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A Transformation-Competent Recombinant between v-*src* and Rous-Associated Virus RAV-1

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The LTR, v-*src*, LTR provirus, which arose by the reverse transcription and integration of *src* mRNA in the H-19 hamster tumor, has been successfully rescued by fusion with chicken fibroblasts infected with Rous-associated virus RAV-1. One rescued virus, E6, acquired 1 kilobase of the 5′ end of the *gag* gene structure. Recombination took place in the region of 15-nucleotide homology exactly between v-*src* exon (position 7054) and *gag* (position 1417). This recombination resulted in the alteration of *src* splice acceptor site sequences, but this site is maintained as a functional splice acceptor site. The nucleotide structure of the long terminal repeat of recombinant E6 virus suggests that it arose by the intermolecular jump of reverse transcription from RAV-1 to *src* mRNA and then the switch of templates between already depicted regions of homology. The second jump of reverse transcription was apparently an intramolecular event. The acquisition of 1 kilobase of the 5′ *gag* by E6 resulted in maintaining the balance of unspliced and spliced E6 RNAs and assured the replication advantage of rescued E6 virus over rescued F6 virus, the genome of which corresponds to that present in ancestral H-19 cells.

We have previously described a functional proviral structure that consists of LTR, v-*src*, LTR sequences integrated in the hamster tumor H-19 and that, according to restriction mapping and sequencing, corresponds to the reverse-transcribed *src* mRNA (4, 10, 30). This provirus can be rescued if the nonpermissive H-19 cells are complemented by fusion with Rous-associated virus RAV-1-infected chicken fibroblasts and the v-*src* proviral transcript is encapsidated in RAV-1 virions (10, 27). The infection of fresh quail cells with such virions leads to the formation of foci of transformed cells. In three individually subcultured foci, the proviral structure corresponded (according to restriction enzyme pattern) to that present in H-19 cells (20, 27, 28). We have noticed, however, that one newly formed provirus, designated E6, acquired 1 kilobase (kb) of additional sequences inserted in front of v-*src*, hybridizing with the *gag* probe (20, 28). To understand the origin of the E6, we cloned the complete provirus together with flanking-cell DNA sequences. For cloning, we utilized the observation that the provirus is located within a single 4.6-kb *Hin*dIII fragment in the nonproducing Japanese quail cell clone E6-NPK1. Nonproducing cell clones lacking RAV-1 were selected from foci produced in Japanese quail fibroblasts infected with terminal dilutions of rescued viruses (10). *Hin*dIII-digested DNA fragments from 4 to 5 kb were isolated by agarose gel electrophoresis and electroelution and then ligated at a 10 M excess with λ 47.1 arms by standard techniques (13). Plaques were screened by using a ^{32}P-labeled pATV-8 DNA (11). From one positive plaque, the 4.6-kb *Hin*dIII insert was subcloned into pBR322, further subcloned into M13mp18 and M13mp19, and sequenced with Sequenase (U.S. Biochemical Corp.) according to the protocol of the manufacturer. First, we verified that the 1-kb structure acquired by E6 contained the 5′ part of the *gag* gene (22). The sequence at the junction of *gag* and v-*src* (Fig. 1) shows that the *gag* gene at position 1417 has been joined to the first nucleotide of v-*src* (position 7054). Recombination occurred within a region of nucleotide homology in which 15 of 17 nucleotides spanning the recombination site match. As a result of recombination, the *src* mRNA splice acceptor site has been altered. However, when compared with a known list of splice acceptor sites (16), its structure is identical (from nucleotide positions +2 to −7) to that of the ovalbumin gene. It complies with additional rules for splice acceptor sequences (16) because the region 5′ of the position −4 is pyrimidine-rich and free of dinucleotide AG (Fig. 1). Two lines of evidence indicate that this newly formed splice acceptor site is functional. First, two viral RNA species are synthesized in E6-NPK1 cells (Fig. 2). The 3.7-kb species hybridizes with the *gag* 2, *src* 11, and LTR 2 probes (10, 15, 19), detecting the nucleotide regions 532 to 1916, 8098 to 8671, and 8992 to 9335, respectively, of Rous sarcoma virus genomic structures (22), and represents the transcript of the whole E6 genome. The smallest species corresponds to the 2.7-kb *src* mRNA and hybridizes only to the long terminal repeat (LTR) and *src* probes. In addition, we found that E6-NPK1 cells synthesize pp60$^{v\text{-}src}$ that has the phosphotyrosine-specific kinase activity (data not shown). Second, using S1 nuclease mapping, we have previously demonstrated that both E6 viral RNAs are protected by a *Sac*I-*Nco*I proviral fragment exactly as expected for spliced *src* mRNA (10). Thus, regular *src* mRNA splicing proceeds in E6 despite reconstruction of the splice acceptor site due to recombination.

