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Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase

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ABSTRACT

A recombinant adenovirus (Ad) expressing Cre recombinase derived from bacteriophage P1 was constructed. To assay the Cre activity in mammalian cells, another recombinant Ad bearing an on/off-switching reporter unit, where a *LacZ*-expression unit can be activated by the Cre-mediated excisional deletion of an interposed stuffer DNA, was also constructed. Co-infection experiments together with the Cre-expressing and the reporter recombinant Ads showed that the Cre-mediated switching of gene expression was detected in nearly 100% of cultured CV1, HeLa and Jurkat cells. These results suggest that the recombinant Ad efficiently expressed functional Cre and offers a basis for establishing a powerful on/off switching strategy of gene expression in cultured mammalian cells and presumably in transgenic animals. The method is also applicable to construction of recombinant Ad bearing a gene the expression of which is deleterious to propagation of recombinant Ad.

INTRODUCTION

A method for activation or inactivation of a transgene introduced into the mammalian genome has recently been developed by using the site-specific recombinases Cre (1) and FLP (2). Cre recombinase derived from bacteriophage P1 is a 38 kDa protein mediating excisional deletion of a DNA sequence flanked by a pair of *loxP* sites, the Cre-specific recognition sequence of 34 nucleotides (nt). The gene-activation strategy involves the excisional deletion of the 'stuffer DNA' that lies between the promoter and the coding region and prevents expression. It has been used in cultured mammalian cells (3,4) and in transgenic mice (5). The strategy of gene inactivation involves disruption of the promoter/coding-region structure required for expression through the excisional deletion and was successfully used for conditional inactivation of a gene in a particular tissue of transgenic mice through tissue-specific expression of Cre recombinase (6). Moreover, a reversed process of excision offered a system for site-specific integration into the mammalian genome (4,7).

However, because the current method of expressing the recombinase in mammalian cells employed either DNA transfection or electroporation, sufficient Cre expression was achieved in only a small fraction of the cultured cell population. Consequently, a step of cell cloning or a drug-selection strategy was normally required to obtain the desired cell population. If a sufficient level of the functional recombinase is obtained simultaneously in 100% of the cultured cells, the recombinase-mediated gene manipulation in mammalian cells can have much wider application. For example, the gene activation strategy can offer a much more powerful and reliable system than the current system using inducible promoters in mammalian cells, because a very powerful promoter could be used for on-switching expression with a very low level of background expression before switching-on.

As the first step towards the above goal, we attempted to obtain efficient Cre expression by using an adenovirus (Ad) expression vector. Use of an Ad vector was shown to be an efficient system to deliver and express foreign genes in virtually 100% of cultured mammalian cells derived from various tissues (8; Y.K. and I.S. *et al.* in preparation). However, to determine the frequency of Cre-mediated gene activation in a given cell line, one normally needs to establish cell clones harboring the on-switching 'reporter' transgene on the chromosome. To monitor such frequency in various cell lines conveniently, we constructed another recombinant Ad bearing the 'reporter' transgene on the Ad genome. Using this system, we showed here that the Cre-expressing recombinant Ad can efficiently process and activate the reporter gene at nearly 100% frequency in three different cell lines, suggesting that the Cre-expressing Ad is a valuable tool for an efficient gene activation–inactivation system in mammalian cells.

MATERIALS AND METHODS

Construction of CALNLZ switching unit

The *loxP* sequence used in this work was a synthesized DNA of 52 nt (5′-CGAacgcgt[*Mlu*I]ATAACTTCGTATAGCATACAT-

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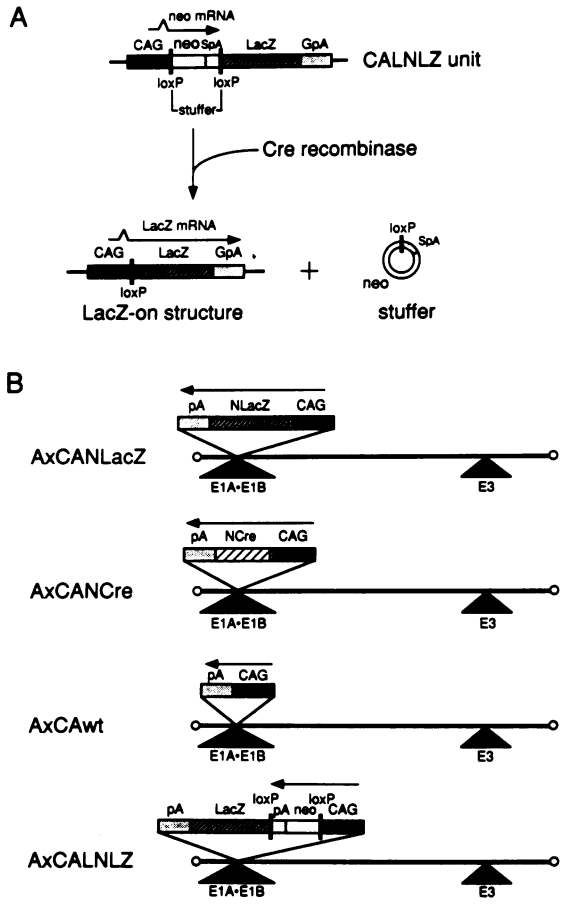


