JOURNAL OF VIROLOGY, July 1984, p. 154–162
0022-538X/84/070154-09$02.00/0
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Vol. 51, No. 1

Characterization of a Rous Sarcoma Virus Mutant Defective in Packaging Its Own Genomic RNA: Biochemical Properties of Mutant TK15 and Mutant-Induced Transformants

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Received 26 October 1983/Accepted 24 February 1984

The accompanying paper (S. Kawai and T. Koyama, J. Virol. 51:147–153, 1984) describes the isolation and biological properties of a mutant, TK15, derived from a Rous sarcoma virus mutant, *tsNY68*. The *cis*-acting defect of the mutant is analyzed biochemically in this paper. TK15 virions released from virus-producing 15c(+) cells were deficient in viral genomic 39S RNA, although comparable amounts of viral RNAs were transcribed in 15c(+) and *tsNY68*-infected cells. Analysis of provirus DNA occurring in 15c(+) cells suggested that the mutant genome had a deletion of ca. 250 bases near the 5′ end of the genome somewhere between the primer binding site and the 5′ end of the *gag*-coding region. These findings indicate that at least part of the sequence lost in the TK15 genome is indispensable for packaging viral genomic RNA into virions. TK15 induces nonvirus-producing 15c(−) transformants at high frequency. Southern blot analysis of DNAs from those 15c(−) clone cells revealed that TK15-derived proviruses contained various extents of internal deletions. Many 15c(−) clones had a provirus carrying only the *src* gene with long terminal repeat sequences at both ends. The mechanism for the segregation of 15c(−) cells is discussed.

A mutant with an extremely low infectivity was isolated from *tsNY68*, a temperature sensitive (*ts*) mutant of Rous sarcoma virus (RSV), grown on cells transformed by UV light-inactivated RSV. The mutant, named TK15, induced two types of transformants, 15c(+) cells producing low infectious TK15 virus and nonvirus-producing 15c(−) cells (12). Biological properties of the mutant and immunoprecipitation and gel electrophoresis of viral proteins in infected cells showed that in 15c(+) cells, four viral genes were all expressed normally; that is, *gag*, the gene coding for the internal structural proteins of the virion, *pol*, coding for the virion-associated RNA-dependent DNA polymerase, *env*, the gene involved in the synthesis of envelope glycoproteins, and *src*, the gene responsible for cell transformation. However, in most 15c(−) cells, only the *src* gene was found to be expressed. The virus particles released from 15c(+) cells appeared to be intact with respect to the capacity of packaging viral genomes, because, when nonvirus-producing transformants, which were induced by the replication-defective avian sarcoma virus, Y73, were cocultured with 15c(+) cells, highly infectious Y73 pseudoviruses were formed.

These findings, together with the fact that the defect of TK15 virus could not be rescued by complementation with gene functions provided by helper viruses, indicate that the mutant had a *cis*-acting defect(s) outside the four viral genes.

To determine the defective function(s) of the mutant, we carried out biochemical analyses of the virus in this study. We found that TK15 had a defect in packaging its own genomic RNA, presumably due to a deletion of ca. 250 bases in the leader sequence (noncoding region at the 5′ end of the genome).

The possible mechanism of induction of various 15c(−) cells besides 15c(+) cells is discussed in relation to the defect in the viral genome.

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MATERIALS AND METHODS

Cells and viruses. Preparations of TK15 and clones of TK15-induced 15c(+) and 15c(−) transformants were described in the accompanying paper (12). *tsNY68*, *tsNY68*α(−), and Rous associated virus 2 (RAV-2) viruses were prepared as described previously (8–11).

Virus purification. Viruses used for RNA extraction and RNA-dependent DNA polymerase assay were purified as described by Smith (27). In brief, after clarification of virus-containing culture fluids, viruses were pelleted by centrifugation in a Beckman type 19 rotor at 18,000 rpm for 2 h. The viruses were then resuspended in STE buffer (0.1 M NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA) and banded in a linear sucrose gradient (15 to 60% [wt/vol]) by centrifugation for 20 h at 24,000 rpm in a Beckman SW28 rotor. Virus bands at a density of 1.16 g/ml were collected, diluted fourfold with STE buffer, and recentrifuged at 24,000 rpm in a Beckman SW28 rotor. The virus pellets were resuspended in STE buffer, layered onto a 20 to 50% linear sucrose gradient, and centrifuged for 5 h at 24,000 rpm in a Beckman SW28 rotor. The bands of virus were collected and diluted with STE buffer. Virus concentrations were measured by the method of Lowry et al. (16).

Polymerase assay. Endogenous activity of purified virions was determined by the method of Mizutani et al. (20). Reaction mixtures contained 10 μl of 1 mM dATP, 10 μl of 1 mM dGTP, 10 μl of 1 mM dCTP, 10 μl of [^{3}H]dTTP (1 mCi/ml), 5 μl of phosphoenol pyruvate (500 μg/ml), 5 μl of pyruvate kinase (2 mg/ml), 0.4 μl of 20 mM ATP, 5 μl of 0.25 M $MgCl_2$–0.5 M KCl, 37.5 μl of 2× buffer (40 mM Tris-hydrochloride [pH 8.0], 0.8 mM EDTA, 10 mM DTT), 25 μl of disrupted virions (400 μg/ml), and 7.5 μl of H_2O. The mixtures were incubated at 40°C, and samples of 25 μl were taken after 0, 20, 40, and 60 min for assay of trichloroacetic acid-precipitable radioactivity.

