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Frequent Segregation of More-Defective Variants from a Rous Sarcoma Virus Packaging Mutant, TK15

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TK15, a mutant derived from a temperature-sensitive mutant of Rous sarcoma virus (*ts*NY68), has extremely low infectivity although it has intact viral genes. Previous analyses of the virus and virus-induced transformants showed that the mutant has a defect in packaging of its own genomic RNA, possibly owing to a deletion near the 5′ end. Another striking feature of TK15 is that it induces various types of virus-nonproducing (NP) transformants, 15c(−), at high frequency. In this work, the mechanisms of frequent segregation of NP cells were examined by molecular cloning of TK15-derived proviruses from NP cell clones and their sequence analysis. The structure of the major type of provirus, found in about half of the NP cell clones, was colinear with *src* subgenomic mRNA and was suggested to be due to infection with virions containing subgenomic mRNA in place of genomic RNA. Other types of proviruses present in 15c(−) cells appeared to contain cellular sequences of various lengths replacing various parts of viral sequences. The mechanism for the generation of these proviruses is discussed in relation to the nature of the packaging mutant.

Retroviruses are known to be genetically unstable. They are unique in showing high frequencies of genetic recombination (21); the frequency of recombination between doubly infected avian retroviruses has been estimated to be as high as 10%, although this may be an overestimate that represents the sum of events in multiple cycles of infection (5, 9, 23). Recombinational events occur not only between virus genomes but also between the virus genome and host cell chromosome sequences. Viral oncogenes of acutely oncogenic retroviruses are known to be derived by transduction of cellular counterparts, cellular oncogenes (1, 21). Except in the case of Rous sarcoma virus (RSV), acutely oncogenic retroviruses are replication defective because cellular oncogenes are transduced into viral genomes at the expense of part of the viral genes. Moreover, even a cloned wild-type RSV, which is replication competent, was found to segregate replication-defective viruses at high frequency in the replication cycle and also always to be associated with a high titer of transformation-defective variants lacking the *src* gene (8, 21). The mechanisms of recombination with viral genomes or with cellular sequences and the high frequency of segregation of defective progeny are not yet fully understood.

TK15 is a packaging mutant of RSV with very low infectivity although it produces a normal level of virus particles. Previous studies (10, 11) showed that this mutant contains three intact viral replication genes (*gag*, *pol*, and *env* genes) and a temperature-sensitive *src* gene derived from the parental virus *ts*NY68 (7, 14), a temperature-sensitive mutant of RSV. The very low infectivity of TK15 was shown to be due to a defect in packaging of its own genomic RNA into virus particles.

Southern blot analysis and partial sequence determination of its provirus DNA clone showed a deletion of 237 bases in the noncoding leader sequence, which is followed by the *gag* coding region, suggesting that this portion of the viral genome contains at least a part of the signal sequences necessary for efficient packaging (11, 13).

In addition to its low infectivity, this packaging-defective mutant, TK15, has another striking feature (10, 11). Namely, it induces virus-nonproducing transformants, 15c(−), at very high frequency, as well as virus-producing transformants, 15c(+); about half of the transformants induced by TK15 infection were found to be 15c(−). Using the Southern blotting technique, Koyama et al. (11) previously analyzed DNAs prepared from 15c(−) cell clones. Interestingly, 20 of 36 independently isolated clones of 15c(−) cells yielded the same *Eco*RI fragment of 2.3 kilobases (kb) which was detected with either a long terminal repeat- or *src*-specific probe, suggesting the existence of a unique mechanism for generating this type of proviral genome. Three clones yielded single *Eco*RI fragments of various lengths; 11 others yielded two *Eco*RI fragments of various lengths, and 2 others produced three fragments of 3.8, 3.1, and 2.15 kilobase pairs (kbp), as did a clone of 15c(+) cells.

Analysis of the mechanism of frequent segregation of more defective variants of TK15 may shed light on the genetic nature of retroviruses. In this study, the provirus genomes of four independent 15c(−) clones were molecularly cloned and subjected to partial sequence analysis. On the basis of the results, we propose a model for the mechanism of frequent segregation of 15c(−) proviruses.

