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Cre-stimulated recombination at *loxP*-containing DNA sequences placed into the mammalian genome

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ABSTRACT

The *cre* gene of coliphage P1 encodes a 38 kDa protein which efficiently promotes both intra- and intermolecular recombination at specific 34 bp sites called *loxP*. To demonstrate that the Cre protein can promote DNA recombination at *loxP* sites resident on a mammalian chromosome, a mouse cell line was constructed containing two directly repeated *loxP* sites flanking a 2.5 kb yeast DNA fragment and inserted between the SV40 promoter and the *neo* structural gene to disrupt expression of the *neo* gene. Expression of the *cre* gene in this cell line results in excision of the intervening yeast DNA and thus permits sufficient expression of the *neo* gene to allow cell growth in high concentrations of G418. Southern analysis indicated that Cre-mediated excision occurred at the *loxP* sites. In the absence of the *cre* gene such excisive events are quite rare. Cre-mediated recombination should thus be quite useful in effecting a variety of genomic rearrangements in eukaryotic cells.

INTRODUCTION

The stable introduction of DNA into mammalian cells by calcium phosphate coprecipitation or by microinjection generally results in seemingly random integration of the DNA into the genome (1-4). Homologous recombination events between the chromosome and introduced DNA's do occur, but at low efficiency (5-8). This is surprising in light of the observation that homologous events occur quite efficiently between extrachromosomal DNA's introduced by gene transfer (4, 9-16) or viral infection (17-21). Indeed, chromosomal context may impede homologous chromosomal recombination events to such an extent that they are undetectable. Similar chromosomal contextural effects have been seen on the expression of cloned genes stably integrated into the genome. The hope that insertion of DNA by homology to a particular chromosomal location should obviate such variation has led to a great interest in the mechanisms of synapsis and recombination of DNA in mammalian cells.

As an alternative and complementary approach to effecting specific homologous recombination events in eukaryotes, we have been studying the prokaryotic Cre-*lox* site-specific DNA recombination system of coliphage P1 (22). The 38 kDa Cre protein efficiently causes both inter- and intramolecular recombination between specific 34 bp sites called *loxP*. Each *loxP* site contains two 13 bp inverted repeats and an 8 bp asymmetric core sequence. No

accessory proteins are required for exchange to occur. Cre-mediated recombination proceeds efficiently with both supercoiled and linear DNA molecules (23-24).

Recently we have shown that the Cre recombinase can be expressed in the yeast *Saccharomyces cerevisiae* and that Cre can efficiently cause DNA synapsis and excisive recombination at *loxP* sites located on various yeast chromosomes (25). We have also shown that Cre can be functionally expressed in cultured mouse cells to perform excisive recombination of extrachromosomal DNA (26). We show here that Cre can perform specific recombination events at *loxP* sites stably located within the genome of cultured mouse cells.

MATERIAL AND METHODS

Animal Cells and Media

The LM*tk⁻ aprt⁻* mouse cell line (27) was cultured as has been described (28) and will be referred to as L*tk⁻* cells.

Plasmid Constructions

All plasmids were constructed and prepared by standard techniques (29). Unless otherwise indicated, all plasmids were propagated in *E. coli* strain DH5Δ*lac* (M. Berman, Bionetics Research, Inc.), a derivative of DH1 (30).

Plasmid pBS69 (Fig. 1) contains two directly repeated *loxP* sites flanking the yeast *LEU2* gene (*lox*^{2} *LEU2*). Importantly, pBS69 was designed so that Cre-mediated recombination at the *loxP* sites would generate a 50 bp *Xho* I-*Hind* III fragment containing *loxP* that did not include the sequence ATG when read from the *Xho* I site toward the *Hind* III site. *Hind* III linkers (Collaborative Research) were ligated to pBS30 (25) which had been treated with *Bam* HI and nuclease S1. This DNA was digested with *Hind* III and religated to generate pBS67. Plasmid pBS52 had been constructed previously by introducing a *Hind* III site at the *Aat* II site of pBS30 (31). The *Hind* III-*Sal* I *loxP*-containing fragment of pBS52 was replaced with the *Hind* III-*Xho* I *loxP*-containing fragment of pBS67 to produce plasmid pBS69.

Plasmid pBS73 (Fig. 2) was derived from pSV2*neo* (32) by treating pBS69 with *Xho* I and the large Klenow fragment of DNA polymerase I, ligating *Hind* III linkers to the resulting blunt DNA ends, digesting that DNA with *Hind* III and then ligating the resulting *lox*^{2} *LEU2*-containing *Hind* III fragment to the *Hind* III site of pSV2*neo*. The *lox*^{2} *LEU2* segment was thus inserted between the SV40 promoter and the *neo* gene. Plasmid pBS74, having only a 50 bp *loxP*-containing insertion at the *Hind* III site, was generated by Cre-mediated recombination at the *loxP* sites by introduction of pBS73 into the Cre$^+$ *E. coli* strain BS591 (31).

