JOURNAL OF VIROLOGY, Aug. 1995, p. 4600–4606 Vol. 69, No. 8
0022-538X/95/$04.00+0

Site-Specific Recombination Mediated by an Adenovirus Vector Expressing the Cre Recombinase Protein: a Molecular Switch for Control of Gene Expression

MARTINA ANTON[1] AND FRANK L. GRAHAM[1,2]*

Departments of Biology[1] and Pathology,[2] McMaster University, Hamilton, Ontario L8S 4K1, Canada

Received 7 March 1995/Accepted 27 April 1995

We have constructed replication-defective human adenovirus (Ad) type 5 vectors containing the gene for the Cre recombinase from bacteriophage P1 under control of the human cytomegalovirus immediate-early promoter (AdCre). Expression of the protein was detected in replication-permissive (293) and in nonpermissive (MRC5) cell lines, and its biochemical activity was demonstrated in a cell-free recombination assay using a plasmid containing two *loxP* sites. To study Cre-mediated recombination in an intracellular system, we constructed an Ad vector (AdMA19) containing the luciferase cDNA under control of the human cytomegalovirus promoter but separated from it by an extraneous spacer sequence flanked by *loxP* sites which blocked luciferase expression. Upon coinfection of 293 or MRC5 cells with AdMA19 and AdCre, luciferase expression was specifically induced by Cre-mediated excision of the intervening sequence. The use of Ad vectors combined with the Cre-*loxP* system for regulation of gene expression and other possible applications is discussed.

Site-specific recombination systems have recently gained attention for the control of gene expression in eukaryotic cells (40–42) and in animals (3, 18, 19, 27, 31, 33, 39). Extensive use has been made of the Cre-*loxP* system of bacteriophage P1, which requires only two well-characterized components: the 38-kDa recombinase protein, Cre, and the 34-bp *loxP* target sequence (1, 22, 23, 25). Cre binds to the two 13-bp inverted repeats of *loxP* and catalyzes precise recombination between the asymmetric 8-bp core regions of two *loxP* sites (24, 25). Recombination between two parallel sites, as defined by the core region, results in excision of intervening sequences, producing two recombination products each containing one *loxP* site (2, 25), whereas recombination between antiparallel sites inverts the bracketed fragment. Intermolecular recombination between *loxP* sites on separate plasmids results in integration of sequences bracketed by *loxP* sites. The Cre-*loxP* system has been shown to function in both bacteria and eukaryotic cells (2, 25, 38, 40–42, 44) and has been exploited for the excision (19, 27, 31, 38, 40, 41) and the integration of fragments in cellular and viral genomes (12, 32, 42, 43). The use of Cre in cell-free systems for construction of recombinant vectors has also been reported (13, 43). Lastly, Cre-*loxP*-based recombination has been used successfully for tissue-specific gene expression or deletion in transgenic mice (3, 10, 18, 27, 31, 33, 39). In the latter cases, the recombinase was delivered by transfection (10, 18, 19) or microinjection (3, 27, 31) of Cre-encoding plasmids into embryonic stem cells or fertilized eggs.

For the applications described above, it would be useful to have methods for the efficient delivery of the Cre recombinase protein to a large number of cells of different origins. Human adenovirus (Ad) vectors could provide such a vehicle, as they have been used extensively for heterologous gene expression in mammalian cells (4, 14, 15) and have attracted considerable attention as potential recombinant vaccines (14, 15, 34) and for use in gene therapy (8, 28, 35, 45, 48). Ad vectors have a number of properties that render them particularly suited for these and other applications. The 36-kbp double-stranded DNA genome is relatively easy to manipulate with recombinant DNA techniques (14), helper-independent vectors can accommodate up to 8 kbp of foreign DNA, depending on the system chosen (5), and Ad virions are physically and genetically stable if the vectors are constructed and propagated appropriately (6). Viruses with a deletion of E1 can be propagated on 293 cells (16) and can infect other human cell lines but are defective for replication. Ad type 5 (Ad5) can be grown to high titers and can infect a wide variety of tissues, such as epithelial and endothelial cells, fibroblasts, stromal cells, and hepatocytes of different species. Moreover, Ad can infect quiescent as well as replicating cells and express proteins therein.

In this report, we describe the construction and use of Ad vectors in which the Cre protein is expressed under control of the human cytomegalovirus (HCMV) immediate-early promoter (AdCre) and provide evidence for the intracellular action of Cre expressed from these vectors. We also demonstrate that the system can be used to induce expression of a reporter gene by coinfection of cells with two Ad vectors, one carrying the luciferase gene (Luc) under the control of a molecular switch that can be turned on by the second vector expressing Cre.

MATERIALS AND METHODS

Construction of recombinant plasmids. Enzymes used for the manipulation of recombinant DNA and molecular weight standards were purchased from Boehringer Mannheim, Inc. (Laval, Quebec, Canada), Pharmacia (Baie d'Urfe, Quebec, Canada), New England Biolabs (Mississauga, Ontario, Canada), GIBCO Laboratories (Grand Island, N.Y.), or Bethesda Research Laboratories (Burlington, Ontario, Canada) and used according to the suppliers' recommendations. Plasmids were constructed by using standard protocols (37). The Magic PCR or Wizard PCR prep kit was used for the isolation of DNA fragments from low- or high-melting-point agarose gels, respectively. Plasmid DNA was prepared by the alkaline lysis method (7) and further purified by CsCl density gradient centrifugation (37) where necessary. Transformation of plasmid DNA into *Escherichia coli* DH5α {*endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* [φ80d*lac*Δ(*lacZ*)*M15*]} was performed by the $CaCl_2$ method (37).

Oligonucleotides were purchased from The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada. A synthetical *loxP* site with compatible *Bam*HI sticky ends, and restriction sites for *Eco*RI and *Sca*I, was obtained by annealing equimolar amounts of two single-stranded oligonucleotides: 5′-GAT CCA ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TAA GTA CTG AAT TCG-3′ and 5′-GAT CCG AAT TCA GTA CTT ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TTG-3′. (The diagnostic *Sca*I site, next to the *Eco*RI site, is under-

* Corresponding author. Phone: (905) 525-9140, ext. 23545. Fax: (905) 521-2955.

lined.) The double-stranded oligonucleotide was 5′ phosphorylated by T4 polynucleotide kinase.

The 5′-CTC CAT AGA AGA CAC CGG GA-3′ primer represents the 3′ end of the HCMV immediate-early promoter and was used for sequencing and in PCR amplification. The second primer for the PCR, 5′-AGA GGA TAG AAT GGC GCC GGG CCT T-3′, binds to the luciferase open reading frame (ORF) from nucleotides 49 to 25. The 5′-CGG ATC CG-3′ oligonucleotide was used to link *Bam*HI sites to blunt-ended fragments.

