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# *In Vivo* Excision and Amplification of Large Human Genomic Segments Using Cre/*loxP*-and EBNA-1/*oriP*-mediated Machinery

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**Excision and amplification of pre-determined, large genomic segments (taken directly from the genome of a natural host, which provides an alternative to conventional cloning in foreign vectors and hosts) was explored in human cells. In this approach, we devised a procedure for excising a large segment of human genomic DNA, the *iNOS* gene, by using the Cre/*loxP* system of bacteriophage P1 and amplifying the excised circles with the EBNA-1/*oriP* system of the Epstein-Barr virus. Two *loxP* sequences, each of which serves as a recognition site for recombinase Cre, were integrated unidirectionally into the 5'-UTR and 3'-UTR regions of the *iNOS* gene, together with an *oriP* sequence for conditional replication. The *trans*-acting genes *cre* and *EBNA-1*, which were under the control of a tetracycline responsive $P_{hcmv*-1}$ promoter, were also inserted into the 5'-UTR and 3'-UTR regions of the *iNOS* gene, respectively, by homologous recombination. The strain carrying the inserted elements was stably maintained until the excision and amplification functions were triggered by the induction of *cre* and *EBNA-1*. Upon induction by doxycycline, Cre excised the *iNOS* gene that was flanked by two *loxP* sites and circularized it. The circularized *iNOS* gene was then amplified by the EBNA-1/*oriP*-system. With this procedure, approximately a 45.8-kb *iNOS* genomic fragment of human chromosome 17 was excised and successfully amplified in human cells. Our procedure can be used effectively for the sequencing of unclonable genes, the functional analysis of unknown genes, and gene therapy.**

**Keywords:** *In vivo* excision and amplification, Cre/*loxP* system, EBNA-1/*oriP* system

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## Introduction

The molecular cloning of DNA segments is a powerful tool in mapping, manipulating, and sequencing genes. Cloning procedures, however, have their intrinsic limitations. The cloned DNA is maintained in a foreign host and copied by a heterologous system. The genetic materials and/or their products can be toxic to foreign hosts, or can carry modifications, which are discriminated against. In addition, selective pressure often favors the unfaithfully copied, deleted, or rearranged clones. This causes clone instability, copying infidelity, and size limitation of the cloned genes.

Directed *in vivo* excision and amplification of genomic fragments in a natural host, which is aimed at overcoming the problems of clone instability, copying infidelity, and size limitation in a foreign host, was explored as an alternative to heterologous cloning (Collins and Hohn, 1978; Frischauf *et al.*, 1983; Burke *et al.*, 1987; Shizuya *et al.*, 1992; Pósfai *et al.*, 1994; Wild *et al.*, 1996, 1998; Yoon *et al.*, 1998a, 1998b). This procedure involved *in vivo* genetic manipulation of a studied organism. This resulted in cloning the desired DNA segment directly from the organism as an amplified, circular form in its own host. Thus, the fidelity of the cloned sequence could be greatly increased. Moreover, this procedure can supply the genomic DNA fragments for the sequencing of unclonable genes because large, contiguous genomic DNA segments can be generated without any gaps. However, the methods developed so far have been focused mainly on microorganisms.

In this report, we developed an *in vivo* excision and amplification system for obtaining large segments of the human genomic DNA in human cells. The developed system utilized the Cre/*loxP*-recombination system for the *in vivo* excision of the targeted genomic segment (Li *et al.*, 1996; Araki *et al.*, 1997; Gagneten *et al.*, 1997; Li *et al.*, 1997; Kellendonk *et al.*, 1999) and the EBNA-1/*oriP* replication machinery for the amplification of the excised circular genomic fragment in human cells (Yates *et al.*, 1985; Reisman

and Sugden, 1986; Kelleher *et al.*, 1998). The Cre recombinase from bacteriophage P1 mediates the precise recombination between two *loxP* sites, each of which is composed of two 13-bp inverted repeats and an asymmetric 8-bp core region (Sternberg and Hamilton, 1981; Abremski *et al.*, 1983; Hoess and Abremski, 1985). Recombination between the two parallel *loxP* sites results in the excision and circularization of the genomic DNA segment intervened between the two *loxP* sites (Kilby *et al.*, 1993). The EBNA-1/*oriP*-mediated amplification system is derived from the Epstein-Barr virus (EBV). The EBNA-1 allows the replication and maintenance of the *oriP* that contains plasmid DNA (Lupton and Levine, 1985; Yates *et al.*, 1984, 1985). Using these two systems, we successfully excised and amplified a 45.8-kb human *iNOS* genomic fragment from chromosome 17 in human cells.