Preparations and blot hybridization analysis of viral and cellular RNAs and DNAs. For extraction of virion RNA,

purified virus was incubated for 30 min at 37°C in the presence of 200 μg of proteinase K per ml and 0.4% sodium dodecyl sulfate and then extracted with equal volumes of a phenol-chloroform-isoamyl alcohol mixture twice and a chloroform-isoamyl alcohol mixture once. RNA was precipitated with ethanol. Total cellular RNA was prepared by the technique of Strohman et al. (31). Polyadenylate-containing RNA was prepared by passing the preparation through a column containing oligodeoxythymidylate-cellulose (ca. 1 ml of packed volume per mg of cellular RNA) (1). Cellular DNAs were extracted with phenol from cell lysates prepared in 0.5% sodium dodecyl sulfate and 200 μg of proteinase K per ml. RNAs prepared as described above were denatured with glyoxal and subjected to electrophoresis in 1.2% agarose gel. Approximately 1 μg of RNA was applied to each lane. Transfer of the RNA to nitrocellulose filters and hybridization with [^{32}P]DNA probes were carried out by the procedure of Thomas (35). After digestion with restriction enzymes, cellular DNAs were separated by gel electrophoresis (about 15 μg of DNA was run in each lane), transferred to nitrocellulose filters, and hybridized with [^{32}P]DNA probes by the procedure of Southern (28).

Two-dimensional polyacrylamide gel electrophoresis of [^{32}P]viral RNA. [^{32}P]virus was prepared as described by Sawyer and Dahlberg (24) except that the infected cells were incubated with 5 ml of phosphate-free minimal essential medium containing 2 mCi of $^{32}PO_4^{3-}$ per 60-mm plate. RNA was promptly extracted from concentrated viruses, and virion RNAs were fractionated on sucrose gradients before polyacrylamide gel electrophoresis. 70S RNA and fractions of free small RNA (4S to 7S) were separated by centrifugation at 48,000 rpm for 2 h in a linear gradient of sucrose (5 to 20%) in a Beckman SW50.1 rotor. The 4S to 7S fractions were collected, pooled, precipitated with ethanol, and separated by two-dimensional polyacrylamide gel electrophoresis by the procedure of Ikemura and Dahlberg (7). All gels were run in an EC-470 (E-C Corp., St. Petersburg, Fla.).

DNA probes. The virus-specific probes used in this study were prepared from the following recombinant plasmids obtained from J. M. Bishop (University of California, San Francisco, Calif.): pSRA2 (4), which contains the entire sequence of the Schmidt-Ruppin strain of RSV, was used as the full-length RSV probe; pBam-C, which contains the 1.35-kilobase pair (kbp) *Bam*HI fragment C derived from RSV DNA in pSRA2, was used as the *gag*-specific probe; pPvuII-E, which contains the 0.8-kbp *Pvu*II fragment E of the RSV DNA, was used as the *src*-specific probe; and pEcoRI-D, which contains all sequences present in the long terminal repeat (LTR) of RSV derived from pSRA2, was used as the LTR probe (32). The relative positions of the fragments of RSV DNA cloned in these recombinant plasmids are shown in Fig. 7A. Viral fragments were recovered from each recombinant plasmid by digestion with appropriate restriction enzymes and were purified by agarose gel electrophoresis and labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by nick translation (23).

RESULTS

Lack of endogenous reverse transcriptase activity of TK15 virions. TK15 virions contained functional polymerase as determined in the presence of an exogenous template primer (12). Assay of exogenous polymerase indicates only the presence of functional polymerase in the virion, whereas assay of endogenous polymerase indicates the presence of viral RNA as a template primer in the virion as well. That is, if viral RNA has some structural change so that it cannot serve as a template primer for reverse transcription, or the virions are deficient in viral genomic RNA, no endogenous polymerase activity will be detected even if the virions contain functional polymerase. Therefore, we first compared the endogenous polymerase activity of TK15 virions with that of RAV-2, an active avian leukosis virus, and that of *tsNY68*α(−), a polymerase-deficient variant of *tsNY68* (11). TK15 virions as well as *tsNY68*α(−) virus particles lack endogenous polymerase activity, whereas RAV-2 virions have high DNA polymerase activity (Fig. 1). This result is in contrast with the finding that SE21Q1b, another packaging mutant of RSV, exhibited endogenous and exogenous polymerase activity (15). This difference between the two mutants was presumably due to a difference in the species of RNA incorporated into the virions, as shown below and discussed in the accompanying paper (12).

Analysis of small RNAs in TK15 virions. A large fraction (up to 30%) of retrovirus virion RNA generally consists of tRNA molecules of 4S in size. Most of this RNA is presumably derived from the host cells (2). However, there is some evidence that the viral 4S RNA population does not reflect the distribution of tRNAs in the cell but instead represents a selected class of host tRNA molecules (14, 25). Among the 4S RNA molecules in RSV particles, tRNA^{trp} has been shown to act as the primer molecule for the initiation of reverse transcription of the genomic RNA (5, 34). Therefore, one possible explanation for the lack of endogenous polymerase activity is that the selective class of small RNAs is not incorporated into virison, resulting in deficiency of primer tRNA. To test this possibility, we analyzed the small RNAs in TK15 virions. For this, TK15- and *tsNY68*-infected cell cultures were labeled with $^{32}PO_4^{3-}$ in phosphate-free

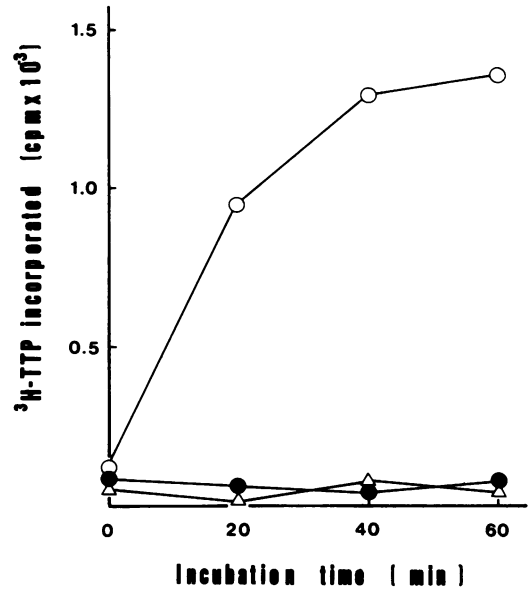

FIG. 1. Lack of endogenous reverse transcriptase activity in TK15 virions. Endogenous reverse transcriptase activity was determined by the procedure by Mizutani et al. (20). Reaction mixtures contained 10 μg of disrupted virions [○, RAV-2; ●, TK15; △, *tsNY68*α (−)], 10 μCi of [^{3}H]dTTP (30 Ci/mmol), 10 nmol of dATP, 10 nmol of dGTP, and 10 nmol of dCTP in 0.1 ml. Samples were incubated at 40°C. Samples of 25 μl were removed at the times indicated and assayed for trichloroacetic acid precipitable radioactivity.

