DRUG DISCOVERY
AND GENOMIC TECHNOLOGIES

Benchmark

In Vitro Ligation-Based Cloning of Foreign DNAs into the E3 and E1 Deletion Regions for Generation of Recombinant Adenovirus Vectors

BioTechniques 30:1112-1116 (May 2001)

Hiroyuki Mizuguchi, Mark A. Kay[1], and Takao Hayakawa
National Institute of Health Sciences, Tokyo, Japan and [1]Stanford University School of Medicine, Stanford, CA, USA

Recombinant adenovirus (Ad) vectors have been used extensively to deliver foreign genes to a variety of cell types in vitro and in vivo (17,19). One problem associated with the use of Ad vectors has been the difficulty of constructing a vector. We recently developed an efficient method for constructing an E1 (E1/E3 or E1/E3/E4)-deleted Ad vector by means of simple in vitro ligation (21,22). In this system, three unique restriction sites, I-*Ceu*I, *Swa*I, and PI-*Sce*I, are inserted into the E1 deletion site of the vector plasmid containing a complete E1 (E1/E3 or E1/E3/E4)-deleted Ad genome; foreign genes can then be easily introduced into the E1 deletion site of the Ad genome by simple in vitro ligation using the I-*Ceu*I and PI-*Sce*I sites. This system is simple and efficient, although it only allows foreign genes to be introduced into the E1 deletion region. Most first-generation Ad vectors in current use are of the E1-substitution type. However, in some cases, it is desirable for foreign genes to be introduced into the E3 deletion region as well as into the E1 deletion region. For example, when heterologous gene expression cassettes inserted into the E1 deletion region are co-expressed, promoter interference can occur [i.e., transcription from one promoter suppresses transcription from another (6,9,10)]. An Ad vector containing foreign genes that can be introduced into both the E1 and E3 deletion regions eliminates such problems because each gene can be efficiently expressed.

