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Structure of the baboon endogenous virus genome: cloning of circular virus DNA in bacteriophage λ

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ABSTRACT

Linear, small and large circular forms of unintegrated viral DNAs were detected in Hirt supernatant fraction of human cultured cells infected with baboon endogenous virus M7. The circular M7 DNAs were cloned in bacteriophage λ, Charon 28. Seventeen independent clones were isolated and analyzed by restriction endonuclease mapping. Nine clones were carrying a viral sequence of 8.6 kilobase pairs (kb) with two tandem repeats of 0.6 kb, which correspond to the large circular form of the unintegrated M7 DNA. Eight other clones had the viral insert of 8.0 kb, i. e., the small circular form, and were deleted one of the repeated sequences. The repeated sequences correspond to the long terminal repeats of 0.6 kb, located at both ends of the linear M7 DNA of 8.6 kb. One of the recombinants of the large circular M7 DNA had an inversion of 2.5 kb. One end of the inverted sequence was near the terminus of the long terminal repeats and the other in the gag gene region. The inversion seems to be occurred by integration of a viral DNA within itself during early periods of infection. The mechanism of the processes leading to integration is discussed from the structure of these unintegrated M7 DNAs as the precursors.

INTRODUCTION

DNA transcripts of retroviruses are integrated into the host genome in early periods of infection. Studies on avian and murine retroviruses have revealed that the DNA transcripts consist of linear, small and large circular double-stranded molecules (for review see reference 1). The linear DNA has identical sequences of 0.34 to 1.4 kb at both ends, called long terminal repeats (LTRs), in which initiation, termination signals of transcription and inverted terminal repeats of 3 to 12 nucleotide pairs are located (2-8). The small and large circular DNAs hold one and two copies of the LTR, respectively (9, 10). The LTR seems to play important roles in the integration and the expression of the viral genome. However, detailed processes of the early retroviral infection remain still obscure because the studies are hampered by related endogenous sequences in uninfected avian and murine cells.

The M7 strain of baboon endogenous virus (11) is a primate retrovirus

related to RD114, an endogenous virus of domestic cats (12, 13). M7 can grow in cultured human cells and does not share any major homology of the nucleotide sequences with the host cell DNA (14). So, this virus provides a good experimental system for studies on the mechanism of integration and expression of the viral genome. We have reported the restriction map of linear M7 DNA extracted from infected human cells (Abstr. 2nd Meet. Mol. Biol. Soc. Japan, 1979; Abstr. Cold Spring Harbor Meet. RNA Tumor Virus., 1980. See Fig. 1). Battula and Todaro (15) have recently reported the map of the linear M7 DNA extracted from infected canine cells. Cohen et al. (16) reported the physical map of BAB8-K virus, another isolate of baboon endogenous virus. The map of BAB8-K and our map of M7 are coincident.

In this report, we describe molecular cloning and detailed structural analysis of the unintegrated circular DNAs of M7.

MATERIALS AND METHODS

Preparation of Viral DNA. A human rhabdomyosarcoma cell line, A204 (17), were infected with baboon endogenous virus M7 in the presence of 8 μg/ml polybrene (Sigma) at 37°C for 60 min. After incubation in Dulbecco's modified minimum essential medium (GIBCO) supplemented with 7.5% newborn calf serum (Mitsubishi Kasei Industrial Co., Tokyo) for 16 hr, DNA was extracted from the Hirt's supernatant fraction as described by Yang et al. (18). The DNA was banded with a marker of closed circular DNA, col E_1 factor, in a CsCl-propidium diiodide gradient (19) and fraction were collected. Propidium diiodide was removed by isopropanol equilibrated with saturated CsCl solution. Then, the fractions were dialyzed against 10mM Tris·HCl, pH 8.0, 1mM EDTA and precipitated with ethanol after additon of sodium acetate to 0.1M.

Restriction Endonucleases. The enzymes were purchased from New England BioLabs, Bethesda Research Laboratories and Takara Shuzo Co., Kyoto. Digestion of DNA was performed under conditions recommended by the suppliers.