The Cre expression plasmid pBS118 (Fig. 3) was constructed as follows. The *Xho* I-*Sal* I *cre*-containing fragment of pBS7 (25) was cloned into the *Sal* I site of the polylinker of pGEM1 (Promega) to generate pBS70. Plasmid pBS70 thus has a *Hind* III site 5' to the *cre* gene

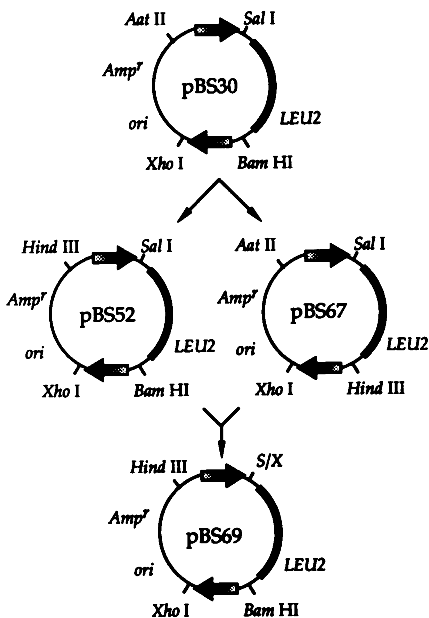

Fig. 1. Construction of the *lox*[2] *LEU2* intervening fragment. The construction of pBS69 is described in detail in Materials and Methods. *S/X* indicates the junction created by ligation of a *Sal* I sticky end to a *Xho* I sticky end. The *loxP* sites and their orientation are represented by the shaded arrows.

and a *Sma* I site 3' to the gene. The *Hind* III-*Sma* I *neo* fragment of pRSV*neo* (33) was then replaced with the *Hind* III-*Sma* I *cre*-containing fragment of pBS70 to generate pBS118.

DNA-Mediated Transformation of Mammalian Cells

The detailed procedure used for $Ca_3(PO_4)_2$ mediated transformation of mammalian cells has been described (34). Electroporation of cells was performed as described (35) using a single pulse of 350 volts at 960μF with the BioRad GenePulser.

Construction of a Mouse Cell Line Containing pBS73

Cell line 12HG-1 was derived from *Ltk*⁻ cells by co-transformation of pBS73 with pY3, a plasmid which confers resistance to hygromycin B (36). Briefly, 10 cm plates containing 5×10^5 *Ltk*⁻ cells were transformed with 5 ng pY3 DNA, 50 ng pBS73 DNA and 10 μg *Ltk*⁻ genomic carrier DNA per plate. Selection for resistance to 100 μg/ml hygromycin B resulted in approximately 31 colonies per plate. Isolates sensitive to 800 μg/ml G418 were screened by Southern blot analysis for the low copy presence of *neo*-specific DNA sequences. Promising candidates were subcloned by limiting dilution in 96 well dishes. Because all subclones were found to plate with equal efficiency in media containing either 200 μg/ml G418 or containing no G418, they were maintained in media containing 50 μg/ml hygromycin B and 200 μg/ml G418. One subclone, 12HG-1, plated at an efficiency of <1% in the presence of 800 μg/ml G418 and contained 3-6 copies of *neo*-specific sequences. No tandem copies of pBS73 were detected after digestion with a number of different restriction enzymes which cleave pBS73 only once.

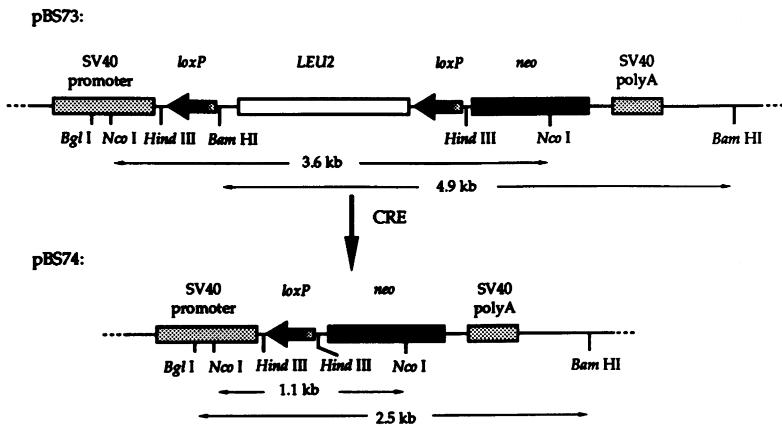

Fig. 2. Activation of the *neo* gene by excision of intervening DNA. Plasmid pBS73 contains the *lox*[2] *LEU2* fragment from pBS69 inserted at the *Hind* III site of pSV2*neo* to disrupt expression of *neo* from the SV40 promoter. Cre-mediated recombination results in deletion of the 2.6 kb intervening fragment to leave a 50 bp fragment at the *Hind* III site containing a single *loxP* site. The sizes of the *Nco* I fragments and the *Bam* HI-*Bgl* I fragments containing the 5' end of the *neo* gene for both plasmids are indicated.

Strain 12HG-1 exhibited no decrease in plating efficiency in 200 μg/ml G418 after 20 generations of growth in the absence of selection.

