Tandem Duplication of the Proviral DNA in an Avian Sarcoma Virus-Transformed Quail Clone

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Previous study has shown that an avian sarcoma virus-transformed quail clone, Q-B77-11, apparently contains full-size proviral DNA and releases virus particles which contain polymerase activity, but are unable to form foci. Recently, we have found that after extended tissue culture of Q-B77-11, the predominant cell type changed from one in which there was a single copy of the integrated viral DNA to one with two copies in tandem. There was approximately one copy of the large terminal redundancy between these two tandem proviruses. Polyadenylic acid-containing RNA species of twice the size of the viral genome were detected in these cells and are interpreted as transcripts of the tandem provirus.

Previous studies have shown that both the unintegrated DNA intermediates and the integrated viral DNA, or provirus, of avian sarcoma virus (ASV) are flanked by a large terminal repeat (LTR) (6, 7, 11, 13). The structural analogy between the provirus and the procaryotic transposable elements has been suggested (15, 17). In this manuscript, we describe an event which may strengthen this analogy between ASV and a transposon. We have observed that after extended tissue culture of the transformed quail clone Q-B77-11 the integrated proviral DNA has changed from one copy to two copies in tandem with only one copy of the LTR in between. In this late passage of Q-B77-11, polyadenylic acid [poly(A)]-containing RNA transcripts which are twice the size of the viral genome have also been detected.

MATERIALS AND METHODS

Cells. Quail embryo fibroblasts were infected at low multiplicity with the Bratislava 77 strain of ASV, and transformed cells were selected by their ability to form colonies in agar. Q-B77-11, which we have studied further in this manuscript, is one of the nonproducer transformed quail clones characterized in a previous report (8). The procedures and media for the isolation and growth of transformed cell clones were described previously (8, 12).

Analysis of integrated proviral DNA. The procedures for analysis of the integrated viral DNA were described in a previous report (12). Briefly, DNA was extracted from Q-B77-11, subjected to restriction endonuclease digestion, and electrophoresed on a 0.7% agarose slab gel. The DNA was then transferred to a nitrocellulose filter by the method of Southern (14), and viral sequences were detected by hybridization with a ³²P-labeled complementary DNA (cDNA)

probe, synthesized with the aid of random calf thymus DNA primers as previously described (6). In some experiments, a nick-translated probe of a 6.8-kilobase (kb) viral DNA fragment was used. This 6.8-kb fragment, which was derived from the *SacI* digest of ASV DNA intermediates and cloned in Charon 16A lambda phage, represents the portion between 0.19 and 6.95 kb from the left-hand side of the unit viral genome. *KpnI* has a single site on ASV, 5.08 kb from the left side. Thus, the cloned probe has 4.89 kb complementary to the left side of the *Kpn* site and 1.87 kb to the right side.

Analysis of poly(A)-containing viral RNA. Total cell RNA was isolated by a guanidinium thiocyanate extraction (2). Briefly, monolayer cultures of Q-B77-11 were rinsed with Tris-saline and extracted with 4 M guanidinium thiocyanate in 0.5% sodium dodecyl sulfate, 25 mM sodium citrate (pH 7.0), 100 mM 2-mercaptoethanol, and 0.1% Antifoam A (Sigma). One gram of cesium chloride was added for each ml of lysate, and the mixture was centrifuged over a cushion of 7.5 M cesium chloride in 50 mM sodium acetate (pH 7) and 1 mM EDTA, using an SW60 rotor at 40,000 rpm for 16 h at 20°C. The RNA band was collected, precipitated with ethanol, and passed through an oligodeoxythymidylic acid-cellulose column. Poly(A)-containing RNA was denatured with glyoxal (9) and subjected to electrophoresis on a slab gel of 0.5% agarose in 25 mM sodium phosphate (pH 6.8) and 2 mM EDTA for 16 h at 0.8 V/cm. The RNA in this gel was transferred to a diazobenzyloxymethyl paper (1) and then hybridized with a ³²P-labeled cDNA probe.

RESULTS

Q-B77-11 is one of the ASV-transformed quail clones we studied previously (8, 12). It releases noninfectious virus particles and contains apparently full-size integrated proviral DNA. These cells were subjected to an extended period in