Agarose Gel Electrophoresis and Blotting Hybridization. DNA samples were re-

Fig. 1 Restriction map of unintegrated linear DNA of baboon endogenous virus, M7. The orientation of the viral RNA is indicated by 5' and 3' marks.

solved by electrophoresis in horizontal slab gel of 0.7 to 1.5% agarose (Bethesda Research Laboratories) according to the procedures of Sharp et al. (20). M7-specific sequences in the gel were detected by Southern's blotting hybridization (21), using [^{32}P]labeled M7 complementary DNA (cDNA). The cDNA was synthesized with purified M7 70S RNA and reverse transcriptase of avian myeloblastosis virus (Life Science Co.) as described by Taylor et al. (22).

Molecular Cloning of M7 Circular DNA. A derivative of bacteriophage λ, Charon 28 (23), was used as a vector. An internal Eco RI fragment (5.8 kb) of this phage DNA was removed by the method of Maniatis et al. (24), and the arms (1.8 μg) were ligated with the Eco RI- and Pvu I-treated circular DNA of the fraction 2 shown in Fig. 2 (0.9 μg), using 0.2 units of T4 DNA ligase, for 16 hr at 4°C in 16.7 μl of a buffer containing 60mM Tris·HCl, pH 7.4, 10mM $MgCl_2$, 200 μg/ml gelatin, 1mM ATP, 1mM spermidine and 3mM dithiothreitol. The ligated DNA was packaged in vitro as described by Enquist and Sternberg (25). The efficiency of packaging of intact Charon 28 DNA was about 3×10^7 plaque-forming units (PFU) per μg DNA. Ligated Charon 28 arms alone gave less than 1×10^2 PFU/μg DNA. Ligation of the M7 DNA and the arms gave 7×10^3 PFU/μg DNA.

Screening for Recombinants. The packaged phage (2×10^4 PFU) was plated with indicator bacteria, LE392 (23). The recombinants containing M7 sequences were detected by plaque hybridization (26) using the [^{32}P]labeled M7 cDNA. Hybridization-positive clones were purified twice.

Restriction Mapping of Cloned DNA. DNA fragment to be mapped was fractionated by agarose gel electrophoresis and eluted from the gel by the method described by Tabak and Flavell (27). One terminus of the fragment was selectively labeled with [^{32}P]nucleotides by reverse transcriptase. The reaction mixture consisted of DNA (12μM as the terminal bases to be labeled), 100mM Tris·HCl, pH 7.5, 10mM $MgCl_2$, 350 units/ml reverse transcriptase, 52μM [α^{32}P]dNTP, and 72μM cold deoxyribonucleotide triphosphates if necessary. The restriction sites on the DNA fragment were determined by the partial digestion method described by Smith and Birnstiel (28).

Containment. All the recombinant DNA experiments were performed in P3-EK1 containment according to the Guidlines of the Japanese Government for Recombinant DNA experiments.

RESULTS

Circular DNA of M7. Three molecular species of unintegrated viral DNA in the Hirt supernatant fraction of A204 cells infected with M7 were detected after

CsCl-propidium diiodide gradient centrifugation (Fig. 2). Linear M7 DNA of 8.6 kb was observed mainly in the fraction 4, where the bulk of the cellular DNA was banded. Two species of circular M7 DNA were detected in the fraction 2, where an internal marker, col E_1 DNA, was also banded. Eco RI digestion of the fraction 2 gave two linear viral DNA bands at 8.0 and 8.6 kb (Fig. 2, slot 6). To define the structures of the viral DNAs, we digested these two fractions with Bam HI (Fig. 3a). Fragment of 2.7, 1.8 and 1.0 kb were commonly detected. These fragments constitute the internal Bam HI fragments of the M7 linear DNA (see Fig. 1). In addition, from the circular DNA fraction, 2.5 and 3.1 kb fragments were recovered, whereas from linear DNA, 1.2 and 1.9 kb fragments were recovered. The fragment of 3.1 kb appeared to be derived from the terminal 1.2 and 1.9 kb fragments joined togather to form circular molecules. Addition of either 2.5 or 3.1 kb fragments to the total of the three internal fragments (5.5 kb) gave 8.0 and 8.6 kb, corresponding to the size of each of the two circular M7 DNAs. The structure of the circular M7 DNAs, suggested by these results, are illustrated in Fig. 3b.

