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Development of a Helper-Independent Human Adenovirus Vector and Its Use in the Transfer of the Herpes Simplex Virus Thymidine Kinase Gene

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Approximately 2 kilobases (kb) of additional DNA can be packaged into wild-type virions of human adenovirus type 5 (Ad5). To extend this limit, a helper independent Ad5 cloning vector was constructed by deleting most of early region 3 (E3) from map coordinates 78.5 to 84.7 and essentially all of early region 1 (E1) from coordinates 1.0 to 10.6. E3 is nonessential for adenovirus replication in cultured cells, and E1 is nonessential when the virus is propagated in 293 cells which constitutively express the E1 gene products. The resulting new virus, *dl*E1,3 is about 5.5 kb shorter than wild-type Ad5 and therefore should be able to accept up to 7.5 kb in foreign DNA. To test the usefulness of this vector, the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (*tk*) along with its regulatory sequences was inserted into the unique *Xba*I site of *dl*E1,3 (at map position 78.5/84.7). The resulting recombinant virus, Ad*tk*, expressed the HSV *tk* at a low level (as compared with HSV-1) in infected cells; however, *tk* expression was markedly enhanced when Ad*tk*-infected cells were superinfected with a tk^- mutant of HSV. Furthermore, the Ad*tk* virus efficiently transformed tk^- mouse cells (line LTA) to the tk^+ phenotype. At a low efficiency, it was also possible to transform tk^- human cells (line 143), and tk^+ transformants of both mouse and human origin have been established as permanent lines.

Human adenoviruses are medium-sized DNA tumor viruses with genomes of linear double-stranded DNA approximately 36,000 base pairs in length (47). Upon infection of permissive human cells, the viral genome is replicated and transcribed in the nucleus in two major stages: an early phase which precedes viral DNA replication, followed by a late phase. The early genes lie in five noncontiguous regions designated as early region 1 (E1), E2a, E2b, E3, and E4 (recently reviewed by Sharp [36]). E1 which maps to the leftmost end of the genome (1.3 to 11.1 map units [m.u.]; see Fig. 3B) contains two transcription units, E1a and E1b, of which E1a encodes the first transcripts to be expressed after infection of permissive cells. Furthermore, one or more of the E1a gene products is required to activate most, if not all, of the remaining viral genes (6, 25, 31). Both E1a and E1b are nonessential for virus growth in 293 cells (18), which constitutively express the E1 gene products (1, 6, 18). Consequently, the 293 cell line has proven very useful for the isolation of various E1 mutants (see review by Young et al. [51]). In contrast, E3, located between map positions 76.6 and 86.0 (see Fig. 3A), appears to be nonessential for virus growth in all types of cultured cells since viruses lacking or defective in E3 are still viable (3, 7, 26, 35).

In addition to the widespread use of adenoviruses as model systems in the study of eucaryotic gene expression, this group of viruses is particularly suited as gene transfer vectors in mammalian cells for several important reasons: first, they are widely studied and well characterized (14); second, they are easy to grow and manipulate, and they exhibit a broad host range in vitro and in vivo; third, copious amounts of virus and viral products can be produced in lytically infected cells. In addition, because only a small portion of the viral genome appears to be required in *cis* (47), adenovirus-derived vectors may ultimately offer excellent potential for the substitution of large fragments once cell lines analogous to 293 cells have been developed which can provide most of the essential viral functions in *trans*. Many of these properties offer distinct advantages for adenoviruses over vectors derived from other DNA viruses or from retroviruses. In particular, with regard to potential applications in gene therapy (reviewed by Anderson [2]), adenovirus-based vectors, unlike those derived from retroviruses, do not carry strong outward-directed promoters such as those of the retrovirus long terminal repeats which can cause neoplastic transformation by a promoter insertion mechanism (13, 20, 30, 32).

Studies on packaging constraints in adenovirus type 5 (Ad5) (Y. Haj-Ahmad and F. L. Graham, manuscript in preparation) suggested that a maximum of about 2 kilobases (kb) of extra DNA can be inserted into the wild-type (wt) viral genome. To increase this limit and enhance the usefulness of the virus as a vector, we undertook the development of deletion mutants and succeeded in constructing helper-independent mutants lacking most of E3 (*dl*E3) or lacking E1 and E3 sequences (*dl*E1,3). The genome of *dl*E1,3 is about 5.5 kb shorter than the wt genome and should theoretically be capable of accepting inserts of up to 7.5 kb in length. Furthermore, *dl*E1,3 is a useful vector not only because genes can be inserted into its unique *Xba*I site with relative ease, but also because it is helper independent if propagated in 293 cells. In this paper we describe the construction of this cloning vector and its use for efficient delivery of the herpes simplex virus (HSV) thymidine kinase (*tk*) gene into mammalian cells.

MATERIALS AND METHODS

Cells and viruses. wt Ad5 and *dl*309 (24) were grown in HeLa or KB cells and titrated on 293 cells (18) as described