medium, and the viral RNAs were purified and fractionated by sucrose gradient centrifugation. Fractions of free small RNA were separated by two-dimensional polyacrylamide gel electrophoresis. Small RNAs from *tsNY68* particles gave a typical pattern (Fig. 2a). TK15 particles also contained a specific subset of the host cell tRNA population (Fig. 2b). There was no apparent difference between the two patterns. However, they were different from those of small RNAs extracted from normal or infected cells (data not shown). A major species of tRNA (Fig. 2, arrow) was identified as $tRNA^{trp}$ by RNase T1 fingerprint analysis (data not shown). This finding excluded the possibility that the defect of the mutant was due to a deficiency of the primer molecule in virions and is consistent with previous reports (21, 25) that the reverse transcriptase mainly determines which tRNAs are incorporated into virions.

Characterization of genomic RNA of TK15 particles. Next, we examined the possibility that the mutant is deficient in viral genomic RNA. The RNA extracted from banded virions was denatured with glyoxal and analyzed by agarose gel electrophoresis (Fig. 3). *tsNY68* particles contained a considerable amount of 39S genomic RNA and two distinct species of low-molecular-weight RNA, one with a molecular weight of 1.1×10^5 (7S) and the other with the mobility of tRNA (4S RNA) (34). However, in TK15 particles, no 39S genomic RNA was detected and only 4S RNA and small amounts of 7S RNA were observed as discrete RNA species. 28S and 18S rRNAs were also observed in each sample, although the bands representing these RNAs in the gel were faint (Fig. 3). The amounts of these rRNAs varied in different virus samples. These results clearly showed that TK15 particles were deficient in 39S RNA. However, the fact that TK15 virus is infectious, although only slightly, indicates that TK15 particles contain some virus-specific RNAs. To determine the concentration of virus-specific RNA in the virions, we extracted the RNAs from RAV-2, *tsNY68*, and TK15 viruses and analyzed them by the Northern transfer technique. Glyoxalated virus RNAs were frac-

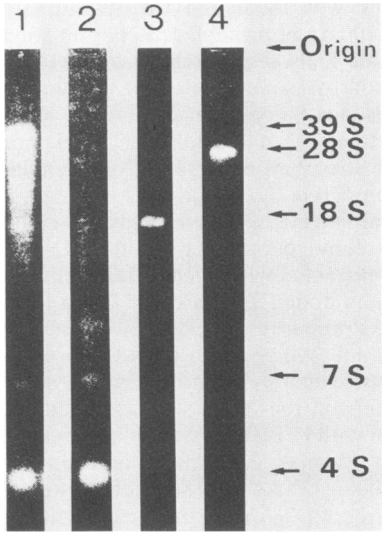

FIG. 3. Agarose gel electrophoresis of glyoxal-denatured RNAs extracted from TK15 virions. Glyoxal-denatured RNAs extracted from purified virions (200 μg of protein) were subjected to electrophoresis on a horizontal 1.2% agarose gel. Samples were run from top to bottom. After electrophoresis, the gel was treated with alkali and stained with ethidium bromide. RNA bands were located under short-wavelength UV light. Lane 1, *tsNY68* RNA; 2, TK15 RNA; 3, chicken 18S rRNA; 4, chicken 28S rRNA.

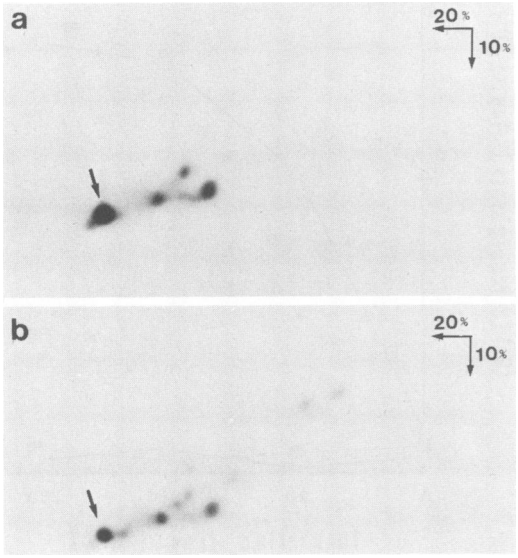

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of free small RNAs in *tsNY68* and TK15 virions. Free small virion RNAs were prepared from ^{32}P-labeled virus particles as described in the text. The free small RNAs were denatured at 90°C for 2 min and analyzed by two-dimensional gel electrophoresis. The first dimension (top to bottom) was in 10% acrylamide; the second dimension (right to left) was in 20% acrylamide. The pattern of 4S to 5S RNAs of *tsNY68* (a) and TK15 (b) particles is shown. Arrows, Position of $tRNA^{trp}$.

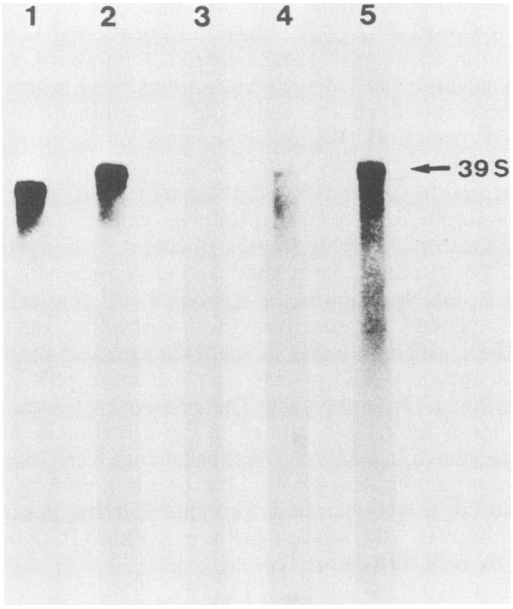

FIG. 4. Characterization of viral RNA in TK15 particles. Glyoxal-denatured RNAs extracted from purified virions were subjected to electrophoresis on a 1.5% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled LTR-specific probe. Size was determined with rRNA markers. Lane 1, virus RNA extracted from RAV-2 virions (200 μg of protein); 2, virus RNA extracted from *tsNY68* (200 μg of protein); 3, 4, and 5, virus RNAs extracted from 200 μg, 2 mg, and 20 mg of protein from TK15 virions, respectively.