MATERIALS AND METHODS

DNA probes and blotting. Plasmid pSRA2, which contains the entire genome of RSV, and a subclone, pPvuIIE, which contains a *src*-specific 0.8-kb *Pvu*II fragment, were kindly supplied by J. M. Bishop (University of California, San Francisco) (3). The inserts of these plasmid DNAs were excised, labeled by nick translation, and used as probes for hybridization experiments.

Molecular cloning and DNA sequencing. Cell cultures were prepared as previously described (10). High-molecular-weight DNAs extracted from TK15-transformed cells by sodium dodecyl sulfate-proteinase K treatment and phenol extraction were digested to completion with *Eco*RI or *Sst*I. Digested DNAs were fractionated by agarose gel electropho-

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resis and recovered from the gels by the glass powder adsorption method (22). The collected DNA fragments were ligated to appropriate λ phage vector arms and then packaged into phage particles in vitro (4). TK15-derived provirus clones were obtained by screening with a ^{32}P-labeled *src*-specific *Pvu*IIE probe (3). The cloned fragments of the proviruses were subcloned into plasmid vectors and subjected to further analyses. DNA sequences were determined by the chemical cleavage method of Maxam and Gilbert (12).

RESULTS

Molecular cloning of the provirus genome of 15c(−) transformants. For analysis of the mechanism of frequent induction of virus-nonproducing transformants by infection with TK15 virus, four provirus genomes of independent 15c(−) transformants that yielded single *Eco*RI fragments of various lengths as the only TK15-derived sequence were subjected to molecular cloning. Among these, no. 60 was one of the most frequently observed types of 15c(−) transformant. A 2.3-kb provirus fragment that contained the *src* gene sequence was generated from DNA of this type of provirus clone by *Eco*RI digestion. As many as about half of the 15c(−) proviruses examined were of this type, suggesting that they were formed by some special mechanism (11). Three other proviruses also seemed to contain large deletions like those of no. 60, but these proviruses may have been generated by a different mechanism(s) from that of the no. 60-type proviruses.

Figure 1 shows the results of Southern blot analyses of proviruses of virus-producing 15c(+) cells and the four 15c(−) cell clones that were subjected to molecular cloning. The DNAs of the transformants were digested with *Eco*RI endonuclease, and the digests were separated by agarose gel electrophoresis and transferred to nitrocellulose. RSV DNA excised from plasmid clone pSRA2 was labeled with ^{32}P and used as a probe. In addition to TK15-derived sequences, the probe also detected the endogenous virus sequence and cellular *src* sequences. However, since these sequences were present in all transformants and also in uninfected cells, the proviral sequences derived from TK15 could easily be identified. Three *Eco*RI fragments of 3.8, 3.1, and 2.15 kb were detected as TK15-specific fragments in DNA of a clone of a 15c(+) transformant by Southern blotting (11) (Fig. 1). The *Eco*RI C fragment of 2.15 kbp is known to originate from the 5′ portion of the provirus and to contain the deletion site of TK15, as described above. The 3.1-kb fragment (*Eco*RI B fragment) of TK15 contains the 3′ one-third of the *env* gene sequence and the entire *src* gene sequence. The largest *Eco*RI A fragment, 3.8 kbp, is considered to be derived from the middle part of the virus genome, which contains the *pol* gene and the 5′ two-thirds of the *env* gene sequence. The four 15c(−) clone DNAs analyzed here each yielded only one fragment of 2.3, 3.1, 3.3, and 4.9 kbp, respectively, as a TK15-derived sequence on *Eco*RI digestion. These fragments were also detected with the *src*-specific probe (data not shown). This result indicates that these proviruses have large deletions eliminating the internal two *Eco*RI sites present in the TK15 provirus. The DNAs of all of the clones except no. 60 were digested with *Eco*RI, size fractionated, packaged into phage particles, and screened with the *src*-specific probe. The proviral genome of no. 60 was cloned by using the *Sst*I site in the cellular flanking sequence. The cloned fragments were subcloned into the plasmid vector and subjected to restriction mapping.