## Materials and Methods

***E. coli* strains, vectors and enzymes** *E. coli* strain XL1-Blue (Stratagene, La Jolla, USA), used as a host for all cloning experiments, was grown at 37°C in a LB medium that was supplemented with ampicillin (50 μg/ml) for ampicillin resistant plasmid-containing strains. pTet-On, pTRE, and pTK-Hyg were purchased from Clontech (Palo Alto, USA). pRH43 was obtained from DuPont (Boston, USA), pIC19R/MC1-TK and pKT1NEO from M.R. Capecchi (University of Utah, Salt Lake City), and p291 from B. Sugden (University of Wisconsin, Madison). Restriction enzymes, T4 DNA ligase and the Lambda DNA mono cut mix (DNA size marker), were purchased from New England Biolabs (Beverly, USA) and *Taq* DNA polymerase from Boehringer Mannheim (Mannheim, Germany). Hybond-N+ membrane and [α-$^{32}$P] dATP were purchased from Amersham (Buckinghamshire, UK). Oligonucleotides were synthesized by Genotech (Taejon, Korea).

**Target sequences for homologous recombination** Upstream and downstream regions of the human *iNOS* gene were selected as the target sequences for homologous recombination. DNA fragments of the upstream region of the *iNOS* gene, 5'-UTR (1.2 kb) and *iNOS* promoter exon 1 (0.8 kb) and DNA fragments of downstream region of the *iNOS* gene, intron 26 exon 27 (1.7 kb) and 3'-UTR (0.5 kb), were prepared from the human genomic DNA by PCR with gene-specific primers. The following primers were used to amplify each of the DNA fragments: for 5'-UTR: 5'-CTCGAGAGGCTCTTGGGTGGGGGCAT-3' and 5'-AAGCTTTGGTGGAATGGCAGGTAGGA-3'; for *iNOS* promoter-exon 1: 5'-AAGCTTACCTAGTGCTAAAGGATGAG-3' and 5'-GGTAAGGACAGTCAAACCAGGAAGAG-3'; for intron 26-exon 27: 5'-TCCCCCGGGCAAGGTGAATAGTGGGTGTA-3' and 5'-TCCCCCGGGTCAGAGCGCTGACATCTCCAG-3'; for 3'-UTR: 5'-CGGGATCCGGGCCTACAGGAGGGGTTATAG-3' and 5'-CCCAAGCTTGATTAAAGTAAAAATGCAAATTC-3'.

Thirty amplification cycles were performed as follows: 1 min at 94°C, 1 min at 55°C, followed by 1 min at 72°C. Each of the PCR