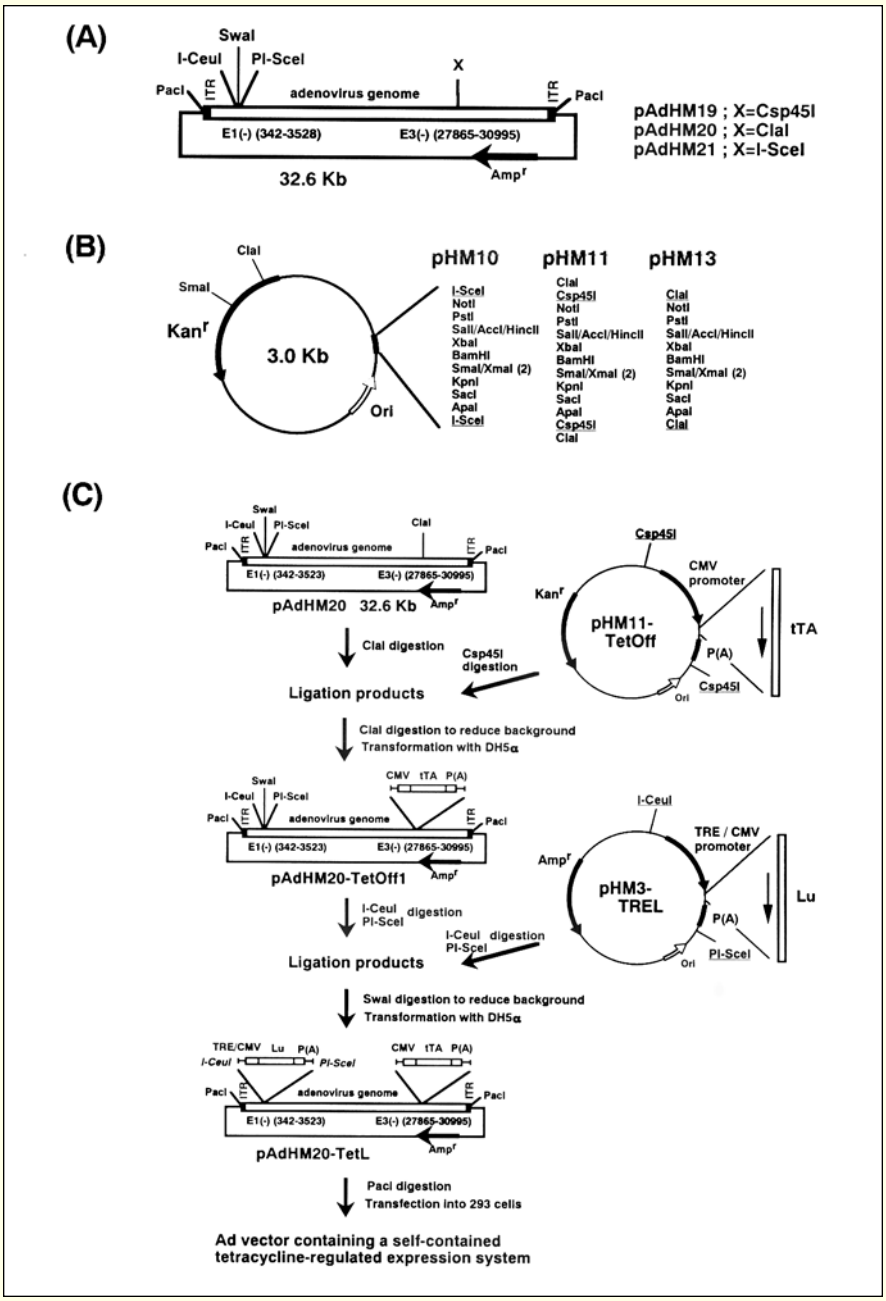
In this study, we developed a system for introducing genes of interest into both the E1 and E3 deletion regions based on simple plasmid construction. A unique restriction site, *Csp*45I, *Cla*I, or I-*Sce*I, was introduced into the E3 deletion region of the Ad vector plasmid containing the unique I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion region. This modification allows for in vitro ligation to construct an Ad vector containing genes of interest into the E3 deletion region as well.

Several systems for constructing Ad vectors have been developed (1–3,5,7, 8,15,18,20,24,25). In several methods, Ad vectors carrying foreign genes into both the E1 and E3 deletion regions can be constructed. In the method developed by Graham et al. (2), a unique *Pac*I site in the E3 deletion region of the plasmid containing the Ad genome can be used for cloning the gene of interest into the E3 deletion region, and then another gene of interest is inserted into the E1 deletion region by homologous recombination in 293 cells. However, inefficient homologous recombination in 293 cells is required to introduce a second foreign gene into the E1 deletion region. Recently, they developed a system that overcomes this inefficiency by using Cre-loxP (FLP-FRT)-based recombination (24,25). Another method for generating an Ad vector that can carry foreign genes into both the E1 and E3 deletion regions is based on homologous recombination in bacteria (3,5,15). However, this method requires specialized bacterial strains (e.g., BJ5183). Thus, the cloning process is complicated. Another method recently developed by Danthinne and Werth (7,8) used in vitro ligation with cosmid, but this was a complicated procedure because it required λ packaging. In contrast, our method is based on only routine, simple plasmid construction, and should be useful as an alternative method in constructing such Ad vectors.

Figure 1, panels A and B, shows new vector and shuttle plasmids. For convenience in cloning, we constructed several vector plasmids with the *Csp*45I, *Cla*I, or I-*Sce*I site in the E3 deletion region. Shuttle plasmids pHM10, pHM11, and pHM13 contain I-*Sce*I, *Cla*I/*Csp*45I, or *Cla*I sites flanked by a multicloning site. Detailed information regarding their construction is available from the authors upon request.