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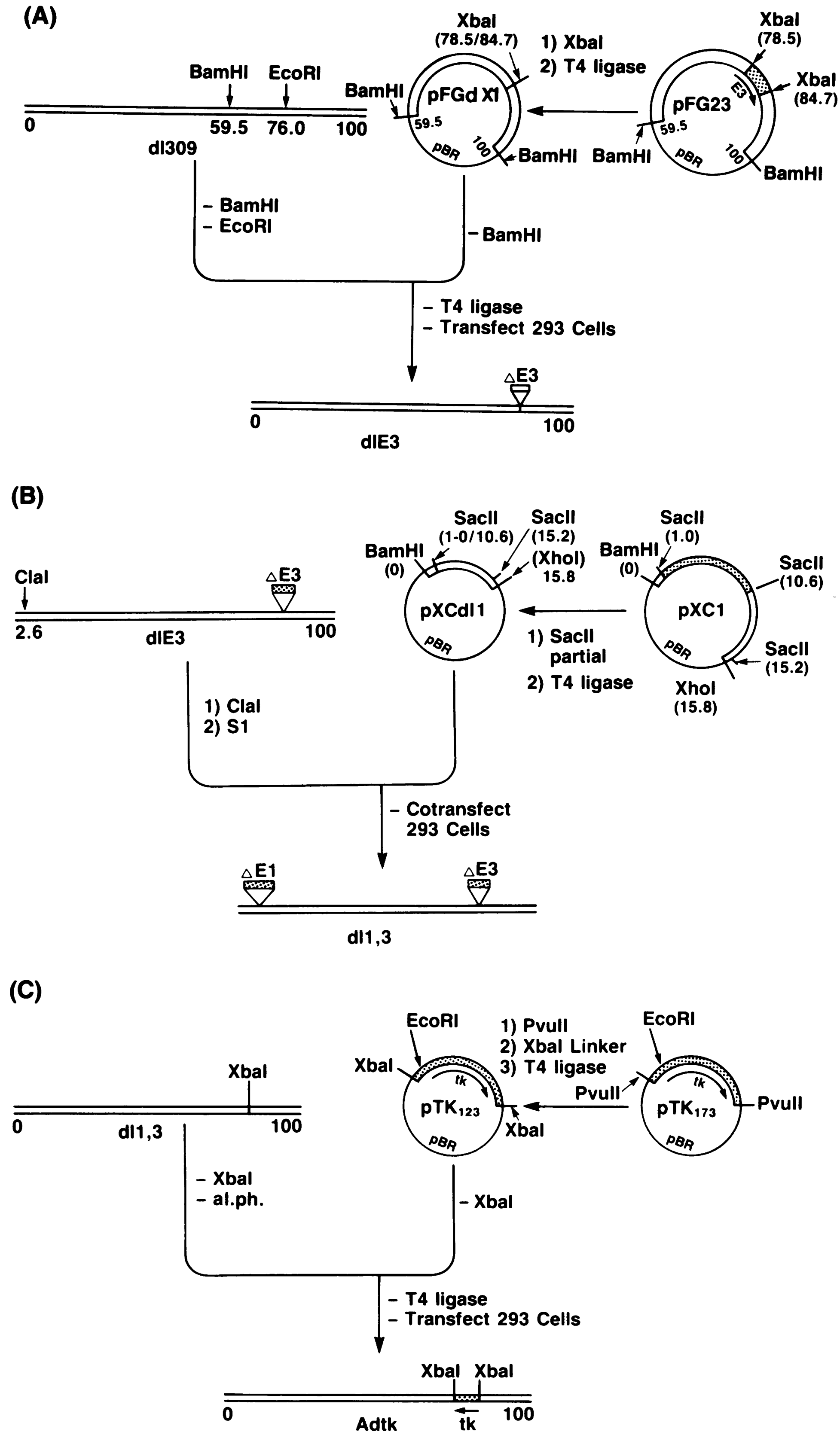
(A)
BamHI
EcoRI
0
59.5
76.0
100
dl309
XbaI
(78.5/84.7)
1) XbaI
2) T4 ligase
BamHI
pFGd X1
BamHI
XbaI
(78.5)
XbaI
(84.7)
pFG23
BamHI
BamHI
- BamHI
- EcoRI
- BamHI
- T4 ligase
- Transfect 293 Cells
ΔE3
0
100
dlE3
(B)
ClaI
ΔE3
2.6
dlE3
100
SacII
(1-0/10.6)
SacII
(15.2)
(XhoI)
15.8
BamHI
(0)
pXCdl1
SacII
(1.0)
BamHI
(0)
SacII
(10.6)
pXC1
SacII
(15.2)
XhoI
(15.8)
1) SacII
partial
2) T4 ligase
1) ClaI
2) S1
- Cotransfect
293 Cells
ΔE1
ΔE3
dl1,3
(C)
XbaI
0
dl1,3
100
EcoRI
XbaI
pTK123
XbaI
1) PvuII
2) XbaI Linker
3) T4 ligase
PvuII
EcoRI
pTK173
PvuII
- XbaI
- al.ph.
- XbaI
- T4 ligase
- Transfect 293 Cells
XbaI XbaI
0
Adtk
tk
100

previously (17). Newly constructed Ad5 deletion and substitution mutants, namely, Ad5 *dl*E3, Ad5 *dl*E1,3 and Ad*tk*, were propagated and titrated on 293 cells. This cell line was maintained in Joklik modified medium supplemented with 10% horse serum (HS). The tk^- human cell line 143 (obtained from K. Huebner and C. Croce and described in reference 16) and the tk^- mouse cell line LTA (16, 27) were grown in alpha-minimum essential medium (α-MEM) (40) supplemented with 10% fetal calf serum. HSV type 1 (HSV-1) and a tk^- mutant of HSV-1 lacking 875 base pairs of *tk*-coding sequences (37) were a gift of J. Smiley.

Transfection of 293 cells. Transfection of 293 cells was carried out as described previously (19), except that 4 to 5 h after transfection the cells were treated with glycerol (12) and overlaid with F11 MEM supplemented with 5% HS and 0.5% agarose. Plaques were picked 8 to 10 days later, and progeny viral DNA was analyzed with appropriate restriction enzymes as described below.