Restriction mapping and nucleotide sequence analysis of 15c(−) clones. Comparison of the restriction map with that of the whole RSV genome suggested that the viral replication genes *gag*, *pol*, and *env* were deleted in the provirus of no. 60. These three structural genes essential for viral replication also seemed to be deleted in the proviral genome of no. 73. There also seemed to be another structural alteration in the downstream portion of the *src* gene of clone 73, and this was confirmed by nucleotide sequence analysis (Fig. 2). The other two proviral clones, no. 49 and 57, had deletions of the *pol* gene and parts of the *gag* and *env* genes, judging by comparison of their restriction maps with that of the RSV genome. To determine the precise structural alterations, we examined the nucleotide sequences of the restriction fragments of each provirus that seemed to contain the possible structural alterations. The nucleotide sequences around the deletion sites of these 15c(−) proviruses are shown in Fig. 3. In clone 60, the deletion was found to have occurred between the splicing donor site (16, 19), which is located 18 bases downstream from the beginning of the *gag* gene sequence, and the acceptor site (16) for the subgenomic *src* mRNA. Therefore, this proviral structure is colinear with the *src* subgenomic mRNA. In clone 73, two structural alterations were found, and the one located upstream of the *src* gene was the same as that of clone 60, namely, colinear with the structure of the 21S mRNA. Unexpectedly, the 3′-terminal portion of the provirus of clone 73 was found to be interrupted by a nonviral sequence (Fig. 2 and 3). The acquired sequence of about 930 base pairs (bp) started from the middle of the U_3 region of the long terminal repeat and ended just before another U_3 sequence. Namely, the 5′ part of the U_3 sequence was duplicated. In the provirus genome of clone 57, a nonviral sequence of 17 bases was inserted between the partly deleted *gag* and *env* gene sequences (Fig.

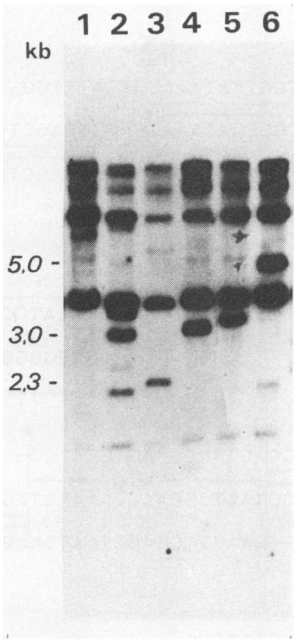

FIG. 1. Southern blot analysis of provirus genomes of 15c(+) and the four independently isolated 15c(−) clones that were subjected to molecular cloning. High-molecular-weight cell DNAs (10 μg) were digested with *Eco*RI, and the digests were separated by electrophoresis on 1% agarose gel and transferred to a nitrocellulose filter. The blot was then hybridized with a ^{32}P-labeled RSV whole-genome probe. The DNAs were isolated from uninfected chicken embryo fibroblasts (lane 1), 15c(+) cells (lane 2), and 15c(−) clones 60 (lane 3), 73 (lane 4), 57 (lane 5), and 49 (lane 6).