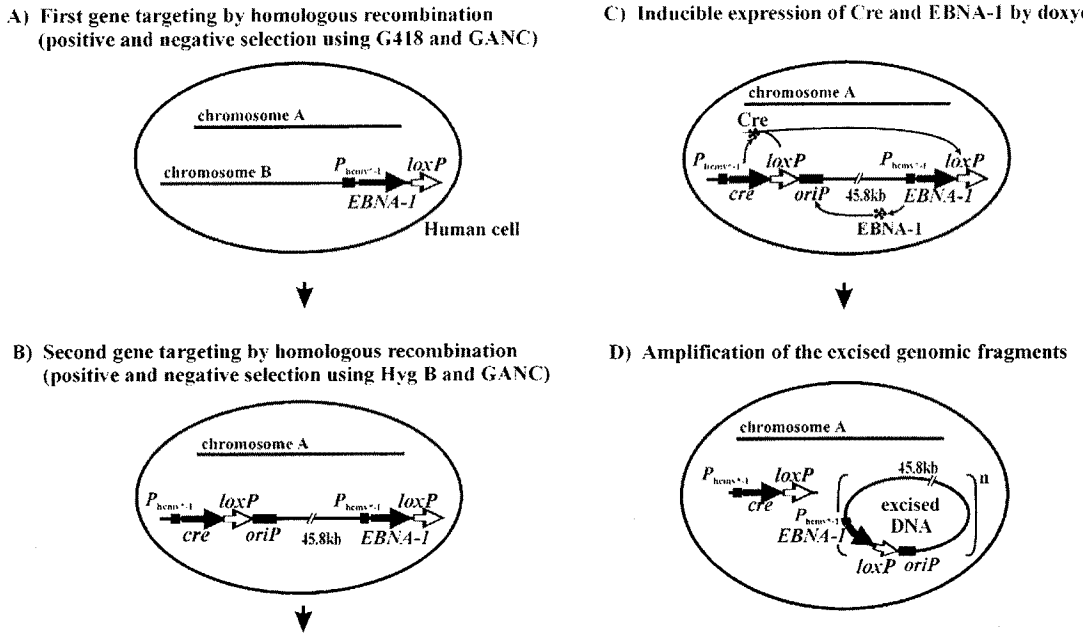


**Fig. 1.** A schematic representation of the Cre/*loxP*-mediated *in vivo* excision and EBNA-1/*oriP*-mediated amplification of large genomic DNA. (A) The target vector containing $P_{hcmv*-1}$/*EBNA-1*, *loxP*, *rtTA* and *neo*$^r$ is inserted into the first target site of the chromosome by homologous recombination. The homologous recombinants are selected by using G418 and GANC. (B) The second analogous insertion, consisting of $P_{hcmv*-1}$/*cre*, *loxP*, *oriP* and *hyg*$^r$ is placed at a 45.8-kb distal second chromosomal target site. The homologous recombinants are selected by using hygromycin B and GANC. (C) and (D) Upon the addition of doxycycline, the $P_{hcmv*-1}$ promoter is induced, resulting in the expression of EBNA-1 and Cre. Cre mediates excision and circularization of the 45.8-kb *loxP*-*loxP* segment. Since such a circular DNA contains an *oriP* sequence, it can be amplified in human cells assisted by EBNA-1.

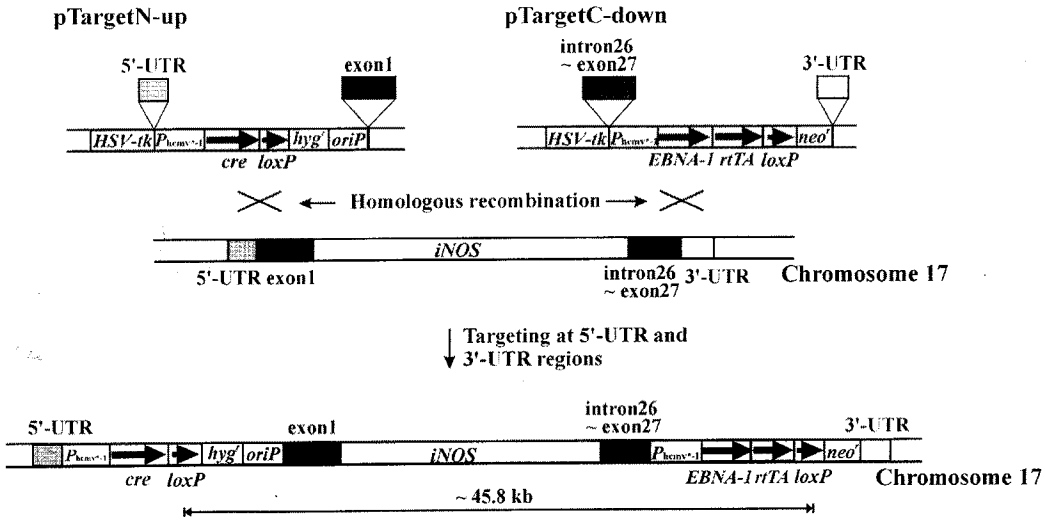


**Fig. 2.** Structures of gene targeting vectors and their target sites on the human *iNOS* gene. The gene targeting vectors, pTargetN-up and pTargetC-down, are integrated into the pre-determined 5'-UTR and 3'-UTR regions of the *iNOS* gene, respectively, by homologous recombination.

products (1.2-kb 5'-UTR, 0.8-kb *iNOS* promoter exon 1, 1.7-kb intron 26 exon 27 and 0.5-kb 3'-UTR) was purified from low-melting point agarose gels (FMC, Rockland, USA) using a Geneclean II kit (BIO 101, Vista, USA) and cloned into pT7BlueT-vector (Novagen, Madison, USA). The nucleotide sequences of the PCR products that were cloned into pT7Blue T-vector were then confirmed by DNA sequencing and used for constructing gene targeting vectors.