^{32}P labeling and extraction of viral DNA. Semiconfluent monolayers of 293 cells in 60-mm dishes were infected with 5 PFU of a given virus per cell; after 1 h of absorption, 4 ml of α-MEM supplemented with 5% HS was added to each dish. At 20 to 24 h postinfection, medium was removed, and 4 ml of phosphate-free α-MEM supplemented with 5% HS and 25 μCi of $^{32}P_i$ per ml was added to each dish. After a further incubation of 8 to 12 h, infected cells were harvested, and viral DNA was isolated by the Hirt extraction procedure (23), digested with appropriate restriction endonucleases, and electrophoresed on 1% agarose gels. Gels were dried on Whatman paper and exposed on Kodak X-Omat R film XR1 or XR5.

***tk* transformation assay.** LTA or 143 cells were plated at densities ranging from 1×10^5 to 5×10^5 cells per 60-mm dish and were infected 24 h later. wt Ad5, *dl*E1,3, or the *dl*E1,3 vector carrying the HSV *tk* gene (Ad*tk*) were used to infect cells at multiplicities of infection ranging from 0.1 to 20 PFU per cell. Each 60-mm dish was infected with 0.2 ml of virus in phosphate-buffered saline at 37°C, and after 1 h, 5 ml of α-MEM plus 10% fetal calf serum was added. After 24 h, the medium was removed and replaced with fresh α-MEM supplemented with 10% fetal calf serum and HAT (43) (15 μg of hypoxanthine per ml, 1 μg of aminopterin per ml, and 5 μg of thymidine per ml), and thereafter the medium was changed every 3 days. At 2 to 3 weeks postinfection, dishes were fixed and stained for determination of transformation efficiencies, and in some experiments tk^+ colonies were isolated and cell lines were established.

[^{3}H]thymidine incorporation and *tk* assay. To measure the incorporation of [^{3}H] thymidine into viral or cellular DNA (an indirect assay for *tk* activity), cells were infected with the appropriate viruses, namely, *dl*E1,3 or Ad*tk* at 10 PFU per cell or HSV at 1 PFU per cell. Superinfection with HSV *tk* was carried out 24 h post-Ad*tk* infection; briefly, medium was removed, cells were superinfected with HSV tk^- virus at 1 PFU per cell in a total volume of 0.2 ml/60-mm dish, and after 1 h of adsorption at 37°C, 5 ml of α-MEM with 5% HS was added to each dish. Subsequently, cells were pulse-labeled at various times for 2 h. Infected cell monolayers were rinsed with phosphate-buffered saline and then lysed in buffer containing pronase and sodium dodecyl sulfate (16) (0.5 ml/60-mm dish) for 6 h at 37°C. The nucleic acid was then ethanol precipitated twice in 1.5-ml Eppendorf tubes and dried at 37°C for 1 h; scintillation fluid was added, and the samples were counted in a scintillation counter. The enzymatic assay for *tk* activity in cell extracts was carried out as described by Summers et al. (41).

RESULTS

Construction of *dl*E3. Figure 1A illustrates the strategy employed for the deletion of region E3 of Ad5. The plasmid pFG23 contains the *Bam*HI B fragment (59.5 to 100 m.u., minus 20 base pairs from the extreme right end [29; R. D. McKinnon, Ph.D. thesis, McMaster University, Hamilton Ontario, Canada, 1984]) of wt Ad5 cloned into the *Bam*HI site of pBR322 DNA. This plasmid contains two *Xba*I sites in the Ad5 sequences in E3 at map positions 78.5 and 84.7 m.u. Since the site at map position 84.7 m.u. is sensitive to methylation, the plasmid pFG23 was grown in Dam$^-$ *Escherichia coli*. Deletion of E3 was then achieved by cleaving pFG23 with *Xba*I followed by ligation, thus eliminating the sequences from 78.5 to 84.7 m.u. The resulting new plamid, pFGdX1, contains a unique *Xba*I site into which additional foreign DNA can be inserted and subsequently rescued into virus. pFGFdX1 DNA was digested with *Bam*HI, and *dl*309 virion DNA was digested with both *Bam*HI and *Eco*RI (the latter digestion was carried out to decrease the likelihood of reconstitution of *dl*309); after inactivation of the restriction enzymes, the digests were mixed and ligated, and the ligation reaction mixture was used to transfect 293 cells as described in Materials and Methods. A total of 40 to 50 plaques were obtained; 3 of these were picked, and their structure was analyzed by restriction enzyme digestion and gel electrophoresis. All three isolates were found to have the desired structure (Fig. 2, lanes 3 and 7).

The E3 deletion which extends from map position 78.5 to 84.7 removes the major parts of all nine E3 messages, leaving the promoter and 5′ initiation site, the polyadenylation sites, and one set of 5′ and 3′ splice sites (Fig. 3A). These signals are utilized in vivo as determined by S1 nuclease mapping (7). E3, however, appears to be nonessential for the adenovirus life cycle at least in cultured cells (3, 7, 24, 26, 35). Indeed, this deletion not only fails to block viral growth in HeLa or in 293 cells, but on the contrary it has been reported that a mutant lacking E3 had a growth advantage relative to wt virus (7).

