JOURNAL OF VIROLOGY, Nov. 1997, p. 8902–8907 Vol. 71, No. 11
0022-538X/97/$04.00+0

Persistence of Recombinant Adenovirus In Vivo Is Not Dependent on Vector DNA Replication

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Received 7 May 1997/Accepted 15 July 1997

Recombinant adenovirus vectors represent an efficient means of transferring genes into many different organs. The first-generation E1-deleted vector genome remains episomal and, in the absence of host immunity, persists long-term in quiescent tissues such as the liver. The mechanism(s) which allows for persistence has not been established; however, vector DNA replication may be important because replication has been shown to occur in tissue culture systems. We have utilized a site-specific methylation strategy to monitor the replicative fate of E1-deleted adenovirus vectors in vitro and in vivo. Methylation-marked adenovirus vectors were produced by the addition of a methyl group onto the N^6 position of the adenine base of *Xho*I sites, CTCGAG, by propagation of vectors in 293 cells expressing the *Xho*I isoschizomer *Pae*R7 methyltransferase. The methylation did not affect vector production or transgene expression but did prevent cleavage by *Xho*I. Loss of methylation through viral replication restores *Xho*I cleavage and was observed by Southern analysis in a wide variety of, but not all, cell culture systems studied, including hepatoma and mouse and macaque primary hepatocyte cultures. In contrast, following liver-directed gene transfer of methylated vector in C57BL/6 mice, adenovirus vector DNA was not cleaved by *Xho*I and therefore did not replicate, even after a period of 3 weeks. Although replication may occur in some tissues, these results show that stabilization of the vector within the target tissue prior to clearance by host immunity is not dependent upon replication of the vector, demonstrating that the input transduced DNA genomes were the persistent molecules. This information will be useful for the design of optimal adenovirus vectors and perhaps nonviral episomal vectors for clinical gene therapy.

Recombinant adenovirus vectors show great promise for clinical treatment of many diseases due to their high efficiency for gene transfer into many target organs (reviewed in reference 20). Recombinant adenovirus vectors were initially designed by removal of the transactivator E1A gene to make space for therapeutic genes and render the vector replication deficient. However, it is now well established that leaky viral gene expression results in vector DNA replication in certain cell culture models and some level of toxicity in vivo (11, 25). Moreover, the production of these viral antigens induces a robust host immune response that limits gene expression in animal models (4, 39, 40). In order to overcome the associated host immunity that is induced, at least in part, by de novo viral antigen production, numerous approaches have been used to produce less antigenic vectors by eliminating or mutating additional genes (36). These second-generation vectors contain additional deletions and/or mutations within the E2 (6, 7, 11, 41, 43) or E4 (1, 3, 10, 21, 42) region and appear to be less toxic than first-generation vectors (10, 36); yet, it remains to be seen whether these additional mutations will ultimately enable sustained gene expression in vivo.

More recently, several groups have removed all or nearly all of the remaining adenoviral genes (9, 12, 19, 22, 25, 26, 28). In these helper-dependent vector systems, the adenoviral gene products that are essential for virus production are supplied in *trans*. Recently, our laboratory has utilized the P1 bacteriophage Cre-*loxP* recombination system to create a mini-adenovirus vector with deletions of the E1, E2, E3, and all late genes (25). Although this vector can transduce cells as efficiently as first-generation vectors, the deletion vector is unstable and is rapidly lost following in vivo administration. Several observations, including the accumulation of E1-deleted vector DNA in noncomplementing cell lines and cultured primary hepatocytes (11, 25) and the loss of vector DNA upon inhibition of virus replication, led to the hypothesis that vector DNA replication may be required for persistence of the episome in vivo. Other factors that may be involved or contribute to episomal DNA stability include nuclear matrix attachment, chromatin structure, DNA sequence length, or specific DNA sequences. These parameters are not mutually exclusive. For example, one could envision that early initial replication may be required to render a stable chromatin structure and/or nuclear matrix attachment which might be necessary for vector persistence. Alternatively, continued replication may be required to achieve an equilibrium between maintenance and loss of virus.

