doi:10.1006/mthe.2001.0278, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Linear DNAs Concatemerize *in Vivo* and Result in Sustained Transgene Expression in Mouse Liver

Zhi-Ying Chen,* Stephen R. Yant,*,† Cheng-Yi He,* Leonard Meuse,* Shiliang Shen,* and Mark A. Kay*,[1]

*Department of Pediatrics and Department of Genetics, Stanford University School of Medicine, Stanford, California 94305
†Program in Molecular and Cellular Biology, University of Washington, Seattle, Washington 98105

Received for publication January 2, 2001; accepted in revised form February 2, 2001.

The short duration of transgene expression remains a major obstacle for the implementation of nonviral DNA vectors in clinical gene therapy trials. Here, we demonstrate stable, long-term transgene expression *in vivo* by transfecting a linear DNA expression cassette (LDNA) into mouse liver. Interestingly, despite similar quantities and cellular distribution of injected DNAs in their livers, mice receiving LDNA encoding human α1-antitrypsin (hAAT) expressed approximately 10- to 100-fold more serum hAAT than mice injected with closed circular (cc) DNA for a period of 9 months (length of study). Furthermore, when a linear human factor IX expression cassette was delivered to factor IX-deficient mice, sustained serum concentrations of more than 4 μg/ml (80% of normal) of the human clotting factor and correction of the bleeding diathesis were obtained. Southern blot analyses indicate that, unlike ccDNA, LDNA rapidly formed large, unintegrated concatemers *in vivo,* suggesting that transgene persistence from plasmid-based vectors was influenced by the structure of the vector in transfected cells. No differences in transgene expression or DNA molecular structures were observed when AAV ITRs were included to flank the hAAT expression cassette in both ccDNA- and LDNA-treated animals. Linear DNA transfection provides an approach for achieving long-term expression of a transgene *in vivo.*

Key Words: **gene therapy; liver; hemophilia; factor IX; α1-antitrypsin; nonviral; AAV.**

INTRODUCTION

Nonviral vector plasmid DNAs have been considered for *in vivo* gene therapy applications for a number of different tissues (1–5). To date, most of the clinical applications have been limited to vaccine strategies because of the relatively low and transient nature of gene expression. Our major interest has been in developing gene transfer strategies into the liver for treatment of inherited and acquired diseases. There have been two major problems that have limited plasmid DNA applications in this setting. The first is related to delivery of the plasmid into the tissue. While hydrodynamic intravascular infusion results in gene transfer into about 40% of hepatocytes, this method of delivery results in transient right-sided heart failure and liver injury (2, 6), making it unlikely to be easily adapted to a safe clinical procedure. Alternative technologies for safe and efficient delivery of vector DNA are under development and needed. Second, in most cases there is a rapid decline in transgene expression and/or the number of cells that express the transgene product over time. This is due to a gradual loss of the episomal plasmid and/or the silencing of the template. Recently, we observed that adding certain *cis* DNA elements to the plasmid constructs not only increased gene expression of factor IX from liver into a curative range, but also maintained therapeutic expression for at least 10 months (7). In addition, Zhang *et al.* demonstrated long-lasting but subtherapeutic transgene expression in mouse liver transfected with plasmid DNA (2). However, the reason some plasmids lose while others maintain activity shortly after gene transfer is not understood. The ability to achieve reproducible, persistent, and therapeutic levels of gene expression has been problematic. Understanding the mechanisms required for persistent gene expression will be important prior to clinical implementation.

We elected to determine if the molecular structure of the DNA vectors delivered into mouse livers affected the level and/or persistence of gene expression *in vivo.* Because episomal concatemers may play an important role

[1] To whom correspondence and reprint requests should be addressed at the Department of Pediatrics, 300 Pasteur Drive, Room G305, Stanford, CA 94305. Fax: (650) 498-6540. E-mail: Markay@stanford.edu.

1525-0016/01 $35.00

in stable rAAV-mediated gene expression in liver (8, 9) and muscle (10–12), we specifically compared the effects of linearized DNA vs circular DNA plasmids with and without AAV ITRs for their ability to transfect hepatocytes *in vivo*. We found that gene expression from circular plasmids was lost soon after delivery, a phenomenon reported in many previous studies (6, 13–16), while linearized DNAs reproducibly expressed high level of transgene product that persisted for up to 9 months. Furthermore, we observed that the uncut plasmid DNA remained intact for at least 10 weeks in mouse liver while (double-cut) linearized DNA formed large concatemers as early as 1 day after entering the mouse liver. The inclusion of the AAV ITRs made no difference in any of the experiments. We hypothesize that the difference in vector structure is responsible for the difference in transgene expression.

METHODS

Plasmid construction. The human α1-antitrypsin (hAAT) cDNA was placed under the control of the Rous sarcoma virus long terminal repeat (RSV) promoter and inserted into the *Xho*I site of pBluescript II KS(+) (pBS.KS II) (Stratagene, La Jolla, CA). The recombinant AAV plasmid, pABsg.RSV.hAAT.bpA, was constructed by *Xho*I release of RSV.hAAT.bpA that was then ligated to *Sal*I-digested pABsg.SENB vector. The integrity of the AAV ITR was confirmed by ethidium bromide gel electrophoresis and DNA sequencing. The pABsg.SENB construct was derived from pALAPSN (17) by adding a *Bsg*I site to the external boundary of each AAV ITR. The plasmids pThAAT, encoding an integrating hAAT transposon, and pCMV-SB, which encodes the *Sleeping Beauty* transposase, were previously described (15). The PBS.ApoE.HCR.hAATp.hFIX+IntA.bpA plasmid was previously described (7) (Fig. 1a).

DNA preparation. Plasmid DNA was amplified in Sure cells (Stratagene) and purified by standard cesium chloride banding or Qiagen column purification. To prepare linear DNA (LDNA) either containing or lacking AAV ITRs, pABsg.RSV.hAAT.bpA and pRSV.hAAT.bpA plasmids were digested with *Bsg*I and *Xho*I, respectively. The LDNA of PBS.ApoE.HCR.hAATp.hFIX+IntA.bpA was prepared by digesting the plasmid with *Spe*I, which cut on both sides of the expression cassette (Fig. 1a). Cut DNA was phenol–chloroform extracted and ethanol precipitated. Both circular and linear DNAs were dialyzed against 1× TE for 24 h.