As a representative example, we constructed an Ad vector containing the tetracycline (tet)-controllable expression system (12). First, a tet-responsive transcriptional activator (tTA) gene driven by the cytomegalovirus (CMV) promoter was introduced into the E3 deletion region of the vector plasmid pAdHM20. Then, a luciferase gene driven by the promoter containing a tet-responsive element (TRE/CMV) was introduced into the E1 deletion region as described previously (22), resulting in pAdHM20-TetL (Figure 1, panel C). In this case, we used the *Csp*45I and *Cla*I sites for cloning into the E3 deletion region, which produces compatible cohesive ends. If the gene of interest does

not have both the *Csp*45I and *Cla*I sites (the tTA expression cassette does not), then recombinant plasmid can be produced easily. That is, when the foreign gene cloned into a shuttle plasmid containing *Csp*45I or *Cla*I is inserted into the *Cla*I or *Csp*45I site of the vector plasmid, recombinant plasmid is produced from recleavable ligation products. Therefore, the generation of parental plasmid that is derived from self-ligated products is prevented by the digestion of ligation products by *Cla*I or *Csp*45I. Moreover, after the digestion of the shuttle plasmid with *Cla*I or *Csp*45I, fragment isolation from agarose gel was not required because a shuttle plasmid has a kanamycin resistance gene, while a vector plasmid has an ampicillin resistance gene. When the gene of interest has both the *Csp*45I and *Cla*I sites, the I-*Sce*I site (i.e., pAdHM21 and pHM10) can be used for cloning. I-*Sce*I recognizes the 18-bp sequence, which makes it a rare cutter and ideal to use as a cloning site.



Recombinant virus AdHM20-TetL was produced by the transfection of *Pac*I-linearized plasmid pAdHM20-TetL into 293 cells and was prepared as described previously (21). Viral titer was determined as previously described (16). DNA restriction analysis showed that the recombinant plasmid and virus contained the expected fragments (data not shown).

Figure 2 shows the functionality of the recombinant Ad vector AdHM20-TetL, which contains the tet-controllable expression system. HeLa (human epitheloid carcinoma of the cervix) and SK HEP-1 cells (endothelial cell line derived from the human liver) were transduced with AdHM20-TetL (MOI = 20) and cultured with medium containing various concentrations of tetracycline. Controllable luciferase production in both cell lines was realized in this Ad vector system. In the medium with a tetracycline concentration of more than 100 ng/mL, luciferase production in the cells was suppressed. However, it must be noted that very low levels of background luciferase production (i.e., HeLa, 4.0%, SK HEP-1, 1.2%, compared with the maximum production) were detectable in both cell lines, even at a tetracycline concentration of 10 μg/mL. Regulatable luciferase production was also observed in other cell lines (i.e., HepG2, human hepatoma, ECV304, endothelial cell line derived from human umbilical vein) (data not shown).

A binary transgene expression sys-

Figure 1. Construction of self-contained Ad vector carrying the tet-regulatable gene expression system. pAdHM20 and pHM11-TetOff, in which the CMV promoter-driven tTA gene was cloned into pHM11, were digested with *Cla*I and *Csp*45I, respectively, and ligated without gel purification of the fragment. Ligation products were digested with *Cla*I to reduce the appearance of a parental plasmid then transformed with *E. coli* (DH5α), resulting in pAdHM20-TetOff1. Then, the luciferase gene driven by TRE/CMV, which had been cloned into the shuttle plasmid containing I-*Ceu*I and PI-*Sce*I sites, was inserted into the E1 deletion site of pAdHM20-TetOff1 as reported previously (22). The resulting plasmid (pAdHM20-TetL) was digested with *Pac*I and transfected into 293 cells, generating a recombinant Ad vector carrying a tet-regulatable gene expression system. (A) Vector plasmids pAdHM19, pAdHM20, and pAdHM21. (B) Shuttle plasmids pHM10, pHM11, and pHM13. (C) Diagram of the scheme for constructing a self-contained Ad vector carrying the tet-regulatable gene expression system.