Cells and viruses. Cell culture media and reagents were purchased from GIBCO. 293 cells were used for growth and titration of Ad vectors as described previously (14, 20). MRC5 cells were grown in alpha minimal essential medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.5 μg of amphotericin per ml, and 10% fetal bovine serum for cell maintenance or 5% horse serum for infection.

Construction and growth of recombinant viruses. Recombinant viruses were obtained by cotransfection (14, 17) of 293 cells with the appropriate plasmids as indicated in Results. Plaques were isolated after approximately 14 days and expanded in 293 cells. Viral DNA was analyzed by restriction enzyme digestion as described previously (14). All viruses were plaque purified and reanalyzed prior to preparation of large-scale stocks.

Western blots (immunoblots). Proteins were extracted by incubating infected cells with radioimmunoprecipitation assay buffer (30) for 30 min on ice. Solutions were then cleared of DNA by centrifugation, and aliquots of the supernatants were used for protein separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (26). Protein transfer from SDS–10% polyacrylamide gels to Millipore Immobilon P polyvinylidene difluoride membranes (Millipore, Mississauga, Ontario, Canada) was performed at 30 V overnight, using a Bio-Rad transblot cell (Bio-Rad Laboratories, Richmond, Calif.). Western blotting was carried out as described by Towbin et al. (46), using a polyclonal Cre-specific antibody (38) at a dilution of 1:2,500 in Tris-buffered skim milk powder (5%) and a mouse anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Pierce, Rockford, Ill.) as the secondary antibody at a dilution of 1:3,333 in Tris-buffered skim milk powder (5%). The horseradish peroxidase reaction was monitored by using the enhanced chemiluminescence reagents for Western blots from Amersham (Oakville, Ontario, Canada) and Kodak XAR5 (Eastman Kodak Company, Rochester, N.Y.) films.

Southern blot analysis. Restricted plasmid DNA was separated on a 0.8% agarose gel and transferred to a Hybond N membrane (Amersham) as described by Sambrook et al. (37). An enhanced chemiluminescence random prime labeling and detection kit (Amersham) was used to label a plasmid DNA probe as recommended by the manufacturer. Hybridization was carried out at 60°C overnight in a Techne HB-1 Hybridiser (Techne Inc., Cambridge, England). High-stringency washes were twice with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS at 60°C for 10 min and twice with 0.5× SSC–0.1% SDS at 60°C for 10 min.

Preparation of cellular extracts for Cre assay. 293 cells (2×10^7) were infected with the indicated viruses at a multiplicity of infection (MOI) ranging from 2.5 to 10 PFU per cell. Eighteen hours postinfection cells were harvested by scraping and centrifuged at 300 × *g* for 10 min at 4°C. Cell pellets were washed once in 500 μl of 20 mM Tris-HCl (pH 7.5)–300 mM NaCl and resuspended in 500 μl of Cre storage buffer (50% glycerol, 20 mM Tris-HCl [pH 7.5], 300 mM NaCl, 1 mM EDTA [pH 7.5]). Extracts were sonicated four times for 30 s, using a Biosonik III sonicator (Bronwill Scientific, Rochester, N.Y.), and cellular debris was removed by centrifugation at 300 × *g* for 10 min at 5°C. The supernatant was removed and used directly for in vitro Cre assays or stored at −20°C.

Cre assay. Assays for Cre function were carried out as described by Abremski and Hoess (1) and Sauer et al. (43), with the following modifications. Fifty-microliter aliquots of cellular extracts, prepared as described above, were mixed with 1 mM phenylmethylsulfonyl fluoride and 1 mM aprotinin. Tris-HCl (pH 7.5), $MgCl_2$, and acetylated bovine serum albumin were added to final concentrations of 50 mM, 10 mM, and 100 μg/ml, respectively. Cre assays were started by addition of plasmid DNA, and the mixtures were incubated for 30 min at 37°C. The reaction was stopped by phenol extraction followed by chloroform extraction and ethanol precipitation. The plasmid DNA was resuspended in 50 μl of Tris-EDTA buffer, and typically 1 μg of plasmid DNA was used for agarose gel electrophoresis. RNase A treatment was performed during digestion of the plasmids with appropriate restriction enzymes. Digestions were terminated by a pronase treatment (0.5 mg/ml) for 5 min at room temperature.

Luciferase assay. Luciferase assays were carried out as described previously (30).

RESULTS

Construction of recombinant adenoviruses expressing the Cre protein. The 3,439-bp *Hin*dIII fragment of pBS185 (42) containing the HCMV immediate-early gene promoter, the *cre* ORF, and the metallothionein-I polyadenylation signal was cloned into the *Hin*dIII site of the shuttle plasmid pΔE1sp1A (5). The resulting plasmids, pMA1 and pMA2 (Fig. 1), contain the left end of the Ad5 genome with the E1 region replaced by the Cre expression cassette in the left-to-right (pMA1) or reverse (pMA2) orientation relative to the Ad genome. These plasmids were cotransfected with pJM17 (29) into 293 cells to obtain the recombinant adenoviruses AdCre1 and AdCre2 by homologous recombination (Fig. 1).

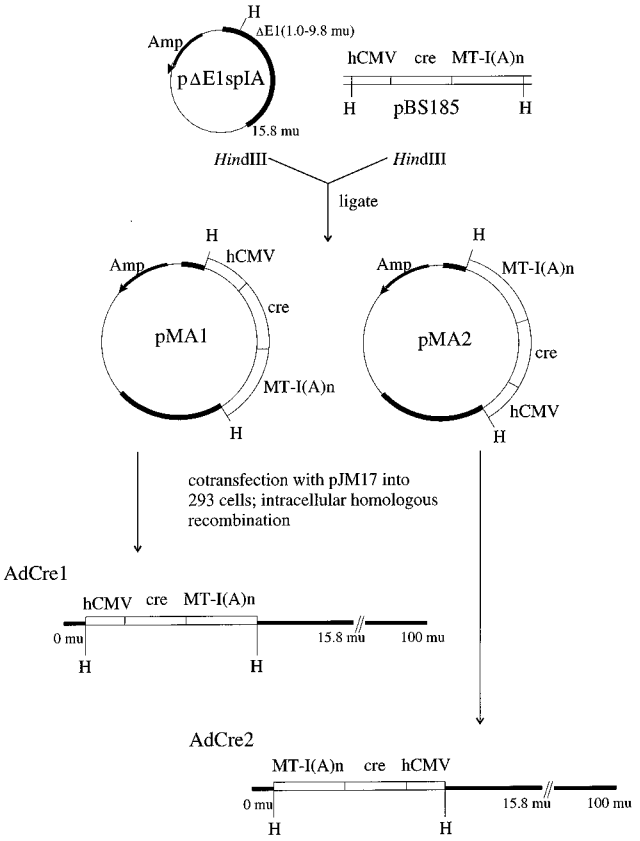

FIG. 1. Construction of Cre-expressing plasmids and viruses. pMA1 and pMA2 were constructed by inserting the *Hin*dIII fragment of pBS185 containing the *cre* ORF (Cre) under control of the HCMV immediate-early promoter (hCMV) and the metallothionein-I polyadenylation signal [MT-I(A)n] into the unique *Hin*dIII site (H) of pΔE1spIA. AdCre1 and AdCre2 were derived by cotransfection of pMA1 and pMA2, respectively, with pJM17 into 293 cells. Thin lines represent plasmid sequences; solid bars represent Ad sequences. Plasmid sizes and the left end of each virus are drawn approximately to scale. mu, map units.