DNA Isolation and Southern Hybridization

M13mp11 *neo*ΔS10 was obtained from N. Sternberg (du Pont) and was used as a probe for *neo*-specific sequences (26). The *LEU2* probe was the 1.3 kb *Cla* I-*Sal* I fragment from pBS30 (25). The *aprt* probe was the 1.2 kb *Pvu* II fragment of pSAM-1 (37). In all cases no sequences derived from pBR322 were included in the probe DNA. Total cellular DNA (38) was digested with the indicated restriction enzyme(s) and analyzed by Southern blotting and hybridization (25, 39) after gel electrophoresis in Tris/borate/EDTA buffer. Cre protein was the gift of K. Abremski (du Pont). Cre-mediated recombination *in vitro* was performed as described (40).

G418-Resistant Derivatives of 12HG-1

Independent spontaneous G418^r derivatives of 12HG-1 were obtained in the following manner. First, Tk$^+$ derivatives of 12HG-1 were obtained by $Ca_3(PO_4)_2$-mediated transformation with pBR*tk*2.0 *Xho* I-C (34), which contains a 2.0 kb *Xho* I fragment of DNA containing the HSV-1 thymidine kinase (*tk*) gene. This plasmid will be referred to as pBR*tk*. Cultures from individual Tk$^+$ colonies were propagated in HAT medium (41) and then plated in media containing 800 mg/ml G418. G418^r colonies were obtained at frequencies ranging from 1×10^{-5} to 3×10^{-4}. A single G418^r colony was picked from each original Tk$^+$ transformant and was subcloned before further analysis.

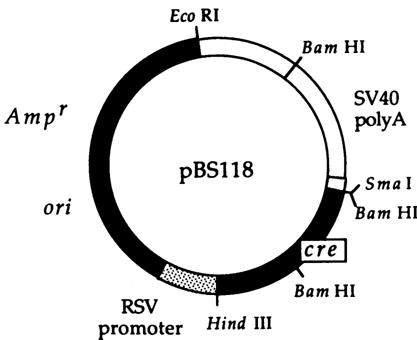

Fig. 3. The Cre expression plasmid pBS118. The 1.2 kb *cre* gene was placed under the control of the RSV LTR as described in Materials and Methods.

Cre was introduced into strain 12HG-1 by $Ca_3(PO_4)_2$-mediated transformation of 5 x 10^5 cells per plate with 100 ng pBR*tk* DNA and also 2 mg of either pBS118 or pRSV*cat* (42). Cells were trypsinized 2 days after DNA transformation and replated in HAT medium to select for Tk^+ transformants. G418 was added to the plates to a final concentration of 800 μg/ml either 4 days or 6 days after HAT selection. Alternatively, 3 x 10^6 cells were electroporated with 15 μg of pBS118 or pRSV*cat* in a volume of 0.8 ml and various aliquots were plated in triplicate. One day later half of the plates were treated with 2.5 mM Na butyrate (pH 7.0) for 20 h. $G418^r$ colonies were selected 4 days after electroporation by either adding 800 μg/ml G418 directly to the plates or by first trypsinizing and replating the cells in 800 μg/ml G418.

RESULTS

Design of a Positive Selection for Cre-Mediated Recombination Events

To develop a positive selection for recombination events at *loxP* sites, plasmid pBS73 was constructed (Fig. 2). This plasmid was derived from pSV2*neo* by inserting a 2.6 kb DNA fragment, containing the yeast *LEU2* gene flanked by two directly repeated *loxP* sites, into the *Hind* III site between the SV40 promoter and *neo* structural gene. We anticipated that this disruption would lead to decreased expression of the *neo* gene in mammalian cells. This could occur, for instance, from the insertion of numerous AUG translation initiation codons upstream of the authentic AUG start signal of the *neo* transcript (43-44). Cre-mediated recombination at the *loxP* sites of pBS73 excises the *LEU2* gene and was used to generate pBS74 (Fig. 2) which is identical to pSV2*neo* except for the 50 bp *Hind* III fragment containing a single *loxP* site. Because no AUG codons are present on this fragment, pBS74 was expected to confer resistance to G418 in mammalian cells about as well as pSV2*neo* and, in particular, much more efficiently than pBS73.

Table 1 shows that *Ltk⁻* cells were transformed to G418 resistance 10 to 20-fold more poorly with the lox^2 pBS73 construct than with pSV2*neo*. The ability of plasmid pBS74 to

TABLE 1. Transformation efficiency of *loxP*-pSV2*neo* derivatives in mammalian cells.

Plasmid	Amount (ng)	G418^r transformants
pSV2*neo*	10	435
pBS73	10	18
pBS74	10	210

Duplicate plates of Ltk⁻ cells were transformed by $Ca_3(PO_4)_2$-mediated DNA transformation in the presence of 10 μg Ltk⁻ genomic carrier DNA (34) to obtain the number of transformants resistant to 400 μg/ml G418 with the indicated amount of plasmid DNA.

confer resistance to G418 was reduced 2-fold compared to pSV2*neo*, perhaps due to secondary structure in the RNA leader sequence from the 13 bp inverted repeats of the *loxP* site which might diminish translation of the *neo* gene (45). Excision of the *LEU2* insert thus allows enhanced expression of the *neo* gene.