Cloning of the M7 Circular DNA. We cloned the Eco RI-digested circular DNA of

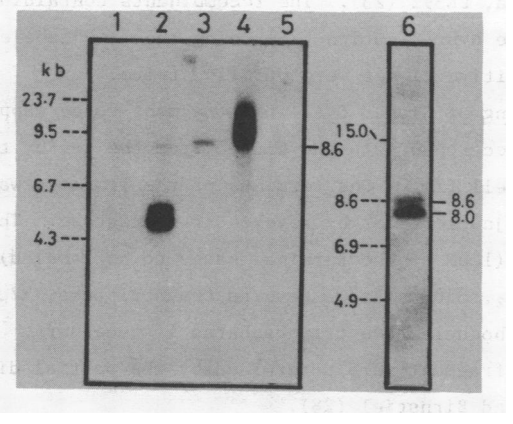

Fig. 2 Detection of unintegrated M7 DNA. Slots 1 to 5: Hirt supernatant DNA extracted from early infected cells was fractionated into 5 fractions by density gradient centrifugation. The DNA of each fraction was resolved by agarose gel electrophoresis (1%), and the viral sequences were detected by Southern's technique. Density of each fraction shown in the slots was; 1, 1.5862; 2, 1.5623; 3, 1.5406; 4, 1.5113; 5, 1.4961 g/cc. Slot 6: The DNA in the fraction 2, shown in slot 2, was digested with Eco RI, and the viral DNAs were detected similarly, except that the agarose concentration was 0.7%. Broken lines indicate the positions of size markers; λDNA digested with Hind III for slots 1 to 5, and with Ava I for slot 6. DNA extracted from the uninfected cells, which had been fractionated similarly, did not exhibit any detectable bands (data not shown).

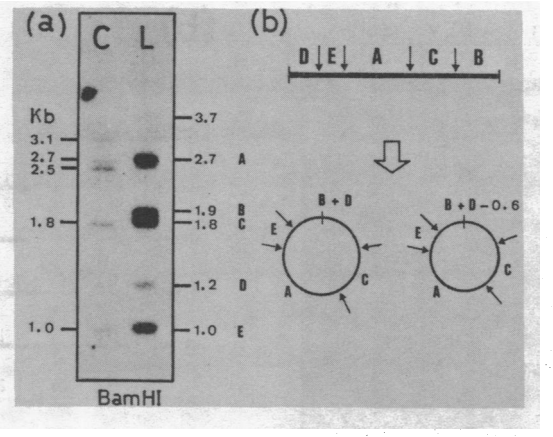

Fig. 3 Bam HI digestion of the linear and circular M7 DNAs. (a) The DNA samples shown in the slots 2 and 4 in Fig. 2 were respectively digested with Bam HI, resolved by agarose gel electrophoresis (1.5%), and detected by Southern's technique. C, circular DNA; L, linear DNA. (b) Interpretation of the cleavage patterns shown in (a). Arrows indicate the cleavage sites for Bam HI.

M7 into a bacteriophage vector, Charon 28, from which an internal 5.8 kb Eco RI fragment has been eliminated. The M7 DNA sample to be cloned, the fraction 2 shown in Fig. 2, was additionally digested with another restriction endonuclease, Pvu I. Because M7 DNA has no Pvu I sites, the number of non-viral recombinants was expected to be reduced by this treatment. Using 0.9 µg of the circular DNA fraction and 1.8 µg of Charon 28 arms, 2 x 10^4 plaques were recovered after *in vitro* packaging. Seventeen independent recombinant phages harboring the M7 sequences were isolated.

