A Novel Mechanism for Achieving Transgene Persistence in Vivo after Somatic Gene Transfer into Hepatocytes

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Infusion of hepatocyte-specific DNA-protein complexes into rats leads to transient recombinant gene expression in vivo. The eventual deterioration of gene expression is due in part to instability of the targeted DNA. In a previous report, we noted retention of transgene sequences in liver and persistent recombinant gene expression when the animals were subjected to partial hepatectomy following in vivo gene transfer. In an attempt to define the mechanism(s) responsible for persistent gene expression following partial hepatectomy, we characterized the molecular state of the retained, liver-associated transgenes. Southern blot analysis of DNA from liver tissues harvested various times after in vivo gene transfer and partial hepatectomy (10 min to 11 weeks) demonstrated high levels of transgene DNA (100–10,000 copies/cell). The predominant form of this DNA appeared to be episomal based on analyses of uncut DNA or DNA restricted by an endonuclease with one site in the plasmid. Livers from several animals contained a small proportion of transgene sequences of unknown structure. The existence of episomal DNA in liver was confirmed in experiments in which intact plasmid was rescued from total hepatocyte DNA by transformation of bacteria. Both strands of DNA in the liver-associated plasmid retained a bacterial pattern of methylation suggesting that the plasmid had not replicated in the eukaryotic cell. These results are consistent with the hypothesis that the majority of transgene sequences are retained as stabilized plasmids. The specific form of DNA which is transcriptionally active was not identified in these studies. This represents a new mechanism for retaining foreign DNA in eukaryotic cells in vivo and has implications both for the development of somatic gene therapies and the pathogenesis of viral diseases.

Somatic cell gene transfer has been a powerful tool in the study of eukaryotic cell biology. Many early techniques, such as transfection, proved to be extremely useful despite the fact that the efficiency of gene transfer was extremely low. The development of more efficient approaches to somatic gene transfer based on recombinant viruses expanded the potential utility of this technology into the clinical arena. The ability to efficiently transduce recombinant genes into cultured human cells suggested a novel approach for treating genetic disease called ex vivo gene therapy. By using this strategy, autologous somatic cells such as lymphocytes or hepatocytes are genetically modified with recombinant viruses ex vivo and subsequently transplanted into the animal/patient from which they were derived (Friedman, 1989). More direct approaches to gene therapy, based on the delivery of the recombinant gene to somatic cells in vivo, are clearly needed. Recombinant viruses have been less useful in the development of these strategies because of the nonspecificity of gene transduction and concerns over safety.

We have designed an approach for reconstituting hepatic expression of a gene in vivo that may have applications in the treatment of human metabolic diseases (Wu and Wu, 1987, 1988; Wu et al., 1989, 1991). A nonviral substrate capable of delivering a recombinant gene directly to hepatocytes is constructed in the following manner. A polypeptide ligand for the hepatocyte-specific asialoglycoprotein receptor is conjugated to poly-L-lysine, and the protein conjugate is complexed through electrostatic interactions with a plasmid-based expression vector. Experiments in cultured cells indicate that the DNA-protein complex is internalized via the asialoglycoprotein-protein receptor and the recombinant gene is expressed transiently (Wu and Wu, 1987). A similar strategy has been developed for targeting genes to cells that express the transferrin receptor (Curiel et al., 1991; Wagner et al., 1990; Wagner et al., 1991; and Zenke et al., 1990).

Early studies on hepatocyte targeting utilized a DNA-protein complex capable of expressing the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Analysis of rats following intravenous administration of the DNA-protein complex showed that 1) the DNA-protein complex is rapidly cleared from the circulation by liver-specific uptake mechanisms (Wu and Wu, 1988), 2) high levels of the recombinant plasmid can be delivered to hepatocytes (Wu and Wu, 1988), 3) the intracellular plasmid is degraded to undetectable levels within 48 h (Wu and Wu, 1988), and 4) the CAT enzyme is expressed transiently in liver for approximately 3 days (Wu and Wu, 1988). The deterioration of recombinant gene expression in liver after in vivo gene transfer is likely due to degradation of the plasmid DNA within the cell. For this approach to have applications in the treatment of most metabolic diseases, it will be necessary to achieve a more prolonged if not permanent expression of the transgene.