**Construction of gene targeting vectors** Gene targeting vectors, pTargetN-up and pTargetC-down, were constructed for homologous targeting as described in Fig. 2. pTargetN-up is a gene targeting vector for the insertion into the upstream region of the *iNOS* gene. It consists of the following: a 34-bp *loxP* sequence, a *cre* gene that is controlled by the Tet-responsive $P_{hcmv*-1}$ promoter that is derived from a pTRE, an *oriP* sequence, a hygromycin resistance gene (*hyg'*, derived from pTK-Hyg), a herpes simplex virus type 1 thymidine kinase gene (*HSV-tk*, derived from pIC19R/MC1-TK), and the homology arms (5'-UTR and *iNOS* promoter exon1). pTargetC-down is a gene targeting vector for the insertion into the downstream region of the *iNOS* gene. It consists of the following: a 34-bp *loxP* sequence, an *EBNA-1* gene that is controlled by the Tet-responsive $P_{hcmv*-1}$ promoter, a reverse tetracycline-controlled transactivator gene (*rtTA*, derived from pTet-On), a neomycin resistance gene (*neo'*, derived from pKT1NEO), *HSV-tk*, and the homology arms (intron 26-exon 27 and 3'-UTR). pTargetN-up and pTargetC-down were linearized with *Xho*I and *Cla*I, respectively, and used for the transfection of BJAB cells by electroporation.

**Cell culture and transfection** The BJAB cell is a EBV-genome-negative B lymphoblast that is derived from a EBV-negative Burkitt lymphoma biopsy. BJAB cells were cultured in a RPMI1640 medium (Gibco BRL, Gaithersburg, USA) that was supplemented with antimycitin (50 μg/ml) in the presence of heat-inactivated 10% fetal bovine serum (FBS) at 37°C in a humidified 5% $CO_2$ incubator. The culture medium was changed every 3 day. Transfection of the cells was performed by the electric field-mediated DNA transfection method using a Bio-Rad Gene Pulser (Hercules, USA). The gene targeting vector (30 μg) was linearized and electroporated into $6 \times 10^6$ cells in 300 μl of RPMI1640 with 10% FBS at 250 V, 960 μF with the time constant 40 ms. The electroporated cells were resuspended with the culture medium and selection pressure (0.5 mg/ml G418 or 0.3 mg/ml hygromycin B) was applied after 48 h.

**Western blot analysis** The inducible expression of Cre and EBNA-1 was confirmed by Western blot analysis (Sambrook *et al.*, 1989). Doxycycline-induced and uninduced cells were lysed with 0.1% SDS (Bollag and Edelstein, 1991) and the total proteins were prepared. Equal amounts of the total proteins (10 μg) were separated by SDS-PAGE and transferred onto the nitrocellulose membranes (0.2 μm pore size, Bio-Rad, Hercules, CA) by semidry electrophoretic transfer (Bio-Rad). Western blot analyses were then carried out. The primary antibody was a rabbit anti-Cre (Novagen, Madison, USA), or a mouse anti-EBNA-1 (Serotic Ltd., Oxford, UK) polyclonal antibody, and used at a 1 : 10,000 or 1 : 5,000 dilution, respectively. The secondary antibody was an anti-rabbit IgG, or an anti-mouse IgG conjugated with horseradish peroxidase (Amersham), and was used at a 1 : 5,000 dilution. Immunoreactivity was visualized by enhanced chemiluminescence (ECL Kit, Amersham).