Construction of *dl*E1,3. As mentioned previously, E1 is nonessential when virus is grown in the 293 cell line, which constitutively expresses the E1 region (1, 6, 18). To delete

FIG. 1. Outline of the construction of *dl*E3 (A), *dl*E1,3 (B), and Ad*tk* (C). (A) Strategy for construction of *dl*E3. pFG23 was digested with *Xba*I and then religated to generate pFGdX1 with a deletion of most of E3 (from map positions 78.5 to 84.7). pFGdX1 was then digested with *Bam*HI and ligated to *Bam*HI- and *Eco*RI-digested *dl*309 viral DNA. The ligation mixture was used to transfect 293 cells as described in Materials and Methods. (B) Strategy for construction of *dl*E1,3. pXC1 containing the left 15.8% of the Ad5 genome was cut with *Sac*II (partial digestion) followed by religation to generate pXCdl1 lacking essentially all of E1. Cotransfection of 293 cells with a mixture of *Cla*I-digested *dl*E3 DNA and undigested pXCdl1 DNA yielded *dl*E1,3 by in vivo recombination. (C) Construction of Ad*tk*. pTK173 contains the entire HSV *tk* gene (*Pvu*II fragment) cloned into pBR322 at the *Pvu*II site. The two *Pvu*II sites flanking the *tk* gene were converted to *Xba*I sites by ligating *Pvu*II-digested pTK173 in the presence of synthetic *Xba*I linkers to create pTK123. The *tk* gene was then inserted into the unique *Xba*I site of *dl*E1,3 via a trimolecular reaction in which pTK123 was digested with *Xba*I and ligated to *Xba*I-digested *dl*E1,3.

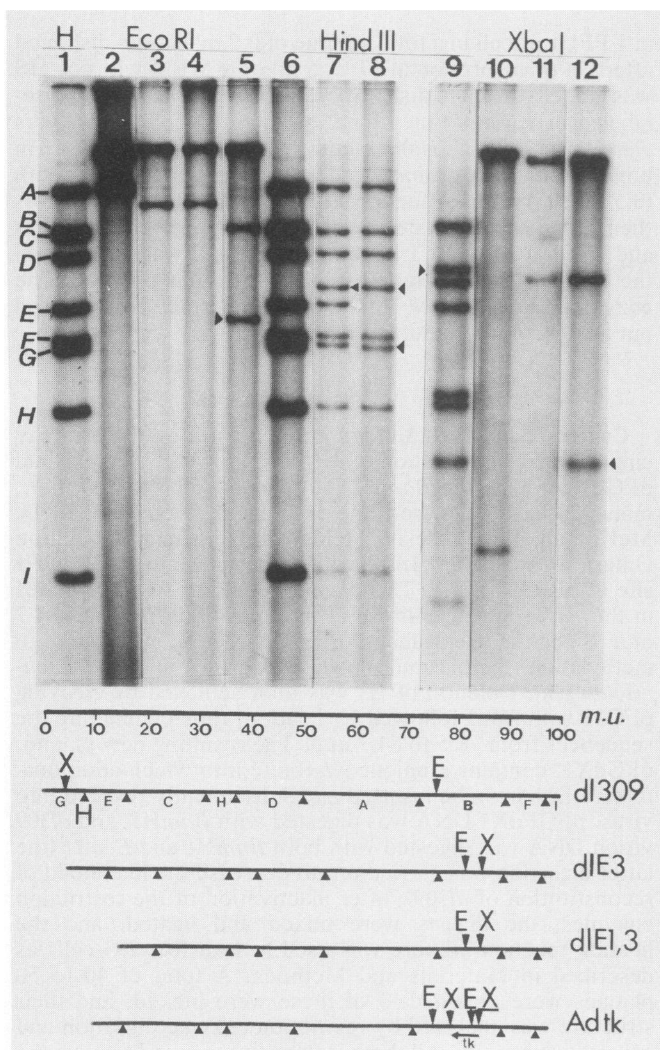

FIG. 2. Restriction endonuclease analysis of *dl*E3, *dl*E1,3, and Ad*tk* viruses. 293 cells were infected with one of the above viruses and then were labeled with $^{32}PO_4$ in phosphate-free MEM plus 5% HS for 8 to 12 h at 20 to 24 h postinfection. The DNA was isolated as described in Materials and Methods, digested with restriction endonucleases, and electrophoresed on 1% agarose gels. Lanes 1 and 6 are *Hin*dIII digests of *dl*309 DNA (run as a molecular weight marker). Lanes 2, 3, 4, and 5 are *Eco*RI digests of *dl*309, *dl*E3, *dl*E1,3, and Ad*tk*, respectively; in lane 5 the small triangle indicates the novel *Eco*RI fragment generated by insertion of the *tk* gene in the orientation shown below. Lanes 6, 7, 8, and 9 are *Hin*dIII digests of *dl*309, *dl*E3, *dl*E1,3, and Ad*tk*, respectively. The E3 deletion reduced the *Hin*dIII B fragment size resulting in a new fragment migrating slightly above the *Hin*dIII E fragment as indicated by the arrows in lanes 7 and 8. Deletion of E1 removed sequences from *Hin*dIII fragments G and E, resulting in a fusion fragment migrating slightly ahead of *Hin*dIII-G (bottom arrow in lane 8). Lanes 10, 11, and 12 are *Xba*I digests of *dl*309, *dl*E1,3, and Ad*tk*, respectively. The arrow in lane 12 indicates the *Xba*I fragment containing the HSV *tk* gene. The *Eco*RI (E), *Hin*dIII (H), and *Xba*I (X) restriction enzyme maps for *dl*309, *dl*E3, dlE1,3, and Ad*tk* are illustrated below (m.u. from reference 42).