Expression of Cre from recombinant adenoviruses. Production of the Cre protein by AdCre-infected cells was analyzed by Western blotting with a Cre-specific polyclonal antibody (38). In initial experiments, 293 cells were infected with AdCre1 or wild-type Ad5 at an MOI of 20 or were mock infected and then were harvested at various times postinfection. Expression of the 38-kDa Cre protein was detectable as early as 6 h after infection with AdCre1 and increased up to 24 h (Fig. 2). No further increase was seen at later times (e.g., 36 h), presumably as a result of the onset of cell lysis since 293 cells are permissive for replication of the E1-deficient AdCre1 vector. Cells infected with wild-type Ad5 or mock infected did not express the Cre protein but contained several proteins which appeared to be nonspecifically stained by the polyclonal serum and which were also detected in AdCre1-infected samples. The 60-kDa species, detected only in infected cells, is likely to correspond to the virus fiber protein which is produced at high levels late in infection. Since levels of Cre expression obtained with AdCre1 and AdCre2 did not differ substantially (data not shown), AdCre1 was chosen for further experiments. Expression of Cre protein was also detectable between 24 and 96 h postinfection

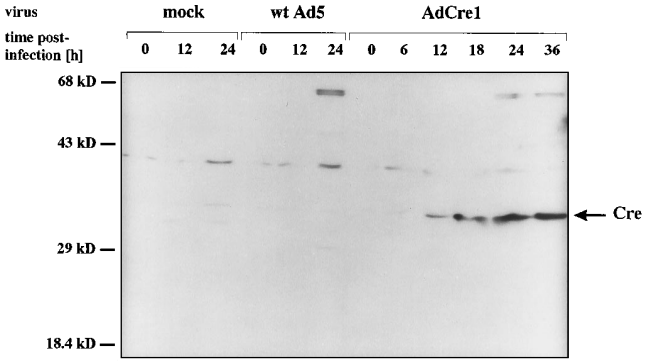

FIG. 2. Detection of Cre expression in 293 cells by Western blot analysis. 293 cells were infected with either AdCre or wild-type (wt) Ad5 at an MOI of 20 or were mock infected and then were harvested for Western blot analysis at the indicated time points. Proteins were separated on an SDS–10% polyacrylamide gel, transferred to an Immobilon membrane, and detected by Cre-specific antibodies. Molecular weights are given on the left, the arrow on the right indicates the position of the 38-kDa Cre protein.

in AdCre1-infected MRC5 cells (MOI of 50) but not in infections with the E1 deletion virus Ad*dl*70-3 (5) at the same MOI or in uninfected MRC5 cells (data not shown).

Recombinant Ad vectors containing the Luc cDNA regulated by a recombination switch. To obtain a protein expression system that could be regulated by Cre-catalyzed recombination, we designed an expression cassette in which the Luc cDNA and the HCMV promoter were separated by a spacer region flanked by *loxP* sites that would prevent luciferase expression unless the spacer was excised. As the recombination product would still contain one *loxP* site between regulatory and coding sequences, we initially investigated whether insertion of *loxP* in this position would allow expression of luciferase. Isolation of such a vector would also confirm that Ad5 could tolerate the palindromic *loxP* sequence in addition to the terminal inverted repeats. Plasmid constructions started with pCA18, which has the E1 region substituted with the HCMV immediate-early promoter and the firefly Luc gene (9) in the left-to-right orientation (2a). A synthetic *loxP* site flanked by *Bam*HI-compatible ends was inserted into the unique *Bam*HI site between the HCMV promoter and the Luc cDNA in such a way that no translational start codon was introduced in any reading frame upstream of the luciferase ATG (Fig. 3A). (If inserted in the opposite orientation, the *loxP* site adds ATGs in two reading frames.) The structure of the resulting plasmid, pMA9, was confirmed by digestion with *Eco*RI and *Sca*I and by sequencing of the insert by using a primer binding in the 3′ region of the HCMV promoter. pMA9 was used with pBHG10 (5) in cotransfection of 293 cells to obtain AdMA9 (Fig. 3A). Our ability to rescue this virus, and its normal growth properties (data not shown), demonstrate that a *loxP* site does not interfere with viral DNA replication.

To generate a construct in which the HCMV promoter is separated from the luciferase ORF by a spacer region, we chose an unrelated sequence with translational start and stop codons in all reading frames that should block luciferase expression. We inserted the 1.3-kbp *Sca*I-*Sma*I fragment of pBS64 (43) into pMA9 that had been linearized by partial digestion with *Sca*I (Fig. 3A). The resulting plasmid, pMA19, contains two *loxP* sites in parallel orientation separated by pBS64 sequences which comprise sequences from pUC12, pBR322, and the SP6 promoter (Fig. 3B). pMA19 was used with pBHG10 to cotransfect 293 cells and obtain the vector AdMA19 (Fig. 3A). AdCA18-3 was obtained by cotransfection of 293 cells with pCA18 and pJM17 (2a). AdMA9, AdMA19,