In previous studies, an attempt was made to obtain pro-

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The abbreviations used are: CAT, chloramphenicol acetyltransferase; HSA, human serum albumin; LDL, low density lipoprotein; bp, base pair(s); LDLR, low density lipoprotein receptor; HBV, hepatitis B virus.
longed transgene expression by introducing the recombinant gene into animals in which the target hepatocytes were dividing actively. This was accomplished by performing a partial hepatectomy immediately following in vivo gene transfer (Wu et al., 1989, 1991). Animals subjected to this procedure retained high levels of the transgene in liver and continued to express the recombinant protein product for the duration of the experiments (at least 4 months; Wu et al., 1988, 1991). Two rodent models, Sprague-Dawley rats expressing CAT (Wu et al., 1989) and Nagase analbuminemic rats (NAR) expressing human serum albumin (HSA, Wu et al., 1991) have been used to demonstrate long term expression of the transgene. The focus of this study is to delineate the molecular basis for transgene persistence following partial hepatectomy.

MATERIALS AND METHODS

Plasmids and DNA-Protein Complexes—A series of three vectors were used in this study. They were expanded in dam’ strain of bacteria (DH5α) and purified on cesium gradients. Each contains a minigene with promoter and enhancer sequences from the rodent albumin gene and a poly(A) tail, and was used to drive expression of one of three genes: 1) the human LDL receptor cDNA (p9-12alb(h)LDLR, Wilson et al., 1992); cDNA for human LDL receptor, p9-12alb(h)LDLR (Wilson et al., 1992); cDNA for human serum albumin, p9-12albHSA (Wu et al., 1991); and the gene for chloramphenicol acetyltransferase, p9-12albCAT (Wu et al., 1989). Plasmids were complexed with protein conjugates of asialoorosomucoid and poly-L-lysine as previously described (Wu et al., 1989, 1991; Wilson et al., 1992).

Animals and Surgical Procedures—Female Sprague-Dawley rats were injected intravenously with 500 μg of plasmid DNA in the form of DNA-protein complexes and subjected to a partial hepatectomy. These animals were injected with p9-12alb(h)LDLR plasmid DNA-protein complex had a partial hepatectomy 10 min after injection and was sacrificed 2 weeks later. The animals injected with p9-12albHSA DNA-protein complex had partial hepatectomies 10 min after injection, lobectomies 2 weeks after injection, and were sacrificed 4 weeks after injection (Wu et al., 1991). The animal injected with p9-12albCAT DNA-protein complex had a partial hepatectomy 30 min after injection, a lobectomy 9 weeks after injection, and was sacrificed 11 weeks after injection (Wu et al., 1989). Liver tissue was frozen in liquid nitrogen immediately after removal from animals and stored at -70°C.

DNA Extractions—Total cellular DNA was extracted in the following manner. Liver tissue was homogenized in 4.5 ml of 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 1 mM EDTA (called TNB) and 1 mg of Proteinase K in a glass Dounce. Sodium dodecyl sulfate (SDS) was then added to a final concentration of 1% and the mixture was placed at 37°C overnight. The tissue was extracted in phenol, and the DNA was precipitated with 2 volumes of isopropanol, air dried, and resuspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA (called TE). DNA enriched for extrachromosomal forms (subsequently called “Extrachromosomal DNA”) was isolated from liver by homogenizing liver tissue in 4 ml of 10 mM Tris-HCl, pH 8.0, containing 10 mM EDTA. SDS was added to a concentration of 0.6%, and the mixture was incubated at room temperature for 15 min. One-fourth volume of 5 M NaCl was added, and the solution was mixed by gentle inversion and placed at 4°C overnight. This solution was centrifuged at 17,000 X g for 45 min at 4°C. The supernatant was recovered and digested with Proteinase K (1 mg/ml) for 60 min at 65°C and extracted sequentially with phenol, phenol-chloroform, and concentrated with butanol. DNA was precipitated by the addition of 0.5 volumes of 7 M ammonium acetate and 2 volumes of ice-cold 100% ethanol. Precipitated DNA was washed once with 70% ethanol, air dried, and resuspended in 0.1 ml of TE. DNA was quantified by measuring absorbance at 260 nm.