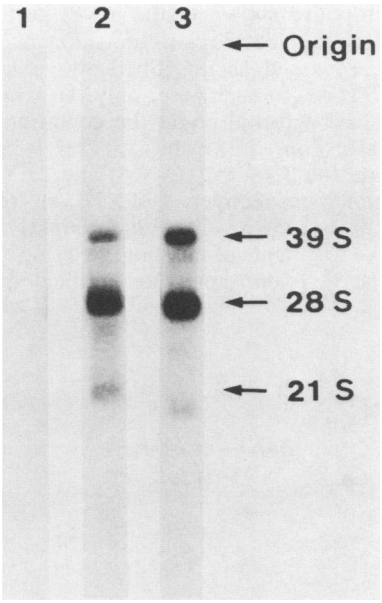

FIG. 5. Viral transcripts in *tsNY68*-infected and TK15-producing cells. Polyadenylated RNAs were extracted from the cytoplasm of uninfected cells (lane 1), *tsNY68*-infected cells (lane 2), and TK15-producing clonal 15c(+) cells (lane 3), fractionated on a 1.5% agarose gel, and transferred to a nitrocellulose filter as described in the text; 1 μg of each polyadenylate-containing RNA was applied to the agarose gel. Virus-specific RNAs were detected with ^{32}P-labeled LTR-specific probe.

tionated on an agarose gel, and after transfer to a nitrocellulose filter, the virus-specific RNAs were detected by hybridization with ^{32}P-labeled probe specific for the LTR (Fig. 4). Comparison of the intensity of the 39S RNA band of TK15 with that of *tsNY68* suggested that the amount of 39S genomic RNA present in TK15 particles was about 0.5% that of parental *tsNY68*. The amounts of subgenomic RNAs present, if any, were below the detectable level.

From the above results it can be concluded that TK15 is a mutant deficient in its own genomic RNAs.

Virus-specific RNAs transcribed in TK15-producing 15c(+) cells. To see whether the deficiency of the viral genomic RNA in TK15 particles reflects any abnormality at the level of transcription of viral RNAs in infected cells, we examined the virus-specific RNAs transcribed in TK15-producing 15c(+) cells. Polyadenylate-containing RNAs extracted from 15c(+) cells, *tsNY68*-infected cells, and uninfected cells were subjected to agarose gel electrophoresis and transferred to nitrocellulose filters. Virus-specific RNAs were detected by hybridization with the LTR-specific probe.

Three different classes of viral RNAs, corresponding to 39S, 28S, and 21S RNAs, which represent the full-length genomic RNA, mRNA for the *env* gene, and mRNA for the *src* gene, respectively (6, 38), were transcribed in *tsNY68*-infected cells (Fig. 5, lane 2). None of these RNA species was detected in uninfected cells. Three classes of viral RNAs were also observed in 15c(+) cells (Fig. 5, lane 3). However, the size of each viral RNA was slightly smaller than that of *tsNY68*-infected cells. Normal amounts and species of viral RNAs were transcribed in TK15-producing cells, except that they were slightly smaller than normal (Fig. 5).

The above data suggest that TK15 is a mutant that cannot package its own genomic RNA, probably owing to a deletion in its genome structure.

Genome structure of TK15 virus. Analysis of viral RNAs transcribed in 15c(+) cells suggested that the genome of TK15 contained a deletion which is shared with its subgenomic mRNAs for the *env* and *src* genes. To determine the location and the size of the deletion, we next analyzed the structure of the provirus integrated into a 15c(+) cell clone.

DNAs extracted from uninfected, *tsNY68*-infected and 15c(+) cells were digested with *Eco*RI, subjected to electrophoresis on 1.5% agarose gel, denatured, and then transferred to nitrocellulose filters. Virus-specific DNA fragments were identified by hybridization with ^{32}P-labeled virus probes. When the probe for full-length RSV genome was used, four major bands were detected in normal cell DNA (Fig. 6, lane 1a). These bands, which were also present in *tsNY68*-infected cells and 15c(+) cells, are considered to be derived from endogenous virus and c-*src* sequences. In addition to these bands, three extra fragments were recognized in DNAs of *tsNY68*-infected cells (fragments of 3.8, 3.1, and 2.4 kbp) and 15c(+) cells (fragments of 3.8, 3.1, and 2.15 kbp) as fragments derived from the respective proviruses (Fig. 6, lanes 2a and 3a). The three fragments derived from the *tsNY68* provirus appear to be identical to *Eco*RI fragments (*Eco*RI fragments A, B, and C) of SRA2 RSV DNA described previously (4). The two larger fragments derived from the TK15 provirus seem to be identical to the corresponding ones of the *tsNY68* provirus. However, the third fragment of TK15 provirus, which is estimated to be about 2.15 kbp long, is much smaller than that of the *tsNY68* provirus. The smallest fragment of each provirus was recognized by the *gag*-specific probe (Fig. 6, lanes 2b and 3b). Therefore, these results suggested that a deletion of ca. 250