**DNA preparation and PFGE (Pulsed-Field Gel Electrophoresis)** Total genomic DNA from BJAB cells was prepared as described by Koob and Szybalski (1992), or by Sambrook *et al.* (1989). The plasmid DNA was isolated from bacteria, or BJAB cells using the alkaline lysis method described by Sambrook *et al.* (1989), or by the modified Hirt extraction procedure (Hirt, 1967; Arad, 1998). The plasmid DNA from BJAB cells was analyzed by PFGE using an LKB 2015 Pulsaphor Electrophoresis Unit (Pharmacia LKB Biotechnology AB, Bromma, Sweden). PFGE was performed under the following

conditions: 17 V/cm field strength, 120° field angle, 0.4s pulse time, 3 h running time and a 1% agarose gel in 0.15× TBE buffer.

## Results and Discussion

**Principle of Cre/*loxP*-mediated *in vivo* excision and EBNA-1/*oriP*-mediated amplification of large segments of genomic DNA** To develop a procedure for the *in vivo* excision and amplification of pre-determined large segments of the human genome, we used the following systems that are shown in Fig. 1: (*i*) Cre/*loxP* site-specific recombination system of bacteriophage P1 for the excision (Abremski *et al.*, 1983), (*ii*) EBNA-1/*oriP*-mediated replication system of EBV (Reisman and Sugden, 1986) for the amplification, and (*iii*) Tetracycline- or doxycycline-inducible Tet-On gene expression system (Gossen *et al.*, 1995) for the conditional expression of Cre and EBNA-1. For the *in vivo* excision and amplification, two targeting vectors (Fig. 2) were constructed. The homology arms for the homologous recombination were PCR-amplified and cloned into the targeting vectors, which were subsequently transfected into cells. Using the two targeting vectors, a *loxP* site, together with an *oriP* sequence or *cre* and *EBNA-1* genes, was inserted unidirectionally by homologous recombination into each of the two pre-determined genomic target sites that flanked the region to be excised and amplified (Fig. 1A and B). Cells, which had targeting vectors at the target sites of the genomic DNA, could be selected by using consecutive selections for both positive and negative markers (Fig. 2). Upon induction by doxycycline, Cre and EBNA-1 were independently expressed under the control of the $P_{hcmv*-1}$ promoter. Cre excised and circularized the genomic fragment that was flanked by the two *loxP* sites, and EBNA-1 assisted the amplification of the excised circular DNA (Fig. 1C and D). The amplified large circular DNA could be used for physical mapping, sequencing, the functional analysis of genes, and gene therapy.

**Construction of the BJAB cell line targeted at the 5'-UTR and 3'-UTR regions of the *iNOS* gene** For the 3'-UTR targeting of the *iNOS* gene, the BJAB cells were transfected with the linearized gene targeting vector, pTargetC-down that contained *loxP, rtTA, EBNA-1, neo*$^r$ and *HSV-tk* genes (Fig. 2). G418- and gancyclovir (GANC)-resistant BJAB cell clones were selected and named BJAB/pTargetC-down. For the 5'-UTR targeting of the *iNOS* gene, the BJAB/pTargetC-down cells were transfected with the linearized gene targeting vector, pTargetN-up that contained *loxP, cre, oriP, hyg*$^r$ and *HSV-tk* genes (Fig. 2). After transfection, hygromycin B- and GANC-resistant BJAB cell clones were picked and named BJAB/pTargetC-down/pTargetN-up. The schematic representations of the chromosomal integration of pTargetC-down and pTargetN-up are described in Figs. 3A and 4A, respectively. The chromosomal integration of the pTargetC-down was confirmed by Southern blot analysis (Sambrook *et al.*, 1989), shown in