E1, we followed the strategy illustrated in Fig. 1B. The plasmid pXC1, which contains the Ad5 *Xho*I C fragment (0 to 15.8 m.u.) minus 21 base pairs from the extreme left end (29; McKinnon, Ph.D. thesis), was partially digested with *Sac*II, which cuts at 1.0, 10.6, and 15.2 m.u., and religated to generate pXCdl1 lacking sequences between map positions 1.0 and 10.6. Infectious *dl*E1,3 was generated by cotransfecting 293 cells with pXCdl1 DNA and *dl*E3 DNA which had been digested with *Cla*I (cuts at 2.6 m.u.) and treated briefly with S1. The S1 treatment was carried out to eliminate or reduce the likelihood of reconstitution of *dl*E3 by in vivo ligation. Approximately 8 days after transfection six plaques were isolated and used to infect 293 cells. After the development of cytopathic effect, infected cell DNA was extracted, and viral DNA was analyzed with restriction endonucleases as described in Materials and Methods. One of six isolates had the expected deletion (Fig. 2, lanes 4, 8, and 11). The deletion between map positions 1.0 and 10.6 removed all of E1a and E1b and about half of the coding sequences for protein IX (Fig. 3B). As expected, *dl*E1,3 was host range for lytic growth in that it grew well in 293 cells but failed to replicate in HeLa cells. It has been reported previously that deletion of the coding sequences for protein IX does not prevent the replication of adenovirus, but capsids lacking protein IX are less stable than wt virions (10). In agreement with these findings we observed that *dl*E1,3 virions are more heat labile than wt virions.

Construction of Ad*tk*. The deletion mutant *dl*E1,3 was constructed to be used as a helper-independent cloning vector in mammalian cells. This mutant contains a unique *Xba*I restriction enzyme site located at the junction of map position 78.5/84.7. Thus, foreign DNA can be inserted at this site by following a strategy similar to that shown in Fig. 1A by which genes can be easily inserted at the *Xba*I site of pFGdX1 and then rescued into virion DNA. Alternatively, after insertion of a gene into pFGdX1, the newly constructed plasmid can be cotransfected along with *dl*E1,3 into 293 cells, thus relying on in vivo recombination as was done for the derivation of *dl*E1,3 from *dl*E3 (Fig. 1A). For insertion of the HSV *tk* gene into *dl*E1,3, we chose yet a third possible strategy involving a trimolecular ligation as outlined in Fig.

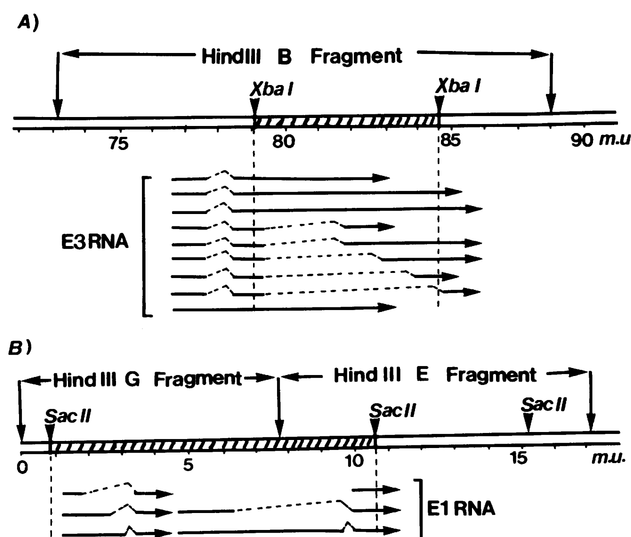

FIG. 3. Schematic representation of E3 transcription units of Ad5 (A) from reference 9 and the E1 transcription units of Ad5 (B) from reference 47. The shaded region between the two *Xba*I sites in panel A represents the segment deleted from *dl*E3, and the region between the two *Sac*II sites in panel B represents the deleted E1 sequences in *dl*E1,3. Introns in E1 and E3 transcripts are represented by dashed lines.