Construction of the Indicator Cell Line

A cell line containing a low copy number of the enfeebled *neo* construct was generated by co-transformation of *Ltk⁻* cells with pBS73 and pY3, a plasmid construct which confers resistance to hygromycin (36). Cell line 12HG-1 contains an intact segment of pBS73 having the SV40 promoter-*lox*^{2} *LEU2-neo*-SV40 poly A region (Fig. 2), but contains no tandem copies of pBS73.

Table 2 shows that 12HG-1 plated well at a low concentration of G418 whereas the parental *Ltk⁻* cells are quite sensitive. At higher concentrations of G418 12HG-1 plated quite poorly. For comparison, 2-4 (5), a *Ltk⁻* derivative containing 3-10 copies of pSV2*neo*, plated at high efficiency in 800 μg/ml G418. Because 12HG-1 plates at an efficiency of 3×10^{-5} in the presence of 800 μg/ml G418, removal of *LEU2* sequences by Cre-mediated site-specific recombination should result in increased resistance to G418. Cell line 12HG-1 was therefore chosen as the desired indicator cell line.

Demonstration *in vitro* that 12HG-1 Contains Functional *loxP* Sites

To show that the genome of 12HG-1 contains functional *loxP* sites, we prepared 12HG-1 genomic DNA and incubated the DNA with purified Cre protein *in vitro*. The product

TABLE 2. Sensitivity of pBS73-containing transformants to G418.

	Efficiency of Plating in G418(μg/ml)				
Cell line	0	200	400	800	1200
Ltk⁻	0.77	$<2 \times 10^{-6}$	$<2 \times 10^{-6}$	$<2 \times 10^{-6}$	$<2 \times 10^{-6}$
12HG-1	0.76	0.74	6×10^{-3}	3×10^{-5}	5×10^{-6}
2-4	0.61	–	–	0.60	–

Duplicate plates (diameter = 10 cm) containing 50, 500, 5×10^3, 5×10^4 and 5×10^5 cells of each cell line were set up with the indicated concentration of G418 in the media. The efficiency of plating was obtained by dividing the number of colonies obtained on each plate by the number of cells with which it was seeded.

of the reaction was digested with *Nco* I and analyzed by Southern blotting using a *neo*-specific probe. The *neo* probe detects only the 3.6 kb *Nco* I fragment of pBS73 containing the lox^2 *LEU2* insert (Fig. 2). As shown in Fig. 4, upon Cre-mediated recombination the 3.6 kb fragment is converted to a 1.1 kb *Nco* I fragment (*rec*). Cell line 12HG-1 contains the 3.6 kb *Nco* I fragment (lox^2), as well as other *Nco* I fragments derived from *neo* rearrangements which presumably occurred during gene transfer. Incubation of genomic DNA with Cre generates the predicted 1.1 kb *Nco* I recombinant fragment. Because Cre-mediated recombination *in vitro* is only ~60% efficient (23), the 3.6 kb band is not completely converted to the 1.1 kb band. These results indicate that 12HG-1 contains functional *loxP* sites flanking the *LEU2* gene and that Cre-mediated recombination can generate the 1.1 kb *Nco* I fragment of pBS74 to allow efficient expression of the *neo* gene.

Cre-Mediated Stimulation of G418-Resistance in Cell Line 12HG-1

To express the Cre protein in mammalian cells, plasmid pBS118 was constructed by placing the *cre* gene under the control of the RSV promoter (Fig. 3). Plasmid pBS118 was introduced into 12HG-1 cells by co-transformation with pBR*tk* using $Ca_3(PO_4)_2$. Tk^+ cells were selected in HAT medium two days after transformation and 800 mg/ml G418 was added to the medium either 4 days or 6 days after HAT selection. The addition of G418 to the medium was delayed to allow expression of the *cre* gene, subsequent recombination of the

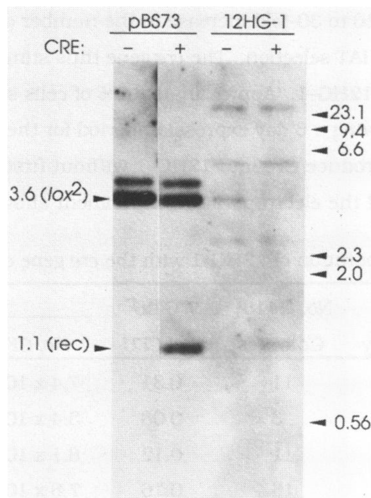

Fig. 4. Cre-mediated recombination of genomic DNA *in vitro*. A Cre reaction *in vitro* was performed with 10µg of genomic DNA from 12HG-1 and then digested with *Nco* I. Analysis by Southern blotting using a *neo*-specific probe allowed detection of the 1.1 kb *Nco* I *neo* fragment (*rec*) which is diagnostic of site-specific recombination and which is derived from the 3.6 kb lox^2 fragment. As a marker, 0.2 ng pBS73 DNA similarly treated with Cre and *Nco* I was run on the same gel. Because the *neo* probe spans the *Nco* I site of pBS73 there is also a 4.0 kb fragment detected with this probe. A *Hind* III digest of phage λ was used to give the indicated set of size markers.