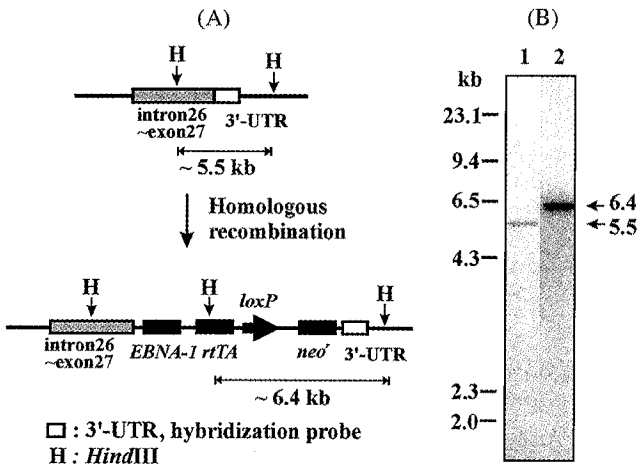


**Fig. 3.** Analysis of the chromosomal integration of pTargetC-down into the 3'-UTR of the *iNOS* gene. (A) Schematic representation of the chromosomal integration of pTargetC-down. A 5.5-kb *Hind*III fragment is detected from the wild type genomic DNA. A 6.4-kb DNA *Hind*III fragment, however, is detected from the genomic DNA containing pTargetC-down at the 3'-UTR of the *iNOS* gene. (B) Southern blot analysis of the chromosomal integration of the pTargetC-down. A 0.5-kb $^{32}$P-labeled 3'-UTR fragment was used as a hybridization probe. Lane 1, genomic DNA from BJAB, digested with *Hind*III (control); lane 2, genomic DNA from BJAB/pTargetC-down/pTargetN-up, digested with *Hind*III. The 6.4-kb DNA fragment in lane 2 indicates the successful integration of the pTargetC-down into 3'-UTR of the *iNOS* gene by homologous recombination.

Fig. 3B. A 5.5-kb band (Fig. 3B, lane 1) was detected from the *Hind*III-cleaved genomic DNA of the wild type BJAB cell after hybridization with the 0.5-kb $^{32}$P-labeled 3'-UTR fragment. A 6.4-kb band (Fig. 3B, lane 2), however, was observed from the *Hind*III-cleaved genomic DNA of the BJAB/pTargetC-down cells. This indicates that pTargetC-down was correctly integrated into the 3'-UTR of the *iNOS* gene (Fig. 3A). The chromosomal integration of the pTargetN-up into the BJAB/pTargetC-down cells was also confirmed by Southern blot analysis, shown in Fig. 4B. A 1.2-kb band (Fig. 4B, lane 1) was detected from the *Hind*III- and *Xho*I- cleaved genomic DNA of the wild type BJAB cell after hybridization with the 1.2-kb $^{32}$P-labeled 5'-UTR fragment. A 7.9-kb band (Fig. 4B, lane 2), however, was detected from the *Hind*III- and *Xho*I-cleaved genomic DNA of the BJAB/pTargetC-down/pTargetN-up cells. The appearance of the 7.9-kb band indicates that pTargetN-up was correctly integrated into the 5'-UTR of the *iNOS* gene in BJAB/pTargetC-down/pTargetN-up cells (Fig. 4A). About 1% of the putative transfectants was the correctly targeted clones. However, the targeting efficiency can be improved by controlling the length and quality of the homology arms (Capecchi and Deng, 1992).

**Expression of EBNA-1 and Cre in the BJAB/pTargetC-down/pTargetN-up cells** In the BJAB/pTargetC-down/

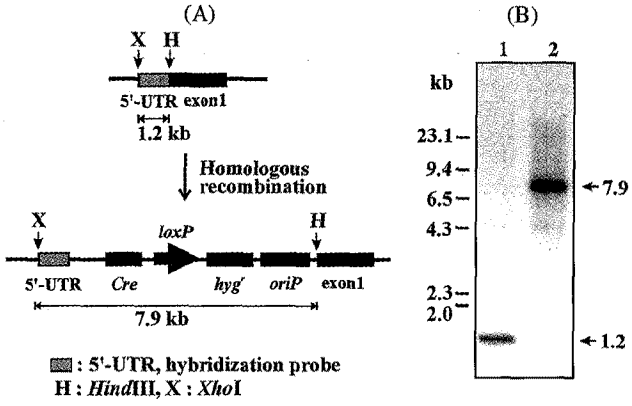


**Fig. 4.** Analysis of the chromosomal integration of pTargetN-up into the 5'-UTR of the *iNOS* gene. (A) Schematic representation of the chromosomal integration of the pTargetN-up. The 1.2-kb *Hind*III-*Xho*I fragment is detected from wild type genomic DNA. The 7.9-kb *Hind*III-*Xho*I fragment, however, is detected after the integration of pTargetN-up into the 5'-UTR of the *iNOS* gene. (B) Southern blot analysis of the chromosomal integration of the pTargetN-up. A 1.2-kb $^{32}$P-labeled 5'-UTR fragment was used as a hybridization probe. Lane 1, genomic DNA from BJAB, digested with *Hind*III and *Xho*I (control); lane 2, genomic DNA from BJAB/pTargetC-down/pTargetN-up, digested with *Hind*III and *Xho*I. The 7.9-kb band in lane 2 indicates the successful integration of the pTargetN-up into 5'-UTR of the *iNOS* gene by homologous recombination.