tissue culture, being passaged approximately once per week. To check for alterations in the proviral DNA, total cell DNA was extracted from both an early passage (the 15th) and a later passage (the 28th), subjected to *Kpn*I digestion, and electrophoresed on 0.7% agarose slab gel. The viral DNA in the gel was assayed by the method of Southern (14) and hybridized with a 32 P-labeled cDNA probe (Fig. 1). The lane on the left in Fig. 1 shows the *Hind*III fragments of 32 P-labeled phage lambda DNA as molecular weight markers. *Kpn*I cleaves viral DNA once, approximately in the middle of the genome (6, 16). Thus, the proviral DNA of Q-B77-11, which integrates at a unique site, should release two cell-virus junction fragments. In Fig. 1, junction bands of 21.00 and 10.68 kb in size were observed in samples from both the early and late passages. As in the previous study (12), the 8.50-kb band is interpreted as containing the endogenous *src*-related sequences. Also, the 17.25-kb band represents ribosomal DNA-related sequences (12) and is labeled because of rRNA in the viral RNA stock which was used to make the cDNA probe. However, the 9.47-kb band, which is indicated by an arrow in Fig. 1, could only be detected in the sample from the late passage; 9.47 kb is the size of a unit-length ASV genome. Another restriction enzyme, *Hpa*I, which also makes only one cut in the ASV genome, likewise released this novel 9.47-kb fragment from the late passage

(data not shown). This fragment, therefore, must arise either from two or more copies of integrated proviral DNA in tandem or from unintegrated circular DNA intermediates. Data from both the Hirt extraction (5), which separates unintegrated viral DNA from high-molecular-weight cellular DNA, and sucrose gradient fractionation of the total DNA indicated that this 9.47-kb novel band was indeed released from the integrated tandem proviral DNA (data not shown).

Figure 2 shows the interpretation of the alteration that occurred in the proviral DNA of Q-B77-11. During the growth of Q-B77-11, the integrated viral DNA changed from a single copy in the early passage (upper line in Fig. 2) to a tandem arrangement in the late passage (lower line in Fig. 2). To determine how many copies of proviruses are in tandem, the *Kpn*I digest of the total DNA from the late passage of Q-B77-11 was studied as described for Fig. 1. However, in this case, after Southern transfer, the proviral DNA sequence was hybridized with the nick-translated recombinant DNA probe described in Materials and Methods (data not shown). Then, using the known location of the *Kpn*I site on this recombinant probe and the relative intensity on the autoradiograph of the 9.47-kb fragment with respect to the cell-virus junctions, we deduced that there were, on the average, two copies of proviral DNA in tandem in the late passage, as shown in Fig. 2. Thirteen subclones obtained from cells of late-passage Q-B77-11 all showed the same tandem arrangement when the *Kpn*I digest of their total DNA was studied as described for Fig. 1.

To understand the structure of the junction between the two tandem proviruses, the size of that junction fragment released by *Xho*I digestion (see Fig. 2) was studied. Previous studies showed that *Xho*I, which makes three cuts in an ASV genome (16), releases 3.23-kb and 2.91-kb

FIG. 1. Agarose gel electrophoresis of *Kpn*I digest of Q-B77-11 DNA. From 4 to 8 μ g of Q-B77-11 DNA from early and late passages was digested with *Kpn*I, followed by electrophoresis for 16 h at 0.8 V/cm on a gel of 0.7% agarose. Viral sequences were detected by the method of Southern (14) using a 32 P-labeled cDNA probe. In a separate experiment, the nick-translated probe referred to in the text was used to identify the left and right cell-virus junctions. Identification of the various bands is given in the text. *Hind*III fragments of 32 P-labeled phage lambda DNA, as shown on the left side, with lengths expressed in kb, were used to deduce the sizes of the virus-related bands, with an estimated error of 5%.

FIG. 2. Interpretation of the alteration in the proviral DNA of Q-B77-11. The upper line shows a single copy of proviral sequence integrated in the cell DNA at early passage. After an extended period in culture, the integrated proviral DNA changed from one copy to two tandem copies, as shown in the lower line. Restriction enzyme sites of *Kpn*I and *Xho*I are also indicated for the ASV genome (6, 16).

3'-5' junction fragments from the large and small circular DNA intermediates of ASV, respectively (6). We have concluded that there are two copies of LTR in the large circular DNA intermediates and only one copy in the small circular species. Lane B in Fig. 3 shows the *Xho*I digest of total cell DNA from late-passage Q-B77-11. The junction fragment between the two tandem proviruses comigrated with a 2.91-kb band released from the small circular DNA intermediates; therefore, between the two tandem proviruses there are not two, but approximately one copy of the LTR, as shown in Fig. 2, lower line. The detailed structure of this copy of the LTR can be obtained by nucleotide sequencing, and such results may provide a clue to the mechanism of generating the tandem duplication. Lane C, Fig. 3, shows the *Xho*I digest of unintegrated linear and circular DNA which was isolated by a Hirt extraction (5) of ASV-infected quail embryo fibroblasts. This sample was contaminated with a small amount of cell DNA. In Fig. 3, two internal fragments of 4.54 and 1.75 kb were released from both the linear and circular DNA intermediates, as well as the integrated proviral DNA, as shown in the previous studies (6, 12). Unintegrated linear DNA also released 2.62-kb and 0.75-kb end fragments; however, the 0.75-kb band was too small to be shown in Fig. 3.