Southern Blots—DNA was digested with the appropriate restriction endonuclease and transferred to Zetabind nitrocellulose filter or Hybond-N nylon filter as described (Wilson et al., 1989). DNA fragments separated on gels were then labeled with [α-32P]dCTP by random priming. A 735-bp EcoRI to HindIII fragment contained in the human LDLR cDNA (Wilson et al., 1992) was used to detect the presence of the p9-12alb(h)LDLR plasmid (probe L, Fig. 1). A 1088-bp BamHI to BgIII fragment from p9-12albHSA (Wu et al., 1991) was used to detect the presence of p9-12albHSA (probe H, Fig. 1). A 301-bp EcoRI to EcoRI fragment from p9-12albCAT (Wu et al., 1989) was used to detect the presence of the p9-12albCAT plasmid (probe C, Fig. 1). In one experiment, transgene sequences in uncultured genomic DNA were detected with a 2308-bp EcoRI to BamHI fragment of p9-12albCAT that spans a portion of the plasmid backbone (probe P, Fig. 1).

RESULTS AND DISCUSSION

Partial Hepatectomy Following In Vivo Gene Transfer Leads to Retention of Transgene Sequences—In previous studies, we observed that animals retain high levels of transgene sequences in liver for prolonged periods of time when infused with DNA-protein complexes and subjected to partial hepatectomy soon thereafter (Wu et al., 1989, 1991). However, the molecular state of the transgene in those animals was not delineated.

Randomly selected animals that received intravenous injection of DNA-protein complexes containing one of three different plasmids were analyzed in detail with respect to the molecular state of the retained DNA. Structures for the plasmids are shown in Fig. 1. In each case, transcriptional elements from the rodent albumin genes were used to drive expression of one of three genes: 1) the human LDL receptor cDNA (p9-12alb(h)LDLR, Wilson et al., 1992); 2) the human serum albumin cDNA (p9-12albHSA, Wu et al., 1991); and 3)
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the bacterial chloramphenicol acetyltransferase (p9-12albCAT) gene (Wu et al., 1989). Experiments with the p9-12alb(h)LDLR plasmid represent a single animal in which liver tissue was removed at 10 min (the time of partial hepatectomy) and at 2 weeks (the time at which the animal was sacrificed) after injection. Two animals that received the p9-12albHSA vector were studied (labeled A5 and A10). For each animal, tissue was harvested at 2 weeks (the time of a second partial hepatectomy) and at 4 weeks (the time at which the animal was sacrificed) after injection. A single animal that received the p9-12albCAT vector was analyzed; liver was removed at 8 weeks (the second partial hepatectomy) and 11 weeks following DNA-protein complex injection (the time at which the animal was sacrificed).

Total cellular DNA was harvested from liver tissues and analyzed by the method of Southern to estimate the relative copy number of the transgene (Fig. 2). The experiments were performed with restriction endonucleases that have at least two recognition sites within each respective plasmid. Digestion of total cellular DNA with this type of endonuclease should release an internal restriction fragment whose size is independent of the molecular state of the transgene.

The animal injected with p9-12alb(h)LDLR complex demonstrated a typical time course of DNA persistence (Fig. 2A). Approximately 2,000 copies of plasmid were taken up by the liver within 10 min. Southern blot analysis indicated that a substantial amount of it was degraded at this early time point (Fig. 2A, lane 3). While the result with p9-12alb(h)LDLR was typical, the amount of liver-associated DNA at 10 min varied with different preparations of DNA-protein complex. The animal injected with p9-12alb(h)LDLR complex retained 1,000 copies of undegraded DNA 2 weeks after gene transfer (Fig. 2A, lane 4). Similar levels of undegraded transgene were detected in tissues harvested 2 and 4 weeks after injection of p9-12albHSA (Fig. 2B, lanes 5–8) and 8 and 11 weeks after injection of p9-12albCAT complex (Fig. 2C, lanes 4 and 5). The apparent increase in transgene sequences between the 2- and 4-week samples of 9-12albHSA animals was unique to this experiment and remains unexplained. This is not due to regional variation within the liver in the uptake of DNA-protein complex (data not shown).