Figure 1. Structures of Cre-mediated activation units of *LacZ* gene and recombinant Ads. (**A**) Activation of a *LacZ* gene in the CALNLZ unit by Cre recombinase. The Cre-mediated excisional deletion removes both a *neo* coding region and a poly(A) sequence, consequently generating a functional *LacZ* expression unit. SpA, SV40 early poly(A) site; GpA, rabbit β-globin poly(A) site. (**B**) Structures of the recombinant Ads. Arrows show the orientation of the transcription. A triangle under the Ad genome represents a deletion of an Ad sequence.

TATACGAAGTTATctcgag[*Xho*I]TCG-3′), where the 34 nt functional *loxP* site (underlined) was flanked by *Mlu*I and *Xho*I sites (small letters) in that order. A head-to-tail dimer of this sequence was cloned at the *Ecl*136II site of pUC119 (named pULL) and used as a donor of the *loxP* sequences for further plasmid construction. The CAG promoter (9), a composite promoter consisting of cytomegalovirus IE enhancer, chicken β-actin promoter and rabbit β-globin polyadenylation [poly(A)] signal, was used for expression of Cre and the reporter construct. pCALNLZ is a plasmid containing an expression-switching reporter unit CALNLZ (Fig. 1A) consisting of the CAG promoter, a *loxP* sequence, *neo*-resistance gene (1.0 kb of the *Bgl*II–*Sma*I fragment derived from pSV2neo), a second *loxP* site, a *LacZ*-coding sequence derived from pSRLacZ (S. Miyake, *et al.*, submitted) and the poly(A) signal from pCAGGS in that order.

Construction of *LacZ*- and Cre-expression units

The initiator-NLS sequence (5′-ctgcag[*Pst*I]CAGACCGTGCATC ATG AGC GGC CCT CCA AAA AAG AAG AGA AAG GTA GAA GAC CCG Ggc ggc cgc[*Not*I]-3′) contains an initiation codon fitting Kozak's rule (10) and a coding sequence of nuclear localization signal (NLS) from SV40 T antigen (underlined) (11). Both strands of this sequence were synthesized and cloned between the *Pst*I and *Not*I sites of the *LacZ*-expressing plasmid pSRLacZ (S. Miyake *et al.*, submitted) as a substitution for the initiation codon and the N-terminal region of the *LacZ* gene. The resulting plasmid, pSRNLacZ, was used as the source of the NLS-tagged *LacZ* gene (denoted as NLacZ).

The coding region of Cre recombinase, from the initiator codon to 7 nt downstream of the terminator codon flanked by *Not*I and *Xba*I, was amplified by the polymerase chain reaction using Vent$_R$ DNA polymerase (New England BioLabs) from bacteriophage P1 DNA (a generous gift from Dr I. Kobayashi). The amplified Cre-coding DNA was then cloned into pSRNLacZ as a substitution for *LacZ*-coding DNA to obtain pSRNCre, a source of the Cre gene with an NLS (denoted as NCre). The sequences of the cloned Cre-coding DNA were confirmed to be identical to the published Cre sequences (12).