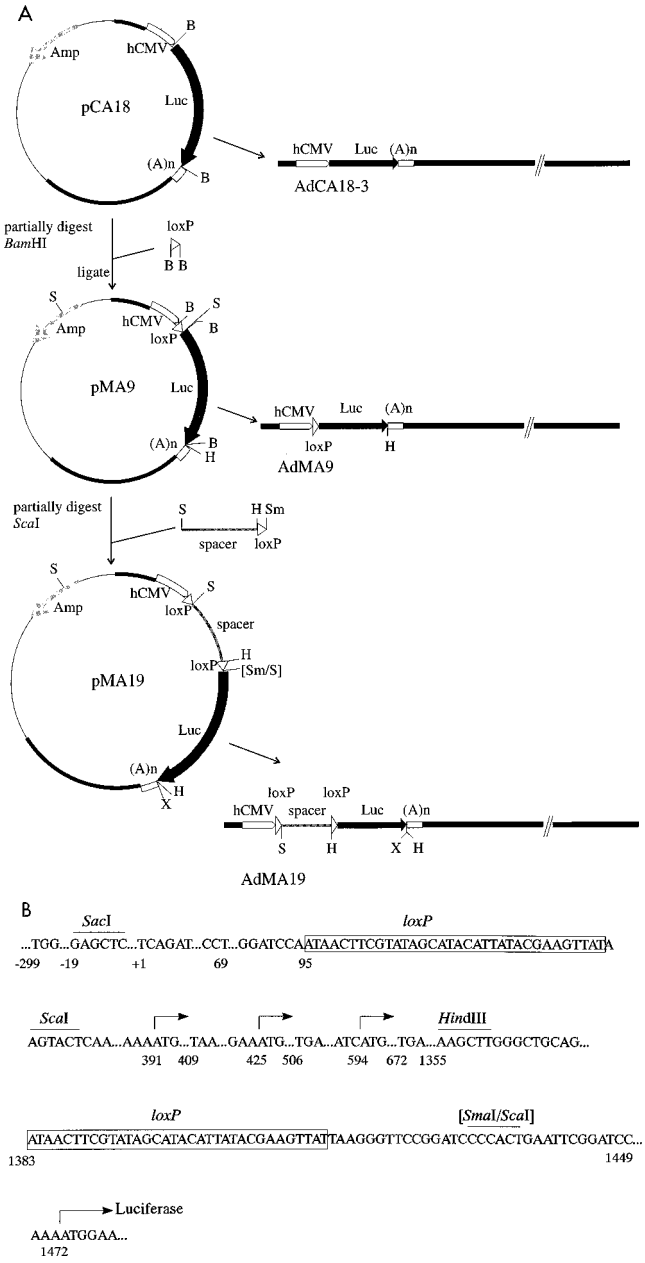

FIG. 3. Construction of regulated reporter plasmids and viruses. (A) pCA18 contains the Luc cDNA under control of the HCMV immediate-early promoter. (A)n designates the SV40 polyadenylation signal. A synthetic *loxP* site was introduced between the promoter and the Luc ORF of pCA18 so that there is no ATG between promoter and Luc cDNA, resulting in pMA9. The spacer region inserted to obtain pMA19 consists of a 1.3-kb plasmid DNA fragment from pBS64 containing a *loxP* site, preceded by start and stop codons in all reading frames. The recombinant Ad vector AdCA18-3 was obtained by cotransfection of pCA18 with pJM17 into 293 cells (2a). To obtain AdMA9 and AdMA19, pMA9 and pMA19, respectively, were cotransfected with pBHG10 (5) into 293 cells. Thin lines represent plasmid sequences; solid bars represent Ad sequences. The left ends of the resulting Ad vectors are shown in detail. B, *Bam*HI; H, *Hin*dIII; S, *Sca*I; Sm, *Sma*I; X, *Xho*I. Plasmid sizes are approximately drawn to scale. (B) Detail of the *loxP*-flanked spacer sequence inserted between promoter and Luc cDNA in pMA19 and AdMA19. Numbering begins at the transcription start (+1) from the HCMV promoter (11). Arrows indicate the luciferase translation start and possible translation starts in all reading frames in the spacer derived from pBS64. Only the first ATG in each reading frame preceded by a purine at the −3 position and the corresponding stop codon in the same frame are shown. Relevant restriction sites are given. *loxP* sequences are boxed.

and AdCA18-3 were further plaque purified before viral stocks were prepared as described previously (20).

Biochemical activity of Cre protein expressed from AdCre1. The biochemical activity of the Ad-encoded Cre protein was first determined in a cell-free assay. Uninfected 293 cells or 293 cells infected with AdCre1 or Ad*dl*70-3 at an MOI of 10 were harvested 18 h postinfection, and cell extracts were prepared by sonication as described in Materials and Methods. Aliquots of 50 μl were incubated with assay buffer and 1 μg of pMA9 or pMA19 at 37°C for 30 min. After phenol and chloroform extractions, samples were digested with *Hin*dIII and analyzed on an 0.8% agarose gel. *Hin*dIII digestion of pMA19 DNA that had been incubated with extracts from mock- or Ad*dl*70-3-infected cells generated a 7.9-kbp band and a 1.8-kbp band (Fig. 4). In contrast, when pMA19 was first incubated with extracts from AdCre1-infected 293 cells, *Hin*dIII digestion resulted in two novel bands of 8.4 and 1.3 kbp (Fig. 4B). These sizes correspond to those expected for the linearized forms of the two circular products of recombination: pMA9′ (identical to pMA9 except for 18 bp due to the cloning procedure) and a second corresponding to the excised spacer (Fig. 4A). *Hin*dIII digestion of the control plasmid pMA9 yielded a single linear fragment of 8.4 kbp, irrespective of the extract used for incubation (Fig. 4), since pMA9 contains only one *loxP* site, which does not allow intramolecular recombination. We estimated the Cre-specific efficiency of recombination to be approximately 50%, as judged by the relative intensities of the bands in Fig. 4B. The fragment of ≈11 kbp present only in the Cre-treated sample of pMA19 may represent a Holliday structure (χ) formed as an intermediate of recombination. χ and α structures, derived from Cre mutants, have been observed previously (21) as bands migrating with reduced electrophoretic mobility relative to the unrecombined, digested form of the plasmid. It is possible that sonication or storage of the extract had functionally altered the Cre enzyme, but production of a protein with altered activity in AdCre1-infected cells cannot be ruled out.

Cre-dependent induction of luciferase expression. The expression of firefly luciferase from the recombinant Ad vectors AdCA18-3, AdMA9, and AdMA19 was measured biochemically by emission of light as described previously (30). Infection of 293 cells with AdCA18-3 (no *loxP*) or AdMA9 (one *loxP*) at an MOI of 5 and preparation of cell extracts 24 h postinfection resulted in a luciferase activity of 0.84 ± 0.14 (AdCA18-3) or 0.61 ± 0.03 (AdMA9) μg/10^6 cells, demonstrating that a single *loxP* site inserted between the HCMV promoter and the translation start for Luc did not significantly alter expression of the gene. In a separate experiment, when 293 cells were infected with AdMA9 at an MOI of 10, luciferase activity of about 1.3 μg/10^6 cells was detected after 24 h (Table 1). In contrast, only a low level of activity (20 ng/10^6 cells) was obtained from AdMA19-infected cells, indicating that expression of luciferase from this virus was effectively suppressed by the spacer DNA interposed between the promoter and ATG of the reporter gene. To assess the ability of Cre protein produced from AdCre1 to act on *loxP*-containing Ad vectors, 293 cells were doubly infected with AdCre1 and AdMA9 or AdCre1 and AdMA19 at various MOIs and harvested after 24 h. AdCre1 had no significant effect on the activity of luciferase expressed from AdMA9 (Table 1). However, upon double infection with AdCre1 and AdMA19, luciferase activity was switched on, and depending on the MOI, levels of activity from 0.6 to 2.9 μg/10^6 cells were obtained (Table 1). Induction of luciferase activity suggested that the *loxP*-flanked spacer, between the HCMV promoter and the luciferase ORF, had been excised.