Animal studies. Eight- to ten-week-old female C57BL/6 mice were obtained from Taconic Farms, Inc. (Germantown, NY). All animal procedures were performed under the guidelines set forth by Stanford University and the National Institute of Health. Forty micrograms of DNA in 2 ml 0.85% saline was injected into mouse tail veins as previously described (6, 16). For each injection, the mass of DNA was the same while the molar ratio varied by twofold. In other studies, small variations in molar ratios did not significantly affect gene expression. Mice were bled periodically by a retro-orbital technique. In some cases, mice were subjected to a surgical 2/3 partial hepatectomy as previously described (18). The bleeding times in mice were determined by measuring the time required for clotting of the blood from a 2- to 3-mm tail snip, as previously described (15).

In situ hybridization. Five-micrometer sections of paraffin-embedded livers of the mice 2 to 3 weeks after receiving 40 μg LDNA or closed circular (cc) DNA via tail vein infusion were processed for *in situ* hybridization according to the protocol described previously (9). Following deparaffinization, rehydration, denaturation, and digestion with proteinase, the sections were incubated with a denatured DNA probe which was the 2.9-kb bacterial backbone of the vectors (Fig. 1a) labeled with digoxigenin using the DIG labeling kit from Roche Molecular Biochemicals (Indianapolis, IN). After hybridization, the sections were incubated with a goat anti-digoxigenin antibody conjugated with alkaline phosphatase, and the alkaline phosphatase-bound vector DNA was visualized by nitroblue tetrazolium chloride-5-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals).

ELISA quantitation. After DNA delivery, mouse blood was collected periodically, and serum hAAT (19) or human factor IX (hFIX) (20) were quantitated by ELISA.

Southern blot analyses. Mice were sacrificed 1 day, 5 weeks, or 10 weeks after DNA injection and total liver DNA was prepared by a salting-out procedure. Twenty micrograms of liver DNA was digested with restriction enzymes, separated by gel electrophoresis, and analyzed by Southern blot hybridization using a 1.35-kb *Eco*RI hAAT cDNA as a probe. Radioactive DNA bands were quantitated by phosphoimager analyses.

RESULTS

Linear DNA-Mediated Gene Expression Persists in Vivo

Single-stranded rAAV genomes have been reported to form stable double-stranded concatemers *in vivo* (8, 10, 12, 21–23). Since these changes in vector structure coincide with stabilized transgene expression in the liver (24), we tested whether double-stranded DNA with AAV ITRs behaved like double-stranded rAAV genomes in this organ. To do this, we delivered 40 μg of linear DNA (both DNA fragments produced by restriction digestion with an enzyme that cleaves twice in the bacterial backbone) and closed circular DNA containing a human α1-antitrypsin cDNA expression cassette with and without flanking AAV ITRs (Fig. 1a) into the livers of mice by a rapid tail vein injection (6, 16). Results from each of three independent studies indicate that despite similar levels of serum hAAT expression 1 day after DNA administration, reporter gene expression was consistently 10- to 100-fold higher 3 weeks later in LDNA-injected mice compared to mice receiving ccDNA (Figs. 1b–1d). Interestingly, serum hAAT levels persisted in animals injected with LDNA without AAV ITRs. Therefore, excision of the transcriptional expression cassette alone was sufficient to mediate prolonged transgene expression from a nonviral vector.

To demonstrate reproducibility and clinical relevance, we delivered an expression cassette containing the human factor IX minigene driven by the ApoE enhancer/HCR and α1-antitrypsin promoter (PBS.ApoE.HCR.hAATp.hFIX+IntA.bpA, Fig. 1a) (7) into hemophilia B mice. The size of this hFIX expression cassette is almost twice that of the one expressing hAAT**.** Five weeks after injection, LDNA-treated animals had serum hFIX concentrations of 12,646 ± 1544 (n = 4) ng/ml, or >2.5 times the wild-type levels (5000 ng/ml), and correction of their bleeding diathesis (not shown). The hFIX expression was stabilized at more than 4000 ng/ml for an additional 10 weeks (Fig. 1e). In contrast, serum hFIX levels fell in ccDNA-treated mice over a 5-week period to concentrations of 393 ± 72 ng/ml (n = 3) and continued to drop in the following 10 weeks to 67 ± 31 ng/ml, more than 60-fold lower than that of LDNA-treated mice.

Circular and Linear DNAs Remain Stable in Vivo

In an attempt to understand the molecular mechanism underlying the differences in transgene expression from LDNA- and ccDNA-based vectors, we quantitated vector genomes in the livers of treated mice by *Eco*RI digestion (two-cutter to release the expression cassette) and Southern blot