**Transgenes Are Retained in Vivo as Episomes**—A series of Southern blot analyses designed to determine the molecular state of the retained DNA were performed with the hypothesis that the DNA was either integrated into the recipient cell’s chromosomal DNA or was retained as a stable or replicating episome.

The first set of experiments, presented in Fig. 3, are Southern blots of total cellular DNA digested with an endonuclease that has a single site within the plasmid. In each case, total cellular DNA isolated at time points greater than 1 week demonstrated a predominant band whose size was indistinguishable from the linearized plasmid. Higher molecular forms were not detected with the p9-12alb(h)LDLR (Fig. 3A, lane 4) or p9-12albCAT complex (Fig. 3C, lanes 3 and 4), except for a single band of approximately 12 kb detected at both time points with p9-12albCAT. Higher molecular forms of DNA in the p9-12albHSA experiment could not be assessed because of cross-hybridization of the human albumin probe to endogenous sequences (Fig. 3B). These findings are consistent with DNA present as an episome or integrated into chromosomal DNA as head-to-tail concatamers. This analysis does not rule out the existence of randomly integrated transgene which would produce a heterogeneous population of DNA-protein complexes containing the indicated plasmids. Liver was harvested at various time points, and DNA was isolated, restricted with an endonuclease, transferred to a filter, and hybridized with a plasmid-specific probe. In each case the restriction enzyme cleaved at more than two sites within the plasmid. Panel A, animals transfected with p9-12alb(h)LDLR. Total cellular DNA was digested with EcoRI and the filter was hybridized with probe L. Plasmid DNA (37.5 ng, lane 1, and 3.75 ng, lane 2) was added to DNA (5 ng) from liver of an untransfected animal. Additional samples include DNA (5 ng) isolated from liver tissue harvested 10 min (lane 3) and 2 weeks (lane 4) following transfection of DNA-protein complex. Panel B, animals transfected with p9-12albHSA. Total cellular DNA was digested with BamHI and the filter was hybridized with probe H. Plasmid DNA (750 pg, lane 1; 75 pg, lane 2; 75 pg, lane 3; 0 pg, lane 4) was added to DNA (10 μg) from liver of an untransfected animal. DNA (10 μg) was isolated from liver of animal A5 harvested 2 weeks (lane 5), and 4 weeks (lane 6) following transfection with DNA-protein complex and from liver of animal A10 harvested 2 weeks (lane 7), and 4 weeks (lane 8) following transfection. Panel C, animals transfected with p9-12albCAT. Total cellular DNA and extrachromosomal HIRT DNA was digested with PstI and filter was hybridized with probe. Plasmid DNA (75 pg, lane 1; 75 pg, lane 2; and 0 pg, lane 3) was added to DNA (10 μg) from liver of an untransfected animal. Total cellular DNA (10 μg) was isolated from liver harvested 8 weeks (lane 4) and 11 weeks (lane 5) following transfection of DNA-protein complex. Extrachromosomal DNA (0.5% of recovered sample from transfected animal plus 2% of recovered sample from an untransfected animal) was isolated from liver tissue harvested 8 weeks (lane 6) and 11 weeks (lane 7) following transfection.

![Fig. 2. Southern blot analysis to assess copy number of the transgene.](image-url)