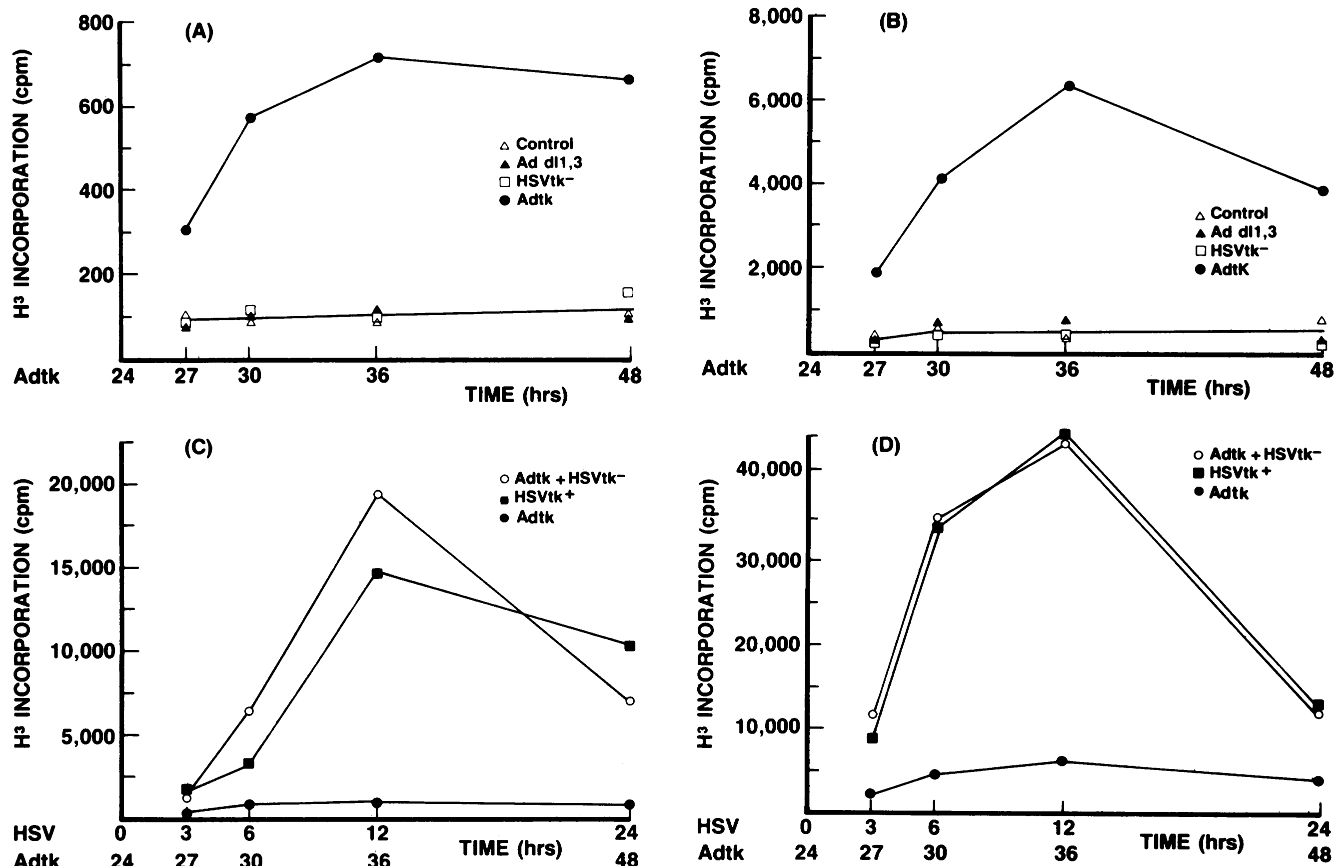

FIG. 4. Kinetics of [^{3}H] thymidine incorporation (counts per minute) in LTA (A and C) and 143 (B and D) cells after viral infection. Cells were infected with 10 PFU of Ad*tk* or *dl*E1,3 per cell or superinfected at 24 h after adenovirus infection with 1 PFU of HSV (either *tk*$^{+}$ or *tk*$^{-}$) per cell (panels C and D). At the indicated times postinfection the cells were harvested, and the levels of ^{3}H incorporation were determined as described in Materials and Methods. Times indicated along the abscissa represent the time after adenovirus infection (all panels) and the time after infection or superinfection with HSV-1 (panels C and D).

1C. To generate Ad*tk*, pTK123, in which the two *Pvu*II sites flanking the HSV *tk* gene in pTK173 (McKinnon; Ph.D. thesis) had been replaced by *Xba*I sites, was cut with *Xba*I and ligated to *Xba*I-digested *dl*E1,3; the ligation mixture was then transfected onto 293 cells. Approximately 8 days later, plaques were picked, and progeny viral DNA was analyzed by restriction enzyme digestion. Of 39 plaques isolated and analyzed, 3 had the *tk* gene inserted in the *Xba*I site (78.5/84.7); all 3 were in the same orientation with the *tk* gene inverted with respect to the direction of transcription of E3 (Fig. 2, lanes 5, 9, and 12).

***tk* expression in Ad*tk*-infected cells.** For [^{3}H] thymidine to be incorporated into DNA the labeled substrate has to be phosphorylated by thymidine kinase. Thus, measurements of [^{3}H] thymidine incorporation into DNA could be used as an indirect measure of the level of *tk* activity. *tk*$^{-}$ cells (143 or LTA) were infected with adenoviruses (*dl*E1,3 or Ad*tk*) at 10 PFU per cell. After 24 h, half of the Ad*tk*-infected dishes were superinfected with HSV *tk*$^{-}$ virus at 1 PFU per cell. Cells were then pulse-labeled with [^{3}H] thymidine (1 μCi/ml) for 2 h at 24, 27, 30, 36, and 48 h after virus infection, the nucleic acids were extracted, and radioactivity was counted as described in Materials and Methods. Figure 4 shows the results obtained in LTA (panels A and C) and 143 cells (panels B and D). In both cell lines the rate of incorporation reached a maximum at 36 h postinfection with Ad*tk* virus; although the absolute levels were approximately 10-fold higher in 143 cells (panel B) than in LTA cells (panel A), the ratio of *tk* activity in infected cells over *tk* activity in mock-infected cells was very similar (sevenfold in LTA compared with eightfold in 143 cells). The rate of incorporation presumably reflects both the levels of *tk* expression and the rate of DNA replication in infected cells. The relatively slow time course of [^{3}H] thymidine incorporation is probably due to the fact that Ad*tk* is defective for growth in both LTA and 143 cells as a result of the E1 deletion. This may also explain, in part, the apparent low levels of *tk* activity seen in Ad*tk*-infected cells. Second, the fact that the *tk* gene is inserted in the *Xba*I site in *dl*E1,3 in the opposite orientation to the direction of transcription of E3 could affect *tk* expression negatively; it is possible, for example, that transcription from the undeleted E3 promoter could generate an antisense *tk* RNA which might inhibit translation of *tk* mRNA. However, a more likely explanation for inefficient expression is that the HSV *tk* gene is under control of the early, or beta class, HSV *tk* gene promoter and therefore requires HSV immediate-early or alpha functions for maximum expression (34, 38). That this may indeed be the case was indicated by the results shown in Fig. 4C and D. In these experiments Ad*tk*-infected LTA (panel A) or 143 (panel B) cells were superinfected at 24 h with a *tk*$^{-}$ mutant of HSV. This resulted in a large increase in *tk* expression (as measured by [^{3}H]thymidine incorporation), raising it to levels comparable to those in cells singly infected with wt HSV.

