

Epstein–Barr Virus/Human Vector Provides High-Level, Long-Term Expression of α_1 -Antitrypsin in Mice

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We have constructed plasmid DNA vectors that contain Epstein–Barr virus (EBV) sequences and the human gene (*SERPINA1*) encoding α_1 -antitrypsin (AAT). We demonstrate that a plasmid carrying the full *SERPINA1* on a 19-kb genomic fragment and the EBV gene *EBNA1* and its family of repeats binding sites undergoes efficient extrachromosomal replication in dividing mammalian tissue culture cells. Therefore, use of a whole genomic therapeutic gene to provide both replication and gene expression may be an effective gene therapy vector design, if the target cells are dividing. The efficacy of this same vector for expression of AAT *in vivo* in the nondividing cells of mouse liver was determined by hydrodynamic injection of naked plasmid DNA by means of the tail vein. A single injection of an EBV/genomic *SERPINA1* vector provided $> 300 \mu\text{g/ml}$ of AAT, which approached normal plasma levels and persisted for the > 9 -month duration of the experiment. These data exceed most previously reported values, probably due to sequences in the genomic DNA that resist silencing of gene expression, possibly in combination with favorable effects on expression provided by the EBV sequences. These results demonstrate that plasmid DNA with the correct *cis*-acting sequences can provide *in vivo* long-term expression of protein at high levels that are therapeutically relevant for gene therapy.

Key words: α_1 -antitrypsin, Epstein-Barr virus, gene therapy, genomic DNA, liver, long-term expression, naked DNA, non-viral vector

INTRODUCTION

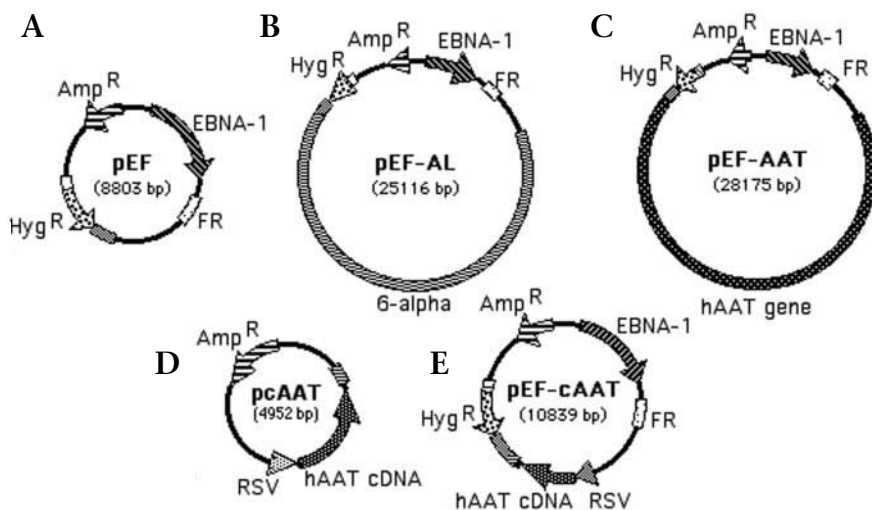
In genetic diseases such as cystic fibrosis, α_1 -antitrypsin deficiency, and hemophilia, successful gene therapy treatments must provide expression of therapeutic levels of protein over the lifetime of the individual. Therefore, persistence of a transgene in mammalian cells, with long-term, adequate expression levels, is necessary. For α_1 -antitrypsin deficiency, which causes liver damage and emphysema, gene therapy has the potential to alleviate the pulmonary symptoms [1]. Normal expression levels are rather high, with plasma levels of 0.8–1.3 mg/ml human α_1 -antitrypsin (AAT, encoded by the gene *SERPINA1*). Integration of gene therapy vectors into the host genome provides permanence for the therapeutic transgene, but most current methods (such as use of retroviruses) produce random integration, resulting in mutagenic interruption of genomic sequences and position effects with associated variable gene expression [2]. An alternative to integration in the host genome is to maintain the gene therapy vector extrachromosomally in the mammalian cell. Adenoviral vectors remain extrachromosomal, but the viral proteins

can be immunogenic and toxic. Although good short-term expression of AAT was observed with first-generation adenoviral vectors, the high expression was short-lived [3]. To avoid some of the drawbacks of viral gene therapy vectors, the use of plasmid DNA as a gene therapy vector has been studied. However, delivery of such vectors is often poor and expression is usually short-lived [2].

