Molecular cloning of unintegrated closed circular DNA of porcine retrovirus

Iwao Suzuka, Nobuaki Shimizu+, Kiichi Sekiguchi, Hiroo Hoshino+, Michi Kodama and Kunitada Shimotohno+

National Institute of Animal Health, Tsukuba Science City, Ibaraki 305 and +*National Cancer Center Research Institute, Chuoh-ku, Tokyo 104, Japan*

Received 10 December 1985; revised version received 28 January 1986

Viral DNA of unintegrated closed circular form was isolated from a swine kidney cell line (SKL) which was infected with a porcine retrovirus Tsukuba-1 (PRetV) produced from a swine malignant lymphoma-derived cell line, Shimozuma-1 and cloned using a λ phage vector; Charon 21A. One of ten independent clones contained the 8.3 kb DNA fragment as an insert, which was thought to be a full length of viral DNA molecule carrying a long terminal repeat (LTR) sequence. We have analyzed this insert by mapping the recognition sites of some restriction endonucleases by Southern blot hybridization with appropriate probes.

closed circular DNA *Cloning* *viral DNA* *Restriction map*

1. INTRODUCTION

In [1] we showed the presence of a new porcine retrovirus which was produced from the swine malignant lymphoma-derived cell line, Shimozuma-1. It was further found that the virus particles contained a high-M_r RNA of 70 S which was converted to 35 S as a viral genome. It was therefore necessary to isolate and clone the unintegrated ccDNA of PRetV from infected cells for an understanding of the molecular characteristics of the viral genome.

As a continuation of our previous work, we report here the first isolation of molecularly cloned unintegrated ccDNA of PRetV from infected SKL cells using λCharon 21A as a vector, and determination of the map for some of the restriction endonuclease recognition sites.

Abbreviations: kb, kilobase pairs; cDNA, DNA complementary to viral mRNA; ccDNA, closed circular DNA; PRetV, porcine retrovirus Tsukuba-1; SKL, swine kidney cell line; LTR, long terminal repeat; EtBr, ethidium bromide

2. MATERIALS AND METHODS

The SKL cell line was originally obtained from Dr A. Fukusho, National Institute of Animal Health. Purification of PRetV and the viral RNA were carried out as described [1,2].

About 10^8 SKL cells were infected with 20 ml PRetV-containing supernatant from 16 h cultured fluid of Shimozuma-1 cells. At 40 h after infection, the cells were harvested and unintegrated viral DNA extracted by the method of Hirt [3]. The extracted DNA (Hirt DNA) was subjected to CsCl/EtBr density gradient centrifugation at 48000 rpm for 46 h at 20°C in an SW 50.1 rotor. After centrifugation, the position at which ccDNA molecules would band was detected by dot blot assay as described in [4], and then the DNA was pooled and precipitated with 75% of ethanol. Restriction cleavage sites of ccDNA were obtained by 0.8% agarose gel electrophoresis after digestion with several restriction endonucleases, following Southern hybridization with PRetV[^{32}P]cDNA$_{rep}$ (see below) used as a probe and autoradiography [5].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

The mixture of linearized ccDNA generated by the single cut with *Xho*I digestion (about 20 ng) and λCharon 21A DNA cut with the same enzyme (150 ng) was ligated with T_4 DNA ligase, then packaged in vitro into infectious particles and amplified by lytic growth of *E. coli*, DP50supF according to Blattner et al. [6]. λCharon 21A used for the in vitro packaging was a genetic variant from original λCharon 21A [7]. The recombinant plaques were screened on the plaque hybridization assay until pure recombinant plaque was obtained. The single recombinant plaque was finally propagated using *E. coli* as a host in an appropriate growth medium. The nature of cloned DNAs was examined by EtBr staining and Southern blotting on agarose gel electrophoresis after digestion with *Xho*I. The sizes of the restriction fragments from the clone were computed from *Hin*dIII-digested λ phage marker in the gel. Further subcloning of cloned PRetV DNA to plasmid vector, pBR322, of which the *Pvu*II site was converted to *Xho*I (denoted $pBR322_{XhoI}$), was carried out by a standard method [8]. The resultant subclone was designated pPRetV8.3.

