain is expected to leave the DNA polymerase and RNase-H functions of the enzyme intact.

A possible mechanism for the generation of this mutation is presented in Fig. 7, based solely on sequence comparisons and identification of direct repeats. Several previous hypotheses postulated involvement of direct repeats in the generation of transformation-defective deletion mutants from RSV (2, 18, 19). Since PR-RSV and BH-RSV (M. Sudol and H. Hanafusa, unpublished data) are homologous in sequence in the *pol* gene, it is reasonable to assume that the PR-RSV-C-like structure, containing the direct repeats A1 and A2, is the parental virus. After having reverse transcribed A1-X-B1-

FIG. 7. Mutation in *pol* in RSV-29 no. 1.2. (A) Comparison of the 3' ends of *pol* in RSV-29 no. 1.2 and in PR-RSV-C (22). A * indicates the same base as RSV-29, and a blank space indicates the absence of that base in PR-RSV-C. A1, A2 and B1, B2 are pairs of direct repeats present in the PR-RSV-C sequence. (B) Diagram of the error in reverse transcription and the deletion using these direct repeats that would generate the mutation sequenced in RSV-29 no. 1.2.

FIG. 8. Comparison of the 3' ends of several avian retroviruses to show the regions across which certain pairs of viruses are related. The arrowed box in each virus corresponds to box Q of Fig. 5 and 6.

A2, the enzyme could have fallen off the template and then reattached, possibly to a second template, at A1 because of the homology between A1 and A2. This would generate the intermediate form shown in the figure, with repeats of X-B1-A2 (Xr-B1r-A2r). A subsequent deletion, using the direct repeats B1r and B2, could generate the variant we have identified.

3' end of RSV-29. Comparison of the sequence at the 3' end of RSV-29 reveals an almost exact homology between RSV-29 and PR-RSV-C in this region, suggesting that the two viruses are closely related (Fig. 5B). In line with the discussion above concerning the deletion in *env*, this might mean that PR-RSV-C is derived from RSV-29 and that the insertion of F2 in the F1C sequence of PR-RSV-C occurred at a later date. However, an alternative possibility is suggested by the reported extensive homology of 3' sequences in SR-RSV-A and MH2 leukemia virus (27). The fact that pairs of viruses like MH2 and SR-RSV-A, which certainly have had different origins and different passage histories, share common 3' sequences, suggests that in the course of passage in chickens, these viruses have been associated with a common pool of helper viruses with which they often exchange their 3' sequences in block. Superimposed on this

are point mutations, deletions, and insertions which do not obscure the relatedness of the 3' sequences. The homology between RSV-29 and PR-RSV-C found in this study could be analogous and might merely reflect the fact that these viruses have acquired their 3' sequences by similar exchanges with a common helper virus. This hypothesis would also propose that F2 was inserted into PR-RSV-C at a later date.

The relationships discussed above and that between BH-RSV and RAV-2 (1, 13), are shown in Fig. 8. A comparison of these 3' sequences revealed a highly homologous short stretch in all these viruses (Fig. 9). This homologous region is present at the 3' end of the *src* sequence in the RSVs, from box Q to the termination codon (Fig. 9). The homology between helper virus sequence and *c-src'* in this region was postulated to be responsible for the recombination at the 3' end required to generate RSV in chicken tumor no. 1 (1, 13) (Fig. 6 and 9). It is interesting to observe that this sequence is relatively well preserved not only in the RSVs, but also in RAV-2 (1), MH2 (27), Y73 (11), and UR2 (17) in their 3' noncoding regions, suggesting that this sequence is present in several helper viruses that have not been associated with any of the RSVs and so its presence could not be an artifact created by exchange of 3' sequences between helper virus and *src*-containing RSV (Fig. 9). This is in concordance with our hypothesis, because it affirms that box Q could have been present in the original helper virus that gave rise to RSV in chicken tumor no. 1.

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	v- <i>src</i>	
PR-RSV-C	GTGTTGCGAGGTGCGTGAG	TAAGTACGAGGC
RSV-29	-----	-----
SR-RSV-A	-----	--GTGCGCGA--
BH-RSV	-----A-----A	--GTA-ACTT-T
RAV-2	A-A-A-----A	--GTA-ACTT-T
Y73	A-A-A-----	--GTA-GCTT--
MH2	A--A-----	-----ACA
UR2	--A-A-----	--GGTA-GCTT--
<i>c-src'</i>	-----GA-CG	ATCTGCAC-TC-
	└─Box Q─┘	

FIG. 9. A highly homologous sequence present at the 3' ends of several avian retroviruses and in *c-src'*. The box labeled v-*src* refers to the 3' end of the *src* gene in the RSVs. A - denotes the same base at that position as in PR-RSV-C. Box Q is the same as in Fig. 5 and 6.

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