Wolff [4] and Liu [5] have developed a simple hydrodynamic procedure for efficient transfection of liver cells by means of tail-vein injections in mice. This procedure has been shown to transfect up to 40% of liver cells after a single injection of naked plasmid DNA. With this method, when 10–50 μg of plasmid DNA expressing the human AAT cDNA were injected, liver cells expressed AAT at plasma levels of 2–5 $\mu\text{g/ml}$ over a period of 6 months [6]. Plasmid DNA was expressed long-term in liver cells, but the level of expression that persisted was not adequate. Although plasmid DNA seemed to be retained, gene expression may have been silenced.

Epstein-Barr virus (EBV) is a human herpesvirus that is capable of maintaining its genome extrachromosomally in

FIG. 1. Plasmids. (A) pEF is a negative control for replication. It contains the EBV family of repeats and *EBNA1* for nuclear retention, but has no sequences that mediate extrachromosomal replication in mammalian cells. (B) pEF-AL is a positive control for extrachromosomal replication and contains 6- α , a 16.2-kb fragment of alphoid repeat human centromeric DNA in a pEF backbone. (C) pEF-AAT contains the 19-kb AAT genomic region (encoded by *SERPINA1*) in a pEF backbone. (D) pcAAT contains the AAT cDNA under control of the RSV promoter in a vector with no EBV sequences. (E) pEF-cAAT contains the AAT cDNA with the RSV promoter in a pEF backbone.



dividing primate cells. Maintenance is accomplished by the viral latent origin of replication, *oriP*, and the EBV nuclear antigen 1, *EBNA1*, which act together to replicate the viral genome and retain it in the nucleus. Inclusion of these EBV sequences on conventional plasmid vectors allows them to replicate and be retained in dividing primate cells [7–9]. These same EBV components have been associated with transcriptional enhancer and antisilencing activity [10,11]. Plasmids containing *EBNA1* and a truncated *oriP* carrying only the tandem array of 21 *EBNA1* binding sites (family of repeats) from *oriP* for retention, but lacking the *oriP* dyad symmetry element for replication, are retained in the nucleus of dividing cells, but cannot replicate efficiently [12]. Consequently, their copy number is reduced over time as the cells divide. We used such replication-defective vectors to identify fragments of human genomic DNA that conferred efficient extrachromosomal replication in dividing mammalian tissue culture cells [12]. These experiments revealed that most large (≥ 16 -kb) fragments of human genomic DNA could mediate replication of an extrachromosomal *EBNA1* family of repeats vector in mammalian cells, with little sequence specificity [13]. Such vectors demonstrated replication and retention ability in both rodent and primate cell lines [14], overcoming the species specificity of the EBV *oriP*, which replicates poorly in rodent cells [8].

To apply the replication ability of large fragments of genomic DNA parsimoniously to create a gene therapy vector, we assayed the ability of full-length therapeutic genes to mediate replication of an extrachromosomal vector. A replicating extrachromosomal gene therapy vector would be advantageous if target cells were dividing [15]. For these experiments, we needed a therapeutically relevant gene whose full genomic length was greater than 16 kb, but was of a reasonably small size for ease of cloning. Based on these criteria, we selected the full-length

SERPINA1 sequence. *SERPINA1* resides on human chromosome 14q32.1 in a cluster of serine protease inhibitor genes. It is highly expressed in liver and cultured hepatoma cells, but transcriptionally repressed in most other cell types [16]. The *SERPINA1* promoters, enhancers, all exons and introns, and 3' untranslated sequences are contained on a 19-kb genomic fragment [17]. This genomic fragment was cloned into a vector carrying the EBV gene *EBNA1* and family of repeats and assayed for replication in human and rodent rapidly dividing cell lines. Additionally, the vector was analyzed for its ability to express therapeutic levels of AAT *in vivo* in nondividing cells, after delivery to mouse liver.