Interestingly, in the absence of antigen-specific immune responses, this episomal vector can persist for more than a year in rodents (2). However, little is known about the mechanism(s) involved in maintenance of the vector. Elucidation of the mechanism for vector maintenance is critical for designing an optimal vector which can persist with minimal immunogenicity and toxicity. Thus, to determine whether replication of vector DNA is indeed required for stability in vivo, we developed a scheme to tag the adenoviral genomic DNA by site-specific methylation of *Xho*I recognition sequences. This was achieved by propagation of vectors in 293 cells expressing the *Xho*I isoschizomer *Pae*R7 methyltransferase (23). Since eucaryotic cells do not contain methylases or demethylases that alter adenine residues, removal of the 6-methyladenine residue, and hence restoration of *Xho*I cleavage, should be a direct indication of adenoviral DNA replication. Thus, by monitoring *Xho*I sensitivity, we were able to determine the replicative state

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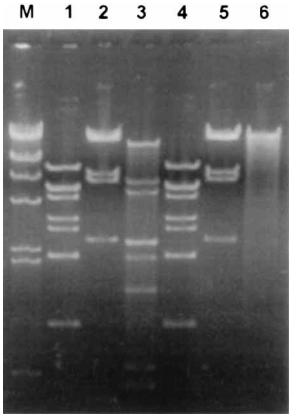

FIG. 1. Restriction analysis of methylated and unmethylated adenovirus vector DNA. Approximately 0.5 μg of purified Ad/RSVhAAT viral DNA grown in 293 (lanes 1 to 3) or 293-PMT (lanes 4 to 6) cells was digested to completion with *Hin*dIII (lanes 1 and 4), *Sal*I (lanes 2 and 5), or *Xho*I (lanes 3 and 6). The digested fragments were resolved on a 1% agarose gel containing ethidium bromide and then visualized by UV fluorescence. *Hin*dIII-digested lambda DNA was resolved in lane M as a size marker.

of input E1-deleted vector DNA following virus administration in vitro and in vivo.

Production of methylated adenovirus vectors. In order to determine if an adenovirus vector with N^6-methylated adenine residues was viable, we created the 293-PMT cell line, which stably expressed the *Pseudomonas aeroginosa* methyltransferase (PMT) gene. To create the plasmid pCEP4-PMT, nucleotides −157 to 1724 relative to the initiation codon of the methyltransferase gene were amplified from plasmid pAOM2.7 (NEB) by amplification with the forward primer 5′-CCGGAT CCGCCGATGATTTAGTG-3′ and the reverse primer 5′-CC GGATCCTTGCCGCCTGTGACG-3′. The amplified fragment was digested with *Bam*HI and then subcloned into the *Bam*HI site of the mammalian expression vector pCEP4 (Invitrogen). This placed the PaeR7 methyltransferase gene (PMT) under control of the cytomegalovirus immediate-early promoter and immediately upstream of the bovine growth hormone polyadenylation signal. The plasmid pCEP4 also contains the hygromycin B gene for selection of stable cell transformants. The correct subclone was confirmed by restriction mapping and DNA sequence analysis.

PaeR7 methylase methylates the adenine residue of *Xho*I recognition sequences (CTCGAG) at the N^6 position, rendering it completely resistant to digestion with *Xho*I (23). As a first screening for methylation, cellular genomic DNA isolated from various clones was found to be resistant to *Xho*I digestion (data not shown). Following E1-deleted adenovirus (Ad/RSVhAAT [18]) infection, 293-PMT cells were essentially indistinguishable from the parental 293 cell line with regard to morphology, kinetics, and total output of adenovirus vector produced. Furthermore, transgene expression from the methylated vector, as detected by hAAT production into the medium of different transduced cells and hepatocytes in vivo, was indistinguishable from unmethylated vector (data not shown). From the results in Fig. 1, restriction analysis of the methylated Ad/RSVhAAT (E1-deleted) vector DNA showed that, as predicted, more than 99% of the methylated DNA was insensitive to digestion with *Xho*I; yet, the presence of the methylation had no effect on the ability to digest the DNA with *Hin*dIII and *Sal*I. Thus, the specific methylation of the seven *Xho*I sites within the Ad/RSVhAAT vector had no detectable effect on the physiology of the virus, including replication, encapsidation, and transcription of the viral genes and transgene expression cassette.