Next, we established the primary structure of the E6 provirus LTRs and the adjacent cellular DNA. The provirus is flanked on both ends by the GCACAG hexanucleotide direct repeat of cellular DNA, providing evidence that the integration of E6 was accomplished by the normal integration mechanism characteristic of avian leukosis-sarcoma viruses (33). This finding is in agreement with an analogous

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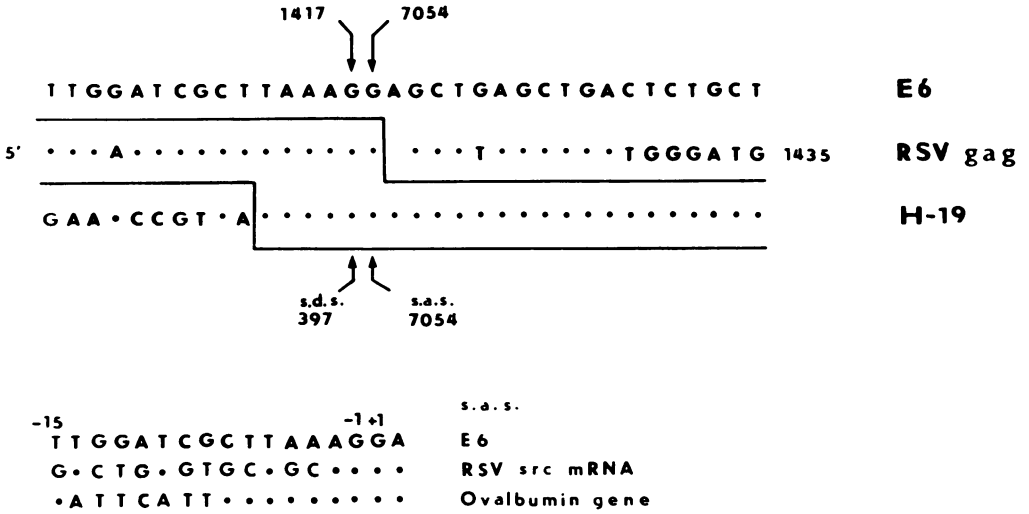


FIG. 1. Nucleotide sequence of the E6 provirus region in which recombination between v-*src* and *gag* genes has taken place. The nucleotide numbering is as described by Schwartz et al. (22). The dots indicate nucleotides matching the E6 sequence. The joining of *gag* (position 1417) to the first nucleotide of v-*src* (position 7054) is indicated by the upper arrows. The lower arrows indicate the junction of the splice donor site (s.d.s.) (position 397) and splice acceptor site (s.a.s.) (position 7054) in the H-19 provirus (4). The *gag* and H-19 genome displaying the best homology with the E6 sequence is emphasized by two solid lines, and the region of recombination appears as a rectangular box. In the stretch of the displayed RSV *gag* sequence, A at position 1407 alternates with G (22). The splice acceptor structures of E6, RSV v-*src* mRNA (22), and the chicken ovalbumin gene (16) are shown at the bottom.

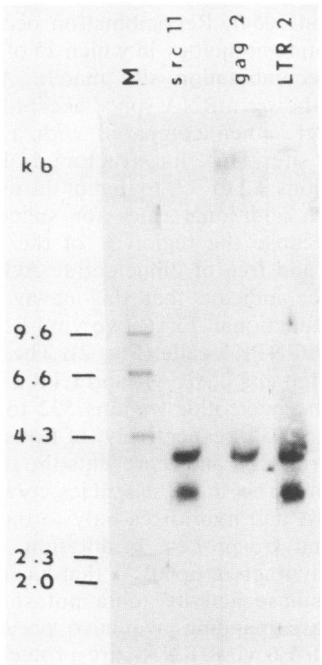
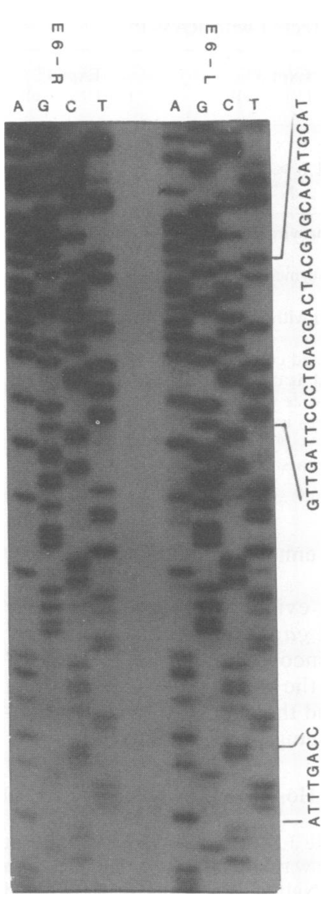