Here we report on the ability of this EBV/genomic AAT vector to replicate extrachromosomally in dividing mammalian tissue culture cells. This vector also conferred enhanced gene expression characteristics to quiescent liver cells, providing high-level expression *in vivo* that persisted for at least nine months. These results demonstrate that a plasmid DNA vector with favorable sequences for gene expression can provide the strong, long-term expression levels necessary for successful gene therapy.

RESULTS

SERPINA1 Sequence Mediates Replication of an Extrachromosomal Plasmid in Dividing Mammalian Cells

To assay the ability of a full gene sequence to supply replication activity to an extrachromosomal vector in dividing mammalian cells, we constructed pEF-AAT (Fig. 1C). This vector contains two sequences from EBV, *EBNA1* and the family of repeats, a tandem array of 21 *EBNA1* binding sites from *oriP*, the EBV latent origin of replication. The vector also carries a 19-kb genomic fragment encoding the complete *SERPINA1*. The replication ability of this vector

was assayed against negative and positive controls for mammalian extrachromosomal replication, pEF and pEF-AL, respectively (formerly pDY⁻ and pDYAL). pEF (Fig. 1A) has sequences for nuclear retention, but does not replicate efficiently [12], whereas pEF-AL has both retention and replication abilities [18]. pEF-AL (Fig. 1B) contains a 16.2-kb fragment of human alphoid DNA from centromere 17 and replicates extrachromosomally once per cell cycle at an efficiency of ~ 100% in mammalian cell lines [18].

The three vectors were transfected into 293 cells and maintained under hygromycin selection. Plasmid DNA was then extracted, digested with *Mlu*I and *Mbo*I, and analyzed for replication by Southern blot. If the transfected vectors did not replicate at least twice in the human cells, then the plasmid DNA will have retained its bacterial methylation pattern and will be resistant to digestion by *Mbo*I. A 6.0-kb *Mlu*I fragment will be released. If the plasmid DNA was able to replicate in 293 cells, it will have lost its bacterial methylation and become sensitive to digestion by *Mbo*I, releasing a 2.8-kb fragment. Plasmid DNA extracted from cells transfected with pEF-AL or pEF-AAT was digested by *Mbo*I and released a 2.8-kb fragment (Fig. 2). This demonstrated that the *SERPINA1* sequence was capable of mediating replication of extrachromosomal plasmids. As expected, pEF did not release any detectable bands, indicating that in the absence of replication the vector was either lost by the cell or, at a low frequency, integrated into the genome.

Replication ability of the three plasmids in dividing mammalian cells was further confirmed by bacterial assay. Extracted plasmid DNA was digested with *Dpn*I or mock-digested, then transformed into *Escherichia coli*. *Dpn*I only cleaves bacterially methylated DNA. Therefore, only plasmids that have replicated in mammalian cells and lost this methylation pattern will resist *Dpn*I digestion and form bacterial colonies after treatment with *Dpn*I. Replication was tested in rapidly dividing 293 human kidney cells and C127 mouse mammary cells. The degree of plasmid replication in mammalian cells was determined by comparing colony numbers in *Dpn*I treated and untreated samples. The majority of pEF-AAT plasmid DNA extracted from both 293 and C127 cells was resistant to *Dpn*I digestion (Table 1), thus confirming the ability of the *SERPINA1* sequence to mediate replication of pEF-AAT in both primate and rodent dividing cell lines.