PRetV[^{32}P]$cDNA_{rep}$ was synthesized from PRetV RNA by the procedure of Taylor et al. [9] for use as a probe. ^{32}P-labeled PRetV cDNA of its 5′-terminal region (PRetV[^{32}P]$cDNA_{5'}$) was prepared from detergent-activated PRetV as in [10]. By digestion with *Sal*I and *Xho*I, two fragments of 1.4 and 2.95 kb were created from $pBR322_{XhoI}$. 0.3 kb fragment was separated from viral DNA insert by digestion of pPRetV8.3 with *Eco*RV. These fragments were used as hybridization probes after nick translation for restriction endonuclease mapping.

Restriction endonucleases were purchased from Toyobo (Tokyo), Bethesda Research Laboratories or Nippon Gene (Toyama, Japan). T_4 DNA ligase and bacterial alkaline phosphatase were from Toyobo. (α-^{32}P)-labeled deoxynucleotide triphosphates were from Amersham or NEN Research Products. *E. coli* DNA polymerase 1 was obtained from New England Biolabs. Reactions were conditioned under those recommended by the suppliers.

3. RESULTS AND DISCUSSION

To isolate the unintegrated ccDNA of PRetV, we extracted Hirt DNA from SKL cells infected with cultured fluid from Shimozuma-1 cells, a spontaneous producer of PRetV, and obtained DNA materials banded at the region of closed circular molecules on the CsCl/EtBr density gradient. The DNA was electrophoresed on agarose gel and subjected to Southern blot analysis with PRetV[^{32}P]$cDNA_{rep}$ as a probe. As shown in lane 1 of fig.1, this DNA revealed a single band corresponding to a hybridization signal of approx. 5.9 kb in size. To confirm that this band is a kind of closed circular molecule, the corresponding DNA was digested with several restriction endonucleases. As shown in lane 2 of fig.1, digestion of the DNA with *Xho*I gave a major band of approx. 8.8 kb. A faint hybridization band of about 2.7 kb was also observed. If the 5.9 kb band consists of the sizes of 8.8 and 2.7 kb, the 2.7 kb band should appear at approx. one-third of the density of the 8.8 kb band. From estimation of the densities of these bands, however, the ratio of the 2.7 to 8.8 kb band was approx. 1:17. This indicates that the majority of the 5.9 kb had a unique *Xho*I site and hence digestion with this enzyme produced the 8.8 kb band. At that time, it was not clear what

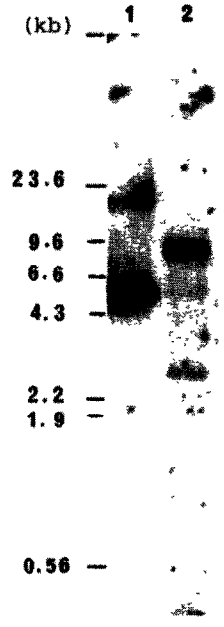
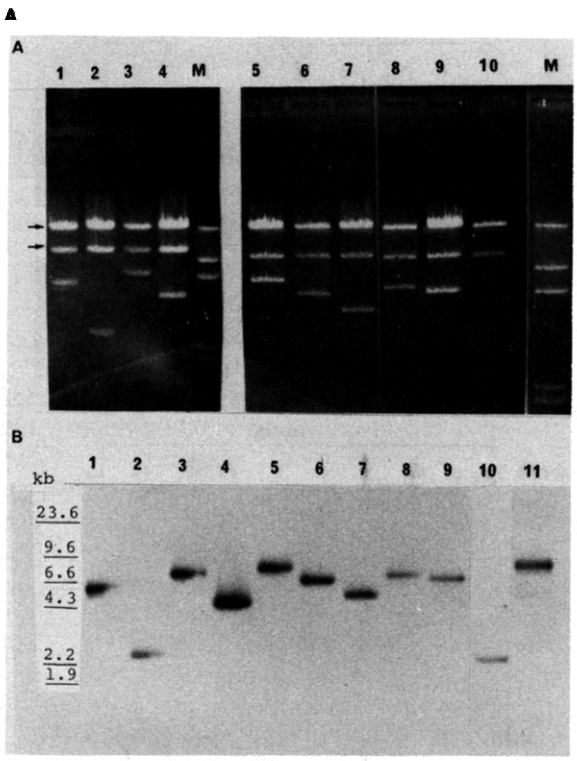