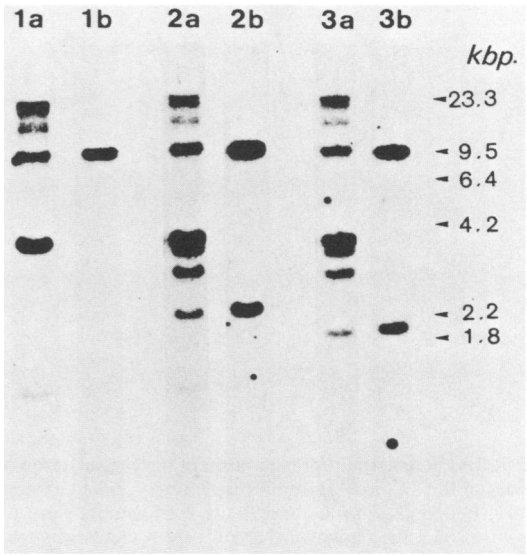

FIG. 6. Analysis of TK15 proviral DNA. Cellular DNAs from uninfected (lanes 1a and 1b), *tsNY68*-infected (2a and 2b), and TK15-producing (3a and 3b) clonal cells were digested with *Eco*RI, subjected to electrophoresis on 1.0% agarose gels, and transferred to nitrocellulose filters. Virus-specific fragments were detected by hybridization with ^{32}P-labeled RSV probe (lanes 1a, 2a, and 3a). The RSV probe was removed and the same filter was then reannealed with ^{32}P-labeled *gag*-specific probe (lanes 1b, 2b, and 3b). *Hin*dIII fragments of bacteriophage lamda DNA in the same gel were used as molecular weight markers.

bp is located on *Eco*RI fragment C, which carries the leader sequence and most of the *gag*-sequence.

To define the location of the deletion more precisely, we recovered the 2.4-kbp fragment of the *tsNY68* provirus and the 2.15-kbp fragment of the TK15 provirus from the gel, digested them with several restriction enzymes, and subjected them to Southern transfer analysis. Based on published information about restriction enzyme cleavage sites of the Schmidt-Ruppin strain of RSV (33), we used *Bst*EII, *Bam*HI, and *Xho*I in this experiment. The presumed cleavage sites of these enzymes on the *Eco*RI fragment of the *tsNY68* provirus are shown in the lower part of Fig. 7A. After digestion with each restriction enzyme, DNA fragments were processed as described above with the ^{32}P-labeled RSV probe. In each lane, only the largest cleavage product could be detected under the conditions employed. On *Bst*EII digestion, 2.25- and 2.0-kbp fragments were generated from the 2.4-kbp (*tsNY68*) and 2.15-kbp (TK15) *Eco*RI fragments, respectively (Fig. 7B, b). On the other hand, when digested with *Xho*I and *Bam*HI, both *Eco*RI fragments gave fragments of the same size (Fig. 7B, c and d). Therefore, the deletion seems to be located somewhere