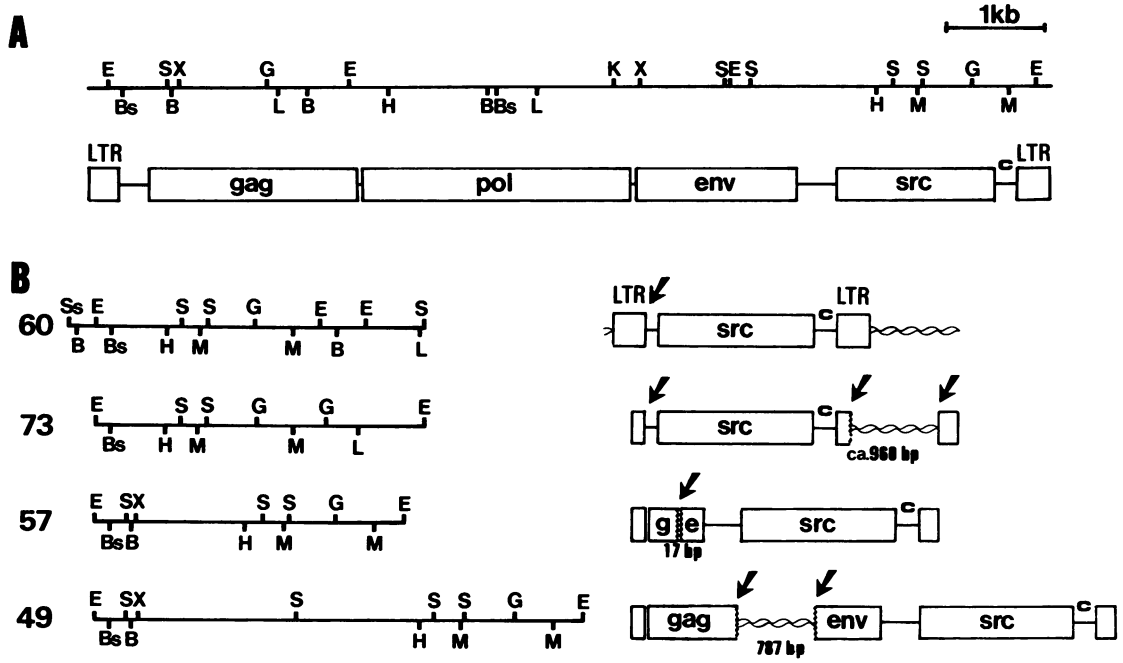

FIG. 2. Physical maps and genome structures of wild-type RSV (A) and the molecularly cloned 15c(−) proviruses (B). These maps were determined by analysis of doubly digested products. Enzymes: Ss, *Sst*I; B, *Bam*HI; E, *Eco*RI; Bs, *Bst*EII; H, *Hin*cII; S, *Sma*I; M, *Mlu*I; G, *Bgl*I; L, *Bgl*II; X, *Xho*I. The genome structures of the proviruses were deduced by comparison of restriction maps and partial nucleotide sequencing. Wavy lines and arrows indicate chicken chromosome-derived sequences and break points found in the provirus genomes, respectively. Abbreviations: g or gag, *gag* gene sequences; pol, reverse transcriptase gene; e or env, *env* gene sequences; src, *src* gene sequences; LTR, long terminal repeat; c, common region.

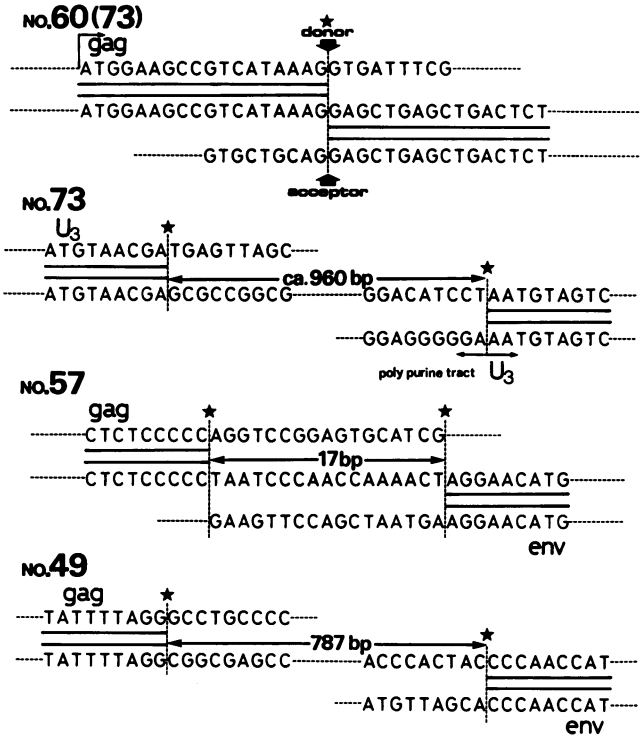

FIG. 3. Nucleotide sequences around the break points found in the cloned 15c(−) provirus genomes. The break points indicated by stars were deduced by comparing the provirus sequences with Prague C strain RSV genome sequences (16), which are shown above and below the 15c(−) provirus sequences. The structure of the 5′ portion of the cloned provirus of clone 73 is identical with that of the corresponding region of clone 60.

2 and 3). This short stretch replaced a viral sequence of about 5.9 kbp containing the *pol* gene and parts of the *gag* and *env* genes. Similarly, in provirus clone 49, a sequence of about 4.9 kb of the viral genome was replaced by a 787-bp G+C-rich sequence (Fig. 2). Interestingly, one open reading frame was found in this sequence, while the other two frames were interrupted by three and five stop codons, respectively. The entire sequence of the substituted sequence of this clone and the structural characteristics of the coding peptide will be published elsewhere.