Structure of Cloned M7 DNA. The DNA of Charon 28-M7 recombinants, designated as "λBEV", were extracted and cleaved with Bam HI. The 17 recombinant phages were classified into five distinctive types by the cleavage patterns with Bam HI. The results on a representative clone of each type are shown in Fig. 4. The 9.2 kb fragments were derived from the vector arm and did not hybridize with the viral cDNA (data not shown). Two fragments of 1.8 and 1.0 kb were detected in all the recombinant DNAs, which corresponded to the fragments C and E of the linear M7 DNA (Fig. 3b). The 2.5 and 3.1 kb fragments seemed to be derived from the joint regions of the terminal Bam HI fragments, B and D, of the linear M7 DNA (Fig. 3b). Therefore, λBEV-11 and -32 that yielded the 3.1 kb fragment should harbor the large circular M7 DNA, while λBEV-12 and -31 that yielded the 2.5 kb fragment should harbor the small circular M7 DNA. The

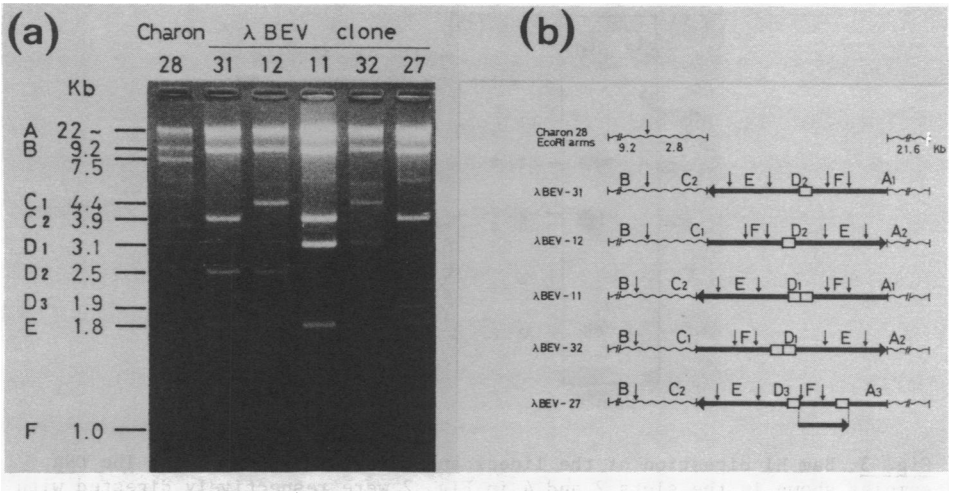

Fig. 4 Genome structure of five representative recombinant phages. (a) The DNAs of λBEV-31, -12, -11, -32, -27 and the parental Charon 28 were digested with Bam HI and resolved by electrophoresis in 1.2% agarose gel. Bam HI splits the Charon 28 DNA into three fragments of 22, 9.2 and 7.5 kb in size. (b) Interpretation of the results shown in (a). Waved and bold lines indicate vector arms and viral inserts, respectively. Open boxes indicate the putative position of the LTRs, whose detailed structure is shown in Fig. 7. Arrows indicate the Bam HI sites. The fragments A to F correspond to the bands shown in (a).

other fragments, A, C_1 and C_2 of Fig. 4a, were resulted from ligation of the M7 DNA to the vector arms. Bam HI cuts the 12.0 kb Charon 28 arm at one site, resulting two fragments of 2.8 and 9.2 kb (see the top line of Fig. 4b). Eco RI splits the 2.7 kb Bam HI fragment of M7 DNA into two fragments of 1.1 and 1.6 kb (Fig. 1). Therefore, the fragment C_1 (4.4 kb) must have been derived from the 1.6 kb M7 fragment and the 2.8 kb fragment of Charon 28 arm, while the fragment C_2 (3.9 kb) from the 1.1 kb M7 fragment and the 2.8 kb Charon 28 fragment. The fragment A of Fig. 4a seemed to be derived from the 21.6 kb Charon 28 arm joined with the M7 end fragments. Thus, the polarity of the M7 DNA to the vector could be determined by the size of the fragment C_1 and C_2. The genome structure of the four representative clones are shown in Fig. 4b. Out of 17 independent clones, 16 were classified into these four types (Table 1). Interestingly, in 12 out of the 16 clones, the M7 DNA was joined with the Charon 28 DNA in the same polarity.