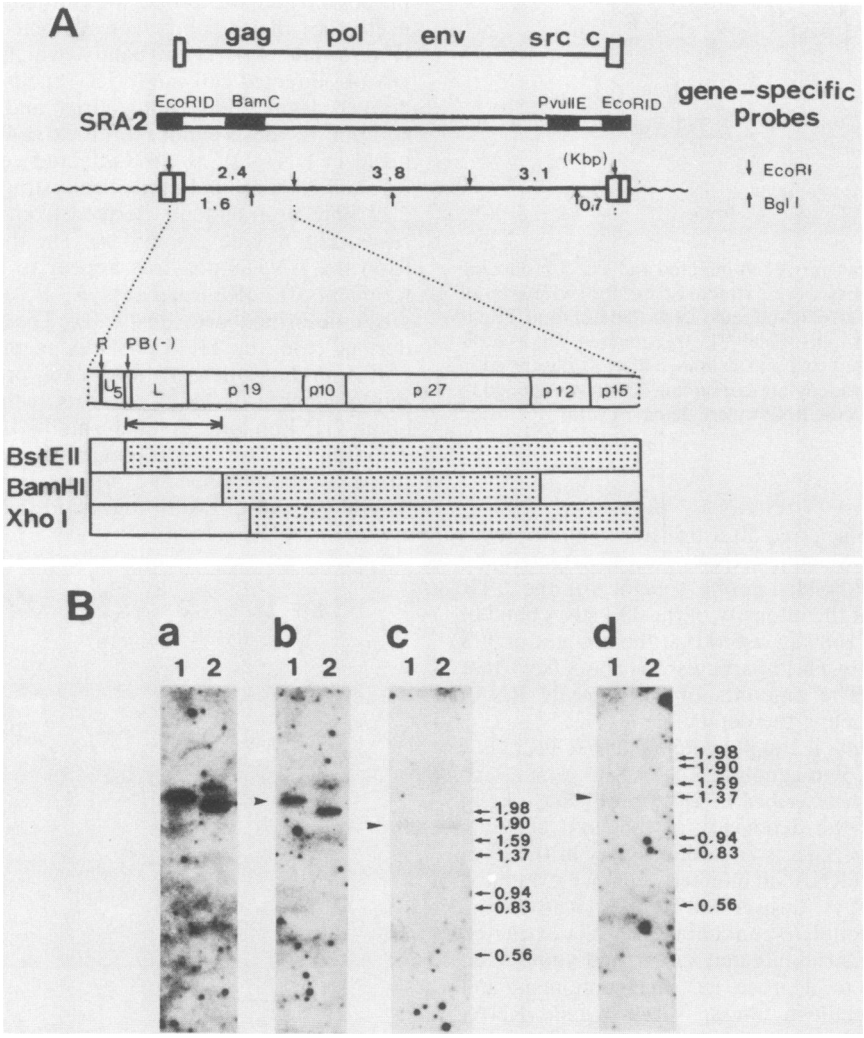

FIG. 7. (A) Schematic representation of the organization of the normal provirus of RSV. Top line, The RNA genome of RSV. Open boxes, Sequences of the 5′ and 3′ termini, respectively, on an expanded scale. Second line, Relative positions on RSV DNA of DNA probes used in this study (*Eco*RI D, *Bam* C, and *Pvu*II E fragments were used as LTR-specific, *gag*-specific, and *src*-specific probes, respectively). Third line, Provirus of RSV. Downward arrows show cleavage sites of *Eco*RI, and upward arrows show those of *Bgl*I. Bottom, Domains and *Bst*EII, *Bam*HI, and *Xho*I cleavage products of the 2.4-kbp *Eco*RI fragment. These restriction enzyme cleavage sites on the Schmidt-Ruppin strain of RSV-subgroup A genome are based on previous reports (3, 4, 26, 33). R, Short sequence repeated at both ends of the genome. U_5, 5′ unique sequence separating R from the primer-binding site. PB(−), Binding site of the primer $tRNA^{trp}$. L, Untranslated leader sequence preceding the coding region for viral proteins. P19, p10, p27, p12, and p15, Regions encoding viral structural proteins. Stippled boxes, Cleavage fragments corresponding to those indicated by arrows to left of lanes b, c, and d in panel B. (B) Location of the deletion in TK15. Cellular DNAs from *tsNY68*-infected cells and TK15-producing clonal cells were cleaved with *Eco*RI and fractionated by electrophoresis on 1.0% agarose gels. The fractionated (2.0 to 2.5 kbp) DNAs were recovered from the gels and cleaved with *Bst*EII, *Xho*I, or *Bam*HI. These DNAs were fractionated by electrophoresis on 2.0% agarose gel and transferred to nitrocellulose filters. Virus-specific DNA fragments were detected by hybridization with ^{32}P-labeled RSV probe. *Eco*RI-*Hin*dIII double-digested fragments of bacteriophage lamda DNA in the same gel were used as molecular weight markers. Lanes 1, DNA of *tsNY68*-infected cells; lanes 2, DNA of TK15-producing clonal 15c(+) cells. *Eco*RI-digested (a) DNA recovered from the 1.0% agarose gel, and the same after digestion with *Bst*EII (b), *Xho*I (c), and *Bam*HI (d).

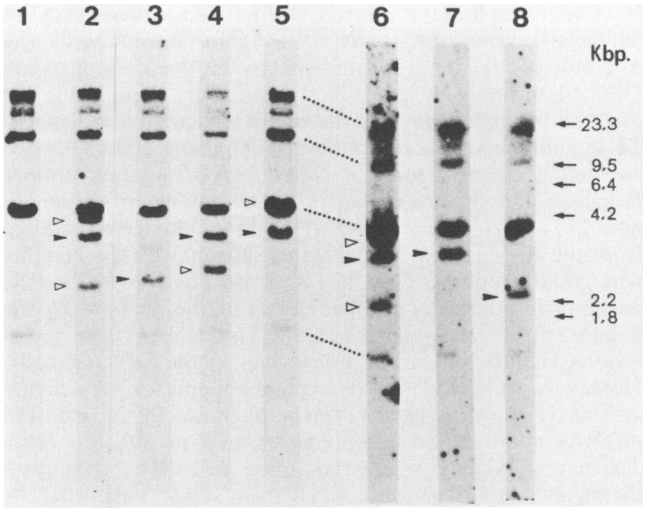

FIG. 8. Analysis of TK15-derived proviral DNA in nonproducer clones. Samples containing 15 μg of total cellular DNA were digested with *Eco*RI, subjected to electrophoresis on 1.0% agarose gels, and transferred to nitrocellulose filters. The blots were then hybridized with ^{32}P-labeled RSV probe. Lane 1, DNA of uninfected cells; 2, 15c(+) cell clone DNA; and 3, 4, 5, 6, 7, and 8, DNAs of 15c(−) clones 1, 2, 3, 4, 5, and 6, respectively. Arrowheads, DNA fragments derived from TK15-related proviruses. Solid arrowheads, Fragments also detected by the *src* probe. The positions of lamda molecular weight markers run in a parallel lane are indicated on the right.

between the *Bst*EII site within the primer-binding site and the first *Bam*HI site within the coding region of p19, as indicated by a biheaded arrow in Fig. 7A. On the one hand, the biological analysis described in the accompanying paper (12) showed that the *gag* gene was intact. Therefore, it is concluded that the deletion of ca. 250 bp in the TK15 provirus is within the region, from the *Bst*EII site to the 5′ end of the *gag*-coding region, which covers ca. 270 bp.

Structure of 15c(−) proviruses. As described in the accompanying paper (12), a unique property of TK15 is segregation of nonvirus-producing 15c(−) transformants at high frequency besides TK15-producing 15c(+) cells. Analysis of viral protein expression in these 15c(−) cells suggested that 15c(−) cells were not all of the same type, although a considerable fraction of 15c(−) transformants appeared to be of the same type. We characterized the structures of TK15-related proviruses in 15c(−) cells by using the same strategy as employed for 15c(+) cells.

15c(−) cell clones isolated from soft-agar suspension cultures of TK15-infected cells were selected by testing for the production of infectious TK15 virus. DNAs of six independently isolated 15c(−) cell clones were digested with *Eco*RI and analyzed as described above with the RSV probe (Fig. 8). The arrowheads show the DNA fragments derived from TK15-related proviruses. Of the six clones examined, three (clones 1, 5, and 6) yielded only one fragment each (2.3-, 3.4-, and 2.3-kbp fragments, respectively) (Fig. 8, lanes 3, 7, and 8), whereas clones 2 and 3 gave two fragments (Fig. 8, lanes 4 and 5) and clone 4 (Fig. 8, lane 6) gave three fragments identical to those of a 15c(+) clone (Fig. 8, lane 2). When the same filters were rehybridized with the *src*-specific probe after the RSV probe had been removed, the same fragments as were detected by the RSV probe were recognized in clones 1, 5, and 6, as shown by solid arrowheads in Fig. 8. With clones 2 and 3, one of the two fragments detected by the RSV probe in each clone was recognized by the *src*-specific probe. These two *src*-containing fragments were also detected by the LTR-specific probe, but not by the *gag*-specific probe (data not shown). These fragments of the two clones appeared to be identical to the 3.1-kbp fragment of a 15c(+) clone (Fig. 8, lane 2). The *gag*-specific probe recognized the other of the two fragments of each clone (2.5- and 4.1-kbp fragments, respectively). These fragments were also found to contain the LTR sequence (data not shown).