**Fig. 2. Southern blot analysis to assess copy number of the transgene.** Animals were transfected with DNA-protein complexes containing the indicated plasmids. Liver was harvested at various time points, and DNA was isolated, restricted with an endonuclease, transferred to a filter, and hybridized with a plasmid-specific probe. In each case the restriction enzyme cleaved at more than two sites within the plasmid. Panel A, animals transfected with p9-12alb(h)LDLR. Total cellular DNA was digested with EcoRI and the filter was hybridized with probe L. Plasmid DNA (37.5 ng, lane 1, and 3.75 ng, lane 2) was added to DNA (5 μg) from liver of an untransfected animal. Additional samples include DNA (5 μg) isolated from liver tissue harvested 10 min (lane 3) and 2 weeks (lane 4) following transfection of DNA-protein complex. Panel B, animals transfected with p9-12albHSA. Total cellular DNA was digested with BamHI and the filter was hybridized with probe H. Plasmid DNA (750 pg, lane 1; 75 pg, lane 2; 75 pg, lane 3; 0 pg, lane 4) was added to DNA (10 μg) from liver of an untransfected animal. DNA (10 μg) was isolated from liver of animal A5 harvested 2 weeks (lane 5), and 4 weeks (lane 6) following transfection with DNA-protein complex and from liver of animal A10 harvested 2 weeks (lane 7), and 4 weeks (lane 8) following transfection. Panel C, animals transfected with p9-12albCAT. Total cellular DNA and extrachromosomal HIRT DNA was digested with PstI and filter was hybridized with probe. Plasmid DNA (75 pg, lane 1; 75 pg, lane 2; and 0 pg, lane 3) was added to DNA (10 μg) from liver of an untransfected animal. Total cellular DNA (10 μg) was isolated from liver harvested 8 weeks (lane 4) and 11 weeks (lane 5) following transfection of DNA-protein complex. Extrachromosomal DNA (0.5% of recovered sample from transfected animal plus 2% of recovered sample from an untransfected animal) was isolated from liver tissue harvested 8 weeks (lane 6) and 11 weeks (lane 7) following transfection.
fragments undetectable by this assay.

In an attempt to discriminate between these two possibilities, the electrophoretic properties of the transgene in total cellular DNA fractions were studied in experiments in which the samples were either not digested or were digested with an enzyme that does not have recognition sites within the plasmid (Fig. 4). In this analysis, integrated head-to-tail concatamers would migrate as heterogeneous populations of high molecular weight bands, while free plasmid would electrophorese as two distinct bands corresponding to nicked circular and supercoiled conformers. Each sample demonstrated two predominant bands that comigrated with the supercoiled and nicked circular forms (Fig. 4A, lanes 3 and 4; Fig. 4B, lanes 3–6; and Fig. 4C, lanes 3 and 4). A variable amount of DNA that comigrated with the linear form of the plasmid was observed in most experiments. The 4-week samples from the p9-12albHSA experiment were more degraded than the 2-week samples, however, this has not been a consistent finding in all experiments (Fig. 4B). These results suggest that a substantial amount of the retained DNA was episomal.

Two additional experiments were performed to confirm this hypothesis. Liver tissue from the animal injected with the p9-12albCAT complex was subjected to the DNA extraction described by Margolskee et al., 1988. This procedure isolates a fraction of DNA that is highly enriched for low molecular weight, non-chromosomal forms of DNA. Southern analysis of aliquots of these preparations that were either undigested (Fig. 4C, lanes 5 and 6), or digested with endonucleases that have a single site (Fig. 3C, lanes 5 and 6) or greater than two sites (Fig. 2C, lanes 6 and 7) within the plasmid, revealed a pattern of bands that were similar to that obtained with total cellular DNA. These experiments support the conclusion that the DNA characterized in total cellular preparations was extrachromosomal.

Southern blot analysis also revealed several discrete bands separate from those representing the input plasmid. The exact structure of these molecular variants is unknown; however, it is unlikely they represent integrated DNA unless the integration occurs in a site-specific manner. It is possible they represent extrachromosomal forms which have undergone rearrangements.

Even more compelling evidence for the existence of episomal DNA in liver tissue was obtained in experiments in which plasmid DNA (p9-12alb(h)HSA and p9-12albCAT) was rescued from total cellular DNA preparations by transformation of bacteria to ampicillin resistance. Plasmids isolated from the resulting bacterial transformants were characterized by restriction endonuclease mapping. Greater than 95% of the rescued plasmids demonstrated restriction patterns indistinguishable from the input plasmid (data not shown). Comparisons to transformations performed with known quantities of the original plasmids permitted an estimate of the total quantity of plasmid in liver (Table I). These estimates generally agreed with those obtained by Southern blot analysis.

The Episome Persists as a Stabilized Nonreplicating Plasmid—Two general mechanisms can explain persistence of episomal DNA in a eukaryotic cell, autonomous replication of the plasmid or stabilization of a nonreplicating plasmid. We employed a strategy based on patterns of DNA methylation to discriminate between these two possibilities. Prior to intravenous injection, the plasmids were grown in a strain of...