Substituted sequences are derived from transcriptionally active chicken genes. For further examination of the origin of the substituted sequences of 15c(−) clones 73 and 49, we subcloned the substituted sequence-specific fragment of each, that is, the 370-bp *Pst*I fragment of no. 49 and the 690-bp *Bgl*I-*Pvu*II fragment of no. 73, into pBR322. DNAs of uninfected chicken fibroblast cells and the 15c(−) cells from which the provirus genomes were derived were doubly digested with *Eco*RI and *Sst*I and analyzed by Southern blotting by using these subcloned fragments as probes. A fragment of about 2.5 kb was detected in normal-cell DNA with the probe of the substituted sequence of the no. 49 provirus, and two fragments of 2.7 and 3.3 kb were detected with the probe specific to the sequence present in the provirus of clone 73 (Fig. 4). This result indicated that these sequences were derived from chicken chromosomal DNA. The transformant DNA of clone 49 yielded two fragments of about 4.9 and 2.5 kbp, which should be derived from the integrated provirus and the normal chromosome, respec-

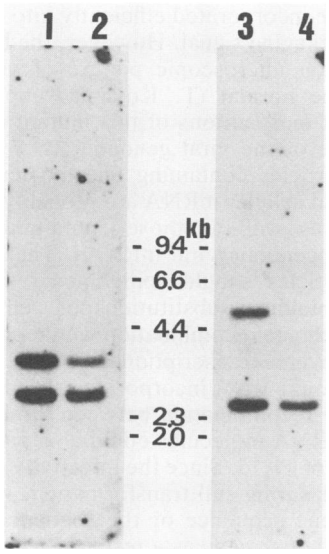

FIG. 4. Chicken chromosome origin of the substituted sequences found in two 15c(−) proviruses. The DNAs of uninfected chick cells and 15c(−) cells from which the provirus molecular clones were isolated were doubly digested with *Eco*RI and *Sst*I. The digests were then fractionated on 1% agarose gel and transferred to a nitrocellulose filter. The blots were hybridized with ^{32}P nick-translated DNA from the subclone of the acquired sequence of either clone 49 (lanes 1 and 2) or 73 (lanes 3 and 4). Lanes: 1, 15c(−) clone 49 DNA; 3, 15c(−) clone 73 DNA; 2 and 4, normal chicken embryo fibroblast DNA. *Hin*dIII-digested λ DNA was used as a size marker.

tively. In no. 73, the band derived from the provirus seemed to be superimposed on one of the two bands (3.3 kb) detected in normal-cell DNA because the intensity of this band of no. 73 was about twice that of normal-cell DNA. Furthermore, we used RNA blot analysis to determine whether two substituted sequences are transcribed in chicken fibroblast cells. Polyadenylated RNA was prepared from normal uninfected chicken fibroblast cells, separated by agarose gel electrophoresis, and transferred to nitrocellulose. A 2.9-kb-long mRNA was detected by using the no. 73 specific fragment as a probe, and transcripts of 2.8 and 3.9 kb were detected with the no. 49 specific probe (Fig. 5). Thus, these substituted sequences are unique cellular sequences and are actively transcribed in normal chicken cells.

DISCUSSION

15c(−) provirus with a structure colinear with subgenomic *src* mRNA. We reported previously that progeny viruses produced from 15c(+) cells induced virus-nonproducing transformants, 15c(−) cells, at high frequency (10). Southern blot analysis of the provirus genomes of 15c(−) clones showed that 20 of 36 independently isolated clones contained a provirus which yielded the same single *Eco*RI fragment of 2.3 kb, while the other proviruses yielded *Eco*RI fragments of various sizes. To clarify the mechanism of frequent induction of 15c(−) cells, we obtained molecular clones of proviruses of four independent 15c(−) transformants and subjected them to restriction mapping and partial nucleotide sequencing. On sequence analysis, the most frequently found type of 15c(−) provirus was colinear with 21S mRNA for the *src* gene.