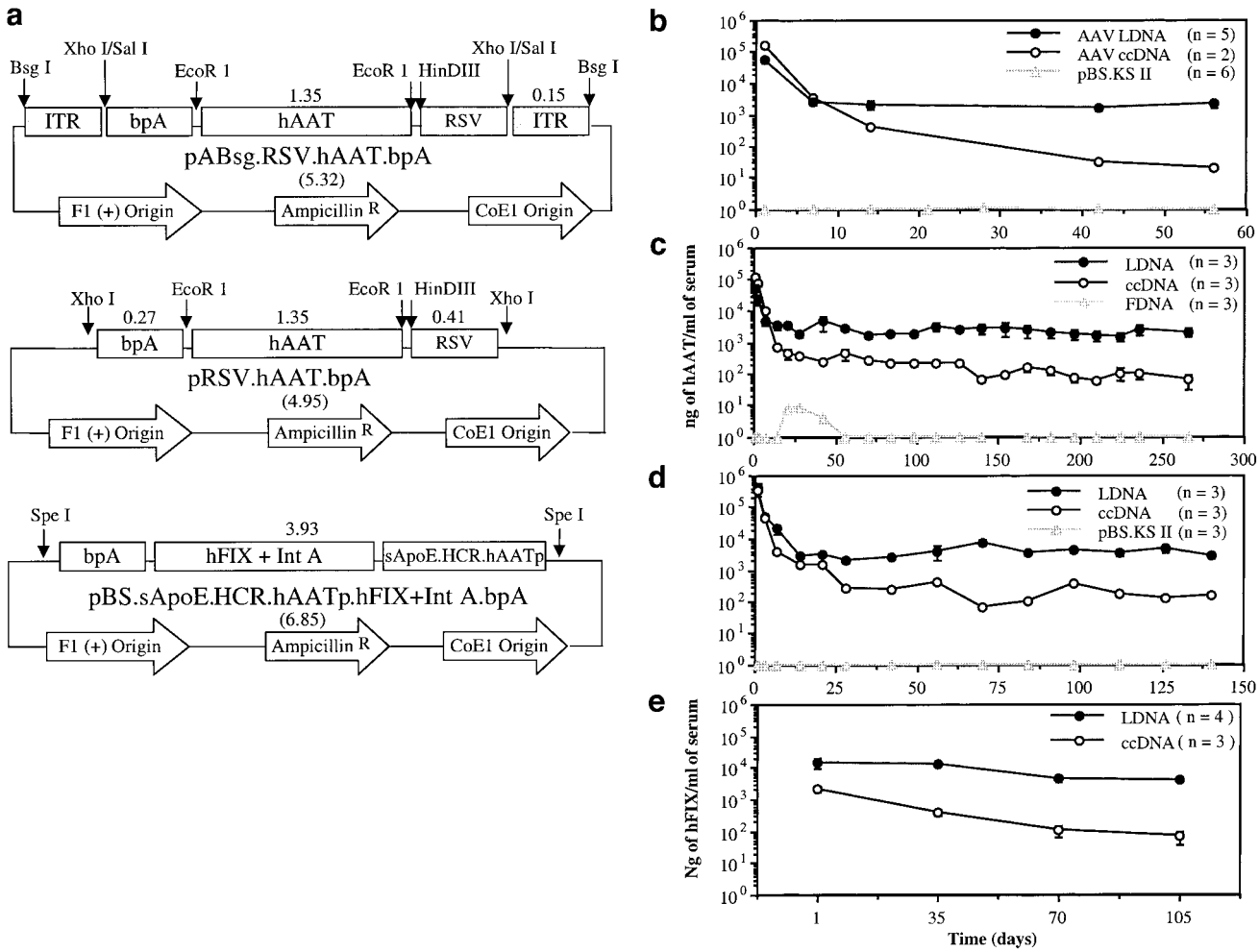

FIG. 1. Human α1-antitrypsin and factor IX expression in LDNA- and ccDNA-injected mice. (a) Schematic of vectors containing a human α1-antitrypsin (hAAT) expression cassette with (pABsg.RSV.hAAT.bpA) and without (pRSV.hAAT.bpA) flanking AAV ITR sequences or a human factor IX expression cassette (pBS.sApoE.HCR.hAATp.hFIX+Int A.bpA). LDNAs were prepared by digesting pABsg.RSV.hAAT.bpA, pRSV.hAAT.bpA, and pBS.sApoE.HCR.hAATp.hFIX+Int A.bpA with *Bsg*I, *Xho*I, and *Spe*I, respectively. RSV, Rous sarcoma virus long terminal repeat promoter; sApoE.HCR, apolipoprotein E locus control region, hAATp, the hAAT promoter; Int A, a 1.4-kb fragment of the first intron of hFIX; bpA, bovine growth hormone polyadenylation signal; ITR, inverted terminal repeat from adeno-associated virus (AAV). (b) Serum hAAT levels in C57BL/6 mice injected with 40 μg of pABsg.RSV.hAAT.bpA (AAV ITR) LDNA (n = 5) or ccDNA (n = 2) or pBS.KS II control DNA (n = 6). Mean values ± standard error are shown. (c) Serum hAAT levels in mice (n = 3 mice per group) injected with 40 μg of pRSV.HAAT.bpA (non-AAV ITR) LDNA or ccDNA or FDNA. FDNA is a control DNA in which the hAAT expression cassette was fragmented by *Bsr*DI digestion prior to injection. (d) An independent repeat of the experiment described in (c) (n = 6 mice per group), with pBS.KS II plasmid DNA used as a negative control. Ten weeks after vector administration, 3 mice in each group were sacrificed for Southern blot analyses. (e) Human FIX expression in hemophilia B mice receiving 40 μg of pBS.sApoE.HCR.hAATp.hFIX+Int A.bpA LDNA (n = 4) or ccDNA (n = 3).

analyses at different times after vector administration. Quantitative phosphoimager analyses indicated similar levels of LDNA (38 ± 4 copies/cell, n = 3) and ccDNA (48 ± 9 copies/cell, n = 3) in treated mice 1 day after DNA delivery (Fig. 2a). In all cases, this initial amount of vector DNA diminished an average of 78% over a 5-week period, but appeared to remain stable for at least an additional 5 weeks. Mice receiving AAV LDNA and AAV ccDNA showed a similar transient loss of vector DNA over the same time period (Fig. 2a). More importantly, the quantities of vector in the livers of LDNA- and ccDNA-treated mice were indistinguishable at all time points, indicating no differential loss of vector DNA in cells transfected with LDNA and ccDNA. Furthermore, *in situ* hybridization of liver sections from LDNA- and ccDNA-treated mice showed that the relative number of hepatic nuclei containing plasmid DNA genomes was similar (~10%) between ccDNA- (Fig. 2b) and LDNA-injected mice (Fig. 2c). Taken together, these results indicate that the profound drop in transgene expression observed in ccDNA- versus LDNA-treated mice cannot be attributed to either the differential loss or the cellular distribution of vector DNA within transfected cells.