The fifth type of the recombinant phage consists of a single isolate, λBEV-27. This clone carries 8.6 kb M7 sequence. The Bam HI-digested DNA of

Table 1. Summary of the λBEV clones isolated.

M7 DNA	Orientation*	Number of clones
small	←[a]	6
small	→[b]	2
large	←[a]	6
large	→[b]	2
inverted	←[a]	1
total		17

[a]An orientation represented by λBEV-11.
[b]An orientation represented by λBEV-32.

the clone gave 5 bands similar to those of λBEV-11, except the 3.1 kb fragment (Fig. 4a). A unique fragment of 1.9 kb was obtained. As shown in the later section, this clone is a recombinant of the large circular M7 DNA with an internal inversion (Fig. 4b, bottom).

Detailed Maps of the Cloned Circular M7 DNA. The M7 sequences cloned in λBEV-31 and -11 were mapped by cleavage with restriction endonucleases. Two fragments of M7 DNA were obtained from each clone by double digestion with Eco RI and Hind III. The ends of the fragments generated by Hind III, which carried a single stranded 5'AGCT- sequence, were selectively labeled by filling up-reaction with [α^{32}P]dCTP and reverse transcriptase, leaving the Eco RI-ends (5'AATT-) unlabeled. These end-labeled M7 fragments were analyzed by electrophoresis after partial digestion with restriction enzymes. In λBEV-11, two Xho I sites were located at 2.8 and 3.4 kb from the Hind III site, while only a single Xho I site was present at 2.8 kb from the Hind III site in λBEV-31 (Fig. 5). Similar experiments were carried out with other restriction enzymes, and the results are summarized in Fig. 7a and b.

λBEV-27 Contains an Inverted Segment Within the Viral Insert. λBEV-27 was analyzed similarly and the results are shown in Fig. 6 and 7c. In this clone, two Xho I sites were separated by about 2.3 kb and the Sma I, Acc I, Bam HI, Sal I and Pst I sites were located within the two Xho I sites. The order of the sites of these enzymes was exactly inverse of that observed in a region between the Pst I and Xho I sites of λBEV-11. Therefore, the M7 sequence in this particular clone contains an inversion of about 2.5 kb in the left end region of the linear M7 map (Fig. 1).

λBEV-11 DNA Contains Two Tandem Direct Repeats of 0.6 kb. To obtain further information about the region around the two Xho I sites of λBEV-11, the viral

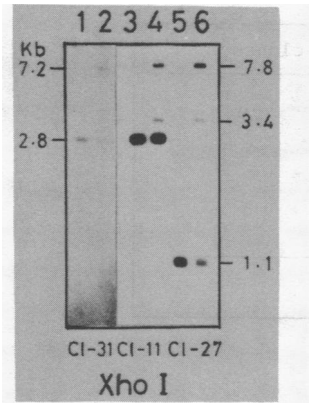

Fig. 5 Restriction mapping of the cloned viral DNAs: Xho I digestion of the viral DNAs of three representative clones is shown as an example. The larger viral DNA fragments generated by double digestion with Eco RI and Hind III were isolated, and the Hind III ends of the fragments were selectively labeled. Each fragment was completely (slots 1, 3, 5) or partially (slots 2, 4, 6) digested with Xho I, and resolved by agarose gel electrophoresis (1.2%). The radioactive bands were detected. The smaller Eco RI-Hind III fragments of these clones were not cleaved with Xho I (data not shown).