TABLE 3. Cre induction of G418-resistance.

Cotransforming DNA	Cre Phenotype	Tk^+ $G418^r$ 4d	6d	Frequency $G418^r$ 4d	6d
none	–	0	2	<0.001	0.0014
pRSV*cat*	–	0	1	<0.001	0.0007
pBS118	+	24	32	0.018	0.023

Each plate of 12HG-1 was transformed with 0.1 μg pBR*tk*2.0 *Xho* C and 2 μg of the indicated cotransforming DNA, as described in Materials and Methods. Two days later cells were trypsinized and replated at a 2:1 dilution in HAT medium. At 4 or 6 days after HAT selection, 800 μg/ml G418 was added to the media and colonies were allowed to form. Total number of colonies from 21 original transformation plates is shown. Transformation of 12HG-1 with 0.1 μg pBR*tk* DNA gave an average of 65 Tk^+ colonies per original transformation plate in either the presence or absence of the cotransforming DNA.
Frequency $G418^r$ = no. $G418^r$ colonies/no. Tk^+ colonies (i.e. 21 x 65).

chromosomal *neo* construct, and resulting expression of the *neo* gene. This procedure determines the ability of Cre to perform recombination in individual Tk^+ transformants. As controls, cells were either transformed with pBR*tk* or cotransformed with pBR*tk* and pRSV*cat* (42). The results are shown in Table 3.

Transformation with pBR*tk* or cotransformation of pBR*tk* and pRSV*cat* resulted in no Tk^+ $G418^r$ transformants with selection at 4 days and very few with selection at 6 days after HAT selection. Those which did occur were small and grew poorly. Cotransformation of pBR*tk* and pBS118 gave a 20 to 30-fold increase in the number of Tk^+ $G418^r$ transformants both 4 days and 6 days after HAT selection. The *cre* gene thus stimulates the generation of $G418^r$ colonies from cell line 12HG-1. Approximately 2% of cells stably taking up the pBR*tk* DNA became $G418^r$ after allowing a 6 day expression period for the *cre* gene.

To more efficiently introduce Cre into 12HG-1 without first selecting for Tk^+ transformation, we performed the electroporation experiment shown in Table 4. Cells were

TABLE 4. Electroporation of 12HG-1 with the *cre* gene construct pBS118.

DNA	Butyrate	Cell Viability	No. $G418^r$ Colonies	%$G418^r$ of CFU	Replated Cells No. CFU	No. $G418^r$	% $G418^r$
none	–	0.016	11	0.31	7.4×10^3	0	<0.02
none	+	0.016	3	0.08	3.4×10^4	12	0.04
pRSV*cat*	–	0.042	11	0.12	8.1×10^4	21	0.03
pRSV*cat*	+	0.044	16	0.16	7.8×10^4	26	0.03
pBS118	–	0.014	281	8.9	2.1×10^4	449	2.1
pBS118	+	0.011	413	17	7.2×10^3	510	7.1

Cells were electroporated with the indicated DNA as described in Materials and Methods. Triplicate plates were seeded with 7.5×10^4 cells and then treated with butyrate as indicated. Selection for $G418^r$ was imposed 4 days after electroporation, either directly or after trypsinization and replating. CFU, colony-forming unit.

electroporated with pBS118 or pRSV*cat* (as a control) and G418^r colonies were selected 4 days later. Because a colony would be scored as resistant if only one cell in the developing colony had undergone recombination, we also trypsinized cells 4 days after electroporation on a second set of plates and replated them in G418 to obtain an estimate of the frequency of recombination on a per cell basis. To further enhance recombination, half of the plates were treated with Na butyrate, which increases gene expression from a variety of promoters, including RSV (46). About 9% of the viable cells which had received pBS118 became G418^r. Treatment with Na butyrate gave an additional 2-fold stimulation but only in cells that had received pBS118. The incidence of G418^r in cells replated and selected 4 days after electroporation was 2% without butyrate and 7% plus butyrate, indicating that recombination and subsequent *neo* expression was occuring in the time between electroporation and selection 4 days later. The data also indicates that the expression of the *cre* gene leads to a 100-fold increase in G418^r colonies among the replated cells.