Molecular Structure of DNA in Vivo

Based on the findings that equal amounts of ccDNA and LDNA remained in liver, we tested whether there was any discernable difference in the molecular structures of

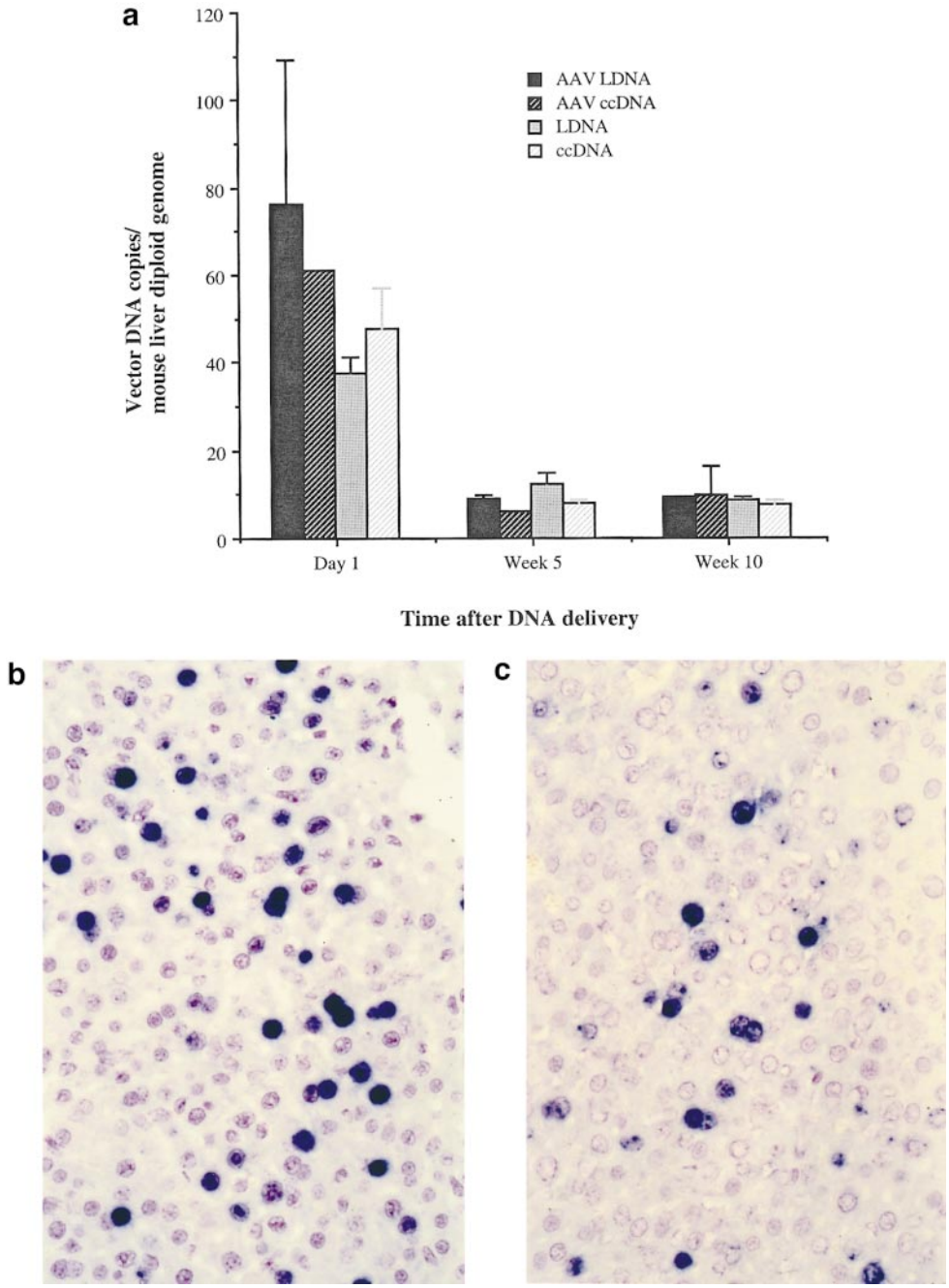

FIG. 2. Vector DNA quantification and distribution in mouse hepatocytes. (a) Vector DNA copy number in each group (n = 2–3 mice per group) over time (mean ± standard error). 20 μg mouse liver DNA was digested with *Eco*RI, separated by gel electrophoresis, and transferred to a nitrocellulose membrane. Blots were hybridized to a 1.35-kb radiolabeled hAAT cDNA probe and subjected to autoradiography. To determine vector copy number, each blot contained a series of standards, consisting of 20 μg *Eco*RI-digested naïve genomic liver DNA spiked with 400, 100, 25, 5, or 0 copies of a 1.35-kb hAAT cDNA fragment. Signals were quantified by phosphoimager analyses. (b and c) DNA *in situ* hybridization. Mice were sacrificed 2 to 3 weeks after injection with either ccDNA or LDNA and their livers sectioned and probed for vector genomes. We examined multiple sections from each mouse (n = 2 mice/group) and determined the relative number of DNA-positive nuclei in each animal. Shown are representative examples from a (b) ccDNA- and a (c) LDNA-treated animal.

ccDNA and LDNA within this tissue. To do this, we performed Southern blot analyses on liver DNA samples from ccDNA-treated mice (Fig. 3a) following digestion with either *Bgl*II (left), which did not cut the input DNA, or *Hin*dIII (right), which cut once through the hAAT expression cassette. Analysis of *Bgl*II-treated ccDNA indicated that the input circular DNA remained intact and migrated as single-copy or multiple-copy aggregates (Fig. 3a, left), which could be converted to a single full-length linear molecule following *Hin*dIII digestion (one-cutter) (Fig. 3a, right). Similar results were obtained with AAV ccDNA (Fig. 3b) and were consistent with the notion that closed cir-