Construction of recombinant Ads

The CALNLZ switching unit described above was excised from pCALNLZ with *Sal*I and *Hin*dIII, filled in with the Klenow enzyme and cloned into the *Swa*I site at the Ad E1-deleted position of a cosmid pAxcw. The pAxcw is a cassette cosmid containing a nearly full-length Ad5 genome with E1 (nt 454–3328) and E3 (nt 28 592–30 470) deletions and is identical to pAdex1w (S. Miyake *et al.*, submitted) except that the cloning *Swa*I site was converted to a polylinker encoding *Cla*I-*Swa*I-*Cla*I-*Sal*I-*Nru*I sites. The resulting cosmid containing the CALNLZ unit with leftward transcription orientation was chosen and named pAxCALNLZ. By using the cosmid DNA, recombinant Ad containing the CALNLZ unit (AxCALNLZ) was generated by the COS-TPC method (S. Miyake *et al.*, submitted). Briefly, the cosmid DNA was mixed with the *Eco*T22I-digested DNA-terminal protein complex of Ad5-dlX (13) and used to transfect 293 cells (14), which support propagation of the recombinant Ad. A recombinant virus AxCALNLZ was generated through homologous recombination in 293 cells.

An expression cassette cosmid, pAxCAwt, was prepared to construct recombinant Ads expressing either the NLacZ or the NCre gene. pAxCAwt is a derivative of pAxcw containing the CAG promoter, the unique *Swa*I site and a rabbit β-globin poly(A) sequence in the leftward orientation at the Ad E1-deleted position. The detailed structure and functions of pAxCAwt will be described elsewhere. The NLacZ gene was excised with *Pst*I and *Sal*I from pSRNLacZ, filled in with the Klenow enzyme and inserted into the *Swa*I site of pAxCAwt to obtain pAxCANLacZ. Similarly, the NCre gene was cut out with *Pst*I and *Xba*I, filled in and inserted into the *Swa*I site of pAxCAwt to prepare pAxCANCre. Recombinant Ads of AxCANLacZ, AxCANCre and AxCAwt (without expressing genes) were generated as described above by using pAxCANLacZ, pAxCANCre and pAxCAwt respectively (Fig. 1B). Virus stocks were prepared by a standard procedure (15). A concentrated and purified virus stock was also prepared as described previously (16) for experiments with high multiplicity of infection (MOI).

Detection of *LacZ* gene expression

To quantify β-galactosidase activity (17), 3×10^5 infected cells were disrupted by sonication and the lysate was subjected to the color reaction with *o*-nitrophenyl-β-D-garactopyranoside (ONPG). For cell staining, three days after infection the cells were washed with phosphate-buffered saline twice, fixed with 0.25% glutaraldehyde and stained with 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (18).

Southern blotting analysis

Total DNAs of infected cells were prepared according to Saito *et al.* (13). The digested DNA was transferred to a nylon membrane Hybond-N (Amersham) by the capillary-transfer method (17). The probe DNA was labeled with digoxigenin-UTP and specific DNA was detected by autoradiography with chemiluminescence of CSPD (Boerhinger).

RESULTS

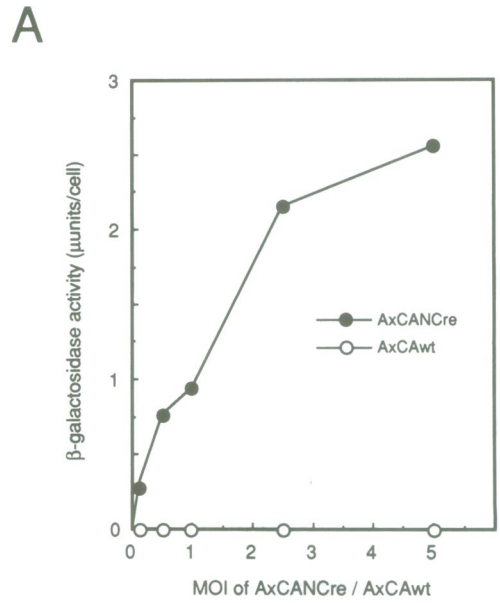
Construction of recombinant Ad expressing NLS-tagged *LacZ* and Cre gene

To express Cre function in the nuclei of mammalian cells efficiently (19), a modified Cre gene, called NCre, was constructed. NCre contained the synthetic NLS sequence of SV40 T-antigen gene connected at the N-terminus of the Cre coding region. To determine if the synthetic NLS is functional, the identical NLS sequence was connected with the *E.coli*-derived *LacZ* gene in the same manner as NCre (called NLacZ) and the NLacZ gene was transferred in the recombinant Ad under the control of the CAG promoter (9) (AxCANLacZ; Fig. 1B). The NLS-tagged β-galactosidase was located almost exclusively in the nucleus, showing that the synthetic NLS sequence was functional (data not shown). Then, we constructed plasmid pSRNCre expressing a Cre protein tagged with the NLS and confirmed that NCre was functional in mammalian cells by co-transfection with pSRNCre and pCALNLZ (see below). Based on these results, recombinant Ad expressing the NCre gene, AxCANCre, was constructed and used for further experiments on Cre expression in mammalian cells. A control virus without any coding sequence was also prepared (AxCAwt, Fig. 1B).