Replication of recombinant adenovirus vectors in cultured cells. Previous studies from our laboratory demonstrate that E1-deleted adenovirus vector DNA accumulates in transformed cells as well as primary hepatocytes in culture, in a multiplicity of infection (MOI)-dependent manner (25). Moreover, vector DNA stability is lost when replication is blocked with hydroxyurea. Therefore, to determine the relationship between stability and replication of the input adenovirus vector DNA in cultured cells, the amounts of replicated and nonreplicated adenoviral DNA were determined in different primary cells or cell lines transduced with methylated or unmethylated Ad/RSVhAAT at various MOIs ranging from 1 to 100. Seventy-two hours later, infected cells were harvested, and the state of the adenoviral DNA was analyzed by Southern analysis. These data are shown for two representative liver-derived cell lines (Fig. 2), and the results for all cultured cells are summarized in Table 1. Cleavage by *Xho*I indicated that the N^6-methyladenine within the *Xho*I site had been removed through replication of the adenoviral DNA whereas noncleaved adenoviral DNA represented the amount of nonreplicating input vector DNA that remained. To simplify the study, one specific *Xho*I site within an 8-kb *Hin*dIII fragment in the E2 region was used for the analysis. *Xho*I cleavage of this fragment results in two fragments with sizes of 6.5 and 1.5 kb. *Xho*I cleavage of unmethylated-vector-transduced cells served as a control for complete restriction enzyme digestion. Similar analysis using total vector DNA as a probe showed that loss of methylation can be detected at each of the seven vector *Xho*I sites (data not shown).

Except for the SK-HEP-1 (ATCC HTB-52) and primary human fibroblasts, each of the cell lines tested supported some level of adenovirus vector replication as determined by loss of methylation. Although the SK-HEP-1 cell line was derived from human liver, these cells are nonparenchymal and have an endothelial origin (13). The inability for adenovirus DNA replication in primary fibroblasts may be due in part to the less than 1% adenovirus transduction rate observed in these cells (data not shown). However, this explanation is not valid for the SK-HEP-1 cell line, since adenovirus transduction of these cells is essentially equivalent to that of HeLa (ATCC CCL-2) and A549 (ATCC CCL-185) cells (data not shown). As previously shown, hepatocytes and hepatoma lines support adenoviral DNA replication in culture (15, 25). In agreement with this, the huH7 (27) human hepatoma cells (Fig. 2) and primary hepatocytes derived from mouse and nonhuman primates (Fig. 3) showed significant loss of vector DNA methylation.

In addition to hepatocytes, cell lines of various origins such as lung, kidney, and cervix were capable of supporting adenovirus vector replication in vitro, but the amount of vector necessary to first detect replication varied between different cells (Table 1). For example, replication was observed in A549 cells at an MOI of only 10. However, replication was not observed in either W162 (37) or HeLa cells until an MOI of 50 was administered. The ratio of unmethylated-to-methylated vectors can be used to estimate the proportion of vector DNA that has replicated. In cells in which loss of methylation was observed at a standard MOI of 100, the variation in the amounts of replication between different cells ranged from 45% in mouse hepatocytes to 88% in the W162 cells (Table 1). The greater loss of methylation in macaque (70%) versus mouse (45%) hepatocytes (Fig. 3; Table 1) may have resulted because the monkey is a more permissive host for this human virus. Nevertheless, taken together, the results suggest that a threshold of E1-deleted virus is required to achieve adenoviral