FIG. 2. Northern (RNA) blot analysis of E6 mRNAs. RNA from E6-NPK1 cells was isolated by the method of Chomczynski and Sacchi (6) and fractionated by electrophoresis on 1% agarose, using 20 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0). Otherwise, the published procedure (32) was followed. RNAs were transferred on a Nytrone membrane (Schleicher & Schuell, Inc.) in 1× SSC (150 mM NaCl–15 mM sodium citrate, pH 7.0), and the membrane was then UV irradiated for 2 min with a Philips TUV 15-W tube at a 12-cm distance. For hybridization, ^{32}P-labeled plasmid probes, *gag* 2, *src* 11, and LTR 2 (10, 15, 19), detecting specifically the viral genome regions spanning nucleotides 532 to 1916, 8098 to 8671, and 8992 to 9335, respectively, were used. Probes were labeled, using hexanucleotide primer pd(N)$_6$ (Pharmacia) and the large fragment of DNA polymerase I (Boehringer Mannheim Biochemicals) according to published procedure (9). The specific activity of labeled probes was around 5×10^8 cpm/μg of DNA. λ *Hin*dIII digest was employed as the molecular weight marker.

arrangement of cellular DNA direct repeats in the ancestral H-19 provirus (4).

The nucleotide composition of both E6 U3 structures was identical to that of PR-RSV-C, based on the sequencing of the pATV-8 proviral clone (11, 22), and matched the ancestral H-19 provirus, with the exception of the nucleotide deletion (position 9283) found in the right-hand LTR of the H-19 provirus (3a). Therefore, E6 U3 differs in 10 single nucleotide substitutions from the U3 region of both RAV-1 and RAV-2 (3, 8). In addition, it retains the characteristic 11-nucleotide continuous deletion demonstrated in the U3 of PR-RSV-C (3). In contrast, both R and U5 sequences contain all eight nucleotides characteristic of RAV-1 or RAV-2 (3, 8) (Fig. 3). With the exception of the G in the R region (found only in RAV-1 and RAV-2), these nucleotides were given as alternatives in the PR-RSV-C map on the basis of the sequencing of cDNAs obtained from viral RNA prepared from virus stocks that had not been cloned for several years (22). However, the entire R and U5 sequences of the H-19 provirus (3a), from which E6 virus had been rescued, correspond exactly to that of PR-RSV-C obtained after the sequencing of cloned provirus (22). Despite ambiguities in the sequence of the PR-RSV-C U5 region, it is clear that E6 U5 differs from the H-19 U5 sequence, corresponding instead to the U5 of RAV-1 or RAV-2. The unique U3-R-U5 sequence of E6 can be explained by the recombination of H-19 with RAV-1, the virus employed for the E6 virus rescue. Such recombination would be likely since it was shown that *src* mRNA transcribed from the LTR, v-*src*, LTR provirus is packaged in RAV-1 virions (27), providing a

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           1                                               40       T           T          G    C A      G   A  80
PrC        GCCATTTTAC  CATTCACCAC  ATTGGTGTGC  ACCTGGGTTG  ATGGCCGGAC  CGTCGATTCC  CTAACGATTG  CGAACACCTG
E6-L       -------G--  ----------  ----------  ----------  ----------  ---T------  --G----C-A  ---G---A--
E6-R       -------G--  ----------  ----------  ----------  ----------  ---T------  --G----C-A  ---G---A--
RAV-1      -------G--  ----------  ----------  ----------  ----------  ---T------  --G----C-A  ---G---A--
RAV-2      -------G--  ----------  ----------  ----------  ----T-----  ---T------  --G----C-A  ---G---A--
                                                               C                                      C