Structure of the on/off switching unit CALNLZ

The on/off switching unit CALNLZ consisted of the CAG promoter, a stuffer, authentic *LacZ* gene and a poly(A) signal (Fig. 1A). The stuffer was made up of the *neo* gene and another poly(A) sequence flanked by a pair of *loxP* sites and was located between the CAG promoter and the *LacZ* gene so that the *LacZ* expression was hampered by the termination of the mRNA at the poly(A) site within the stuffer sequence. Consequently, the CALNLZ unit should initially express the *neo* gene but does not express the *LacZ* gene. However, after a sufficient amount of functional Cre is supplied to the switching unit, the stuffer DNA is excised as circular DNA and then the CAG promoter and the *LacZ* gene are joined together via a single *loxP* site. The resulting structure on the recombinant Ad now expresses the *LacZ* gene under control of the CAG promoter (*LacZ*-on structure).

To test whether the strategy shown in Figure 1A is working as expected, CV1 cells were co-transfected with pCALNLZ and an NCre-expressing plasmid, pSRNCre. Three days later, ~20% of the CV1 cells co-transfected with pSRNCre were stained with



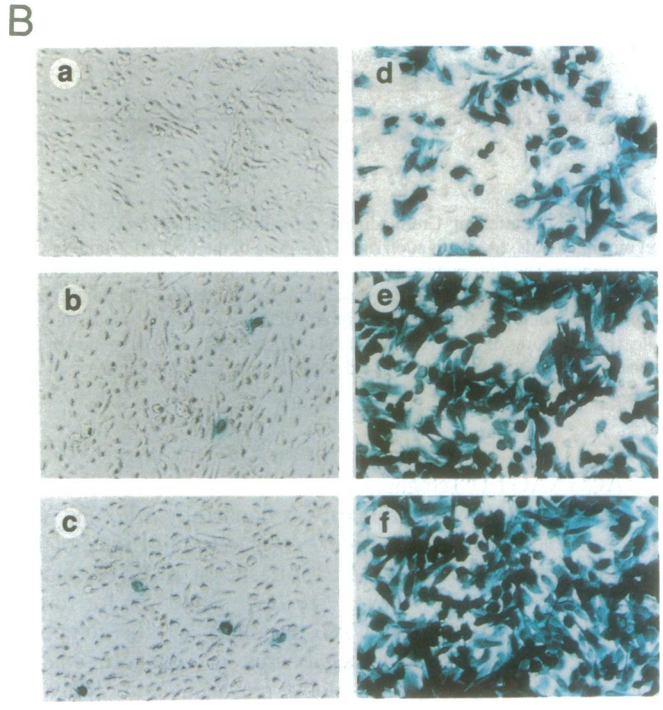


Figure 2. Cre-mediated *LacZ* gene activation on the reporter Ad genome in CV1 cells. (**A**) Quantification of the expressed β-galactosidase activity. CV1 cells were doubly infected with AxCALNLZ at a constant MOI 2.5 and AxCANCre at the MOIs indicated. As a control, AxCAwt was used instead of AxCANCre. The β-galactosidase activity was measured by using ONPG. (**B**) The X-gal staining of the CV1 cells doubly infected with the reporter Ad and the NCre-expressing Ad. Cells were stained 3 days after infection. The MOI of AxCALNLZ was a constant 2.5. (a) Mock-infected. (b) AxCALNLZ only. (c) AxCALNLZ + AxCAwt (MOI 2.5). (d) AxCALNLZ + AxCANcre (MOI 0.1). (e) AxCALNLZ + AxCANcre (MOI 0.5). (f) AxCALNLZ + AxCANcre (MOI 2.5). Photos containing stained cells are shown in (b) and (c).

X-gal and very few cells, if any, transfected with pCALNLZ only were stained (data not shown). The result suggested that the CALNLZ unit indeed expressed *LacZ* through Cre expression and that the synthetic *loxP* sequence of 52 nt did not hamper the *LacZ* expression when it was located between the promoter and the coding region. Then we constructed the reporter recombinant Ad (AxCALNLZ, Fig. 1B, bottom) carrying the CALNLZ unit on the Ad genome in order to examine the functional Cre expression of AxCANCre in various cultured cells conveniently.