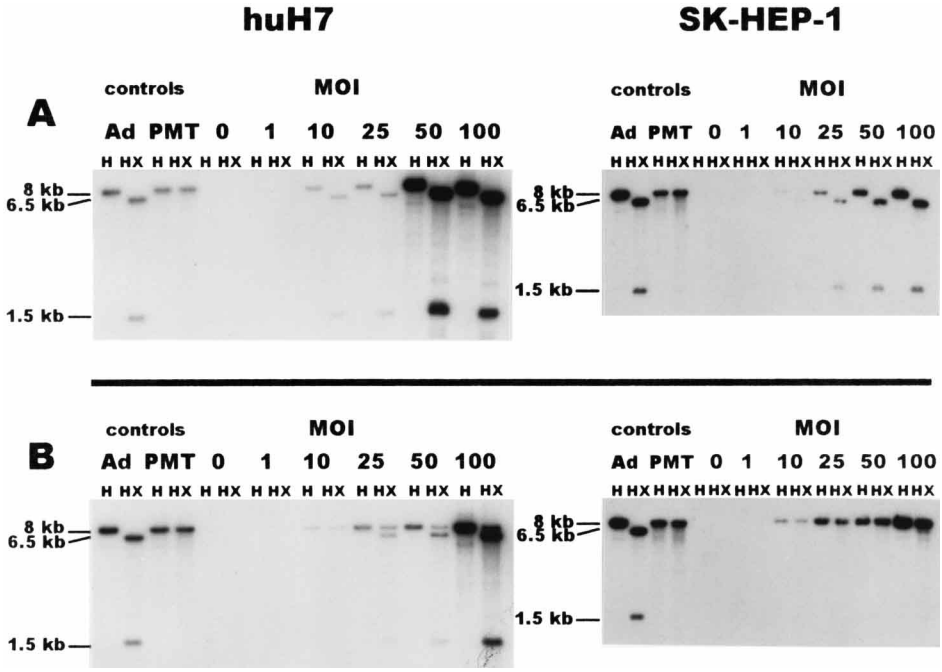

FIG. 2. In vitro adenovirus vector replication assay of liver-derived cell lines infected with either methylated or unmethylated virus. Southern analysis was performed on total genomic DNA from huH7 hepatoma cells and SK-HEP-1 liver-derived endothelial cells infected with either the Ad/RSVhAAT control virus (A) or the methylated virus PMT-Ad/RSVhAAT (B) at various MOIs (indicated above the lanes). Purified DNA (6 μg) was digested to completion with either *Hin*dIII alone (lanes H) or *Hin*dIII and *Xho*I (lanes HX). Digested DNAs were electrophoretically resolved in 1% agarose gels, alkaline transferred to nylon membranes, and hybridized to a radiolabeled 8-kb *Hin*dIII fragment of Ad/RSVhAAT (nucleotides 18318 to 26328). The sizes of the resulting hybridizing bands are shown on the left. Adenovirus replication is indicated by the cleavage of the 8-kb band into the 6.5- and 1.5-kb bands in the cells infected with the methylated virus PMT-Ad/RSVhAAT (B). For controls (first four lanes of each blot), the corresponding cellular DNA was spiked with 600 pg (representing 15 viral genomes/diploid genome) of either Ad/RSVhAAT (Ad) or PMT-Ad/RSVhAAT (PMT) DNA.

DNA synthesis and that this threshold differs between cell types.

In vivo analysis for adenovirus vector replication. The observations described above support only previous studies of adenoviral DNA accumulation or synthesis in cell lines and do not address the question of whether replication is required for vector genome persistence in vivo. Following adenovirus-mediated gene transfer in vivo, the bulk of adenoviral DNA is lost in liver (and other) tissues within hours of infusion (25, 38), most likely due to nontransduction events in nonparenchymal cells, making it difficult to determine whether replication of vector DNA occurs within hepatocytes in vivo. To determine if replication occurs to any appreciable degree and/or if nonreplicated DNA molecules were stable in vivo, methylated vectors were studied in C57BL/6 mice because adenovirus-mediated gene expression was stable for at least 8 months in this mouse strain (2). Following infusion with methylated or unmethylated control Ad/RSV-hAAT via the portal vein cannula (35), expression of the marker hAAT gene was detected in the sera of mice beginning on day 1, and the levels of expression were similar with both vectors. At specific times postinfection, genomic liver DNA was isolated, digested with *Hin*dIII or *Hin*dIII-*Xho*I, and analyzed by Southern analysis. As shown in Fig. 4, and consistent with previous observations (25, 38), the majority of the viral DNA is lost by day 2, but the amount then remains stable for at least 3 weeks regardless of the methylation status

TABLE 1. Summary of adenovirus vector replication in cultured cells[a]