tem in which heterologous genes can be inserted into both the E1 and E3 regions has certain advantages over a system in which heterologous genes can be inserted only into the E1 deletion region. Several reports on tet-regulated expression systems using Ad vectors have appeared (4,11,13,14,23). In most of these experiments, two kinds of E1-substituted Ad vectors were co-transduced, one expressing the tTA and the other, driven by a tet-responsive promoter, expressing the gene of interest (11,13,14,23). The single vector system described in this study has the advantage of eliminating extra labor to generate vectors and does not require co-transduction.

Our system, which can introduce foreign genes into both the E1 and E3 deletion region by means of simple in vitro ligation, combined with a variety of vectors and shuttle plasmids (21,22), would be a powerful tool for constructing recombinant Ad vectors for gene therapy and gene transfer experiments.

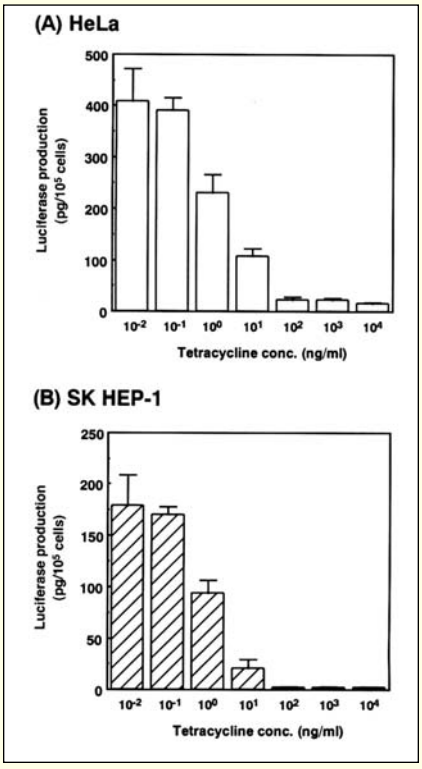


Figure 2. Tet-dependent luciferase expression by AdHM20-TetL. (A) HeLa and (B) SK HEP-1 cells were transduced with AdHM20-TetL and cultured with medium containing various concentrations of tetracycline. After being cultured for 48 h, luciferase production in the cells was determined. All data represent the mean ± SD of experiments performed in triplicate.

ACKNOWLEDGMENTS

We would like to thank Jun Murai for technical assistance. This work was supported by grants from the Ministry of Health and Welfare of Japan to T.H. M.A.K. was supported by NIH 49022.