AdCre1-specific recombination, measured as induction of luciferase expression, was also detected in double infections of

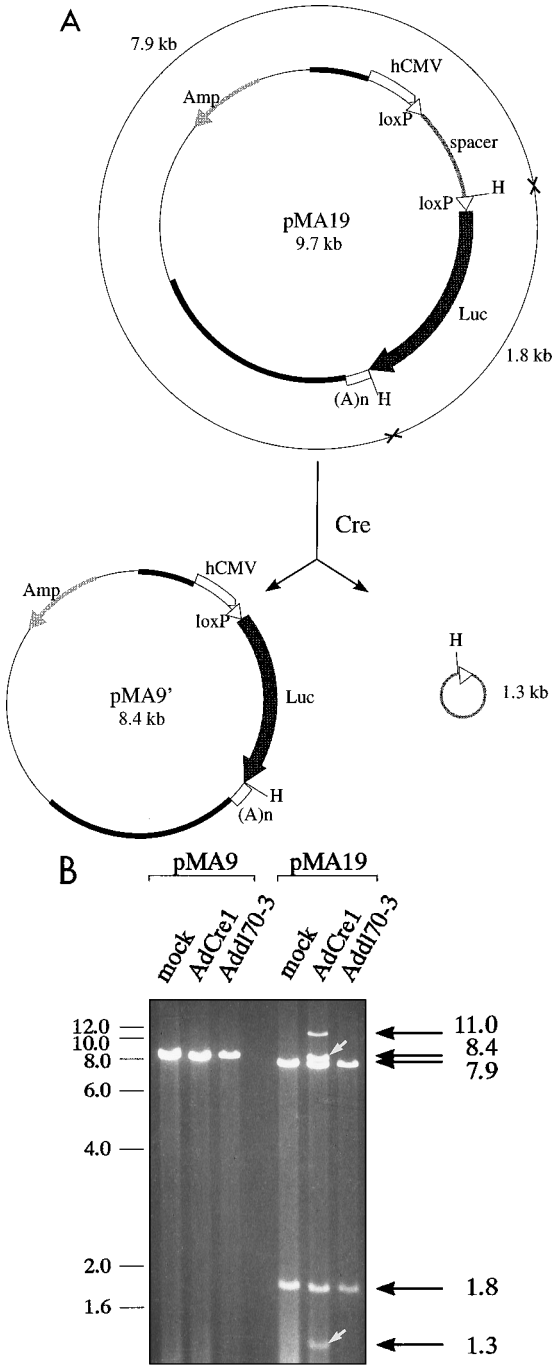

FIG. 4. In vitro recombination assay with pMA9 and pMA19. (A) The expected products after incubation of pMA19 with Cre protein are shown. The product of Cre-mediated recombination of pMA19, indicated as pMA9′, should be identical to the structure of pMA9 except for an additional 12 bp derived from cloning. Open arrowheads represent *loxP* sites. The lightly stippled bar represents the spacer region derived from pBS64, inserted into pMA19 and the expected circular molecule resulting from excision. *Hin*dIII restriction sites (H) and fragment sizes are shown. For further details, see the legend to Fig. 3. (B) One microgram of plasmid DNA was incubated with crude extracts prepared from cells infected 18 h previously with viruses indicated at the top. Molecular sizes (in kilobases) are given on the left margin, and the sizes (in kilobases) of unrecombined and recombined bands are given on the right. Arrows indicate the recombination products of Cre-treated pMA19, as predicted from recombination events shown in panel A.

TABLE 1. Cre-dependent luciferase expression in infected 293 cells

Virus[a] (MOI)	Amt (μg) of luciferase/10^6 cells[b]
None (mock infection)	0
AdCre1 (1)	0
AdCre1 (5)	0
AdCre1 (10)	0
AdMA9 (10)	1.3 ± 0.07
AdCre1 (1) + AdMA9 (10)	1.7 ± 0.4
AdCre1 (5) + AdMA9 (10)	1.2 ± 0.5
AdCre1 (10) + AdMA9 (10)	1.0 ± 0.1
AdMA19 (10)	0.02 ± 0.01
AdCre1 (1) + AdMA19 (10)	0.6 ± 0.2
AdCre1 (5) + AdMA19 (10)	1.8 ± 0.3
AdCre1 (10) + AdMA19 (10)	2.9 ± 0.4

[a] 293 cells were singly or doubly infected with viruses as indicated in a total volume of 200 μl for each 60-mm-diameter dish and harvested 24 h postinfection as described previously (30).

[b] Mean values and standard deviations were obtained from two independent experiments with two dishes for each sample.

the nonpermissive cell line MRC5 (Table 2). No luciferase activity was observed in cells infected with AdCre1 or Ad*dl*70-3 or when cells were mock infected. As was the case with 293 cells, expression of Cre did not alter the luciferase activity produced by infection with AdMA9, but mixed infections with AdMA19 and AdCre1 increased luciferase expression from a background activity of 2 ng/10^6 cells to 0.6 or 1.6 μg/10^6 cells. Double infections with Ad*dl*70-3 and AdMA19, which did not result in increased luciferase activity, confirmed that the induction was dependent on the recombining activity of Cre expressed from AdCre1. Although MRC5 cells were infected at a higher MOI than 293 cells and harvested after longer times, they expressed lower luciferase activity than infected 293 cells, even at the highest MOI tested. This finding was consistent with the results of Western blotting, which indicated that Cre was produced at lower amounts in MRC5 than in 293 cells (data not shown).

The background activity of luciferase observed after infection of 293 or MRC5 cells with AdMA19 alone could be due to spontaneous homologous recombination between the *loxP* sites, resulting in excision of the spacer in a small fraction of viruses. To examine this possibility, we performed PCR analyses that would allow detection of recombined viruses. Amplification of parental AdMA19 DNA by using primers binding to the HCMV promoter and the Luc cDNA (see Materials and Methods) should result in a fragment of ≈1.5 kbp, whereas amplification of AdMA19 viral DNA, from which the 1.3-kbp spacer had been excised by spontaneous or Cre-mediated recombination, should yield a 182-bp fragment derived from the excised spacer. In addition to the 1.5-kbp fragment, a faint band of ≈180 bp was detected after amplification of AdMA19 viral DNA (data not shown), in support of our hypothesis that spontaneous excision may have occurred in a small subpopulation of AdMA19 virions.