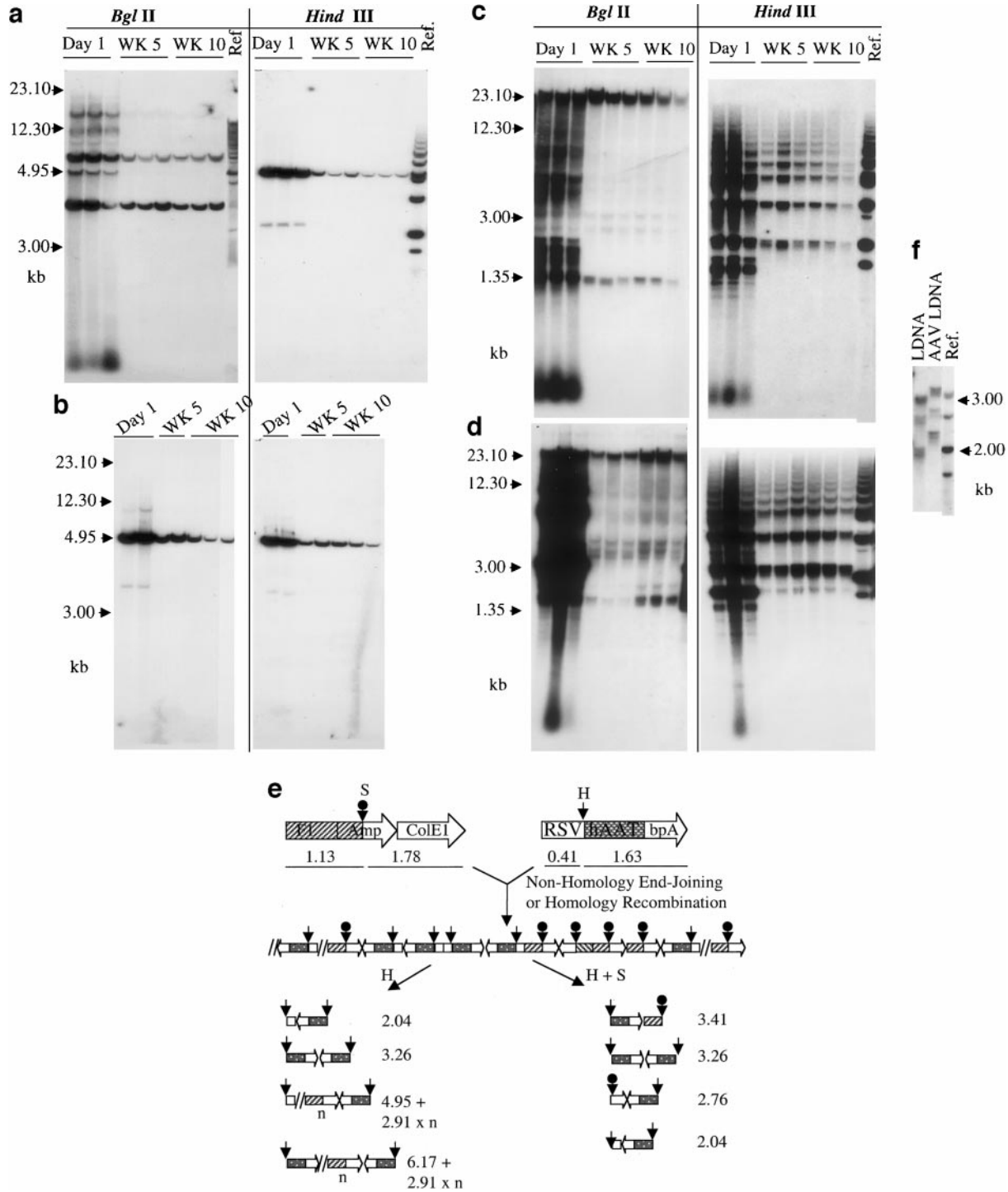

FIG. 3. Molecular structure of ccDNA and LDNA after delivery to mouse liver. (a and b) Southern blot analyses of (a) pRSV.hAAT.bpA (non-AAV ITR) ccDNA- and (b) pABsg.RSV.hAAT.bpA (AAV ITR) ccDNA-treated animals. 20 μg of total liver DNA was digested with either *Bgl*II, which does not cut the vector DNA (left), or *Hin*dIII, which cuts the vector DNA once (right). Digested samples were separated by gel electrophoresis, transferred to a membrane, and hybridized to a 1.35-kb hAAT cDNA fragment. Sizes are in kb. Ref, reference DNA was produced by adding *in vitro* ligated *Xho*I-digested pRSV.hAAT.bpA (LDNA), 20 μg naïve mouse liver DNA, followed by *Hin*dIII digestion. (c and d) Southern blot analyses of (c) pRSV.hAAT.bpA (non-AAV LTR) LDNA- and (d) pABsg.RSV.hAAT.bpA (AAV LTR) LDNA-injected animals. 20 μg total liver DNA was digested with either *Bgl*II (left) or *Hin*dIII (right). (e) Schematic illustration of possible concatemers formed in mouse livers after random end-joining of two linear DNA fragments. Assuming random linkage of DNA fragments, treatment with *Hin*dIII (H) and *Sca*I (S) should produce the four hAAT-containing fragments of the denoted length (in kb). (f) Representative liver DNA samples from a week 5 LDNA- and AAV LDNA-injected animal after digestion with both *Hin*dIII and *Sca*I. The decreased mobility of the fragments produced by AAV LDNA compared to the reference DNA (*in vitro*-ligated LDNA cut with *Hin*dIII and *Sca*I) corresponds to the presence of one or two 145-bp AAV ITRs. The top band represents the 3.41- and 3.26-kb products, which cannot be separated under these electrophoresis conditions.

cular plasmid DNA remains as an episomal, circular monomer in the liver from day 1 onward.

Interestingly, *Bgl*II-treated LDNA samples produced a prominent high-molecular-weight (>20-kb) band in all day 1 samples, which appeared to remain stable over a 10-week period (Fig. 3c, left). These results were consistent with either the integration of linear DNA into the mouse genome or the rapid formation of concatemers *in vivo* from small linear DNA fragments. To distinguish between these two possibilities, we digested liver DNA samples with *Hin*dIII, which cuts once through the hAAT expression cassette (Fig. 3c, right). This converted the high-molecular DNA signal into a DNA ladder composed of fragments ranging from ~2 to >12.3 kb, each of which comigrated exactly with fragments produced by *in vitro* ligation of LDNA (Fig. 3c, Ref. lane). Similar results were obtained with liver DNA from AAV LDNA-treated mice digested with enzymes that do not cut or cut once within the expression cassette (Fig. 3d). Therefore, LDNA-based vectors can efficiently concatemerize *in vivo* and remain transcriptionally active for at least 9 months.