TABLE 1. *tk* activity in infected 143 cells[a]

Viruses	Sp act at (h postinfection):			
	6	12	24	36
Mock infected	ND	ND	90, 65	80, 90
Ad*tk*	ND	ND	140, 120	300, 270
HSV	580, 450	950, 820	ND	ND

[a] Cells were infected with either 10 PFU of Ad*tk* per cell or 1 PFU of HSV1 per cell. At the times indicated postinfection, the cells were harvested, and the specific activity of *tk* in cell extracts was determined. Each value represents the average of two reactions calculated as counts per minute of [^{14}C]thymidine phosphorylated per microgram of total cellular protein. ND, Not determined.

TABLE 3. *tk* activity in Ad*tk*-transformed or control cells

Cell line	Sp act[a]
143	110, 90
143[b]	970, 760
LTA	36, 40
TLTA[b]	780, 810
293	1,100, 950

[a] Specific *tk* activity in counts per minute of [^{14}C]thymidine phosphorylated per microgram of protein, average of two reactions.
[b] T143 and TLTA are *tk*$^+$ human and mouse cell lines established after transformation of *tk*$^-$ 143 and LTA cells with Ad*tk*.

Finally, data obtained by direct measurement of *tk* activity in cell extracts prepared from Ad*tk*-infected 143 cells (Table 1) were in qualitative agreement with results obtained by assaying thymidine incorporation into DNA. In particular, the enhancement of *tk* activity in infected-cell extracts was similar to that shown in Fig. 4 for the rates of incorporation.

Transformation of *tk*$^-$ cells to *tk*$^+$. wt Ad5 will replicate in both mouse and human cells with the result that the lytic response typically prevents the detection and isolation of transformants. However, because the Ad*tk* mutant lacked E1, it seemed likely that it could be used to transform *tk*$^-$ cells to a *tk*$^+$ phenotype and to establish *tk*$^+$ cell lines.

Both LTA and 143 cells were used in transformation assays, but the efficiency of transformation with 143 cells was very low, and quantitative results were obtained only for the LTA cells. Colonies of LTA cells resistant to HAT were visible 1 to 2 weeks postinfection and were counted 3 weeks postinfection with Ad*tk*. Table 2 shows the transformation efficiency obtained in two independent experiments after infection of LTA cells with 0.1, 0.5, 10, and 20 PFU per cell. The results indicate that over this range the transformation efficiency was approximately linear with respect to multiplicity of infection, resulting in 1×10^{-6} to 2×10^{-6} transformants per PFU, a value similar to that reported for an adenovirus mutant carrying the neomycin resistance gene (49) or the simian virus 40 early region (48). The transformation efficiency was also dependent upon the recipient cell used, and as mentioned above the 143 cell line transformed at very low efficiency compared with LTA cells: in contrast to LTA cells for which up to 100 transformants per plate were readily obtained, only 1 to 2 transformants were detected in several dishes of 143 cells infected with Ad*tk*. It seems likely that this difference results from differences in the efficiency of integration or some other factor rather than differences in the efficiency of delivery of the gene into the cells, since levels of *tk* expression were similar in the two lines.

TABLE 2. Transformation of LTA cells[a]

Expt no.	Virus	Multiplicity of infection	No. of foci[b]	Mean
1	Mock infected		0, 0, 0, 0	0
	*dl*E1,3	0.5	0, 0, 0, 0	0
		20	0, 0, 0, 0	0
	Ad*tk*	0.1	1, 0, 1, 0	0.5
		10	48, 33, 63, 60	51
		20	80, 100, 93, 105	94
2	Mock infected		0, 0, 0	0
	*dl*E1,3	0.5	0, 0, 0	0
		20	0, 0, 0	0
	Ad*tk*	0.1	1, 0, 0, 0	0.3
		0.5	5, 6, 7, 3	5
		10	23, 19, 25, 16	21
		20	40, 36, 48, 33	39

[a] Cells were infected with the virus and multiplicity of infection indicated, incubated in selective (HAT) medium, and fixed and stained at 2 to 3 weeks postinfection.
[b] Colonies of transformed cells per 60-mm dish.

tk$^+$ cell lines of both mouse and human origin were established by picking single colonies and passaging them in selective medium (HAT). *tk* activity in the resulting transformed cells was measured by the enzymatic assay as decribed by Summers et al. (41). The results (Table 3) indicate that the level of *tk* activity in Ad*tk*-transformed human (T143) and mouse (TLTA) cells was significantly higher than that of the respective *tk*$^-$ parents (143 and LTA) and was comparable to levels expressed in a wt (*tk*$^+$) human line (293).

DISCUSSION

We constructed a helper-independent adenovirus vector (*dl*E1,3) which theoretically should be able to accept up to 7.5 kb of foreign DNA. We used this vector to clone the HSV thymidine kinase gene and showed that this gene could be expressed upon infection of cells with the recombinant virus (Ad*tk*) but still responded to signals provided in *trans* by HSV *tk*$^-$ virus. Moreover, the recombinant Ad*tk* efficiently transformed *tk*$^-$ cells to the *tk*$^+$ phenotype, and permanent *tk*$^+$ lines have been established from both mouse and human *tk*$^-$ lines.