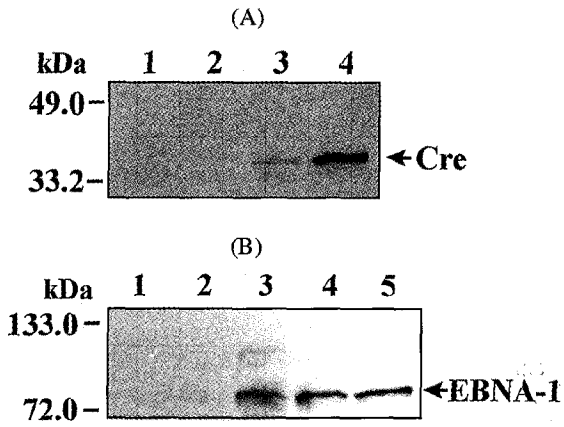


**Fig. 5.** Western blot analysis of the expression of Cre and EBNA-1 by doxycycline. (A) Expression of Cre. Total proteins were prepared from the doxycycline-induced and uninduced cells, fractionated by SDS-PAGE on a 12.5% gel and an immunoblot analysis was carried out using a rabbit anti-Cre polyclonal antibody. Lane 1, total proteins from BJAB cells; lane 2, total proteins from BJAB/pTargetC-down/pTargetN-up cells, uninduced control; lanes 3, 4, total proteins from BJAB/pTargetC-down/pTargetN-up cells, induced for 12, 24 h, respectively. (B) Expression of EBNA-1. Total proteins were prepared from the doxycycline-induced and uninduced cells, fractionated by SDS-PAGE on a 10% gel. An immunoblot analysis was carried out using a mouse anti-EBNA-1 polyclonal antibody. Lane 1, total proteins from BJAB cells; lane 2, total proteins from BJAB/pTargetC-down/pTargetN-up cells, uninduced control; lanes 3-5, total proteins from BJAB/pTargetC-down/pTargetN-up cells, induced for 12, 24, 48 h, respectively.

pTargetN-up cells, the *cre* and *EBNA-1* genes were under the control of the tetracycline-responsive $P_{hcmv*-1}$ promoter. The transcription of these genes was activated by binding the rtTA regulatory protein with doxycycline. To examine whether or not *cre* and *EBNA-1* could be expressed conditionally by doxycycline induction, Western blot analyses were carried out using mouse anti-EBNA-1 and rabbit anti-Cre polyclonal antibodies, respectively. As shown in Fig. 5, 38-kDa Cre (Fig. 5A, lanes 3 and 4) and 88-kDa EBNA-1 (Fig. 5B, lanes 3, 4 and 5) were expressed only after doxycycline induction. The expression levels reached their maximum 24 h after induction. However, Cre and EBNA-1 were not expressed in both the BJAB cells (Fig. 5A and B, lane 1) and in the uninduced control cells (Fig. 5A and B, lane 2). These results show that the *cre* and *EBNA-1* are under the tight control of the tetracycline-responsive $P_{hcmv*-1}$ promoter and are efficiently induced by doxycycline.