Plasmid Replication Mediated by the *SERPINA1* Sequence Occurs in the Presence of Transcription

Previous studies have indicated that transcription through an origin of replication inhibits the ability of the sequence to function as an origin [19]. *SERPINA1* on pEF-AAT is under control of its endogenous, hepatocyte-specific promoter. It was not expected to be expressed in the non-liver 293 and C127 cell lines used above [17]. We wished to determine if transcription through *SERPINA1* would eliminate the ability of pEF-AAT to replicate and be maintained

in dividing liver cells. The same three replication test vectors were transfected into Hepa1a cells, a line derived from mouse hepatocytes, and maintained under hygromycin selection. Plasmid DNA was extracted, digested with *Dpn*I or mock-digested, and transformed into *E. coli*. Resistance of the DNA to *Dpn*I digestion is an indication of two or more rounds of replication in the mammalian cells (Table 1). Although the ability of pEF-AAT to replicate was reduced in liver cells, it still showed a substantial degree of replication in Hepa1a cells, as evidenced by the formation of bacterial colonies transformed with *Dpn*I-resistant Hirt DNA. To verify that *SERPINA1* was being expressed in these cells, medium was sampled and AAT levels were analyzed by ELISA. A 100-mm tissue culture dish of Hepa1a (9.3×10^6 cells) secreted AAT into the medium at a concentration of 1.3 ng/ml/ 10^6 cells/day, but was barely detectable in 293 and C127 cells (data not shown). This analysis confirmed that the gene was being expressed and that the AAT protein was being made and secreted properly. Expression of *EBNA1* was not directly verified in this study, but has been functionally verified in human and mouse cells using similar plasmid constructs [14,20].

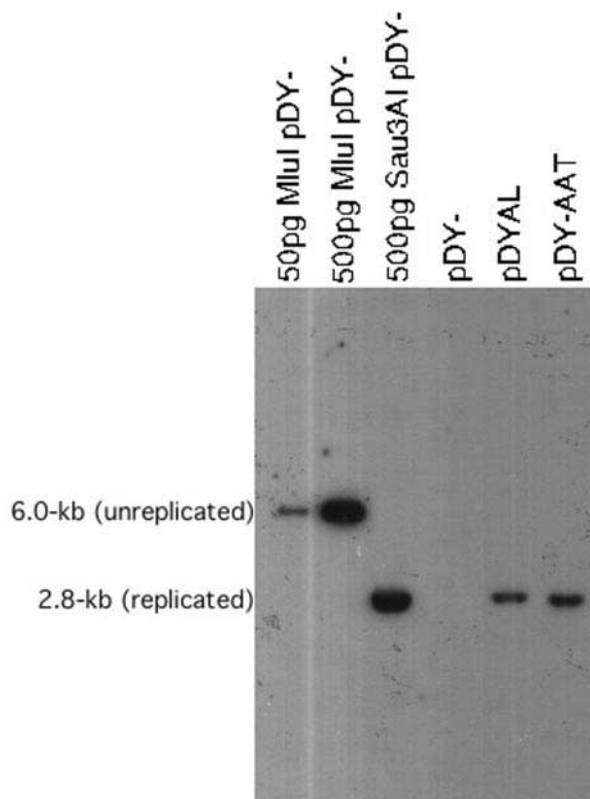


FIG. 2. pEF-AAT replicates in human 293 cells. Plasmid DNA was extracted from human cells five weeks after transfection, digested with *Mbo*I and *Mlu*I, run on a gel, subjected to Southern blotting, and probed with a radiolabeled *Sau*3AI fragment from *EBNA1*. A 2.8-kb band released by digestion with *Mbo*I indicates that the plasmid DNA has replicated in mammalian cells. pEF is a negative control for extrachromosomal replication, whereas pEF-AL is a positive control. pEF-AAT shows strong replication in 293 cells.