sequence was isolated after Eco RI digestion, and cleaved with Xho I and Sma I. Resulted four viral fragments of 4.6, 3.4, 0.52 and 0.08 kb were isolated by agarose gel electrophoresis and radio-labeled at the Xho I ends. Then, these labeled fragments were analyzed by digestion with restriction enzymes

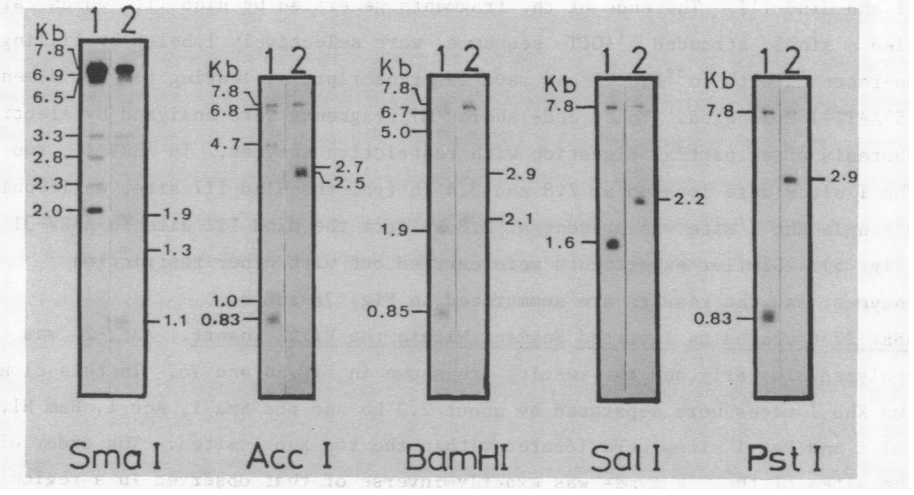

Fig. 6 Restriction mapping of the inverted region of λBEV-27. The end-labeled 7.8 kb Eco RI-Hind III fragments of λBEV-11 (slots 1) and -27 (slots 2), prepared as described in Fig. 5, were partially digested with restriction enzymes indicated below each plate, and analyzed as described in Fig. 5. A complete digestion with each enzyme gave only the smallest band shown in the figure. The results are summarized in Fig. 7a and c.

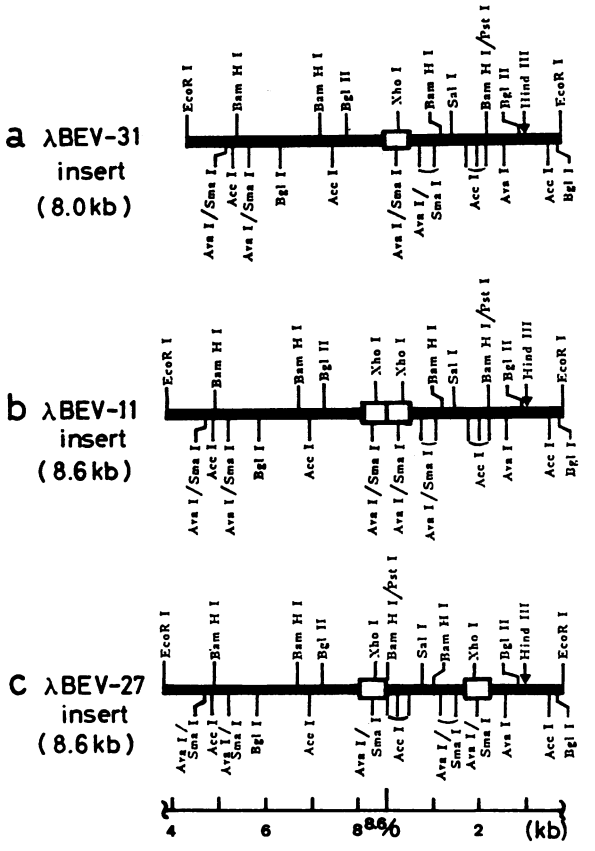

Fig. 7 Restriction maps of the cloned circular DNA of baboon endogenous virus. The results shown in Fig. 5 and 6, and those described in text are summarized. The arrows indicate the positions of [^{32}P] labeling used in the mapping. Open boxes indicate the LTRs. The position "0" on the map scale corresponds to the extreme left end of the linear M7 DNA (see Fig. 1).