           C                  100
PrC        AATGAAGCAG  AAGGCTTCAT  T
E6-L       C---------  ----------  T
E6-R       C---------  ---------G  CACAG
RAV-1      C---------  ----------  T
RAV-2      C---------  ----------  T
```

FIG. 3. Nucleotide sequence of R and U5 regions of the left-hand (L) and right-hand (R) E6 LTRs. An autoradiogram of the sequencing gel depicting these regions is shown on the left. The nucleotide sequences of E6 left (E6-L) and right (E6-R) R and U5 regions are compared with PR-RSV-C (PrC) (based on the pATV-8 clone [22]) and RAV-1 and RAV-2 (3, 8) sequences. The alternative nucleotides found in PR-RSV-C and RAV-2 are shown above and below the corresponding sequences, respectively. As a result of integration, the E6 right-hand U5 is deleted in two nucleotides and then followed by a GCACAG direct repeat of cell DNA.

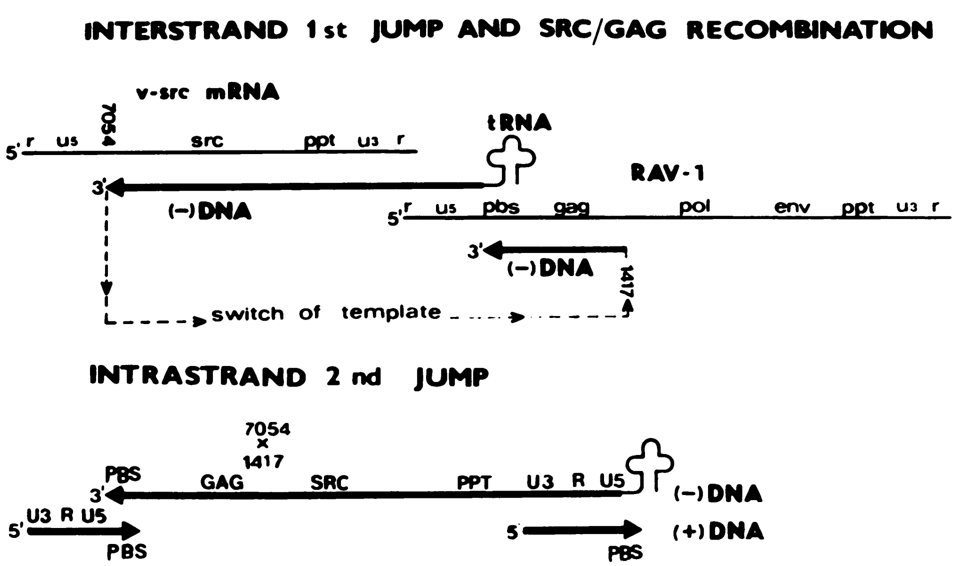

FIG. 4. Proposed way of recombination between *src* mRNA and RAV-1 leading to E6 genesis. Reverse transcription is initiated at the RAV-1 primer binding site (pbs), and a newly synthesized minus-strand DNA then jumps to the 3′ end of *src* mRNA because of the homology in the sequence repeated terminally (r). It copies the *src* mRNA template until the region of homology (position 7054) with RAV-1 *gag* (position 1417) is reached (Fig. 1). The homology between v-*src* and *gag* regions makes possible the switch of the template for minus-strand DNA synthesis, which then follows the RAV-1 template until the primer binding site is reached. The synthesis of plus-strand DNA starts at the polypurine track (PPT) and copies the LTR of minus-strand DNA, comprising a sequence unique for the 3′ end (U3), R, and a sequence unique for the 5′ end (U5). It also copies the primer binding site of viral RNA which makes possible the second jump because of the attachment on the 3′ end minus-strand DNA primer binding site. The second jump is therefore intramolecular. The structures on RNA and DNA are given in lowercase and uppercase letters, respectively. For an explanation and references, see the text.

TABLE 1. Production of transforming virus by E6 and F6 nonproducing cell clones superinfected with RAV-1[a]

Cell clone	Expt 1[b]		Expt 2[b]		Expt 3[c] (FFU/10^6 cells)	Expt 4[d] (FFU/10^6 cells)
	FFU/10^6 cells[e]	IC/10^6 cells[f]	FFU/10^6 cells	IC/10^6 cells		
F6-NPK1	1.1×10^3	1.4×10^4	1.0×10^3	9.3×10^3	1.6×10^2	1.0×10^3
E6-NPK1	1.4×10^5	3.7×10^5	1.5×10^5	1.0×10^6	1.6×10^5	5.6×10^4
16Q[g]	3.4×10^6	1.1×10^6	1.3×10^6	1.2×10^6	NT	NT

[a] For the experiments, F6-NPK1 and E6-NPK1 quail cells containing LTR, v-*src*, LTR and LTR, Δ*gag*, v-*src*, LTR proviral sequences, respectively, were used. Two sets of plates were used in all virus tests. FFU, focus-forming units; IC, infectious centers; NT, not tested.

[b] Cells were infected with RAV-1 at a multiplicity of infection of 1. Virus harvest and infection center assay were performed 2 days after infection.

[c] Cells were infected with RAV-1 at a multiplicity of infection of 10. Virus was harvested 14 days after infection.

[d] The 10^6 nonproducing cells were cocultivated with 10^6 avian leukosis virus-free chicken embryo fibroblasts preinfected with RAV-1. Virus was harvested 21 days later.

[e] The number of focus-forming units was obtained by dividing the total amount of transforming virus released by infected cultures into supernatant medium (4 ml) by the actual number of cells (in millions) at the time of virus harvest. Titers of transforming viruses were assayed as described earlier (29).