Fig.1. Identification of PRetV ccDNA. Lanes: 1, PRetV ccDNA; 2, *Xho*I-digested PRetV ccDNA. The marker DNA fragments (left) are wild-type λDNA digested with *Hin*dIII.

the minor, moving band represented and investigation of this band was deferred. On the other hand, digestion with other enzymes (*Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I) generated smaller hybridization bands than 8.8 kb (not shown). Thus, we concluded that the 5.9 kb DNA isolated by the Hirt method from infected SKL cells was the unintegrated PRetV ccDNA and use of the single *Xho*I site was available for molecular cloning of PRetV ccDNA.

The *Xho*I-digested PRetV ccDNA was ligated into the same sites of λCharon 21A vector, then packaged in vitro and amplified in *E. coli* as a host. Fifty positive signals were obtained in the primary screening of about 10^5 plaques. To determine whether recombinants contained DNA inserts of full size PRetV ccDNA, 10 positive clones were purified by successive screening and the resultant clones designated λPRetV-1, -2, -8, -9, -10, -11, -21, -22, -24 and -26, respectively. The DNA from these clones was digested with *Xho*I, electrophoresed on agarose gel and analyzed by EtBr staining followed by Southern blot hybridization. Fig.2A and B shows that all 10 recombinants carried a single DNA insert and hybridized with PRetV[^{32}P]cDNA$_{rep}$. Of these recombinants, the insert from λPRetV-2 revealed a size of 8.3 kb. PRetV ccDNA, however, had migrated at 8.8 kb as a linear molecule cleaved with *Xho*I (fig.1). It is well known that the retroviral LTRs have a wide range in size from 0.33 to 1.33 kb [11]. One possible explanation for the discrepancy in size between ccDNA and its clone is that the DNA insert may correspond to a full length of viral DNA carrying one copy of LTR, although the possibility that 0.5 kb of the *Xho*I fragments was deleted cannot be excluded. The appearance of the various sizes of



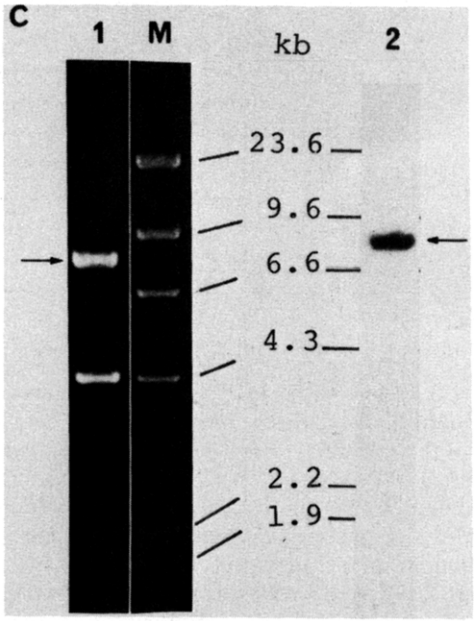


Fig.2. Viral DNA insert in recombinants. DNAs of recombinants were digested with *Xho*I and electrophoresed on 0.8% agarose gel. They were then stained with EtBr (A), and hybridized with PRetV[^{32}P]cDNA$_{rep}$ as a probe (B). Lanes in (A,B): 1, λPRetV-21; 2, λPRetV-24; 3, λPRetV-26; 4, λPRetV-22; 5, λPRetV-2; 6, λPRetV-8; 7, λPRetV-9; 8, λPRetV-10; 9, λPRetV-1 and 10, λPRetV-11. In (A), the two bands indicated by arrows correspond to the fragments of λDNA arms for cloning. Lane 11 in (B) indicates *Xho*I-digested PRetV ccDNA. In (C), recombinant plasmid (pPRetV8.3) digested with *Xho*I was stained with EtBr after electrophoresis (lane 1) and applied to blot hybridization with PRetV[^{32}P]cDNA$_{rep}$ (lane 2). The arrows in (C) represent the 8.3 kb viral insert subcloned in pPRetV8.3. M in (A,C): standard markers of *Hin*dIII-digested wild-type λDNA.