Some signal sequences on genomic RNA, such as the primer-binding site and the polypurine tract required for initiation of plus-strand DNA synthesis, are reported to be necessary for complete reverse transcription and integration into cellular DNA (1, 20, 21). However, all of these signal sequences are considered to be present in the 21S subgenomic *src* mRNA of RSV.

Previously the 28-base sequence forming a possible dimer linkage, which is present approximately 160 bp downstream of the *gag* initiation codon of avian retrovirus, was reported to be necessary for efficient packaging of the viral genome (15).

However, Ikawa et al. (6) recently found that the genome structure of S1 virus, a newly isolated avian sarcoma virus, resembles that of RSV subgenomic *src* mRNA. The 5′ portion of S1 virus is the spliced form, just like that of *src* mRNA. Therefore, the genome does not contain a possible dimer linkage structure. However, the packaging efficiency of this transforming virus is not very low compared with that of wild-type RSV, although it is about 1/10 as much as the latter. Furthermore, Wang and Stacey (24) found that subgenomic *env* mRNA could be packaged into virus particles and converted to a subgenomic provirus. These observations suggest that, in the case of wild-type viruses, subgenomic *src* mRNA could also be packaged but could not be detected because of its low content relative to that of genomic RNA. However, in the case of the packaging mutant, because of reduction of its packaging efficiency, nonspecific packaging of subgenomic RNA should increase relative to specific packaging of genomic RNA. Thus, the provirus colinear with subgenomic *src* mRNA that was most frequently detected in 15c(−) transformants should be formed by infection of virions that contain the subgenomic RNA molecule in place of genomic RNA (Fig. 6).

15c(−) proviruses containing cell-derived sequences. Nucle-

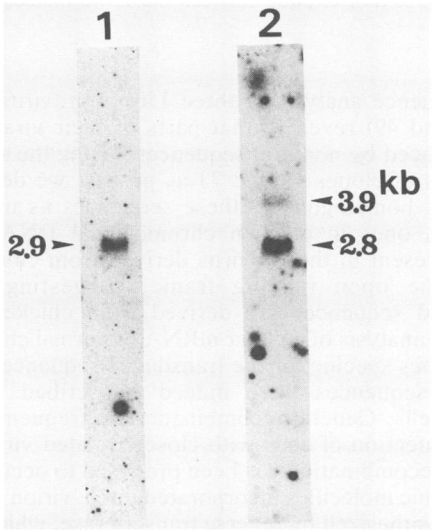

FIG. 5. Transcripts detected with probes specific to the transduced sequences found in the provirus genomes of two 15c(−) cells. Total cellular RNA was extracted from normal chicken embryo fibroblast cells by the cesium chloride-guanidine thiocyanate method (2). Polyadenylated RNA selected on an oligo(dT)-cellulose column (5 μg) was subjected to electrophoresis on a 1.2% agarose gel containing 2.2 M formamide. RNAs were transferred to nitrocellulose filters and hybridized. The probes used for hybridization were as follows: lane 1, a *Bgl*I-*Pvu*II fragment of about 470 bp excised from the provirus genome of no. 73 and subcloned into a pBR vector with an *Eco*RI linker; lane 2, the *Pst*I 370-bp fragment of clone 49 also subcloned into a pBR vector.