Efficient NCre-mediated on-switching in CV1 cells

To determine the efficiency of on-switching mediated by NCre-expressing recombinant Ad, CV1 cells were doubly infected with the reporter AxCALNLZ and the NCre-expressing AxCANCre, the reporter Ad at a constant MOI 2.5 and the NCre-expressing Ad at various MOIs from 0.1 to 5.0. Three days later, the expressed β-galactosidase activity was quantified (Fig. 2A). An almost linear dependency between the MOI of NCre-expressing Ad and the expressed β-galactosidase activity was observed up to the MOI 2.5 of NCre-expressing Ad infection. This finding suggested that the NCre recombinase expressed by recombinant Ad switched on the *LacZ* expression of the CALNLZ unit in mammalian cells in a dose-dependent manner.

To determine the frequency of gene activation in the cultured cells, cells infected under each condition were prepared in parallel and stained with X-gal (Fig. 2B). The percentage of the stained cells again increased in a dose-dependent manner according to the MOI of the NCre-expressing recombinant Ad. At MOI 0.1, 20% of the cells were already stained. (The percentage is not too high because 1 PFU generally corresponds to 10 or more viral particles.) Nearly 100% of the cells were stained at MOI 2.5 (Fig. 2B, f) and MOI 5 (data not shown), showing that the gene activation by the NCre-expressing Ad was achieved in nearly 100% of the cultured mammalian cells. The staining frequencies are comparable to the AxCANLacZ infection (data not shown). In contrast, only ~20% of the cells were stained when NCre was expressed by plasmid transfection (data not shown). About 0.2% of the cells infected with the reporter AxCALNLZ alone were reproducibly stained by X-gal (Fig. 2B, b and c) and this was similarly observed when independently-prepared virus stocks were used (data not shown). The background *LacZ* expression was, however, much lower than the detectable level of quantification using ONPG (Fig. 3A) and the *LacZ*-on structure was not detected by Southern blotting analysis of the AxCALNLZ-infected cells (see below). The reason for the background expression is not clear at present, though a similar number of stained cells were also observed when CV1 cells were transfected with a reporter plasmid of similar structure, pNEOβGAL (5) (Stratagene) (data not shown).