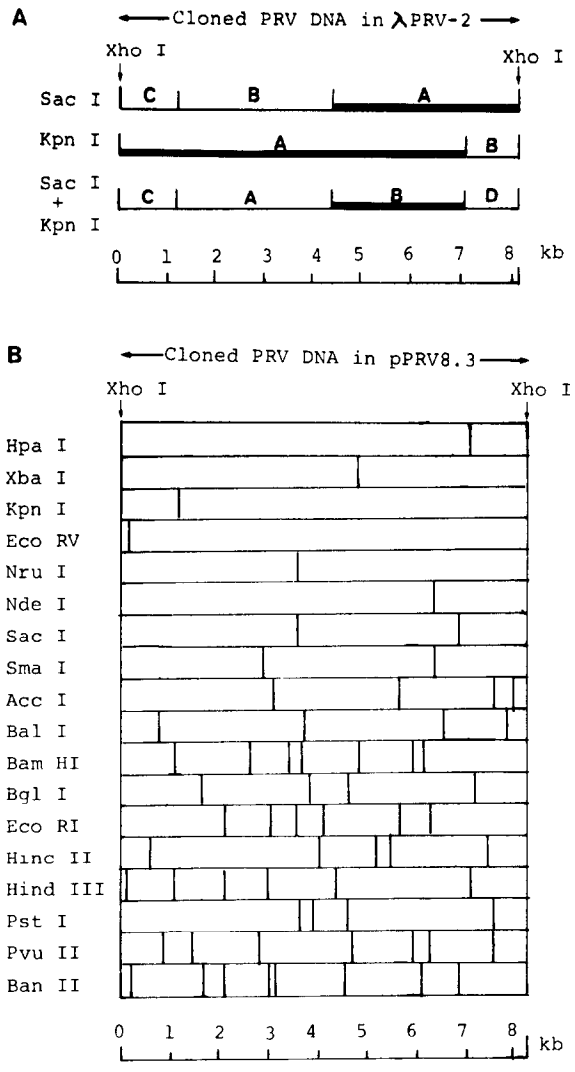


Fig.3. (A) Preliminary map of recognition sites for some restriction endonucleases on the 8.3 kb insert in λPRetV-2. The thick lines represent the fragments hybridized to PRetV[^{32}P]cDNA$_5$'. (B) Restriction endonuclease map of PRetV ccDNA subcloned in pPRetV8.3. Because of the lack of an *Sal*I site on the 8.3 kb insert, nick-translated 1.4 and 2.95 kb fragments from pBR322$_{Xho\,I}$ digested with *Sal*I and *Xho*I were used as probes for hybridization. *Sal*I-cut pPRetV8.3 was partially cleaved with each enzyme on time course, then electrophoresed on 1.2 or 1.5% agarose gel and examined by Southern blot hybridization using the same probe as above. In some cases, 0.3 kb *Eco*RV fragment from the 8.3 kb insert was used as a probe.

the DNA inserts may be due to defective provirus or deleted ones during the cloning process.

Fig.3A shows a restriction cleavage map of the 8.3 kb insert in λPRetV-2. To locate the position of LTR sequences on this clone, we hybridized each fragment generated by these digestions to ^{32}P-labeled strong stop PRetV DNA. As shown in fig.3A, this probe hybridized with fragments A by *Sac*I or *Kpn*I digestion and fragment B by *Sac*I plus *Kpn*I double digestion, suggesting the possible location of LTR on fragment B generated by digestion with *Sac*I and *Kpn*I.