**Fig. 3. Southern blot analysis with restriction endonucleases containing one recognition site within the plasmid.** The experiment was performed as described in the legend to Fig. 2. Panel A, animals transfected with p9-12alb(h)LDLR. Total cellular DNA was digested with KpnI and the filter was hybridized with probe L. Plasmid DNA (5.75 ng, lane 1; 3.75 ng, lane 2) was added to DNA (5 µg) from liver of an untransfected animal. DNA (5 µg) was isolated from liver tissue harvested 10 min (lane 3) and 2 weeks (lane 4) following transfection of DNA-protein complex. Panel B, animals transfected with p9-12albHSA. Total cellular DNA was digested with KpnI or XhoI and the filter was hybridized with probe H. Plasmid DNA (0 pg, lanes 1 (KpnI) and 7 (XhoI); 750 pg, lanes 2 (KpnI) and 8 (XhoI)) was added to DNA (13.5 µg) from liver of an untransfected animal. DNA was isolated from liver of animal A5 harvested 2 weeks (10.8 µg, lanes 3 (KpnI) and 9 (XhoI)) and 4 weeks (2.5 µg plus 10 µg of DNA from liver of an untransfected animal, lanes 4 (KpnI) and 10 (XhoI)) following transfection of DNA-protein complex. DNA from liver of animal A10 harvested 2 weeks (16.2 µg, lanes 5 (KpnI) and 11 (XhoI)) and 4 weeks (2.5 µg plus 13.6 µg of DNA from liver of an untransfected animal, lanes 6 (KpnI) and 12 (XhoI)) following transfection. Panel C, animals transfected with p9-12albCAT. Total cellular DNA and extrachromosomal HIRT DNA was digested with XbaI and the filter was hybridized with probe C. Plasmid DNA (75 pg, lane 1; 7.5 pg, lane 2) was added to DNA (5 µg) from liver of an untransfected animal. Total cellular DNA (0.1 µg from transfected animal with 5 µg of DNA from untransfected animal) from liver harvested 8 weeks (lane 3) and 11 weeks (lane 4) following transfection. Extrachromosomal DNA (0.5% of recovered sample from transfected animal plus 2% of recovered sample from an untransfected animal) from liver tissue harvested 8 weeks (lane 5) and 11 weeks (lane 6) following transfection of DNA-protein complex.
bacteria (dam+) that methylates all adenine residues contained within the GATC sequence. One round of replication of plasmid sequences in a eukaryotic cell results in the formation of duplex that is hemimethylated. The restriction enzyme DpnI can be used to determine the fraction of the episomal DNA that had undergone replication in the hepatocyte because methylation of both strands is required for endonuclease digestion (Geier and Mondrich, 1979). This strategy assumes that eukaryotic cells are incapable of methylating adenine residues at this sequence and that loss of methylation cannot occur in the absence of eukaryotic replication.

Total hepatocyte cellular DNA preparations were analyzed in the following way. Each preparation was digested with a methylation-independent endonuclease that contains at least two sites contained within the fragments were completely digested in the internal restriction fragments. Digestion of aliquots of plasmids used to produce the DNA-protein complex with the methylation-independent enzyme released unique fragments of the appropriate size (Fig. 5A, lanes 1 and 2; Fig. 5B, lane 2; and Fig. 5C, lane 1). GATC sites contained within the fragments were completely digested.
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A.

B.

C.