(Fig. 8). Apparently, the Hha I, Hpa II, Xho I and Hinf I sites were repeatedly located at 0.6 kb intervals in a region from 0.4 kb left to 0.2 kb right of the two Xho I sites. Therefore, this region shown by two rectangles in Fig. 7 consists of two tandem direct repeats.

DISCUSSION

Three molecular species of unintegrated M7 DNA were detected in the virus-infected human cells; one linear molecule of 8.6 kb and two supercoiled molecules of 8.0 and 8.6 kb. Battula and Todaro (15) have recently reported

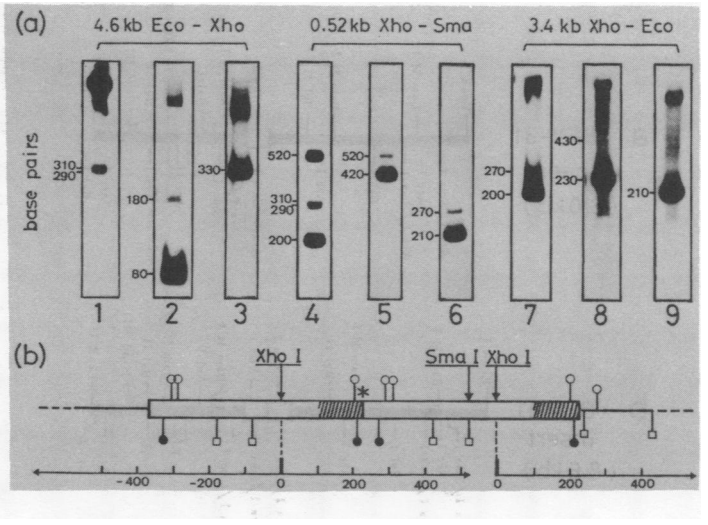

Fig. 8 Fine mapping of the LTR of baboon endogenous virus. (a) The viral insert of λBEV-11 was separated into three fragments with Eco RI and Xho I (see Fig. 7). The 0.6 kb fragment between the two Xho I sites was further digested with Sma I and the resulted larger fragment (0.5 kb) was isolated. The three fragments indicated at the top of each slot were partially digested with Hha I (slots 1, 4, 7), Hpa II (slots 2, 5, 8) and Hinf I (slots 3, 6, 9) after labeling the Xho I ends. The digested samples were analyzed and detected as described in Fig. 5, except that the electrophoresis was done in 6% polyacrylamide gel. The complete digestion of these fragments with excess amounts of each restriction enzyme gave only the smallest bands shown in the figure. The 0.08 kb fragment between the Sma I and the right Xho I sites (see (B)) was not cleaved with these enzymes (data not shown). (b) Summary of the results shown in (a). Sites for Hha I (○), Hpa II (□) and Hinf I (●) are indicated by vertical lines. The rectangles represent the units of 0.6 kb direct repeats, i. e., the LTRs. The shaded portions correspond to the extreme 5' end of the viral RNA genome. The asterisk indicates the junction of the ends of the linear M7 DNA. In the scale below, "0" points represent the positions of [^{32}P] labeling, and the numbers represent the length of nucleotide pairs.

similar findings on the unintegrated M7 DNAs extracted from infected canine thymus cells. To study the structures of these viral DNAs, we have cloned the Eco RI-digested circular M7 DNAs into phage λ. The structures of the cloned viral DNAs represent those of the unintegrated M7 DNAs detected in the virus-infected cells.