Cell type	Origin	Source	Adenovirus replication	MOI for initial replication	% replication at 100 MOI
A549	Lung carcinoma	Human	Yes	10	84
HeLa	Cervix carcinoma	Human	Yes	50	81
huH7	Hepatoma	Human	Yes	25	77
SK-HEP-1	Liver adenocarcinoma	Human	No	NA	0
W162	Kidney	African green monkey	Yes	50	88
Primary fibroblasts	Skin	Human	No	NA	0
Primary hepatocytes	Hepatocytes	C57BL/6 mouse	Yes	25	45
Primary hepatocytes	Hepatocytes	Pig-tailed macaque	Yes	5	70

[a] The species and tissue origins and characteristics of adenovirus replication are summarized for each of the cell types used in this study. Cells were infected with a range of MOI from 1 to 100 for 72 h and then assayed for adenovirus vector replication by Southern analysis as described in the text and in the legend to Fig. 2. Phosphorimager analysis was used to determine a numerical signal for each hybridizing band in the double-digested samples. The percentage of replication was estimated by dividing the sum of the values for the 6.5- and 1.5-kb bands, indicative of vector replication, by the sum of all three bands and then multiplying by 100. NA, not applicable.

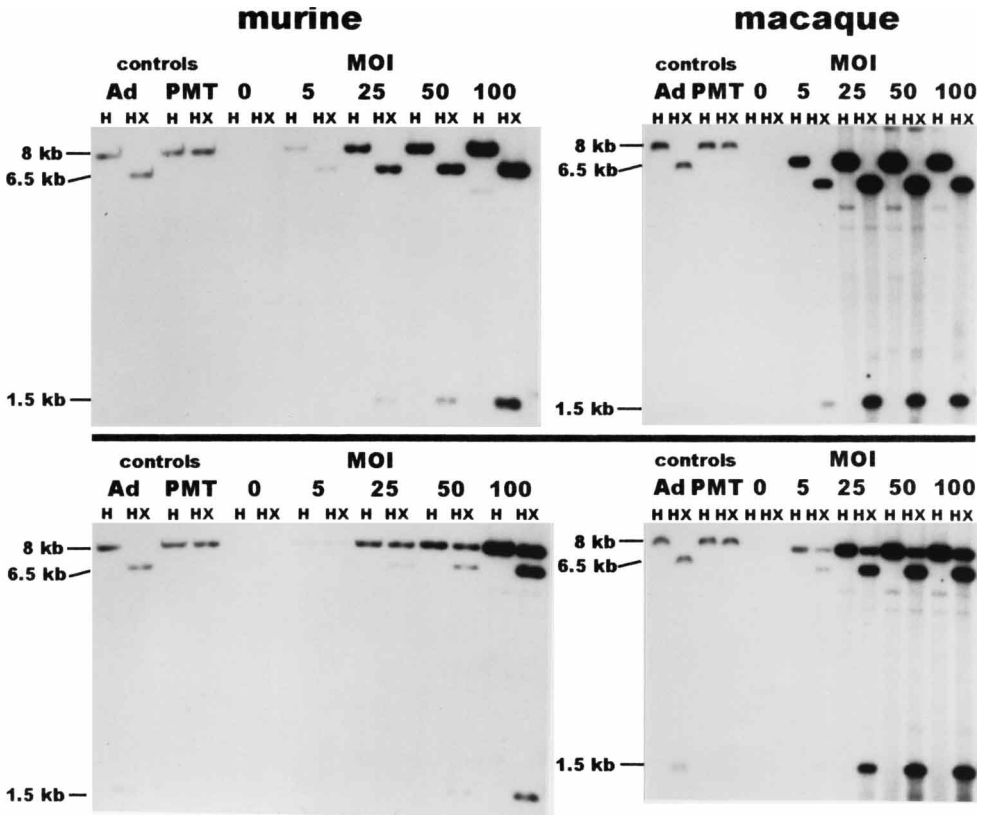

FIG. 3. In vitro adenovirus vector replication assay in nonhuman primate and mouse primary hepatocytes infected with either methylated or unmethylated virus. Southern analysis was performed with total genomic DNA isolated from pig-tailed macaque (*Macaca nemestrina*) and C57BL/6 congenic mouse primary hepatocytes isolated as described previously (20). Hepatocytes with >90% viability were infected 12- to 18-h postisolation with either the Ad/RSVhAAT control virus (A) or the methylated virus PMT-Ad/RSVhAAT (B) at various MOIs (indicated above the lanes). DNA (6 μg) was digested to completion with either *Hin*dIII alone (lanes H) or *Hin*dIII and *Xho*I (lanes HX). The Southern blots were processed and analyzed as described in the legend to Fig. 2. The sizes of the resulting hybridizing bands are shown on the left.