TABLE 2. Cre-dependent luciferase expression in infected MRC5 cells

Virus[a] (MOI)	Amt (μg) of luciferase/10^6 cells[b]
None (mock infection)	0
AdCre1 (5)	0
AdCre1 (25)	0
AdMA9 (25)	2.3 ± 0.3
AdCre1 (5) + AdMA9 (25)	1.5 ± 0.9
AdCre1 (25) + AdMA9 (25)	2.1 ± 1.0
AdMA19 (25)	0.002 ± 0.001
AdCre1 (5) + AdMA19 (25)	0.6 ± 0.1
AdCre1 (25) + AdMA19 (25)	1.6 ± 0.2
Ad*dl*70-3 (5)	0
Ad*dl*70-3 (25)	0
Ad*dl*70-3 (5) + AdMA9 (25)	1.8
Ad*dl*70-3 (25) + AdMA9 (25)	2.4 ± 0.03
Ad*dl*70-3 (5) + AdMA19 (25)	0.003 ± 0.0007
Ad*dl*70-3 (25) + AdMA19 (25)	0.002 ± 0.0005

[a] MRC5 cells were singly or doubly infected with the viruses as indicated in a total volume of 200 μl for each 60-mm-diameter dish for 30 min at 37°C. Cells were harvested and extracts were prepared 72 h postinfection.

[b] Mean values and standard deviations were obtained from two independent experiments with two dishes for each sample.

Direct demonstration that induction of luciferase activity in cells coinfected with AdMA19 and AdCre1 was due to Cre-specific excision of the 1.3-kbp *loxP*-flanked spacer in AdMA19 was obtained by Southern blot analysis. Viral DNA was extracted 24 h after double infection of 293 cells with AdMA19 and AdCre1 (both at an MOI of 5) and digested with *Hin*dIII. Southern hybridization was carried out by using as a probe the 2.9-kbp *Sca*I-*Xho*I fragment of pMA19 containing Luc cDNA and the spacer segment derived from pBS64 (Fig. 3A). Unrecombined AdMA19 is represented by the 1.9- and 1.8-kbp bands (from the left end of the virus) obtained after double infection of cells with AdMA19 and Ad*dl*70-3 or after mock infection (Fig. 5). Upon coinfection with AdCre1, the virus-encoded enzyme should mediate recombination, resulting in excision of a 1.3-kbp circle from the viral genome (indicated in Fig. 5A as a 1.3-kbp miniplasmid), leaving behind a 2.4 kbp *Hin*dIII fragment. All of the predicted fragments were detected by Southern hybridization (Fig. 5B). From visual comparison of the intensity of the 2.4 kbp band with that of the 1.8- or 1.9-kbp fragment, we estimate that approximately 50% of the AdMA19 viral DNA had undergone recombination under the experimental conditions used (Fig. 5B). Underrepresentation of the 1.3-kbp fragment is due to the fact that the excised miniplasmid does not replicate, in contrast to the viral vector. Thus, the relative intensity of this band does not reflect the efficiency of the recombination process. The faint bands of 3.1 and 3.4 kbp represent viral DNA fragments and are detected because of contamination of the probe with viral DNA sequences from pMA19, from which the probe was derived. Since no unexpected fragments were generated in this in vivo assay, we assume that the Cre protein is produced correctly in AdCre1-infected cells and that the presence of the 11-kbp band, seen in the in vitro assay (Fig. 4B), was likely due to functional alteration of the Cre enzyme for technical reasons.

DISCUSSION

As mentioned in the introduction, delivery of genes by means of Ad-based vectors is highly efficient and applicable to a wide variety of cell types, both in culture and in vivo. Combining the Cre-*loxP* site-specific recombination system with Ad vectors could therefore provide a powerful new tool for inducing DNA rearrangements and regulating gene expression both in cultured cells and in transgenic animals that contain *loxP* sites engineered in their genomes. As a first step in exploring this approach, we have constructed an E1-deleted vector, AdCre1, containing the coding sequences for Cre under the control of the HCMV immediate-early gene promoter, and a second vector, AdMA19, containing a reporter gene whose expression was regulated by Cre-mediated recombination. We were able to demonstrate the production of the Cre protein in two different AdCre1-infected cell lines: replication-permissive 293 cells and nonpermissive MRC5 cells. Functionality of the Cre protein produced in Ad vector-infected cells was demon-