Viral RNA transcribed in the cells of late-passage Q-B77-11 was also studied. Total RNA of these cells was extracted by guanidinium thi-

ocyanate and passed through a column of oligodeoxythymidylic acid-cellulose. The poly(A)-containing RNA was treated with glyoxal and subjected to electrophoresis on a slab gel of 0.5% agarose. The RNA in the gel was transferred to a diazobenzyloxymethyl paper and then hybridized with a 32 P-labeled cDNA probe. Three exposures of the same autoradiograph of this sample are shown in lanes B through D in Fig. 4. Relative to the molecular weight markers in lane A, the poly(A)-containing RNA has virus-related species of 10.2, 5.37, and 3.34 kb. Based on the work of others (19), these are identified as genome-sized, *env*, and *src* messengers, respectively. Longer exposures showed an additional 19.9-kb band, as indicated in Fig. 4 by an arrow. This 19.9-kb band, which is about twice the genome size and unique to late-passage Q-B77-11, is interpreted to be transcribed from the tandem proviral DNA. From densitometer scanning of various autoradiogram exposures we deduced that the amount of this transcript relative to the genome-sized RNA is approximately 0.6%. Other faint bands which migrated between 19.9 and 10.2 kb may have arisen by splicing of tandem transcripts.

If, as we have interpreted, the 19.9-kb fragment is in fact a double-length transcript, then we have detected transcription which proceeds

FIG. 3. Agarose gel electrophoresis of *Xho*I digest of Q-B77-11 DNA from the late passage. Lane A shows the same molecular weight markers as in Fig. 1. Lane B shows the *Xho*I digest of DNA from Q-B77-11 late passage, and lane C contains mainly the *Xho*I digest of nondefective linear and circular DNA intermediates, which were contaminated with a small amount of cellular DNA (see text). Electrophoresis and transfer to nitrocellulose paper were carried out as described in the legend to Fig. 1. The bands indicated by the size in kilobases are discussed in the text. The additional bands arose because of ribosomal DNA contamination of the cDNA probe (12).

FIG. 4. Agarose gel electrophoresis of poly(A)-containing RNA from the late passage of Q-B77-11. After glyoxylation, samples were electrophoresed on a gel of 0.5% agarose for 16 h at 0.8 V/cm, the nucleic acids were transferred to diazobenzyloxymethyl paper (1), and viral sequences were detected with 32 P-labeled cDNA probe. Lanes B, C, and D are three exposures of the same autoradiograph. Lane A shows *Hind*III fragments of phage lambda DNA, terminally labeled with [α - 32 P]ATP and T4 polynucleotide kinase and used as molecular weight markers.

through the central LTR sequence. The number, 0.6%, is most likely an underestimate, since we were only quantitating those which failed to be spliced; nevertheless, it gives us a minimum estimate of the extent with which induced transcription of cellular sequences might be detected downstream from integrated proviruses.

DISCUSSION

In this work we observed the tandem duplication of the proviral DNA in a transformed quail clone, Q-B77-11. The integrated proviral DNA changed from one copy in the early passage to two copies in tandem in the late passage. During extended tissue culture, those cells with two copies of proviruses took over the population. Thirteen subclones obtained from the late passage of Q-B77-11 all contained the tandem proviruses; therefore, at least 13 of 14, i.e., 93% of that cell population, had the tandem proviruses at the time of subcloning. Selective pressures must have favored the outgrowth of those cells that had acquired an extra copy of the proviruses. A double dosage of the transforming gene might provide some growth advantage in ASV-transformed cells; however, we have not yet determined whether late-passage Q-B77-11 produces more *src* mRNA or gene product.

The integrated viral DNA with its flanking LTR in the early passage of Q-B77-11 is analogous to the transposable elements of procaryotes (15, 17). After tandem duplication, two copies of integrated proviral DNA were in tandem with only one copy of LTR in between. Similar tandem duplication has been reported for the Tn9 insertion in lambda, which also results in two copies of the chloramphenicol resistance element and three copies of the insertion sequences (IS1), i.e., only one copy of IS1 in between (11). O'Rear et al. (10), with recombinant clones of integrated retroviral DNA, reported a tandem duplication which arose either during the infection or as a consequence of cloning.

Several models have been proposed to interpret the amplification of the tetracycline resistance determinant on plasmid pAMa1 in *Streptococcus faecalis* (3). The same duplication and recombination pathways may account for the alteration that occurred in Q-B77-11, and one of these pathways involves an unequal crossing-over between homologous sequences.