In addition to the high-molecular-weight concatemers, there was a ~1.35 kb hAAT-containing DNA band present in liver DNA isolated from LDNA-treated mice, digested with an enzyme (*Bgl*II) that did not cut within the vector (Fig. 3c, left). The structure of this small DNA band was not definitively identified but was likely a circular hAAT expression cassette because it was not present in *Hin*dIII-digested DNA. The role, if any, that this molecular DNA form played in the persistence of gene expression is not known.

If we assume that linkage of the DNA fragments occurred randomly in a head-to-head, tail-to-tail, and head-to-tail orientation, then digestion of LDNA and AAV LDNA liver samples with a pair of restriction enzymes that cut once in the hAAT expression cassette (*Hin*dIII) and once in the bacterial plasmid (*Sca*I) should produce four fragments that would hybridize with the hAAT cDNA probe as illustrated in Fig. 3e. Therefore, we compared the restriction patterns produced after *Hin*dIII/*Sca*I double digestion of *in vitro*-ligated LDNA to those of representative liver samples from LDNA- and AAV LDNA-treated mice. Results are shown in Fig. 3f and are consistent with random linkage of DNA fragments in mouse liver (the top band is the combination of the 3.21- and 3.41-kb bands, the 1.6-kb band in the reference lane corresponds to an unligated *Hin*dIII-digested LDNA fragment, and the decreased mobility of the bands in the AAV LDNA lane corresponds to the presence of one or two 145-bp AAV ITRs in these vectors). The two lower DNA bands in both samples are not as sharp as the corresponding bands of the reference, suggesting a difference in the size of the DNA fragments joined by the same two DNA ends, probably reflecting the differences in end modification and/or in the mechanisms of end-joining. We are currently conducting experiments to further characterize these DNA junctions.

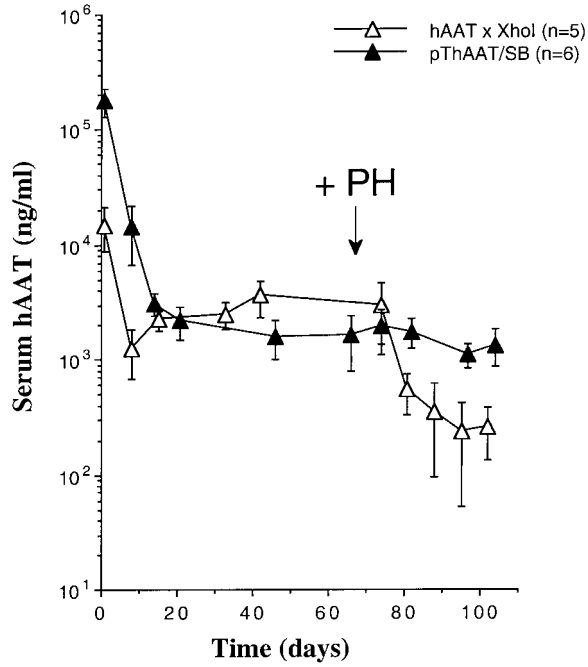

FIG. 4. Extrachromosomal status of LDNA in mouse liver. Animals (n = 5–6 mice per group) received 25 μg *Xho*I-treated pRSV.hAAT.bpA LDNA or a mixture of 25 μg pThAAT and 1 μg pCMV-SB, which results in integration of the hAAT expression cassette (15). This served as a positive control for vector integration *in vivo*. Approximately 10 weeks after DNA administration, treated animals were subjected to a 2/3 surgical partial hepatectomy to facilitate hepatocellular division and loss of extrachromosomal DNA. Reporter gene expression was monitored before and after partial hepatectomy by serum ELISA (19).

Linear DNAs Are Not Integrated

Based on the Southern analyses, the majority of the high-molecular-weight DNA originated from concatemers. These studies could not distinguish between extrachromosomal and integrated concatemers in the mouse genome. To distinguish between these two possibilities, we subjected mice injected with either LDNA or an integrating hAAT transposon (15) to a 2/3 partial hepatectomy. In the transposon group, the transposase, expressed from one plasmid, mediated the release of the hAAT expression cassette flanked by the transposon ITR from a second plasmid and the insertion of the released transgene expression cassette into the mouse genome. The partial hepatectomy results in one or two rounds of hepatocyte cell division and a significant loss of extrachromosomal DNA. Results of these studies indicate that in contrast to integrating plasmid-infused mice, whose transgene expression was unchanged by the induction of hepatocyte proliferation, LDNA-treated mice showed a 10-fold drop in gene expression after partial hepatectomy over the same time period (Fig. 4). Although we cannot exclude the possibility that some low level of LDNA integration occurred *in vivo*, these data were consistent with our Southern blot analyses and together they indicate that transcriptionally active LDNA vectors remained predominantly extrachromosomal in the liver.

DISCUSSION

Concatemerization of exogenous DNA in mammalian cells is a well-characterized biological process (25–28) that has been used for the production of transgenic mice (29) and stable cell lines (26). The introduction of linear plasmid DNAs has been shown to be more efficient than circular plasmids to stably transform cells in culture (26). In a separate study, when linear molecules were ligated into concatemers prior to gene transfer into tissue culture cells an approximately 14-fold increase in transient gene expression was observed although persistence of gene expression was not addressed (30). As a result of stable transfection of cells in culture with circular or linear DNAs integrated head-to-tail concatemers are formed (26). In contrast, in our study, concatemers were detected when linear but not circular DNAs were injected into mice. Moreover, the concatemers were randomly linked and not integrated. Some of the observed differences may be due to the fact that in culture, stably transfected cells are usually selected for and represent rare events. However, at this time, we cannot establish if somatically transfected hepatocytes *in vivo* and cells in culture undergo similar mechanistic processes.