Initially, we constructed a nondefective Ad5 variant lacking E3 (*dl*E3). Sequence analysis of this region indicates that E3 could theoretically encode about seven to nine proteins (8, 9, 22), but thus far, only two glycoproteins have been identified (33). As mentioned above, E3 seems to be nonessential for virus growth in cultured mammalian cells, but the fact that this region remains undeleted in natural adenovirus populations suggests that it may confer selective advantages for growth or persistence in vivo. The additional deletion of E1 between nucleotides 354 and 3827 from *dl*E3 yielded the *dl*E1,3 virus which lacks approximately 5.5 kb of the wt genome in total but retains the sequences between nucleotides 200 and 350 needed in *cis* for packaging (21). In previous work, we have found that wt Ad5 can package up to approximately 38 kb of DNA, i.e., up to about 2 kb over and above the size of the wt genome (Haj-Ahmad and Graham, in preparation). Theoretically, therefore, the *dl*E1,3 vector should accept up to 7.5 kb of foreign DNA (comparable to the size rescuable into retrovirus vectors). We have not yet attempted to clone inserts approaching this theoretical limit but have recently inserted a DNA segment of 4 kb into *dl*E1,3 (G. Ghosh Choudhury, Y. Haj-Ahmad, and F. L. Graham, unpublished data).

Several adeno-expression vectors engineered by Solnick (39), by Thummel et al. (44–46), and by Mansour et al. (28) are all missing essential information and therefore must be grown in the presence of nondefective helper adenovirus. Recently, a conditionally helper-independent adenovector (E1/X) was developed in which 2.9 kb had been deleted from E1 (49). This vector might be expected to accept inserts of foreign DNA up to about 5 kb. Other workers have deleted approximately 2 kb of DNA from E3 of Ad5 (7, 35) to create vectors analogous to *dl*E3 described in this report which are nonconditionally helper-independent and should accept inserts of up to 4 kb.

As expected, the phenotype of *dl*E1,3 was similar to that of other E1 mutants (24) in that it grew normally in 293 cells but did not produce detectable cytopathic effect in HeLa or 143 cells unless high multiplicities of infection (over 100 PFU per cell) were used. Consequently, it was possible to use this vector to transform human cells, albeit at a low efficiency. The vector *dl*E1,3 is a useful mutant not only because of its helper independence but also because it may offer the possibility of identifying genes with E1-like functions. That is, it may be possible to clone in various viral and cellular genes and determine whether their functions can substitute for E1 proteins in allowing viral replication in HeLa cells.

For the cloning and expression of foreign genes the mutants and procedures described in this report offer considerable flexibility. There are three different possible strategies for insertion of a DNA fragment into the unique *Xba*I site of *dl*E1,3, and each of these methods was used at different stages in the work. First, genes can be inserted into the *Xba*I site of pFGdX1 and the resulting plasmid spliced to the *Bam*HI A fragment of Ad5 DNA (either wt or *dl*E1,3) to generate infectious virus as was done for construction of *dl*E3 (Fig. 1A). It should be noted that splicing to the *Eco*RI A fragment (left 76%) of Ad5 DNA is an option if the inserted DNA has one or more *Bam*HI sites. Second, DNA segments inserted into pFGdX1 can be rescued into infectious virus by coinfecting cells with overlapping viral DNA fragments and relying on in vivo recombination or marker rescue, analogous to the method used to obtain *dl*E1,3 (Fig. 1B). Finally, genes flanked by *Xba*I sites can be inserted directly into *dl*E1,3 by trimolecular ligation as outlined in Fig. 1C.

To demonstrate the usefulness of *dl*E1,3 as a vector, we inserted into its unique *Xba*I site the HSV *tk* gene. The HSV *tk* gene was chosen for several reasons: it was readily available and is very well characterized, its expression is easily detected, and it has been extensively used in studies on control of gene expression and in transformation (38). Characterization of the new virus, Ad*tk*, revealed that it behaved like its progenitor, *dl*E1,3, with respect to growth in various cell lines (unpublished observations). Furthermore, the *tk* insert remained stable and unmodified during successive passages of the recombinant Ad*tk*. This was in sharp contrast to the retrovirus vector systems in which partial or total deletion or rearrangements of foreign DNA sequences during virus replication have been found to occur at high frequency (4, 5, 11).

Determination of *tk* expression in Ad*tk*-infected tk^- cells (143 and LTA) by [^{3}H]thymidine incorporation into replicating DNA or by direct enzymatic assays of *tk* activity revealed that the HSV *tk* gene, although under control of its own regulatory sequences and inserted in the opposite orientation to the direction of transcription of the deleted E3, was expressed at detectable levels in the absence of any positive selection. Superinfection with tk^- HSV significantly enhanced this expression, indicating that the HSV *tk* gene embedded in Ad5 DNA could still respond to positive regulation by HSV functions. Expression of the HSV *tk* in Ad*tk*-infected cells contrasts with results of a recent study in which the hepatitis B virus surface antigen gene was cloned into an adenovirus vector similar to *dl*E3 (35). Although the inserted gene was transcribed, expression of surface antigen was not detectable in infected cells. High-level expression of the HSV *tk* gene has recently been achieved with a helper-dependent adenovirus vector in which the *tk* gene was under control of the adenovirus major late promoter (50).

With regard to transformation of cells, our results indicated that Ad*tk* efficiently converted tk^- cells of the mouse line LTA to a tk^+ phenotype at a rate similar to that reported for transformation by an adenovirus mutant carrying the neomycin resistance gene (49) or the simian virus 40 early region (48). However, Ad*tk*-mediated transformation efficiencies seemed to depend on the cell line tested: the human cell line 143 transformed extremely inefficiently as compared with mouse cells. This difference must not be due to differences in the efficiency of delivery or expression of *tk* since levels of *tk* activity in Ad*tk*-infected 143 cells were at least as high as those in LTA cells. It is interesting that tk^- mouse and human cells also differ greatly in the efficiency with which they can be transformed to tk^+ in DNA-mediated transfection assays (16) and that similar differences between human and rodent cells have been observed for oncogenic transformation (discussed in reference 15).

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