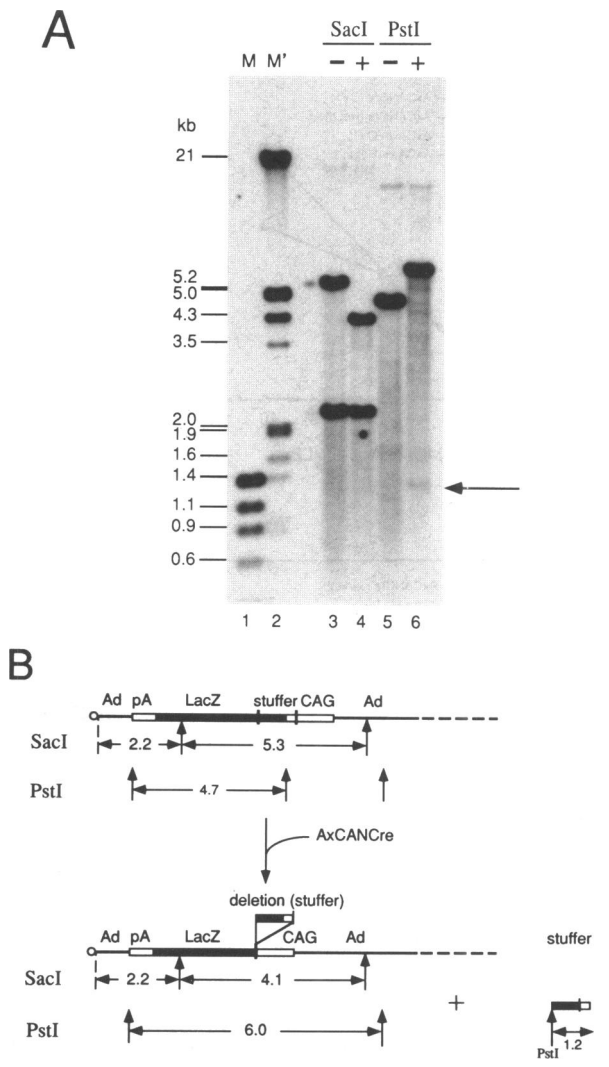


Figure 3. Southern blotting analysis of the processed reporter Ad genome. (**A**) Autoradiogram of the reporter Ad genome in infected CV1 cells. Cells were infected with AxCALNLZ alone at MOI 5 (lanes 3 and 5) or doubly with AxCALNLZ at MOI 5 and AxCANCre at MOI 10 (lanes 4 and 6). M (lane 1) and M′ (lane 2) represent size markers of double-strand ϕX174 DNA digested with *Hae*III and lambda phage DNA double-digested with *Eco*RI plus *Hin*dIII respectively. The 1.2 kb band indicated by the arrow represents the *Pst*I-linearized DNA of the excised stuffer. (**B**) Maps of the reporter Ad genomes. Lengths of the DNA fragment shown in (A) are shown in kb below the Ad genome map. The *neo-LacZ* probe (filled box) was the 4.2 kb *Pst*I–*Eco*RI fragment from pCALNLZ spanning most of the stuffer DNA and *LacZ* coding region. Vertical bars on the Ad genome map show *loxP* sites and vertical arrows show cleavage sites.

Detection of the *LacZ*-on structure by Southern blotting analysis

To obtain more direct evidence supporting that the *LacZ* expression occurs through the *LacZ*-on structure as illustrated in Figure 1A, total DNA was prepared from the CV1 cells doubly infected with both the reporter Ad at MOI 5 and NCre-expressing Ad at MOI 10 and the DNA structure of the *LacZ*-expressing unit was examined by Southern blotting analysis (Fig. 3A and B). The number of copies of the reporter Ad DNA introduced was estimated as ~15 per cell judged from the intensity of the detected band. In the *Sac*I digestion, the 5.3 kb band derived from the initial CALNLZ unit (lane 3) was shifted to the 4.1 kb band (lane 4) when the NCre-expressing Ad was used for co-infection, suggesting that virtually all (>95%) of the CALNLZ copies were processed in the expected manner through the Cre-mediated deletion. The sizes of bands detected in the *Pst*I digestion confirmed the expected *LacZ*-on structure, while the corresponding bands were shifted upward (from 4.7 kb in lane 5 to 6.0 kb in lane 6) due to the deletion of a *Pst*I site within the stuffer. These data suggest that MOI 10 of the NCre-expressing Ad was