The introduction of linear DNA into cells likely induces the enzymatic systems that are responsible for DNA double-strand break repair. Two general processes are possible, homologous recombination or nonhomologous DNA end-joining (NHEJ). NHEJ is a complex process believed to be the major mechanism involved in ligating DNA ends in mammalian cells (31). At least 11 enzymes and proteins are involved in the process of NHEJ (27). The process is complex because for intermolecular joining to occur, the protein complexes must form and join the free ends. The final ligation step involves the interaction of the protein DNA end with DNA ligase IV (27, 32–34). It is likely that the concentration of linear DNA molecules could affect the efficiency of the process and in some cases intra- vs intermolecular ligations may be favored. The final proportion of linear versus circular concatemers will require further study. Molecular analyses of the ligated ends as well as the ability to form concatemers with noncompatible ends will give insight as to which mechanism is more predominant in liver. Our preliminary studies with linear DNA molecules generated by digestion with two enzymes producing noncompatible ends show similar concatemer formation *in vivo* (not shown).

Our study clearly demonstrates that mouse liver is highly efficient in linking linear DNAs into complex and random unintegrated high-molecular-weight concatemers. This correlates with 10- to 100-fold higher levels of gene expression compared with the equivalent circular DNA. Because there was no difference in the total amount of DNA or the relative number of hepatic nuclei containing vector DNA in mice injected with linear or circular DNAs, we hypothesize that the large concatemers are more likely to be in a transcriptionally active conformation than circular plasmid. Our hypothesis is consistent with the recent finding that rAAV proviral vector genomes concatemerize *in vivo,* resulting in stable gene expression from multiple tissues, including liver (8, 9, 22) and muscle (10–12). We compared the effects of linearization of circular DNAs with and without AAV ITRs on gene transfer and expression in the livers of mice and found no difference. This suggests that the AAV ITRs may be dispensable for the DNA concatemerization or the mechanisms involved in the process are different between linear double-stranded DNAs and AAV genomes.

The central question arises with linear episomal vectors if concatemer formation itself or other factors are responsible for maintaining transcriptional activity. Nonreplicating episomal linear adenoviral vector genomes are transcriptionally persistent (in the absence of an immune response) in liver (35, 36) and are believed to primarily exist as monomers, not concatemers (8, 37). Random concatemer formation has been observed with Ad-E4-deficient adenoviral genomes in cultured cells but this may be linked to the process of viral DNA replication (38–39). Perhaps the presence of the preterminal protein on the end of non-replication-competent vector genomes somehow also interferes with the cellular machinery responsible for concatemerization. Whether the process that maintains transcriptional activity of adenoviral genomes is similar to that observed with linear DNAs is not known.

The sequences and positioning of the bacterial backbone may play an important role in inhibiting transcription from plasmid DNA that is altered and somehow lost in random concatemeric DNAs. The inhibitory process may influence chromatin assembly and/or methylation of the DNA. The process of random concatemer formation might physically disrupt the inhibitory process, resulting in the maintenance of transcriptional activity. As demonstrated by Kass *et al.*, a methylation center can result in chromatin condensation that can spread to a downstream promoter and result in silencing of the gene (40). Conceivably, the silencing effect will be disrupted if the methylation center has been physically distanced from the promoter as might occur in random concatemers. Furthermore, the rare plasmids that have resulted in persistent gene expression (2, 7) *in vivo* may contain insulators or other elements that block the inhibitory effects of the bacterial backbone. Further experiments are required to examine this hypothesis. Nevertheless, the elucidation of the involved mechanisms will be important for further predictive design of plasmid-based vectors. More experiments to determine if this phenomenon occurs in other tissues will need to be conducted. Nonetheless, with the combination of optimal *cis* DNA elements and the linearization of plasmid DNA prior to gene transfer, clinical application of non-viral-mediated gene transfer becomes a reasonable approach for the treatment of genetic and acquired diseases.

ACKNOWLEDGMENTS

This work was supported by a grant from Hepavec, a company in which M.A.K. holds equity, and by NIH Grant DK49022.

REFERENCES

[1] Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* **247:** 1465–1468.

[2] Zhang, G., Song, Y. K., and Liu, D. (2000). Long-term expression of human alpha 1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using hydrodynamics-based procedure. *Gene Ther.* **7:** 1344–1349.

[3] Hofland, H., and Huang, L. (1995). Inhibition of human ovarian carcinoma cell proliferation by liposome-plasmid DNA complex. *Biochem. Biophys. Res. Commun.* **207:** 492–496.

[4] Foglieni, C., Bragonzi, A., Cortese, M., Cantu, L., Boletta, A., Chiossone, I., Soria, M. R., and Monaco, L. (2000). Glomerular filtration is required for transfection of proximal tubular cells in the rat kidney following injection of DNA complexes into the renal artery. *Gene Ther.* **7:** 279–285.

[5] Zou, S. M., Erbacher, P., Remy, J. S., and Behr, J. P. (2000). Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* **2:** 128–134.

[6] Liu, F., Song, Y., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6:** 1258–1266.

[7] Miao, C. H., Ohashi, K., Patijn, G. A., Meuse, L., Ye, X., Thompson, A. R., and Kay, M. A. (2000). Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression *in vivo* but not *in vitro*. *Mol. Ther.* **1:** 522–532.

[8] Miao, C. H., Snyder, R. O., Schowalter, D. B., Patijn, G. A., Donahue, B., Winther, B., and Kay, M. A. (1998). The kinetics of rAAV integration in the liver. *Nat. Genet.* **19:** 13–15.

[9] Miao, C. H., Nakai, H., Thompson, A. R., Storm, T. A., Chiu, W., Snyder, R. O., and Kay, M. A. (2000). Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes in vivo: Cell cycling does not influence hepatocyte transduction. *J. Virol.* **74:** 3793–3803.

[10] Yang, J., Zhou, W., Zhang, Y., Zidon, T., Ritchie, T., and Engelhardt, J. F. (1999). Concatemerization of adeno-associated virus circular genomes occurs through intermolecular recombination. *J. Virol.* **73:** 9468–9477.

[11] Duan, D., Sharma, P., Dudus, L., Zhang, Y., Sanlioglu, S., Yan, Z., Yue, Y., Ye, Y., Lester, R., Yang, J., Fisher, K. J., and Engelhardt, J. F. (1999). Formation of adeno-associated virus circular genomes is differentially regulated by adenovirus E4 ORF6 and E2a gene expression. *J. Virol.* **73:** 161–169.