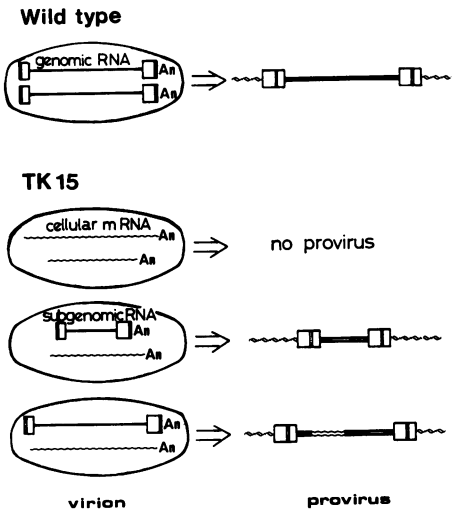

FIG. 6. Possible mechanism for frequent segregation of 15c(−) transformant cells. In the case of wild-type retrovirus, two genome RNA molecules are selectively packaged into a virus particle and reverse transcribed into a provirus DNA molecule. However, since the TK15 genome lacks the signal sequence for efficient packaging of its own genomic RNA, cellular mRNAs or subgenomic RNAs would be packaged into virions in place of the genomic RNA. Infection with the virions that contain only cellular mRNA molecules would not result in formation of any provirus, but infection with virions containing subgenomic *src* mRNA molecules would result in formation of provirus which is colinear with the subgenomic mRNA, since the subgenomic mRNA molecules contain a full set of the signal sequences necessary for complete reverse transcription. On the other hand, infection with virions containing one molecule each of viral genomic RNA and cellular mRNA would probably often result in formation of aberrant proviruses probably generated by copy choice reverse transcription between these two molecules.

otide sequence analysis of three 15c(−) proviruses (clones 73, 57, and 49) revealed that parts of their viral genomes were replaced by nonviral sequences. Using the substituted sequences of clones 49 and 73 as probes, we detected the sequences homologous to these sequences as unique, not repetitive, ones in chicken chromosomal DNA. The sequence present in the provirus derived from clone 49 contained one open reading frame, suggesting that the transduced sequences are derived from chicken mRNA. RNA blot analysis of cellular mRNA of normal chicken cells using probes specific for the transduced sequences revealed that the sequences were indeed transcribed in normal chicken cells. Genetic recombinants are frequently formed by dual infection of cells with closely related viruses (5, 8, 23). The recombination has been proposed to occur between two genomic molecules incorporated into a virion when viral DNA is synthesized by reverse transcriptase, which is apt to guide a nascent DNA strand from a template to another template (copy choice) (21). Furthermore, microinjection of *env* mRNA into cells transformed by *env*-deficient Bryan RSV was found often to result in the appearance of replication-competent viruses after long-term culture. These replication-competent recombinants are thought to be generated by copy choice recombination between *env*-deficient virus genome RNA and *env* mRNA, which are incorporated together into one virus particle (17, 18, 24).

Wild-type RSV very selectively incorporates two molecules of viral genomic RNA into virions, but TK15 genomic RNA cannot be incorporated efficiently into virions because of lack of a packaging signal. However, the buoyant density and the electron microscopic picture of virions of TK15 appeared to be normal (T. Koyama, unpublished data), suggesting that most virions of this mutant contain cellular RNAs in place of the viral genomic RNA (Fig. 6). Thus, some virus particles containing one molecule each of genomic RNA and cellular mRNA are probably produced from 15c(+) cells, as well as those containing only cellular mRNAs or subgenomic viral mRNAs. Therefore, infection with such particles should sometimes result in aberrant proviruses containing substitutions of cellular sequences because copy choice recombination would presumably often occur during reverse transcription between a cellular mRNA and a viral genomic RNA incorporated together into virions.

Copy choice recombination between viral genomic RNA and cellular mRNA molecules could also partly explain the low infectivity of TK15. Since the infectivity of the virus was assayed by measuring cell transformation, viruses that had lost the *src* gene sequence or the sequences essential for expression of the *src* gene as a result of recombination with cellular sequences would not be detected as infectious virus. The fact that the reduction in infectivity of the mutant (10^{-3} to 10^{-4} of that of wild-type virus) was considerably lower than the reduction in the content of viral genomic RNA (about 0.5% of that of the wild type) suggests that such abortive proviruses were quite frequently formed in the TK15 system.

Acute transforming viruses are known to result from recombination between cellular oncogene sequences and viral genomes (1). In normal-packaging viruses, the mechanism of recombination leading to the formation of acute transforming viruses might be packaging of cellular mRNA molecules and subsequent copy choice recombination. Unlike in packaging mutants, however, the amount of cellular mRNAs incorporated into viruses should be very small and the chance of incorporation of transcripts of cellular oncogenes into virions should be very low. However, as proposed on the basis of structural analyses of cellular and viral oncogenes (21), if chance recombination occurs at the DNA level between a provirus sequence and a cellular oncogene, which would produce a fused transcript of the oncogene with the viral packaging signal, the cellular oncogene transcripts would be incorporated into virions together with viral genomic RNAs very efficiently and the rate of recombination between the viral genome and the cellular oncogene would be as high as that of recombination between viral genomes and cellular sequences observed in the packaging mutants.

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