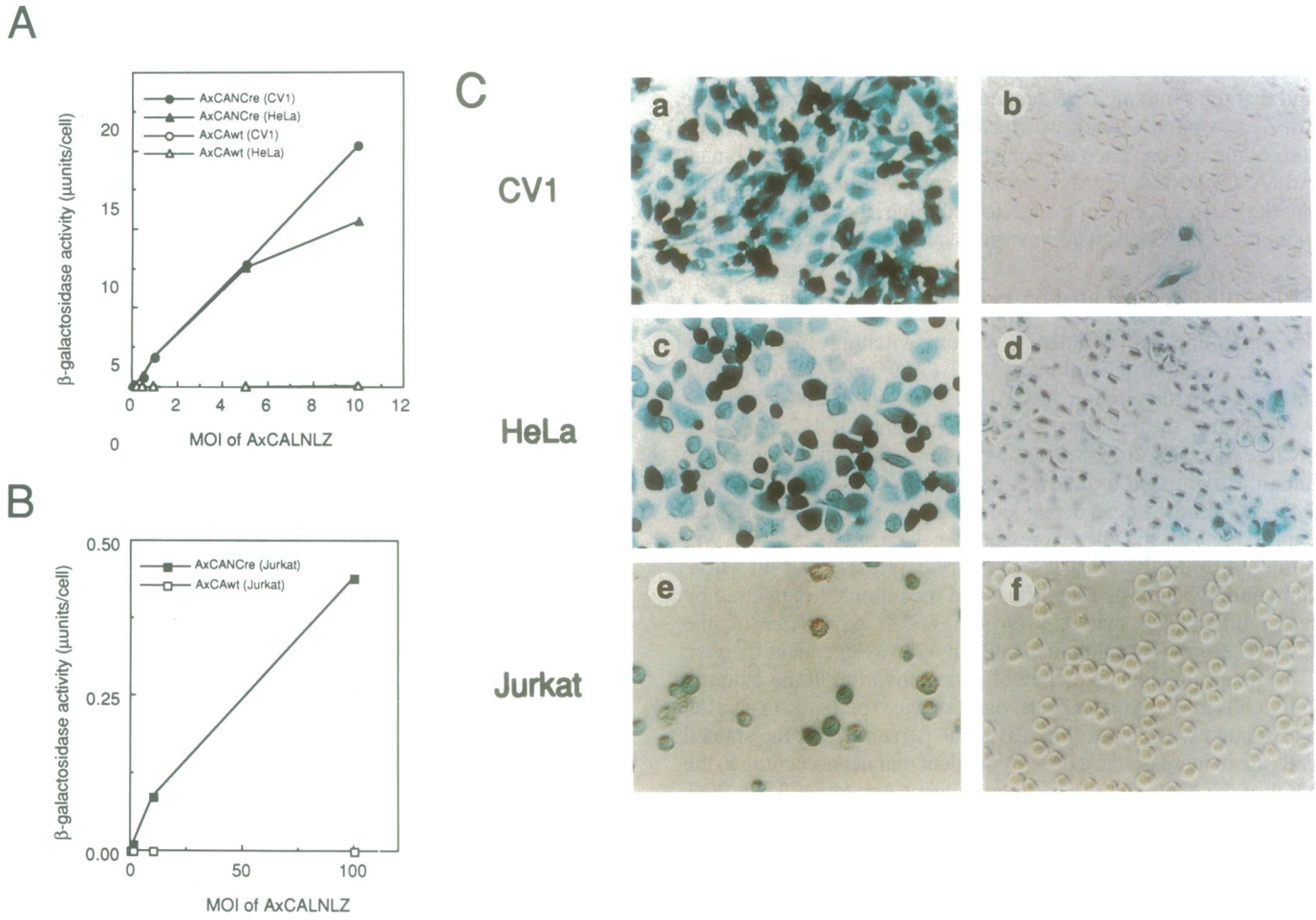


Figure 4. Cre-mediated LacZ gene activation in CV1, HeLa and Jurkat cells. (**A**) Quantification of the expressed β-galactosidase activity in CV1 and HeLa cells. Cells were doubly infected with AxCANCre at a constant MOI 10 and AxCALNLZ at the MOIs indicated at the bottom. As a control, AxCAwt was used at MOI 10 instead of AxCANCre. (**B**) Quantification of the expressed β-galactosidase activity in Jurkat cells. Cells were doubly infected with AxCANCre at a constant MOI 100 and AxCALNLZ at the MOIs indicated at the bottom. As a control, AxCAwt was used at MOI 100 instead of AxCANCre. (**C**) The X-gal staining of CV1 (a and b), HeLa (c and d) and Jurkat (e and f) cells doubly infected with the reporter Ad and the NCre-expressing Ad. (a and c), AxCALNLZ (MOI 5) + AxCANCre (MOI 10); (b and d), AxCALNLZ (MOI 5) + AxCAwt (MOI 10); (e), AxCALNLZ (MOI 100) + AxCANCre (MOI 100); (f), AxCALNLZ (MOI 100) + AxCAwt (MOI 100). The other conditions were the same as for Figure 3B.

sufficient to process virtually all of the reporter Ad DNA introduced at MOI 5 or ~15 copies/cell within 3 days. When the NCre-expressing Ad was used at MOI 5 instead of MOI 10, at most 10% of the reporter Ad DNA remained unprocessed (data not shown). The excised stuffer DNA was barely observed in the *Sac*I digestion but the linearized form with *Pst*I digestion was detected as a 1.2 kb band (lane 6). The copy number of the excised stuffer appeared much lower than the processed reporter Ad DNA. The reason is not clear but the difference might be due to a shorter half life of the excised small DNA than that of the Ad genome.

NCre-mediated on-switching in HeLa and Jurkat cells

To investigate whether the NCre-mediated efficient switching of gene expression can be applied to cells other than CV1 (monkey kidney-derived fibroblasts), we chose HeLa (human cervical cancer-derived cell line with an epithelial shape) and Jurkat cells (human T-cell line) for further analysis. Of these cell lines, Jurkat cells showed much lower *LacZ*-expression than CV1 cells when infected with AxCANLacZ (unpublished data), therefore higher doses of both the NCre-expressing Ad and the reporter Ad were used. To determine whether efficient gene activation can be achieved in these cells, the β-galactosidase activity was measured in extracts of cells doubly infected with a constant high dose (MOI 10 in HeLa and CV1 cells and MOI 100 in Jurkat cells) of NCre-expressing Ad and various doses (MOI 0.1–10.0 in HeLa and CV1 cells and MOI 1–100 in Jurkat cells) of the reporter Ad (Fig. 4A and B). The β-galactosidase activity was increased almost linearly up to MOI 5 in HeLa and CV1 cells (Fig. 4A), suggesting that the NCre activity expressed from AxCANCre of MOI 10 appeared enough to process most of the copies of the reporter recombinant Ad up to MOI 5 to the *LacZ*-on structure. The β-galactosidase activity was also increased in Jurkat cells up to MOI 100 (Fig. 4B). These results suggest that the on-switching of gene expression can be achieved efficiently not only in adherent cell lines but also in some lymphoid cell lines.