of the vector. As expected, DNA from mice which received the unmethylated Ad/RSVhAAT vector was completely digested by *Xho*I, indicated by the conversion of the 8-kb band to 6.5- and 1.5-kb bands with *Hin*dIII-*Xho*I double digestion (Fig. 4A, lanes HX). However, as shown in Fig. 4B, *Xho*I digestion of the 8-kb *Hin*dIII fragment was not observed at any time in any of the mice receiving the methylated virus. This finding indicates that the methylated vector genomes persisted for at least 3

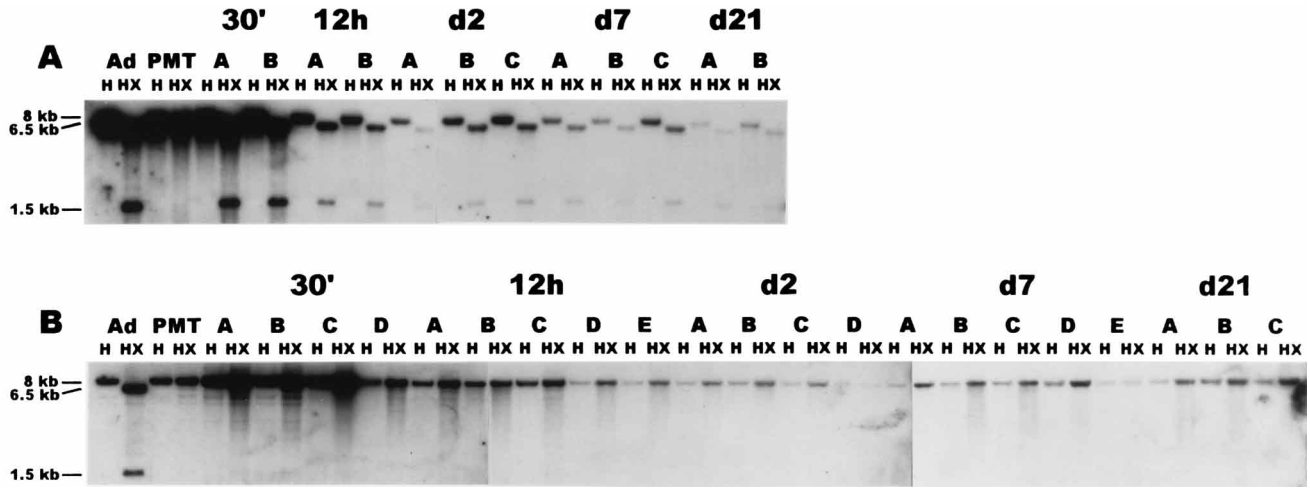

FIG. 4. Southern blot assay for in vivo adenovirus vector replication. C57BL/6 mice were injected via portal vein cannulas with 5×10^9 PFU of either Ad/RSVhAAT control virus (A) or methylated virus PMT-Ad/RSVhAAT (B). At the specific time points indicated, mice were sacrificed and liver genomic DNA was purified. DNA (10 μg) was digested to completion with either *Hin*dIII alone (lanes H) or *Hin*dIII and *Xho*I (lanes HX) and then resolved in a 1% agarose gel. Individual animals at each time point are represented by separate letters. The Southern blots were processed and analyzed as described in the legend to Fig. 2. The sizes of the resulting hybridizing bands are shown on the left. ′, minutes; d, days.

weeks without any viral DNA replication. Although low-level replication cannot be entirely eliminated, these results demonstrate that virus replication is not required for establishing stable persistence of adenovirus vectors in the absence of host immunity.

Implications for gene therapy. DNA replication, as evidenced by the loss of methylation, occurred in most cells in culture but was not observed in vivo and thus does not appear to be required for vector stabilization. Although DNA repair could result in loss of methylation, it is highly unlikely that it had an effect on the adenoviral DNA in these studies because loss of methylation (i) was not observed at lower MOIs in the same cells, (ii) was not observed in all cells, and (iii) correlated with the results of our earlier studies showing vector DNA accumulation in the same cells.