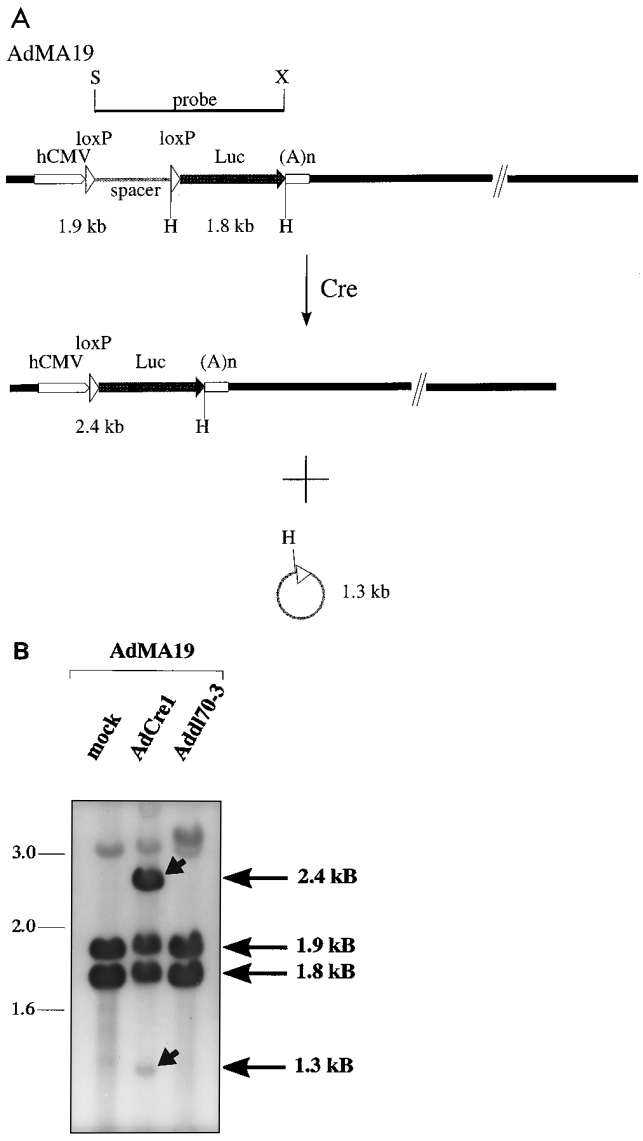

FIG. 5. Detection of Cre-specific recombination of AdMA19 in vivo. (A) The expected recombination products derived from AdMA19 upon coinfection with AdCre1 are shown. Solid bars represent viral sequences, and *loxP* sites are shown as open arrowheads. The spacer sequence blocking luciferase expression is shown as the stippled bar in AdMA19 and as the stippled circle generated by recombination. *Hin*dIII restriction sites (H), approximate sizes of the *Hin*dIII fragments of the left end of AdMA19, the recombined virus, and the excised circle are indicated. (B) Viral DNA extracted from 293 cells doubly infected as indicated at the top was digested with *Hin*dIII. The Southern blot of separated DNA was probed with the 2.9-kb *Sca*I-*Xho*I fragment of pMA19 (see also Fig. 3A). Molecular sizes (in kilobases) are given on the left, and the sizes of fragments derived from unrecombined and recombined viral DNA are indicated on the right. Arrowheads represent the recombination products of AdMA19, as predicted from recombination events shown in panel A. Only relevant restriction sites are shown. For more details, see the legend to Fig. 3.

strated in several assays. Cellular extracts of AdCre1-infected cells specifically mediated recombination of a plasmid containing two *loxP* sites, resulting in precise excision of sequences flanked by them. Double infections with AdCre1 and AdMA19 revealed the recombinatory function of virus-produced Cre protein on a second Ad vector, as excision of the *loxP*-flanked stop sequence was shown by Southern hybridization and, more important, by specific induction of luciferase expression from the recombined AdMA19. The luciferase assays also indicated that the expression of the reporter gene in AdMA19 could be efficiently blocked by the presence of the *loxP*-flanked spacer. We have therefore demonstrated that a system consisting of two Ad vectors, one expressing the Cre protein the other containing a suitably silenced reporter gene, can be used to switch on gene expression in a very specific and efficient manner.

Under the experimental conditions chosen, we estimated that approximately 50% of AdMA19 viral DNA had undergone Cre-dependent recombination. It may be possible to improve on this efficiency by altering the relative MOIs or by staggering the infection times with the two viruses. As the virus-encoded Cre protein contains no nuclear localization signal, addition of this sequence may enhance the efficiency of recombination by increasing the concentration of the protein in the nucleus. It is also possible that immediately after excision of the 1.3-kb DNA spacer from AdMA19, the molecule is efficiently reinserted by Cre (reverse of the reaction diagrammed in Fig. 5A), in which case there may be an upper limit to the fraction of AdMA19 genomes that can be resolved. We detected very low levels of luciferase activity in cells infected with AdMA19 alone and demonstrated that this probably resulted from spontaneous, homologous recombination between the two *loxP* sites in the vector. Consistent with this result, very low levels of Cre-independent recombination in *loxP*-containing molecules have been reported (41). It also has been shown (36) that homologous sequences as short as 14 and 56 bp can be substrates for spontaneous intramolecular recombination of simian virus 40 DNA in mammalian cells, albeit infrequently. If the spontaneous homologous recombination between *loxP* sites should present a problem, its frequency might be lowered by altering the sequence of one of the target sites to reduce the degree of homology, as has been described previously (22, 24).

The system that we have developed might be exploited for the regulated expression of toxic gene products in cells or animals. Cellular or viral genes that currently cannot be expressed at high levels because of the detrimental activities of their protein products could be efficiently delivered into many cells in a silent form and then turned on by infection with AdCre vectors. This system should allow production of toxic proteins at levels sufficient for their characterization and purification. The combination of the Cre-*loxP* and Ad vector systems may also be a particularly useful tool in experiments with transgenic animals in which timed and/or tissue-specific expression of genes is critical. Such studies could include the investigation of developmental processes involving proteins that are suspected of having different functions in embryogenesis and in the adult and that are lethal if expressed in the embryo. Providing Cre protein by a recombinant Ad vector might also prove an easier and more efficient method for the characterization of transgenic mice with Cre-regulated genes than crosses with Cre-containing strains. Lastly, infection with AdCre could be used to knock out genes or gene fragments by site-specific excision of sequences previously flanked by *loxP* sites. This method would allow characterization of proteins with multiple functions and, more important, may overcome the lethality associated with complete knockout mutations since the knockout could be appropriately timed and could be induced in specific organs.

In contrast to retroviruses and adeno-associated virus, which have been used or are being considered for gene therapy, adenoviruses integrate only rarely into the genomes of eukaryotic cells. There is evidence that Ad DNA can persist as extrachromosomal molecules (45), but nonreplicating Ad genomes should eventually be lost from dividing cells. We are interested in exploring whether site-specific integration sys-

tems can be used to increase integration of viral DNA into the genomes of transduced cells. Toward this end, we have successfully used the AdCre1 vector for the site-specific integration of a *loxP*-containing plasmid into the genomes of cultured human cells containing an engineered *loxP* site (47). AdCre may be similarly exploited to target integration of whole Ad genomes or of genes delivered by Ad vectors into chromosomal *loxP* sites of target cells.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health, the Natural Sciences and Engineering Research Council, the Medical Research Council, and the National Cancer Institute of Canada. F.L.G. is a Terry Fox Research Scientist of the National Cancer Research Institute.

We thank C. Addison for providing pCA18 and AdCA18-3, B. Sauer for providing pBS64, pBS185, and the Cre-specific antibody, J. Rudy for excellent technical assistance, M. Rudnicki for helpful comments, and S. Bacchetti for critically reading and revising the manuscript.

REFERENCES

1. **Abremski, K., and R. Hoess.** 1984. Bacteriophage P1 site-specific recombination; purification and properties of the Cre recombinase protein. J. Biol. Chem. **259:**1509–1514.
2. **Abremski, K., R. Hoess, and N. Sternberg.** 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. Cell **32:**1301–1311.

2a. **Addison, C.** Personal communication.