Previous studies have shown that Q-B77-11 releases polymerase-containing particles which are negative in a focus-forming assay (8). Five days after quail embryo fibroblasts were incubated with this virus, the amount of reverse transcriptase activity in the tissue culture fluids was at background levels, i.e., less than 10^{-4} of wild-type virus control. However, 18 h after in-

cubation, both linear and circular DNA intermediates could be detected by the Southern technique at about 5% of the control. Integrated proviral DNA was not detected (unpublished data). Therefore, we consider it unlikely that tandem proviruses in the late passage of Q-B77-11 are via an integration of viral DNA into an already integrated provirus. This interpretation is consistent with previous studies which have been unable to detect tandemly integrated proviruses in ASV-transformed cells (4, 7, 12, 18). In a preliminary study we have also observed what might be an excision event which reversed the integrated proviruses from two copies in tandem to a single copy, together with the release of an unintegrated unit-length DNA fragment (unpublished data). Further experiments are being carried out to obtain culture conditions which will facilitate the induction or selection, or both, of the tandem duplication or excision.

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LITERATURE CITED

1. Alwine, J. C., D. J. Kemp, and G. R. Stark. 1977. A method for transferring RNA or DNA from agarose gels to diazobenzoyloxymethyl paper. *Proc. Natl. Acad. Sci. U.S.A.* 74:5350-5354.
2. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
3. Clewell, D. B., and Y. Yagi. 1977. Amplification of the tetracycline resistance determinant on plasmid pAMa1 in *Streptococcus faecalis*, p. 235-246. In A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), *DNA insertion elements, plasmids, and episomes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. Collins, C. J., and J. T. Parsons. 1977. Integration of avian sarcoma virus DNA sequences in transformed mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 74:4301-4505.
5. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
6. Hsu, T. W., J. L. Sabran, G. E. Mark, R. V. Guntaka, and J. M. Taylor. 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. *J. Virol.* 28:810-818.
7. Hughes, S. H., P. K. Shank, D. H. Spector, H. J. Kung, J. M. Bishop, and H. E. Varmus. 1978. Proviruses of avian sarcoma virus are terminally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. *Cell* 15:1397-1410.
8. Mason, W. S., T. W. Hsu, C. Yeater, J. L. Sabran, G. E. Mark, A. Kaji, and J. M. Taylor. 1979. Avian sarcoma virus-transformed quail clones defective in the production of focus-forming virus. *J. Virol.* 30:132-140.
9. McMaster, G. K., and G. G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine.

- dine orange. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4835-4838.
10. O'Rear, J. J., S. Mizutani, G. Hoffman, M. Fianndt, and H. M. Temin. 1980. Infectious and noninfectious recombinant clones of the provirus of SNV differ in cellular DNA and are apparently the same in viral DNA. *Cell* **20**:423-430.
 11. Rosner, J. L., and M. M. Gottesman. 1977. Transposition and deletion of Tn9: a transposable element carrying the gene for chloramphenicol resistance, p. 213-228. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 12. Sabran, J. L., T. W. Hsu, C. Yeater, A. Kaji, W. S. Mason, and J. M. Taylor. 1979. Analysis of integrated avian RNA tumor virus DNA in transformed chicken, duck, and quail fibroblasts. *J. Virol.* **29**:170-178.
 13. Shank, P. R., S. Hughes, H.-J. Kung, J. E. Majors, N. Quintrell, R. V. Guntaka, J. M. Bishop, and H. E. Varmus. 1978. Mapping unintegrated forms of avian sarcoma virus DNA: both termini of linear DNA bear a 300 nucleotide sequence present once or twice in two species of circular DNA. *Cell* **15**:1383-1396.
 14. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
 15. Taylor, J. M. 1979. DNA intermediates of avian RNA tumor viruses. *Curr. Top. Microbiol. Immunol.* **87**:23-41.
 16. Taylor, J. M., T. W. Hsu, and M. M. C. Lai. 1978. Restriction enzyme sites on the avian RNA tumor virus genome. *J. Virol.* **26**:479-484.
 17. Taylor, J. M., W. S. Mason, T. W. Hsu, J. L. Sabran, C. Yeater, G. E. Mark, A. Kaji, R. V. Guntaka, and M. M. C. Lai. 1979. Avian RNA tumor virus DNA before and after integration into the host genome. *Cold Spring Harbor Symp. Quant. Biol.* **43**:865-867.
 18. Weinberg, R. A. 1980. Integrated genomes of animal viruses. *Annu. Rev. Biochem.* **49**:197-226.
 19. Weiss, S., H. E. Varmus, and J. M. Bishop. 1977. The size and genetic composition of virus-specific RNAs in the cytoplasm of cells producing avian sarcoma-leukosis viruses. *Cell* **12**:983-992.