To facilitate the construction of a finer map for restriction endonuclease recognition sites, this 8.3 kb insert of λPRetV-2 was further subcloned into plasmid vector pBR322$_{Xho\,I}$. The resultant plasmid was designated pPRetV8.3 (fig.2C). The map of cleavage sites was obtained by Southern blot hybridization of single- or double-digested fragments of the plasmid. The results are summarized in fig.3B. *Hpa*I, *Xba*I, *Kpn*I, *Eco*RV, *Nru*I and *Nde*I gave a unique site on this insert, whereas no site for *Aat*I, *Aat*II, *Ban*III, *Bcl*I, *Mlu*I, *Pvu*I, *Sal*I, *Sca*I, *Sph*I and *Stu*I was observed.

In summary, we isolated the unintegrated form of PRetV ccDNA from SKL cells infected with PRetV and accomplished the cloning of the ccDNA into λCharon 21A using a single site on it for a restriction endonuclease, *Xho*I. Thus, this is the first report of cloning of the PRetV genome. The obtained recombinant fragment had 8.3 kb as a viral insert (λPRetV-2) and may correspond to almost a full length of PRetV ccDNA carrying an LTR sequence. The preliminary physical map of the λPRetV-2 insert also suggests a possible location of LTR on fragment B generated by digestion with *Sac*I and *Kpn*I. Subsequently, we constructed the subclone, pPRetV8.3, harboring the 8.3 kb insert in pBR322$_{Xho\,I}$ and determined 65 restriction sites for the 18 endonucleases.

Direct involvement of PRetV in swine malignant lymphomas has thus far been unclear. Molecular cloning of the unintegrated PRetV DNA offers the prospect of studying the structural organization of the PRetV genome and its relationship with trans-activating retroviruses (HTLVs [12,13] and BLV [14]). These studies are also important for an understanding of the pathogenicity of PRetV.

REFERENCES

[1] Suzuka, I., Sekiguchi, K. and Kodama, M. (1985) FEBS Lett. 183, 124–128.

[2] Kodama, M., Sekiguchi, K., Kudo, M., Mitani, K., Osada, M., Sonoda, A., Kashiwazaki, M. and Saito, T. (1981) The Abstract of the 92nd Meeting of the Japanese Society of Veterinary Science, pp.126.

[3] Hirt, B. (1967) J. Mol. Biol. 26, 365–369.

[4] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.

[5] Southern, E.M. (1975) J. Mol. Biol. 38, 503–517.

[6] Blattner, F.R., Williams, B.G., Denniston-Thompson, K., Faber, H.E., Furlong, L.-A., Grunwald, D.J., Kiefer, D.O., Moore, D.D., Schumm, J.M., Sheldon, E.L. and Smithies, O. (1977) Science 196, 161–169.

[7] Shimotohno, K. (unpublished).

[8] Boliver, F.R., Rodriguez, L., Greene, P.J., Betlach, M.C., Heyneker, H.L. and Boyer, H.W. (1977) Gene 2, 95–113.

[9] Taylor, J.M., Illmensee, R. and Summers, J. (1976) Biochim. Biophys. Acta 442, 324–330.

[10] Friedlich, R., Kung, H.-J., Baker, B., Varmus, H.E., Goodman, H.M. and Bishop, J.M. (1977) Virology 79, 198–215.

[11] Kennedy, N., Knedlitschek, G., Groner, B., Hynes, N.E., Berrlick, P., Michalides, R. and Van Ooyen, J.J. (1982) Nature 295, 622–624.

[12] Seiki, M., Hattori, S., Hirayama, Y. and Yoshida, M. (1983) Proc. Natl. Acad. Sci. USA 80, 3618–3622.

[13] Shimotohno, K., Takahashi, Y., Shimizu, N., Gojobori, T., Golde, D.W., Chen, I.Y.S., Miwa, M. and Sugimura, T. (1985) Proc. Natl. Acad. Sci. USA 82, 3101–3105.

[14] Sagata, N., Yasunaga, T., Ozawa, Y., Tsuzuku-Kawamura, J. and Ikawa, Y. (1984) Proc. Natl. Acad. Sci. USA 81, 4741–4745.