3. **Araki, K., M. Araki, J.-I. Miyazaki, and P. Vassalli.** 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc. Natl. Acad. Sci. USA **92:**160–164.
4. **Berkner, K. L.** 1992. Expression of heterologous sequences in adenoviral vectors. Curr. Top. Microbiol. Immunol. **158:**39–66.
5. **Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham.** 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early region 1 and 3. Proc. Natl. Acad. Sci. USA **91:**8802–8806.
6. **Bett, A. J., L. Prevec, and F. L. Graham.** 1993. Packaging capacity and stability of human adenovirus type 5 vectors. J. Virol. **67:**5911–5921.
7. **Birnboim, H. C., and J. Doly.** 1978. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
8. **Crystal, R. G., N. G. McElvaney, M. A. Rosenfeld, C.-S. Chu, A. Mastrangeli, J. G. Hay, S. L. Brody, H. A. Jaffe, N. T. Eissa, and C. Danel.** 1994. Administration of an adenovirus containing the human *CFTR* cDNA to the respiratory tract of individuals with cystic fibrosis. Nat. Genet. **8:**42–51.
9. **De Wet, J., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
10. **DiSanto, J. P., W. Mueller, D. Guy-Grand, A. Fischer, and K. Rajewsky.** 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. Proc. Natl. Acad. Sci. USA **92:**377–381.
11. **Fickenscher, H., T. Stamminger, R. Rueger, and B. Fleckenstein.** 1989. The role of a repetitive palindromic sequence element in the human cytomegalo major immediate early enhancer. J. Gen. Virol. **70:**107–123.
12. **Fukushige, S., and B. Sauer.** 1992. Genomic targeting with a positive-selection *lox* integration vector allows highly reproducible gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA **89:**7905–7909.
13. **Gage, P. J., B. Sauer, M. Levine, and J. C. Glorioso.** 1992. A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. J. Virol. **66:**5509–5515.
14. **Graham, F. L., and L. Prevec.** 1991. Manipulation of adenovirus vectors. Methods Mol. Biol. **7:**109–128.
15. **Graham, F. L., and L. Prevec.** 1992. Adenovirus-based expression vectors and recombinant vaccines, p. 363–390. *In* R. W. Ellis (ed.), Vaccines; new approaches to immunological problems. Butterworth-Heinemann, Boston.
16. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
17. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
18. **Gu, H., J. D. Marth, P. C. Orban, H. Mossmann, and K. Rajewsky.** 1994. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science **265:**103–106.
19. **Gu, H., Y.-R. Zou, and K. Rajewsky.** 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-*loxP*-mediated gene targeting. Cell **73:**1155–1164.
20. **Hitt, M., A. Bett, C. Addison, L. Prevec, and F. L. Graham.** Methods Mol. Genet., in press.
21. **Hoess, R., A. Wierzbicki, and K. Abremski.** 1987. Isolation and characterization of intermediates in site-specific recombination. Proc. Natl. Acad. Sci. USA **84:**6840–6844.
22. **Hoess, R., M. Ziese, and N. Sternberg.** 1982. P1 site-specific recombination: nucleotide sequence of the recombining sites. Proc. Natl. Acad. Sci. USA **79:**3398–3402.
23. **Hoess, R. H., and K. Abremski.** 1984. Interaction of the bacteriophage P1 recombinase Cre with the recombining site *loxP*. Proc. Natl. Acad. Sci. USA **81:**1026–1029.
24. **Hoess, R. H., and K. Abremski.** 1985. Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system. J. Mol. Biol. **181:**351–362.
25. **Kilby, N. J., M. R. Snaith, and J. A. H. Murray.** 1993. Site-specific recombinases: tools for genome engineering. Trends Genet. **9:**413–421.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lakso, M., B. Sauer, B. Mosinger, Jr., E. J. Lee, R. W. Manning, S.-H. Yu, K. L. Mulder, and H. Westphal.** 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. USA **89:**6232–6236.
28. **Le Gal La Salle, G., J. J. Robert, S. Berrard, V. Ridoux, L. D. Stratford-Perricaudet, M. Perricaudet, and J. Mallet.** 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. Science **259:**988–990.
29. **McGrory, J., D. Bautista, and F. L. Graham.** 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology **163:**614–617.
30. **Mittal, S. K., M. R. McDermott, D. C. Johnson, L. Prevec, and F. L. Graham.** 1993. Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. Virus Res. **28:**67–90.
31. **Orban, P. C., D. Chui, and J. D. Marth.** 1992. Tissue- and site-specific DNA recombination in transgenic mice. Proc. Natl. Acad. Sci. USA **89:**6861–6865.
32. **Peakman, T. C., R. A. Harris, and D. R. Gewert.** 1992. Highly efficient generation of recombinant baculoviruses by enzymatically mediated site-specific *in vitro* recombination. Nucleic Acids Res. **20:**495–500.
33. **Pichel, J. G., M. Lakso, and H. Westphal.** 1993. Timing of SV40 oncogene activation by site-specific recombination determines subsequent tumor progression during murine lens development. Oncogene **8:**3333–3342.
34. **Prevec, L., J. B. Campbell, B. S. Christie, L. Belbeck, and F. L. Graham.** 1990. A recombinant human adenovirus vaccine against rabies. J. Infect. Dis. **161:**27–30.
35. **Ragot, T., N. Vincent, P. Chafey, E. Vigne, H. Gilgenkrantz, D. Couton, J. Cartaud, P. Briand, J.-C. Kaplan, M. Perricaudet, and A. Kahn.** 1993. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. Nature (London) **361:**647–650.
36. **Rubnitz, J., and S. Subramani.** 1984. The minimum amount of homology required for homologous recombination in mammalian cells. Mol. Cell. Biol. **4:**2253–2258.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Sauer, B.** 1987. Functional expression of the *cre-lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2087–2096.
39. **Sauer, B.** 1993. Manipulation of transgenes by site-specific recombination: use of the Cre recombinase. Methods Enzymol. **225:**890–900.
40. **Sauer, B., and N. Henderson.** 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc. Natl. Acad. Sci. USA **85:**5166–5170.
41. **Sauer, B., and N. Henderson.** 1989. Cre-stimulated recombination at *loxP*-containing DNA sequences placed into the mammalian genome. Nucleic Acids Res. **17:**147–161.
42. **Sauer, B., and N. Henderson.** 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. New Biol. **2:**441–449.
43. **Sauer, B., M. Whealy, A. Robbins, and L. Enquist.** 1987. Site-specific insertion of DNA into a pseudorabies virus vector. Proc. Natl. Acad. Sci. USA **84:**9108–9112.
44. **Sternberg, N., B. Sauer, R. Hoess, and K. Abremski.** 1986. Bacteriophage P1 *cre* gene and its regulatory region; evidence for multiple promoters and for regulation by DNA methylation. J. Mol. Biol. **187:**197–212.
45. **Stratford-Perricaudet, L. D., I. Makeh, M. Perricaudet, and P. Briand.** 1992. Widespread long-term gene transfer to mouse skeletal muscle and heart. J. Clin. Invest. **90:**626–630.
46. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
47. **Wang, P., M. Anton, F. L. Graham, and S. Bacchetti.** Unpublished data.
48. **Zabner, J., L. A. Couture, R. J. Gregory, S. M. Graham, A. E. Smith, and M. J. Welsh.** 1993. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. Cell **75:**207–216.