Occurrence of Recombination at *loxP* Sites in the G418-Resistant Derivatives of 12HG-1

To verify that recombination at the *loxP* sites had occurred in the Cre-induced G418^r derivatives of 12HG-1, G418^r transformants from the experiment in Table 3 were analyzed by Southern blotting. Cre-mediated recombination at the *loxP* sites of pBS73 should result in the loss of the *neo*-containing 3.6 kb *Nco* I fragment and the generation of a new 1.1 kb *Nco* I fragment lacking *LEU2* sequences (Fig. 2). Southern blot analysis of *Nco* I digested DNA from 5 of the Cre-induced G418^r 12HG-1 derivatives using both *neo* and *LEU2* probes, shows that this is exactly what has occurred (Fig. 5). Cell line 12HG-1 (lane 1) displays a 3.6 kb *Nco* I band (*lox*^{2}) containing both *neo* and *LEU2* sequences. The five Cre-induced transformants (lanes 2-6) no longer contain the 3.6 kb *lox*^{2} fragment. Instead a new 1.1 kb fragment is present which contains *neo* sequences but not *LEU2* sequences. This is exactly the mobility of the predicted 1.1 kb *Nco* I recombinant fragment. Analysis of two additional Cre-induced G418^r transformants gave a similar result (data not shown). Although high level resistance to G418 could also result from amplification of the *neo* gene (47-48), no large differences in copy number of *neo* sequences in the Cre-induced transformants were observed, using a probe homologous to the endogenous adenosine phosphoribosyl transferase (*aprt*) gene (37) to control for the amount of DNA in each sample.

In addition to loss of the 3.6 kb *Nco* I *lox*^{2} fragment, some of the five Cre-induced G418^r clones show loss of other fragments containing *neo* or *LEU2* sequences. These other DNA fragments may represent rearranged pieces of pBS73 which also contain a *loxP* site. This issue is addressed in greater detail below.

Because G418^r derivatives of 12HG-1 can be obtained in the absence of Cre, it was important to determine whether or not they also occurred by recombination at the *loxP* sites. This could occur by homologous recombination between the 50 bp of repeated sequences containing the *loxP* sites, although recombination is inefficient with such small regions of

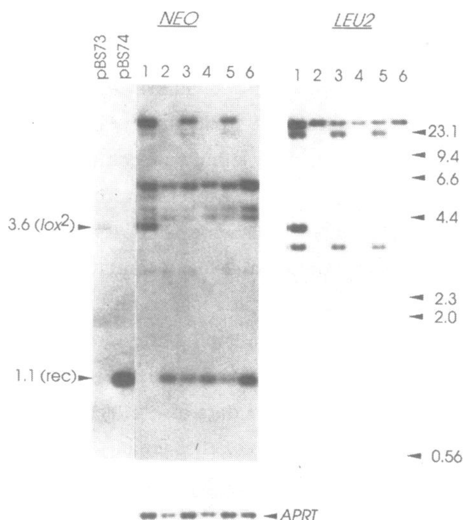

Fig. 5. *Nco* I analysis of Cre-induced G418 resistance. DNA from the parental 12HG-1 (lane 1) and five independently obtained G418^r derivatives (lanes 2-6) were digested with *Nco* I and analysed by Southern blotting and sequentially probing the resulting filter with *neo*-specific, *LEU2*-specific and *aprt* specific probes.

homology (26, 49). A large number of independent spontaneous G418^r derivatives were obtained by first selecting Tk$^+$ transformants of 12HG-1 following transformation with pBR*tk*. Cultures of each Tk$^+$ clone were then selected for resistance to 800 μg/ml G418 to obtain independent G418^r colonies. Fig. 6 shows the analysis of nine spontaneous G418^r derivatives after DNA digestion with *Nco* I and Southern blotting. None of the 9 spontaneous G418^r clones (lanes 2-10) had lost the 3.6 kb *lox*^{2} band visualized with either *neo* or *LEU2* probes. Only one of the spontaneous G418^r clones (lane 2) exhibits the 1.1 kb *neo*-specific band characteristic of Cre-mediated recombination, although this band is faint and may be submolar. In general, the spontaneous G418^r clones do not exhibit loss or rearrangements of either *neo* or *LEU2 Nco* I fragments. In some cases G418^r may have occurred by amplification of *neo* sequences. This is clearly so for the spontaneous G418^r clone of lane 2 when compared to the parent 12HG-1 (lane 1) and adjusting for the relative amount of DNA in each lane with the *aprt* probe.

To confirm that the recombination event had occured at the *loxP* sites, a similar Southern blot analysis of the Cre-induced and spontaneous G418^r derivatives of 12HG-1 was performed after digestion of genomic DNA with *Bam* HI and *Bgl* I (Fig. 7). As diagrammed in Fig. 2, 12HG-1 should exhibit a 4.9 kb *lox*^{2} fragment from pBS73 containing both *neo* and