[12] Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K. J., and Engelhardt, J. F. (1998). Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol.* **72:** 8568–8577. [Published erratum appears in *J. Virol.,* 1999, **73:** 861]

[13] Wu, G. Y., and Wu, C. H. (1987). Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* **262:** 4429–4432. [Published erratum appears in *J. Biol. Chem.,* 1988, **263:** 588]

[14] Nishikawa, M., Yamauchi, M., Morimoto, K., Ishida, E., Takakura, Y., and Hashida, M. (2000). Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. *Gene Ther.* **7:** 548–555.

[15] Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z., and Kay, M. A. (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat. Genet.* **25:** 35–41.

[16] Zhang, G., Budker, V., and Wolff, J. A. (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10:** 1735–1737.

[17] Alexander, I. E., Russell, D. W., and Miller, A. D. (1994). DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J. Virol.* **68:** 8282–8287.

[18] Park, F., Ohashi, K., Chiu, W., Naldini, L., and Kay, M. A. (2000). Efficient lentiviral transduction of liver requires cell cycling in vivo. *Nat. Genet.* **24:** 49–52.

[19] Kay, M. A., Graham, F., Leland, F., and Woo, S. L. (1995). Therapeutic serum concentrations of human alpha-1-antitrypsin after adenoviral-mediated gene transfer into mouse hepatocytes. *Hepatology* **21:** 815–819.

[20] Walter, J., You, Q., Hagstrom, J. N., Sands, M., and High, K. A. (1996). Successful expression of human factor IX following repeat administration of adenoviral vector in mice. *Proc. Natl. Acad. Sci. USA* **93:** 3056–3061.

[21] Snyder, R. O., Spratt, S. K., Lagarde, C., Bohl, D., Kaspar, B., Sloan, B., Cohen, L. K., and Danos, O. (1997). Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum. Gene Ther.* **8:** 1891–1900.

[22] Nakai, H., Storm, T. A., and Kay, M. A. (2000). Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver in vivo. *J. Virol.* **74:** 9451–9463.

[23] Yang, C. C., Xiao, X., Zhu, X., Ansardi, D. C., Epstein, N. D., Frey, M. R., Matera, A. G., and Samulski, R. J. (1997). Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration in vivo and in vitro. *J. Virol.* **71:** 9231–9247.

[24] Snyder, R. O., Miao, C. H., Patijn, G. A., Spratt, S. K., Danos, O., Nagy, D., Gown, A. M., Winther, B., Meuse, L., Cohen, L. K., Thompson, A. R., and Kay, M. A. (1997). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet.* **16:** 270–276.

[25] Wilson, J. H., Berget, P. B., and Pipas, J. M. (1982). Somatic cells efficiently join unrelated DNA segments end-to-end. *Mol. Cell. Biol.* **2:** 1258–1269.

[26] Folger, K. R., Wong, E. A., Wahl, G., and Capecchi, M. R. (1982). Patterns of integration of DNA microinjected into cultured mammalian cells: Evidence for homologous recombination between injected plasmid DNA molecules. *Mol. Cell. Biol.* **2:** 1372–1387.

[27] Critchlow, S. E., and Jackson, S. P. (1998). DNA end-joining: From yeast to man. *Trends Biochem. Sci.* **23:** 394–398.

[28] Wu, X., Wilson, T. E., and Lieber, M. R. (1999). A role for FEN-1 in nonhomologous DNA end joining: The order of strand annealing and nucleolytic processing events. *Proc. Natl. Acad. Sci. USA* **96:** 1303–1308.

[29] Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K., and Palmiter, R. D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA* **82:** 4438–4442.

[30] Leahy, H., Carmichael, G. G., and Rossomando, E. F. (1997). Transcription from plasmid expression vectors is increased up to 14 fold when plasmids are transfected as concatemers. *Nucleic Acids Res.* **25:** 449.

[31] Labhart, P. (1999) Nonhomologous DNA end joining in cell-free systems. *Eur. J. Biochem.* **265:** 849–861.

[32] Grawunder, U., Zimmer, D., Kulesza, P., and Lieber, M. R. (1998). Requirement for an interaction of XRCC4 with DNA ligase IV for wild-type V(D)J recombination and DNA double-strand break repair in vivo. *J. Biol. Chem.* **273:** 24708–24714.

[33] Grawunder, U., Zimmer, D., and Leiber, M. R. (1998). DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. *Curr. Biol.* **8:** 873–876.

[34] Grawunder, U., Zimmer, D., Fugmann, S., Schwarz, K., and Lieber, M. R. (1998). DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. *Mol. Cell* **2:** 477–484.

[35] Morral, N., O'Neal, W., Rice, K., Leland, M., Kaplan, J., Piedra, P. A., Zhou, H., Parks, R. J., Velji, R., Aguilar-Cordova, E., Wadsworth, S., Graham, F. L., Kochanek, S., Carey, K. D., and Beaudet, A. L. (1999). Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc. Natl. Acad. Sci. USA* **96:** 12816–12821.

[36] Barr, D., Tubb, J., Ferguson, D., Scaria, A., Lieber, A., Wilson, C., Perkins, J., and Kay, M. A. (1995). Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes in vivo: Comparisons between immunocompetent and immunodeficient inbred strains. *Gene Ther.* **2:** 151–155.

[37] Lieber, A., He, C. Y., and Kay, M. A. (1997). Adenoviral preterminal protein stabilizes mini-adenoviral genomes in vitro and in vivo. *Nat. Biotechnol.* **15:** 1383–1387.

[38] Weiden, M. D., and Ginsberg, H. S. (1994). Deletion of the E4 region of the genome produces adenovirus DNA concatemers. *Proc. Natl. Acad. Sci. USA* **91:** 153–157.

[39] Boyer, J., Rohleder, K., and Ketner, G. (1999). Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. *Virology* **263:** 307–312.

[40] Kass, S. U., Landsberger, N., and Wolffe, A. P. (1997). DNA methylation directs a time-dependent repression of transcription initiation. *Curr. Biol.* **7:** 157–165.