REFERENCES

1. **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 regions 1 and 3. Proc. Natl. Acad. Sci. USA *91*:8802-8806.
2. **Bramson, J., M. Hitt, W.S. Gallichan, K.L. Rosenthal, J. Gauldie, and F.L. Graham.** 1996. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: in vitro and in vivo production of biologically active interleukin-12. Hum. Gene Ther. *10*:333-342.
3. **Chartier, C., E. Degryse, M. Gantzer, A. Dieterle, A. Pavirani, and M. Mehtali.** 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J. Virol. *70*:4805-4810.
4. **Corti, O., O. Sabate, P. Horellou, P. Colin, S. Dumas, D. Buchet, M.H. Buc-Caron, and J. Mallet.** 1999. A single adenovirus vector mediates doxycycline-controlled expression of tyrosine hydroxylase in brain grafts of human neural progenitors. Nat. Biotechnol. *17*:349-354.
5. **Crouzet, J., L. Naudin, C. Orsini, E. Vigne, L. Ferrero, A. Le Roux, P. Benoit, M. Latta et al.** 1997. Recombinational construction in *Escherichia coli* of infectious adenoviral genomes. Proc. Natl. Acad. Sci. USA *94*:1414-1419.
6. **Cullen, B.R., P.T. Lomedico, and G. Ju.** 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis. Nature *307*:241-245.
7. **Danthinne, X.** 1999. New vectors for the construction of double recombinant adenoviruses. J. Virol. Methods *81*:11-20.
8. **Danthinne, X. and E. Werth.** 2000. New tools for the generation of E1- and/or E3-substituted adenoviral vectors. Gene Ther. *7*:80-87.
9. **Emerman, M. and H.M. Temin.** 1984. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. Cell *39*:459-467.
10. **Emerman, M. and H.M. Temin.** 1986. Quantitative analysis of gene suppression in integrated retrovirus vectors. Mol. Cell. Biol. *6*:792-800.
11. **Ghersa, P., R.P. Gobert, P. Sattonnet-Roche, C.A. Richards, E. Merlo Pich, and R. Hooft van Huijsduijnen.** 1998. Highly controlled gene expression using combinations of a tissue-specific promoter, recombinant adenovirus and a tetracycline-regulatable transcription factor. Gene Ther. *5*:1213-1220.
12. **Gossen, M. and H. Bujard.** 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA *89*:5547-5551.
13. **Harding, T.C., B.J. Geddes, D. Murphy, D. Knight, and J.B. Uney.** 1998. Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system. Nat. Biotechnol. *16*:553-555.
14. **Harding, T.C., B.J. Geddes, J.D. Noel, D. Murphy, and J.B. Uney.** 1997. Tetracycline-regulated transgene expression in hippocampal neurones following transfection with adenoviral vectors. J. Neurochem. *69*:2620-2623.
15. **He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein.** 1998. A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. USA *95*:2509-2514.
16. **Kanegae. Y., M. Makimura, and I. Saito.** 1994. A simple and efficient method for purification of infectious recombinant adenovirus. Jpn. J. Med. Sci. Biol. *47*:157-166.
17. **Kay, M.A. and S.L. Woo.** 1994. Gene therapy for metabolic disorders. Trends Genet. *19*:253-257.
18. **Ketner, G., F. Spencer, S. Tugendreich, C. Connelly, and P. Hieter.** 1994. Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. Proc. Natl. Acad. Sci. USA *91*:6186-6190.
19. **Kozarsky, K.F. and J.M. Wilson.** 1993. Gene therapy: adenovirus vectors. Curr. Opin. Genet. Dev. *3*:499-503.
20. **Miyake, S., M. Makimura, Y. Kanegae, S. Harada, Y. Sato, K. Takamori, C. Tokuda, and I. Saito.** 1996. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. Proc. Natl. Acad. Sci. USA *93*:1320-1324.
21. **Mizuguchi, H. and M.A. Kay.** 1998. Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. Hum. Gene Ther. *9*:2577-2583.
22. **Mizuguchi, H. and M.A. Kay.** 1999. A simple method for constructing E1 and E1/E4 deleted recombinant adenovirus vector. Hum. Gene Ther. *10*:2013-2017.
23. **Molin, M., M.C. Shoshan, K. Ohman-Forslund, S. Linder, and G. Akusjarvi.** 1998. Two novel adenovirus vector systems permitting regulated protein expression in gene transfer experiments. J. Virol. *72*:8358-8361.
24. **Ng, P., D.T. Cummings, C.M. Evelegh, and F.L. Graham.** 2000. Yeast recombinase FLP functions effectively in human cells for construction of adenovirus vectors. BioTechniques *29*:524-528.
25. **Ng, P., R.J. Parks, D.T. Cummings, C.M. Evelegh, and F.L. Graham.** 2000. An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. Hum. Gene Ther. *11*:693-699.

Received 23 October 2000; accepted 26 February 2001.

Address correspondence to:

Dr. Hiroyuki Mizuguchi
Division of Biological Chemistry and Biologicals
National Institute of Health Sciences
1-18-1 Kamiyoga
Setagaya-ku, Tokyo 158-8501, Japan
e-mail: mizuguch@nihs.go.jp