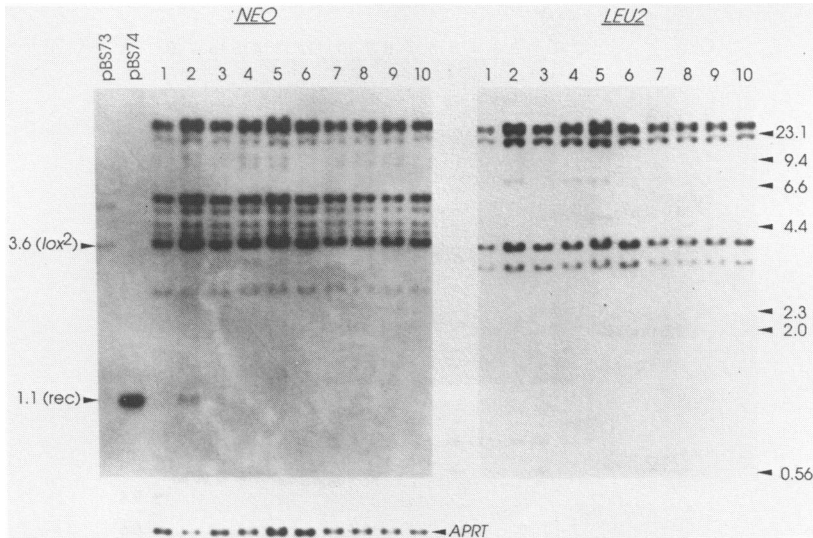

Fig. 6. *Nco* I analysis of spontaneous G418 resistance. DNA from 12HG-1 (lane 1) and nine independently obtained spontaneous G418^r derivatives was analysed as described for Fig. 5.

LEU2 sequences. Cre-mediated recombination results in loss of the 4.9 kb fragment and the generation of a 2.5 kb recombinant fragment which hybridizes only to the *neo* probe. Fig. 7 shows that of seven Cre-induced G418^r clones analyzed (lanes 2-8), all have lost the 4.9 kb *lox*^{2} band present in 12HG-1 (lane 1) and have gained the predicted 2.5 kb band. As was observed with the *Nco* I analysis, certain rearranged *neo* and *LEU2* containing fragments present in 12HG-1 also disappear in the Cre-induced G418^r derivatives. Of the ten spontaneous G418^r clones, none have lost the 4.9 kb band and only one (lane 9) shows a faint 2.5 kb recombinant band. This is the same spontaneous G418^r clone which contained the 1.1 kb recombinant *Nco* I fragment (Fig. 6, lane 2). Comparison of the intensities of the *neo*-specific bands with the *aprt* band in 12HG-1 and the spontaneous G418^r clones indicates that some amplification of *neo* sequences may have occurred in the spontaneous G418^r derivatives. Little or no amplification of *neo* sequences is apparent in the Cre-induced G418^r colonies.

The *Bam* HI-*Bgl* I analysis confirms the *Nco* I data and shows that all of the Cre-induced G418^r clones have undergone recombination at the *loxP* sites, as does digestion with two other sets of diagnostic restriction enzymes (data not shown). However, spontaneous G418^r derivatives of 12HG-1 have not excised the *LEU2* fragment by recombination at the 50 bp *loxP*-containing sequences or have done so only very inefficiently. In fact, in only one of

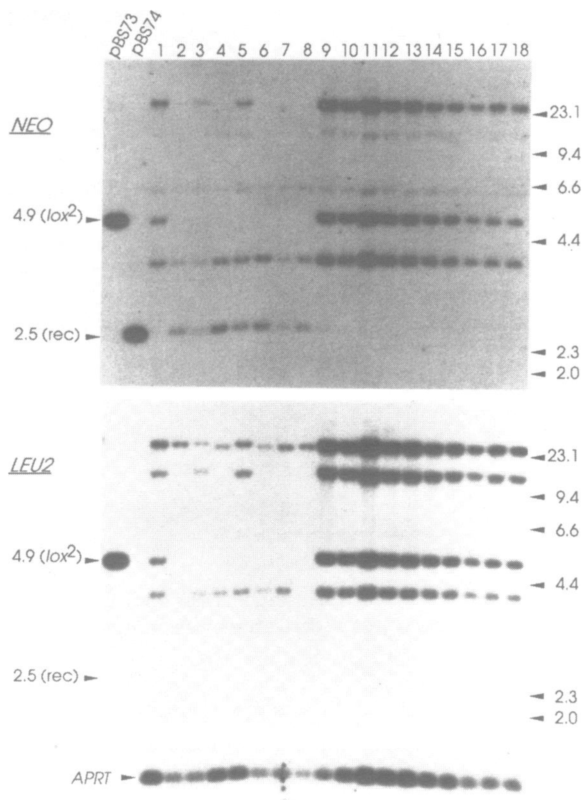

Fig. 7. *Bam* HI-*Bgl* I analysis of G418 resistance. DNA from 12HG-1 (lane 1), seven Cre-induced G418^r derivatives and ten spontaneous G418^r derivatives was digested with both *Bam* HI and *Bgl* I and then analysed by Southern blotting as described for Fig. 5.

the ten spontaneous clones analyzed is the 1.1 kb *Nco* I recombinant fragment detected (Fig. 6, lane 2), and it is clearly less abundant (<10%) than the amplified 3.6 kb fragment from which we presume it is derived. The recombinant fragment could, for instance, be present in only a subpopulation of these cells. The occurence of spontaneous homologous recombination at the 50 bp sequences containing the *loxP* sites is therefore at least 10 x 10 x 20 = 2000 times less efficient than Cre-induced recombination events. Cre thus greatly stimulates recombination at *loxP* sites placed into the genome of cultured mouse cells.