In addition to these 6 clones, we examined 30 other independently isolated clones of 15c(−) cells. Of these 30 clones, 20 yielded one fragment of 2.3 to 5.0 kbp. Interestingly, as many as 18 of these 20 clones yielded 2.3-kbp fragments, like clones 1 and 6 in Fig. 8. Nine other clones produced two fragments, and two of these clones appeared to have the same provirus as clone 2 in Fig. 8. The other clone was found to produce three fragments that appeared to be the same as those in clone 4 and 15c(+) cells.

Defective proviruses in 15c(−) cells containing LTR structures at both ends. The results described above suggested that the proviruses in clones 2 and 3, which produced two fragments on *Eco*RI digestion, contained LTRs at both ends. Furthermore, it was apparent that proviruses of 15c(−) cells that produced one fragment on *Eco*RI digestion contained at least one LTR structure. To see whether these proviruses also had LTRs at both ends, we carried out the following experiments on the DNAs of clones 5 and 6. The DNAs were doubly digested with *Eco*RI and *Bgl*I, which cleave sites within LTR and on the 3′ side of the *src* gene (Fig. 7A), and the fragments were analyzed by the Southern transfer technique as described above with the LTR-specific and *src*-specific probes. These DNAs yielded a 0.7-kbp fragment, detectable with the LTR probe (Fig. 9, lanes 4a and 5a),

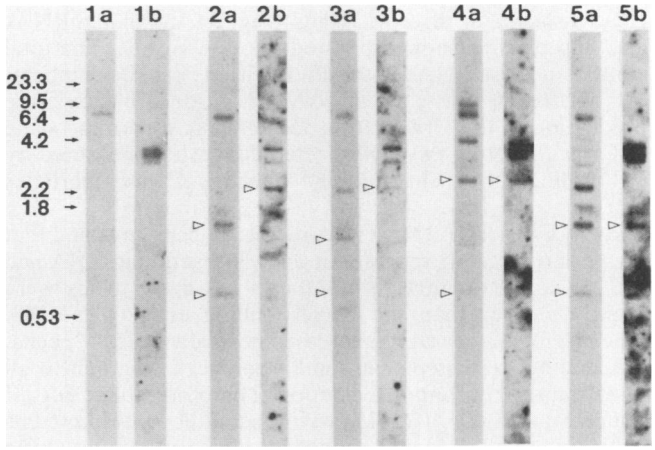

FIG. 9. Analysis of *Eco*RI-*Bgl*I double digests of DNA from 15c(−) clones. Samples of total cellular DNA were doubly digested with *Eco*RI and *Bgl*I, subjected to 1.0% agarose gel electrophoresis, and transferred to nitrocellulose filters as described in the text. The filters were hybridized with ^{32}P-labeled LTR-specific probe (lanes 1a through 5a). The LTR probe was removed, and the filters were rehybridized with the *src*-specific probe (lanes 1b through 5b). Lanes 1a and 1b, DNA from uninfected cells; 2a and 2b, DNA from *tsNY68*-infected cells; 3a and 3b, DNA from 15c(+) clonal cells; 4a and 4b, DNA from 15c(−) clone 5; and 5a and 5b, DNA from 15c(−) clone 6. Numbers at the left indicate bp × 10^{-3}. Arrowheads, 5′ and 3′ terminal fragments derived from internal proviral DNA (lanes 1a through 5a) and the *Eco*RI-*Bgl*I fragments containing the *src* gene of provirus DNA (lanes 1b through 5b).

which was presumably derived from the right-hand portion of the proviruses, judging from the restriction map shown in Fig. 7A. This fragment was also obtained from the *tsNY68* and TK15 proviruses (Fig. 9, lanes 2 and 3a). These 0.7-kbp fragments showed no detectable hybridization with the *src*-specific probe in this experiment, probably because these small fragments may have come off the filter when the first LTR probe was removed. The fragments detectable with the *src*-specific probe did not hybridize with the LTR-specific probe in *tsNY68* and TK15 proviruses as expected (Fig. 9, lanes 2b and 3b), but *src*-containing fragments of clones 5 and 6 (2.7 and 1.6 kbp, respectively) were also recognized by the LTR-specific probe, suggesting that LTR structures were present at both ends of the proviruses of these two clones. Several other fragments were detected by the LTR probe in DNAs from clonal cells (Fig. 9, lanes 3a, 4a, and 5a). These may represent cellular flanking sequences containing part of the LTR structure and some products resulting from incomplete digestion with the restriction enzymes. A further 10 15c(−) clones of this type were examined in the same way, and 9 of them appeared to have LTR sequences at both ends.

These results of analyses of 15c(−) cell DNAs showed that a variety of defective proviruses containing wide-range deletions occurring at many different sites resulted from TK15 virus infection.

DISCUSSION

The *cis*-acting defect causing the extremely low infectivity of TK15 was analyzed biochemically in this study. Analysis of RNA species present in the virions demonstrated that the virus particles were deficient in 39S genomic RNA. The relative concentration of genomic RNA in TK15 virions estimated by Northern transfer analysis was about 0.5% that of *tsNY68* particles (Fig. 4). The particles contained small 7S and 4S RNAs as detectable RNA species. Although not clearly shown in discrete bands (Fig. 3), cellular mRNAs also appeared to be incorporated into the virions. The total content of these RNAs in the TK15 virions may be comparable to that in the parental *tsNY68* virions, because the production of the virions from 15c(+) cells was in the same order as that from *tsNY68*-infected cells when measured by [^{3}H]uridine- or [^{35}S]methionine-labeling of virus particles (12).

Analysis of RNA transcripts in 15c(+) cells revealed that the viral transcripts representing the 39S genomic RNA and the 28S and 21S mRNAs for the *env* and *src* genes were slightly shorter than the corresponding ones of *tsNY68*. Since all four genes were expressed normally in 15c(+) cells, this finding suggested that some sequences common to all three transcripts contain a deletion. Therefore, the results of structural analysis of TK15 provirus could be interpreted as indicating that the leader sequence, which is located at the left end of the provirus and is transposed to the 5′ ends of mRNAs, contained the deletion. A similar deletion near the 5′ end of the genome was reported in the provirus of another packaging mutant of avian sarcoma virus, SE21Q1b (26).