[f] At 2 days after infection, cells were treated with mitomycin C (40 μg/4 ml of medium per plate) for 2 h and washed twice to remove the drug, and after an additional 2 h of cultivation, cells were trypsinized and plated on secondary chicken embryo fibroblasts. After 24 h, cultures were overlaid with agar-containing medium (29). Infectious centers were scored 7 to 14 days later.

[g] Japanese quail cells containing the BH-RSV provirus (17) were used as a positive control in RAV-1 complementation assays.

chance for the formation of the heterozygous particles required for recombination. Recombination would occur during the first jump of reverse transcription, since only U5 sequences of RAV-1 were incorporated in the E6 provirus (Fig. 4). This interpretation agrees with recently published data (18) documenting that the first jump of reverse transcription represents an intermolecular event. Assuming the involvement of the copy-choice mechanism in retroviral recombination (7, 34), the minus-strand DNA would have copied *src* mRNA until the region of homology with the RAV-1 *gag* sequence (positions 7054 and 1417, respectively) was reached, making possible the switch of templates and the acquisition of *gag* in the E6 virus. Because the U3 region of E6 was clearly derived from PR-RSV-C, the second jump of reverse transcription should have been accomplished as an intrastrand event in which the plus-strand DNA comprising U3, R, U5, and the primer binding site attached to the 3′ end of the minus-strand DNA primer binding site (14, 21, 23, 31) of the recombinant. The structure of LTRs of the recombinant provirus therefore suggests that the reverse transcription between two parent RNAs consists of the first intermolecular and the second intramolecular jumps.

Finally, we characterized some parameters of E6 virus expression. The ratio of unspliced to spliced E6 RNAs obtained by densitometric measurements of both bands hybridized to the *src* 11 probe was 1 (average of two measurements) (Fig. 2), indicating that the E6 provirus can be employed as a suitable splicing vector. This finding agrees with recently published data (1, 26) showing that the *gag* region spanning nucleotides 630 to 1149 acts as a negative regulator of splicing, maintaining the balance of unspliced and spliced RNAs.

There is evidence that the 5′-end half of the avian leukosis-sarcoma virus *gag* gene contains the enhancer regions (2, 5, 12, 25) and increases virus replication (25). We attempted to verify whether the acquisition of the 5′-end half of *gag* gene sequences will be reflected in an increase of transforming virus production. Therefore, we compared transforming virus production in E6-NPK1 and F6-NPK1 (a cell clone containing one LTR, v-*src*, LTR provirus) (27) quail cells superinfected with RAV-1 or cocultivated with RAV-1-infected chicken fibroblasts. The E6 cells produced ten thousand times more transforming virus than F6 cells (Table 1), thus documenting the replication advantage of the E6 recombinant. This finding provides the required proof (24) that the acquisition of a significant portion of the 5′-end *gag* gene sequences, including the enhancer, increases the virus titers.

Our results provide the first evidence that the v-*src* gene can recombine with the RAV-1 *gag* gene without altering *src* mRNA splicing. Whether the incorporation of 1 kb of the 5′ *gag* in the v-*src* mRNA opens the way for the acquisition of further retroviral structures and the genesis of a replication-competent retrovirus is now being investigated.

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