To determine the frequency of gene activation in these cells, HeLa cells together with CV1 cells as a control were doubly infected with both the reporter Ad at MOI 5 and the NCre-expressing Ad at MOI 10 and stained with X-gal 3 days later. Similarly, Jurkat cells were infected with the reporter Ad and the NCre-expressing Ad at MOI 100 each (Fig. 4C). Most, if not all of the infected cells of each cell line, even Jurkat cells, were stained blue, suggesting that at least one copy of the reporter DNA per cell was processed in almost all cells and accordingly that efficient switching of expression by means of the NCre-expressing Ad can be applied to these cell lines.

DISCUSSION

Here we showed that the functional site-specific recombinase Cre was efficiently expressed in mammalian cells by using a recombinant Ad. The expression is efficient enough to switch on the gene expression of a reporter construct introduced by another recombinant Ad in nearly 100% of CV1, HeLa and Jurkat cells. We have found, by using AxCALacZ, that most adherent mammalian cell lines, even those having differentiated cell functions, show much higher *LacZ*-expression than lymphoid cell lines, owing to the different efficiencies of gene transfer by the Ad vector (Y.K. *et al.*, in preparation). Therefore, the results obtained in this study suggest that efficient Cre-mediated gene activation can be achieved in most of the adherent mammalian cell lines and some lines of hematopoietic origin.

We determined the frequency of Cre-mediated gene activation by using a recombinant Ad bearing the 'reporter' gene on the Ad genome. This strategy offers an efficient and rapid assay to test and screen out the best cell line for efficiency of Cre-mediated gene manipulation among cell lines suitable for a particular research purpose, without establishing cell clones harboring the reporter gene on the chromosome. The strategy described here also offers a new approach to construction of recombinant Ad. A gene difficult to express in an Ad vector because of inhibition of Ad growth could be introduced onto the Ad genome as a switch-off state and be expressed in cells by co-infecting the cells with the NCre-expressing Ad.

We used NCre, a Cre gene tagged with NLS. Gu *et al.* (19) first reported that a modified Cre tagged with NLS at its amino terminal was more active in mammalian cells than the authentic Cre. Recently we constructed a recombinant Ad expressing the authentic Cre (AxCACre) and showed that AxCANCre was ~3-fold more effective than AxCACre (data not shown).

Can Cre recombinase expressed by the recombinant Ad efficiently activate a reporter gene having been introduced on the mammalian chromosome? Recently we showed that a cell line containing the CALNLZ unit having been introduced on the cell chromosome expressed the *LacZ* gene in 100% of the cells by infecting the cells with AxCANCre (Y.K. *et al.* in preparation). Such a gene activation system seems much better than the strategy using conventional inducible promoters because of the very high expression after switching-on using a potent CAG promoter and a very low level of background expression before switching-on.

The Cre-expressing recombinant Ad can be used for purposes other than the on/off switching of gene regulation in cultured cells. For knock-out mouse construction, a Cre-expressing plasmid was used to remove a selectable marker gene located on the chromosome of ES cell clones (19). The Cre-expressing Ad could remove such a marker gene flanked by a pair of *loxP* sites much more efficiently, because a recombinant Ad was able to express a foreign gene in ES cells efficiently (T. Tsukui and Y. Toyoda, unpublished data) and in mouse preimplantation embryos (21). Moreover, because the Ad vector was shown to express a foreign gene *in vivo* in animals (8) it also could be possible for a gene to be switched on or off only in a specific region of transgenic animals by inoculating the Cre-expressing Ad positionally (for example, in the liver administrated via the portal vein).

All the plasmids and recombinant Ads are available upon request.

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Note added in proof

In 1995 M. Anton and F. L. Graham (*J. Virol.*, **69**, 4600–4606) reported recombinant adenoviruses expressing the Cre recombinase and an inducible luciferase gene.

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