DISCUSSION

We have shown that the Cre recombinase of coliphage P1 is capable of recognizing chromosomal *loxP* sites placed into a mammalian genome to cause recombination at those

loxP sites. These results extend our previous observations that Cre can promote recombination on extrachromosomal DNA introduced into mammalian cells either by calcium phosphate precipitation or by viral infection (26). We believe that the Cre-mediated events which we observe in mammalian cells occur by the efficient catalysis of recombination at *loxP* sites by the Cre recombinase. Alternatively, Cre would have to stimulate >2000-fold the homologous recombination of 50 bp sequences containing the *loxP* site. Facilitation of homologous recombination at such short DNA sequences might occur, for instance, by Cre-mediated synapsis of DNA. Future experiments using appropriately marked sites should distinguish between these two possibilities. Our results also suggest that Cre-mediated recombination should be of use in the genetic manipulation of any eukaryote.

Other prokaryotic DNA-binding proteins have been functionally expressed in mammalian cells. In a number of cases these proteins have been shown to act on chromosomal DNA in the nucleus of the cell. For example, the *Pae*R7 methylase is capable of methylating chromosomal DNA rendering it resistant to restriction enzyme cleavage (50). Recently it has also been shown that the *lac* repressor of *E. coli* can regulate expression of a stably resident chloramphenicol acetyl transferase gene in the genome of cultured monkey cells, presumably by binding to a *lac* operator site placed in the promoter region (51). It is remarkable, however, that the Cre protein not only can bind to a specific DNA sequence(s) in the eukaryotic genome, but that it can also cause synapsis of two such DNA sequences and lead to precise recombination between them. The Cre protein may thus prove to be quite useful as a tool to understand the rules governing the interaction of chromosomal sequences.

The experiments we present here indicate that only 2-7% of the Tk^+ cells had undergone site-specific recombination 4-6 days after introduction of the Cre expression vector. It is quite possible that allowance of a longer expression period for the *cre* gene would result in the enhanced generation of $G418^r$ colonies. In preliminary experiments we have observed that >50% of subclones from Tk^+ colonies obtained after cotransformation of pBR*tk* and pBS118 exhibit high levels of G418 resistance and display the characteristic 1.1 kb *Nco* I recombinant *neo* fragment (data not shown). This suggests that the concentration of Cre may be limiting in these cells. For instance, 1) a high concentration of Cre may be required to search the large mammalian genome efficiently for *loxP* sites; 2) Cre's lack of a nuclear localization sequence may result in a reduced efficiency in entry of Cre into the nucleus; 3) chromosome structure in mammalian cells may inferfere with recombination.

In addition to the predicted restriction fragments of pBS73 the 12HG-1 cell line contains portions of the *LEU2* and *neo* gene which were rearranged upon integration after gene transfer (Fig. 3). Some of the DNA fragments containing rearranged portions of *LEU2* and *neo* were also deleted from the genome in the Cre-induced $G418^r$ derivatives. It is likely that *loxP* sites from pBS73 are present on these rearranged DNA fragments. The presence of one or more such *loxP* sites upstream of the unrearranged copy of the enfeebled SV40-*LEU2*-

neo DNA segment could lead to deletion of the intervening DNA and the resulting loss of DNA fragments containing *neo* or *LEU2* homology. Because Cre is capable of intermolecular recombination both in *E. coli* and in yeast (B. S., unpublished data), it is anticipated that Cre would perform excisive recombination not only between *loxP* sites located 2.5 kb apart (as in the pBS73 construct) but also between *loxP* sites many kilobases away, as long as the sites are not sequestered by some feature of the nuclear architecture.

Site-specific recombination in mammalian cells is likely to be quite useful in a variety of situations. Intermolecular recombination events, for instance, may allow the relatively efficient insertion of engineered gene constructs to a particular predetermined *lox*-containing genome location after gene transfer. Conversely, a gene could be efficiently removed from the genome by Cre-mediated recombination. Alternatively, a single exon of a gene could be removed by placing the *loxP* sites within adjacent introns of a gene. In this latter context, using excisive recombination, it is particularly interesting to speculate on the utility of the Cre system to understand cell lineage in multicellular organisms. For example, Cre-mediated excision of an appropriate gene could result in a easily detectable (visible) phenotypic change in a cell and its decendents. If Cre-mediated excision is suitably inefficient cell lineages could be established in a relatively straightforward manner, using techniques similar to those which have been employed in *Drosophila* (52-53).

Finally, site-specific recombination may influence the nature of homologous recombination events in the vicinity of a *loxP* site. For example, either synapsis of DNA by the Cre protein or subsequent Cre-mediated breakage of DNA could promote the occurrence of homologous events at sequences adjacent to the *loxP* site. In yeast Cre stimulates the occurrence of crossover events adjacent to a *loxP* site at the *ILV2* locus (B. S., unpublished data). Such modulation of recombination between homologous sequences could contribute to an understanding of the mechanism(s) of recombination in higher eukaryotes.

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