Adenovirus replication is dependent on three viral proteins encoded in the E2 region: preterminal protein (pTP), DNA binding protein, and the viral polymerase (Pol). In addition, at least three host cellular factors, termed nuclear factors I (NFI) and III (NFIII/Oct-1) and topoisomerase I (NFII), are also required. The initial transcription of the three virus replication proteins utilizes the E1A-dependent E2 promoter. For this reason, first-generation E1A-deficient adenovirus vectors have been presumed to be nonreplicative and therefore safe for human gene therapy. However, a number of viral (8, 14, 29, 30, 33, 34) and cellular proteins (5, 15, 24, 31, 32) have been demonstrated to possess E1A-like transactivating activity that induces the activation of E1A-dependent adenoviral promoters such as the E2 promoter in a variety of cells including the human hepatoblastoma cell line HepG2 (31), mouse preimplantation embryos (5), and F9 embryonal carcinoma cells (24) and in two of the cell lines used in this study, HeLa (15, 17) and A549 (11, 15). Furthermore, cellular E1A-like activity that is sufficient to support replication of E1-deleted adenoviral mutants has been demonstrated in several noncomplementing cell lines including HepG2 (31, 32), HeLa (15, 17, 29), and A549 (11, 15). We have observed adenovirus replication in human hepatoma huH7 cells as well as mouse and macaque primary hepatocytes. Interestingly, we did not observe vector replication in vivo in C57BL/6 mouse livers. This may be related to significant differences in gene expression observed in cultured hepatocytes (16) and/or differences in the actual transducing titer after intraportal injection compared with that after administration of the vector in culture.

Based on our recent observations, we had expected to detect virus replication in vivo. Leiber et al. (25) had shown that a small 9-kb vector deleted for most of the viral genome, including the entire E2 region, using the Cre/*loxP* recombinase system was rapidly lost after transduction. Metabolic labeling experiments indicated that the parental E1-deleted virus, but not the deleted vector, could replicate within transduced hepatocytes, suggesting a possible explanation for the early loss of the deletion vector was due to its inability to replicate and establish a stable conformation in the transduced cell. The seemingly conflicting results of these two studies can be reconciled in terms of several methodological differences. In the former metabolic labeling studies, twice the titer of virus was administered to the mice and five times more DNA was analyzed. Furthermore, preliminary evidence suggests that in a direct comparison of the replication assays of each study, there is at least 10-fold-increased sensitivity in the metabolic labeling method compared with that of the Southern assay used here (data not shown). Taken together, we predict that the level of detection observed in the metabolic labeling experiment is on the order of 100 times more sensitive than the Southern blotting method described herein. Therefore, this study cannot exclude the possibility that a very low level of replication may occur in vivo. This possibility may become more relevant when the more-permissive human hepatocytes are targeted in clinical gene therapy. Further studies are needed to determine what effect, if any, the possible low level of virus replication would have on the majority of the nonreplicating genomes in *trans*. However, with more-crippled vectors it becomes less likely that this will be of major concern. Most importantly, these results clearly show that vector genomes that are stable and persistent for 3 weeks in vivo have not undergone replication. This is consistent with recently published data demonstrating equal periods of persistence of an E1 and E1/E2A-deleted Ad/RSVhAAT vector in C57BL/6 mice (43). Therefore, vector replication is not a major effector in the establishment of vector stability.

In light of the results presented here, the future of adenovirus vectors for clinical use is more encouraging, since virus replication is probably the one biological property of adenoviruses that would be difficult to incorporate into a safe and nonimmunogenic vector. It remains to be determined what property or combination of factors is required to maintain the adenoviral genome within quiescent tissue such as the liver. The uncovering of these principles may also be important for developing stable nonviral episomal DNA vectors.

We thank Thomas Gingeras at New England Biolabs for kindly providing plasmid pAOM2.7 and Marshall Horwitz (University of Washington) for the 11A1 fast fibroblasts. We also thank the following people for technical assistance: Leonard Meuse, Carol Miao, Gijsbert Patijn, and Brian Winther. We thank Andre Lieber for helpful discussions.

This work was supported by NIH grant DK49022. J.E.N. is supported by a CF Foundation fellowship.

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