It has also been found with spleen necrosis virus (37) and murine leukemia virus (17) that a certain sequence near the 5′ end of the genome is necessary for packaging. The recognition signal for packaging must explain the mechanism by which the genomic RNA is distinguished from subgenomic mRNAs and packaged selectively into virions. In spleen necrosis virus and murine leukemia virus, the sequences near the 5′ end of the genomes that are necessary for selective packaging are removed from subgenomic mRNAs by splicing. Therefore, mRNAs of these viruses are not packaged into virions. However, in avian sarcoma virus, the 5′ donor splice site is located within the *gag*-coding region (33). Accordingly, it seems likely that some sequence in the *gag*-coding region beyond the donor splice site, which the TK15 genome appears to retain, is also involved in selective packaging of the genomic RNA. Supporting this notion, Pugatsch and Stacey recently found that when sequences near 150 nucleotides 3′ of the splice donor site were removed from the RSV genome, packaging efficiency of the genome was greatly reduced (22). If TK15 had completely lost the capacity for the selective packaging of the genome by the deletion in the leader sequence, the viral RNAs contained in virions should reflect the transcripts in the infected cells. However, we could not detect subgenomic RNAs in the virions (Fig. 4), whereas the subgenomic 28S and 21S mRNAs could be readily demonstrated in infected cells. These results may suggest that the selective mechanism distinguishing 39S genomic RNA from subgenomic mRNAs is still operating in TK15, although the efficiency of packaging is greatly reduced. If this is the case, it would probably be because of partial retention of the packaging sequence in the TK15 genome, which could distinguish the genome RNA from the subgenomic mRNAs.

TK15 induced two types of transformants, TK15-producing 15c(+) cells, and nonvirus-producing 15c(−) cells. The previous study on gene expression in 15c(−) transformants revealed the existence of subtypes of 15c(−) transformants, although most 15c(−) cells expressed only the *src* gene. Southern transfer analysis of proviruses in many 15c(−) clones confirmed this previous conclusion. Of 36 15c(−) clones examined, 23 clones contained TK15-derived proviruses that produced only one DNA fragment detectable by the RSV and *src* probes when digested with *Eco*RI. Of these 23 clones, 20 yielded the same *Eco*RI fragment of 2.3 kbp, whereas the other 3 clones yielded fragments of different sizes ranging from 3.4 to 5.0 kbp. Most of these proviruses appeared to contain LTR structures at both ends. Preliminary analysis of the nucleotide sequence of proviruses suggested that the provirus yielding the 2.3-kbp fragment is colinear with 21S mRNA for the *src* gene. Furthermore, a provirus yielding a larger *Eco*RI fragment of 3.4 kbp was found to be a variant of this type which had lost part of the right-hand LTR, including the *Eco*RI site, and had acquired, instead, a cellular flanking sequence at the right end (M. Nishizawa, unpublished data).

The two proviruses in clones 2 and 3 yielded two internal *Eco*RI fragments, one detectable with *src*-specific probe and the other with the *gag*-specific probe. It appears that these proviruses have an internal deletion such that the *Eco*RI site in the *gag* gene is removed but another site in the *env* gene remains, since the *src*-containing *Eco*RI fragments of these proviruses seemed to be identical in size to that of the *tsNY68* provirus.

Proviruses of the third type (clone 4) yielded three *Eco*RI fragments seemingly identical to those derived from the *tsNY68* provirus. These proviruses may contain point mutations or small deletions undetectable by this analysis.

It has been found that nondefective RSV segregated defective mutants at a relatively high frequency (10 to 15% of the total virus population) during the replication cycle (13, 19). Therefore, some of these defective proviruses, particularly proviruses of the third type, may be formed from TK15 in a similar way. However, most defective proviruses are considered to be induced by an as yet unknown mechanism relevant to the unique properties of TK15 or the unusual

structure of TK15 genome. Since most of the proviruses of the first type appeared to be colinear with 21S mRNA for the *src* gene, it seems most likely that they were derived from subgenomic 21S mRNA incorporated into TK15 virions, as discussed in the accompanying paper (12). Stacey (29, 30) and Wang and Stacey (36) demonstrated with an *env*-mRNA assay system that viral mRNAs are packaged into RSV virions. Furthermore, they presented evidence that these mRNAs were reverse transcribed to form active provirus-like molecules. Therefore, conceivably at least some of 15c(−) transformants may have arisen through the mRNAs and also possibly through misprocessed mRNA molecules for the *src* gene which had been incorporated into TK15 virions. However, we could not detect any subgenomic mRNAs in TK15 virions, probably because their concentrations were too low relative to that of the 39S genomic RNA.

On the other hand, the concentration of genomic RNA in TK15 virions is still much higher than that estimated from the infectivity of TK15 virus (10^{-3} to 10^{-4} that of *tsNY68*). This suggests that TK15 is defective not only in packaging but also in some step of replication of its genome. For example, the genomic RNA may not be faithfully reverse transcribed due to the unusual structure of the genome. Most of it may be inactive for reverse transcription and faithful reverse transcription of the genome may occur only by chance. The various defective proviruses found in 15c(−) cells might have been formed through such unfaithful reverse transcription of the genome. In this connection, it is interesting that Martin et al. (18) found that UV-irradiated RSV induced defective proviruses containing a deletion of up to two-thirds of the genome.

The above explanations for the segregation of various types of 15c(−) transformants are highly speculative. Moreover, at present we cannot exclude the possibility that the TK15 genome may contain an additional structural defect(s) related to the instability of the replication of the genome. A reconstruction experiment with cloned fragments derived of TK15 provirus is under way to see whether the deletion in the leader sequence can explain all the properties of the mutant.

ACKNOWLEDGMENTS

We are grateful to K. Toyoshima, T. Yamamoto, A. Nomoto, and M. Shibuya for helpful discussions and to H. Shinno for excellent technical assistance.

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

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