A clone which carries a 8.6 kb viral insert, λBEV-11, seems to have been originated from the large circular M7 DNA of the infected cell. The restriction mapping of this clone suggests that the large circular M7 DNA must be an end-to-end joined molecule of the linear M7 DNA. As shown in Fig. 8, two tandem, directly repeated sequences of 0.6 kb are located in the joining

point. These repeated sequences represent the LTRs of the baboon endogenous virus, which locate at both ends of the linear M7 DNA. On the other hand, a clone, λBEV-31, contains a viral insert of 8.0 kb, which must represent the small circular M7 DNA found in the infected cell. In the viral insert of this clone, only one Xho I site was observed. Therefore, the small circular form of the viral DNA seems to be deleted one of the LTRs. The structural features of these unintegrated M7 DNAs are quite analogous to those of other well-characterized retroviruses (9, 10).

From the arrangements of the restriction sites shown in Fig. 8, we concluded that the joining point of the two LTRs must be located at the site indicated by an asterisk. On the other hand, Lovinger and Schochetman (13) deduced the nucleotide sequence of the 5'-terminal 133 bases of the M7 RNA genome by analysis of the cDNA synthesized in vitro. It has been shown that the 5'-end region of retroviral genome corresponds to the right end portion of LTR (1, 29). Thus, this sequence of M7 represents the region from about 100 to 230 bases right from each Xho I site, i. e., the shaded protion of the rectangles shown in Fig. 8. From the sequencing data, they have suggested that clustered two Hinf I, an isolated Hha I and clustered five Hha I sites are located in this region. We have detected the isolated Hha I and the clustered Hinf I sites as a single site each. However, the clustered Hha I sites were not observed in our cloned DNA. Since the 5'-terminus of the retrovirus genome is suggested to play a key role in the formation of LTR (29, 30), some structural changes might occur in this region during reverse transcription of the genome. Alternatively, this might be one of the characteristics of this particular clone, λBEV-11.

The mechanism which generates these unintegrated viral DNAs are presently not known. In an avian sarcoma virus, the circular DNA is predominantly found in the nucleus of the early infected cells (31). Recently Yang et al. reported that formation of the closed circular DNAs and the virus production of a murine leukemia viruses were inhibited by treating the infected cells with cycloheximide (32). Taking advantage of low homology of the M7 sequence to the host DNA, we have done similar experiments with M7-infected human cells and found that the amount of integrated viral sequences as well as that of the circular DNAs were markedly reduced by cycloheximide (our unpublished data). These findings suggest that the circular DNA formation and the integration of retroviruses must be closely related. Two assumptions might be entertained; one or both species of the circular DNAs are the immediate intermediates leading to integration, or alternatively, the linear DNA might

be the precursor for integration and the circular DNAs might be formed only if the linear viral DNA has integrated into the host DNA.

We obtained a recombinant phage which might serve as a clue to studies on the mechanism of integration. In this clone, λBEV-27, the insert contained an internal inversion occurred at or near the edge of a LTR. The other end of the inversion located in the gag gene region. Shoemaker et al. reported a similar inverted clone which was found among the twelve recombinant phages carrying the circular DNAs of Moloney murine leukemia virus (33). They interpreted that such a structure had resulted from a circular viral DNA molecule integrating within itself. Their sequencing data suggested that two terminal base pairs are lost at the ends of the inversion. We are analyzing the sequences of the end region of the LTR in each recombinant DNA obtained in this study. Recently, Cohen et al. (34) proposed that the expression of the endogenous viral information in baboon cells are suppressed by base modification or small insertion which occurs in the gag gene region. Such structural changes might also be resulted from the "LTR-derived recombination" as observed in the inverted clones we obtained.

The circular DNAs of baboon endogenous virus were first cloned systematically and analyzed in this study. The clones we obtained were stable and no deletions nor inversions of the inserted viral sequences were observed during passages of the recombinant phages. Cohen et al., and Battula and Todaro have independently cloned the integrated proviral sequences of baboon cells, and the M7 DNA, respectively (cited in the discussion sections of their recent papers, 15, 34).

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