***In vivo* excision and self-amplification of the target genomic segment as a 45.8-kb plasmid** The *iNOS* gene-targeted BJAB/pTargetC-down/pTargetN-up cells ($1 \times 10^7$ cells) were grown for 3 days in 20 ml of RPMI1640 that contained 10% FBS with G418 (0.5 mg/ml) and hygromycin B (0.3 mg/ml). To induce *cre* and *EBNA-1* using doxycycline, the cells were resuspended in 20 ml of a RPMI1640 medium with 10% FBS containing doxycycline (2 μg/ml) after being washed twice with a RPMI1640 medium that contained 10% FBS, then cultured again for 24 h in a humidified $CO_2$ incubator at 37°C. The 45.8-kb *iNOS* genomic DNA was isolated directly from the uninduced and doxycycline-induced BJAB/pTargetC-down/pTargetN-up cells by the modified Hirt extraction method, respectively. The isolated DNA was analyzed by PFGE. A Southern blot analysis was carried out using the $^{32}$P-labeled *loxP* fragment as a hybridization probe (Fig. 6). To further verify that the amplified 45.8-kb fragment is the *iNOS* gene, the exon 1 and exon 27 regions of the isolated 45.8-kb genomic fragment were sequenced and found to be identical to those in the EMBL Data Bank. The excised and amplified 45.8-kb *iNOS* gene was detected from the DNA that was isolated from the doxycycline-induced cells (indicated by the arrows in Fig. 6, A and B, lane 4). However, it was undetected in the DNA that was isolated from the uninduced cells (Fig. 6A and B, lane 3).

Our results showed that a large human genomic DNA fragment could be isolated as circular DNA directly from the human cell using the Cre/*loxP*- and EBNA-1/*oriP*-mediated *in vivo* excision and amplification machinery. Most of the excised and circularized DNA exists as monomers in the human cells, shown in Fig. 6 (lane 4). The DNA sequence of the *in vivo* excised and amplified fragments should reflect the original native sequence with high fidelity. It should also be

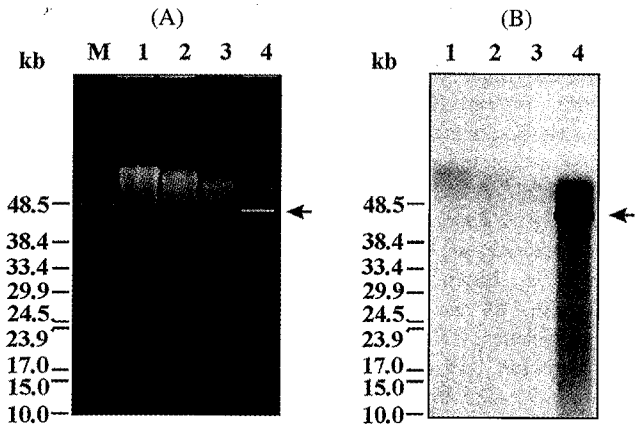


**Fig. 6.** PFGE and Southern blot analyses of the excised and amplified, circular DNA that was isolated from the BJAB/pTargetC-down/pTargetN-up cells. (A) PFGE analysis. DNA from the uninduced and doxycycline-induced BJAB/pTargetC-down/pTargetN-up cells was isolated using the modified Hirt extraction method and fractionated by PFGE on a 1% agarose gel. (B) Southern blot analysis of the excised and amplified DNA fragments. The DNA fractionated by PFGE was transferred onto a Hybond-$N^+$ membrane and probed by $^{32}$P-labeled *loxP* fragments. Lane M, Lambda DNA-mono cut mix (size marker); lane 1, DNA from BJAB cells; lane 2, DNA from BJAB/pTargetC-down cells; lane 3, DNA from BJAB/pTargetC-down/pTargetN-up cells, uninduced control; lane 4, DNA from BJAB/pTargetC-down/pTargetN-up cells, induced with doxycycline for 24 h. Arrows indicate the excised, circular DNA.

free of cloning artifacts, because the pre-determined large genomic DNA fragment is isolated directly from the human cells without intermediate cloning in a foreign host. Our procedure can be used effectively for the sequencing of unclonable genes, the functional analysis of unknown genes, and gene therapy. In our procedure, the amplification level of the excised large circular DNA was less than 5 copies per cell. This is confirmed by comparing the band intensities of the Southern blot analysis. The low amplification level might be ascribed to the EBNA-1/*oriP* system, which maintains a low copy number of an episomal DNA (Aiyar *et al.*, 1998). Currently, we are working on increasing the amplification level of the excised genomic DNA fragment to 100 copies per cell by using the high-copy-number large T antigen/SV40 *ori* replication system of Simian Virus 40 (Cooper *et al.*, 1997).

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