FIG. 5. Southern blot analysis to assess replicative state of the transgene. The experiment was performed as described in the legend to Fig. 2. Panel A, animals transfected with p9-12alb(h)LDLR. Total cellular DNA was digested with EcoRI or EcoRI/DpnI and the filter was hybridized with probe L. Plasmid DNA (37.5 ng, lanes 1 (EcoRI) and 5 (EcoRI/DpnI)) and 7.5 ng, lanes 2 (EcoRI) and 6 (EcoRI/DpnI)) was added to DNA (5 µg) from liver of an untransfected animal. DNA (5 µg) was isolated from liver tissue harvested 10 min (lanes 3 (EcoRI) and 7 (EcoRI/DpnI)) and 2 weeks (lanes 4 (EcoRI) and 8 (EcoRI/DpnI)) following transfection. Plasmid DNA (75 pg, lanes 9; 7.5 pg, lane 10; 0 pg, lane 11) was added to DNA (10 µg) isolated from untransfected 3T3 fibroblasts. DNA (10 µg) was isolated from a population of 3T3 fibroblasts stably transfected with p9-12alb(h)LDLR (lane 12). The autoradiograph on the right was developed for 5.5 h while the one on the left was developed for 0.5 h. Panel B, animals transfected with p9-12albHSA. Total cellular DNA was digested with BamHI or BamHI/DpnI, and the filter was hybridized with probe H. Plasmid DNA (0 pg, lanes 1 (BamHI) and 8 (BamHI/DpnI); 750 pg, lanes 2 (BamHI) and 9 (BamHI/DpnI)) was added to DNA (13.5 µg) from liver of an untransfected animal. DNA (5.4 µg) was isolated from a population of 3T3 fibroblasts stably transfected with p9-12albHSA (lanes 3 (BamHI) and 10 (BamHI/DpnI)). DNA was isolated from liver of animal A5 harvested 2 weeks (10.8 µg, lanes 4 (BamHI) and 11 (BamHI/DpnI)) and 4 weeks (2.6 µg plus 13.6 µg of DNA from liver of an untransfected animal, lanes 5 (BamHI) and 12 (BamHI/DpnI)) following transfection. DNA from liver of animal A10 harvested 2 weeks (16.2 µg, lanes 6 (BamHI) and 13 (BamHI/DpnI)) and 4 weeks (2.6 µg plus 13.6 µg of DNA from liver of an untransfected animal, lanes 7 (BamHI) and 14 (BamHI/DpnI)) following transfection. Panel C, animals transfected with p9-12albCAT. Total cellular DNA and extrachromosomal HIRT DNA was digested with XbaI or XbaI/DpnI, and the filter was hybridized with probe C. Plasmid DNA was added to total cellular DNA (5 µg) from liver of an untransfected animal and digested with either XbaI (75 pg, lane 1) or XbaI/DpnI (75 pg, lane 2, and 7.5 pg, lane 3). Total cellular DNA (0.1 µg from transfected animal with 5 µg of DNA from untransfected animal) from liver harvested 8 weeks (lane 4) and 11 weeks (lane 5) following transfection was digested with XbaI/DpnI. Extrachromosomal DNA (0.5% of recovered sample from transfected animal plus 2% of recovered sample from an untransfected animal) from liver tissue harvested 8 weeks (lane 6) and 11 weeks (lane 7) following transfection was digested with XbaI/DpnI.

with DpnI (>99%) indicating that the plasmid was appropriately and completely methylated in bacteria (Fig. 5A, lanes 5 and 6; Fig. 5B, lane 9; and Fig. 5C, lanes 2 and 3). Cell lines stably transfected with p9-12alb(h)LDLR or p9-12albHSA were used as controls to demonstrate the effect of eukaryotic DNA replication on the methylation status of the plasmids and its effect on DpnI restriction. Southern analysis of total cellular DNAs isolated from the transfected cell lines demonstrated the presence of the transgenes (Fig. 5B, lane 3). However, DpnI failed to digest recombinant sequences in these preparations indicating that the transfected plasmid had lost its bacterial methylation as a result of replication in a eukaryotic cell (Fig. 5A, lane 12 and Fig. 5B, lane 10).

DNA (total cellular and extrachromosomal) from livers of transfected animals demonstrated the appropriate size fragments when digested with the methylation-independentendonucleases (Fig. 5A, lanes 3 and 4 and Fig. 5B, lanes 4–7), and virtually all GATC sites within the internal restriction fragments were completely digested with DpnI (Fig. 5A, lanes 7 and 8; Fig. 5B, lanes 11–14; and Fig. 5C, lanes 4–7). These studies indicated that the plasmid had not replicated in the eukaryotic cell. Its persistence in vivo, therefore, must be explained by enhanced intracellular stability.

Potential Mechanism(s) for Episomal Stabilization Following in Vivo Gene Transfer—Gene transfer into an animal whose liver is relatively quiescent leads to transient gene expression and eventual degradation of the incorporated DNA (Wu and Wu, 1988; Wu et al., 1989). The original aim of this project was to achieve stable gene expression by integrating the transgene into the recipient cell's chromosomal DNA. It was hypothesized that the frequency with which transgene integration would occur could be improved by stimulating the hepatocytes to divide at the time of gene transfer (i.e. subjecting the animal to partial hepatectomy). We felt it unlikely, however, that this process would be efficient enough to achieve meaningful levels of stable transfection. Partial hepatectomy, in fact, resulted in the retention of substantial quantities of transgene sequences. However, this was not due to integration of the plasmid into chromosomal DNA. The majority of plasmid DNA exists as an episome whose structure is identical to that of the input plasmid. Methylation analysis of the DNA suggests that the plasmid has not replicated in the animal; its persistence must be explained by enhanced stabilization.

Our results were somewhat unexpected based on previously held dogma that transgene sequences must integrate into chromosomal DNA or replicate autonomously as episomes if they are to persist in eukaryotic cells. The literature in this
area is largely based on transfection experiments in actively growing cultured cells or microinjection of DNA into procytosomes obtained to become transgenic animals (Roth et al., 1985; Wilkie and Palmiter, 1987; Roth and Wilson, 1988; Bishop and Smith, 1989). In each system DNA is introduced into a cell which continues to proliferate for an extended period of time. This differs from the model described in this study in which DNA is transferred into a large population of cells which undergo one to two rounds of replication and subsequently become post-mitotic.

We propose the following model for the persistence of transgene sequences in our system. Partial hepatomecytomy at the time of gene transfer leads to stabilization of the intercalated DNA by either partitioning it into cytoplasmic and nuclear compartments that are protected from nuclease digestion or by altering its structure to render it resistant to nuclease digestion. Once stabilized the DNA would persist for the lifetime of the hepatocyte which has been estimated to be extremely long (Sell, 1990).

Two important questions remain unanswered. The first relates to the occurrence of other molecular forms of DNA in the recipient cell. These studies unambiguously demonstrate the existence of episomal DNA in liver long after in vivo gene transfer. However, they do not rule out the possibility of infrequent, random integration. The only experiments that may be relevant to this question are the DpnI sensitivity studies. Virtually complete digestion of transgene sequences with DpnI are consistent with little, if any, integration if one assumes that integration is associated with replication of one strand of DNA. These experiments are less informative in quantifying integration if this assumption is wrong and integration can occur in the absence of replication (e.g., via DNA repair mechanisms).

In addition, these studies do not identify the form of DNA which is transcriptionally active. The copy number of retained DNA is out of proportion to the level of recombinant gene expression suggesting that some of the DNA is not transcriptionally active. Expression could, therefore, result from a subpopulation of nuclear localized plasmid or from integrated transgene which has escaped detection in our analyses.

Implications for Liver Biology and Somatic Gene Therapy—The demonstration of stabilized, nonreplicating episomes in hepatocytes in vivo has possible implications in the pathobiology of viral hepatitis. Chronic forms of hepatitis due to infection with hepatitis B virus (HBV) are associated with extrachromosomal forms of the HBV genome in hepatocytes (Blum et al., 1989). Persistence of double-stranded, circular forms of the HBV genome is required for ongoing production of virions (Pugh and Bassendine, 1990). A better understanding of the factors which contribute to the maintenance of this molecular form of HBV could provide insight into the variable expression of the clinical syndrome and potential therapeutic approaches. The findings described in this report raise the notion that stabilization of the viral genome as an extrachromosomal element may be a factor in the pathogenesis of hepatitis following infection with HBV or other hepatotropic viruses.

These findings also have implications in the development of somatic gene therapies. Effective treatment of inherited diseases by gene transfer requires prolonged if not permanent expression of the transgene in vivo. This has been accomplished in animal models by integrating the recombinant gene into chromosomal DNA of a stem cell or relatively long-lived differentiated cell. Disadvantages of this approach are 1) the target cell must be dividing to achieve persistence, and 2) random integration of the transgene is associated with the risk of malignancy due to insertional mutagenesis. The use of non-viral gene transfer substrates to achieve persistence of transgene sequences in vivo as stabilized, nonreplicating episomes provides an attractive alternative.

REFERENCES
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