Table 1: Bacterial assay for plasmid replication in mammalian cells

Mammalian cell type	Plasmid	Number of bacterial colonies ^{a,b,c}	
		- DpnI	+ DpnI
293	pEF	2	1
	pEF-AL	1290	1274
	pEF-AAT	715	782
C127	pEF	0	0
	pEF-AL	548	511
	pEF-AAT	360	242
Hepa1a	pEF	50	7
	pEF-AL	1242	1212
	pEF-AAT	332	86
Liver ^c	pcAAT	2366	0
	pEF-cAAT	1992	0
	pEF-AAT	64	0

^aPlasmid DNA that has replicated in mammalian cells is resistant to *DpnI* digestion and will give rise to bacterial colonies when transformed into *E. coli*.

^bExperiments were repeated at least four times, with consistent results.

^cExtrachromosomal DNA present in liver preparations will give rise to bacterial colonies when transformed into *E. coli*. Colonies formed are per 1 μ g of genomic DNA transformed.

Tail-Vein Injection of EBV/Human Plasmid DNA Results in Long-Term, High-Level Expression of AAT *in Vivo*

We observed successful maintenance of pEF-AAT in dividing tissue culture cells. We next wanted to determine the relative abilities of various AAT plasmids to be stably maintained and expressed *in vivo*, in this case in the largely quiescent cells of the normal liver. For delivery of naked plasmid DNA to mouse liver, we used the hydrodynamic tail-vein injection procedure [4,5], followed by determination of serum levels of AAT by ELISA. For these experiments, two cDNA control plasmids were used: pcAAT (4.9 kb; Fig. 1D) contains the human AAT cDNA under control of the RSV promoter and carries no EBV sequences; and pEF-cAAT (Fig. 1E) is a 10.8-kb plasmid containing the EBV family of repeats and *EBNA1*, as well as the AAT cDNA under control of the RSV promoter. As described previously, pEF-AAT (28.2 kb; Fig. 1C) contains the same plasmid backbone and EBV components as pEF-cAAT, but carries the 19-kb human genomic fragment encoding AAT instead of the cDNA (Fig. 1C). A single-25- μ g tail-vein injection of each plasmid DNA was delivered to mice.

Average serum AAT levels from all three vectors were in the range of ~ 150–1150 mg/ml one day after injection (Fig. 3). The cDNA-containing plasmids pcAAT and pEF-

cAAT dropped to serum AAT levels of ~ 1 μ g/ml and ~ 0.1 μ g/ml, respectively, within three weeks of injection. In contrast, pEF-AAT, which contained the 19-kb *SERPINA1* and EBV sequences, maintained serum AAT levels exceeding 300 μ g/ml at two months post-injection and maintained these high levels through the more than nine-month duration of the experiment.

Analysis of Plasmid DNA in Liver Samples

The expression levels of AAT from the three plasmids tested differed over time. We wanted to quantify the levels of intracellular plasmid DNA, to address whether expression levels correlated with levels of plasmid DNA remaining in liver tissue. We also wished to analyze the state of the injected plasmid DNA in liver tissue, in particular to examine whether it remained extrachromosomal. Representative mice that had received pcAAT, pEF-cAAT, and pEF-AAT were sacrificed seven months after injection of plasmid DNA. The liver was removed and total DNA prepared from

liver tissue. The DNA was analyzed by transformation into *E. coli* to assess the presence of extrachromosomal plasmid DNA and by Southern blot to quantify the amount of plasmid DNA present.

The total DNA sample from mouse liver might contain extrachromosomal plasmid DNA or integrated plasmid DNA as part of the high-molecular-weight chromosomal component. Only intact free plasmid DNA from this DNA sample would produce bacterial colonies on plates containing ampicillin, the resistance marker present on all three injected plasmids, after transformation into *E. coli*. Therefore, bacterial colony numbers are diagnostic for the presence of free intact plasmid DNA remaining in the liver cells and also provide a rough quantification of the amount of extrachromosomal plasmid DNA present. The results of this analysis (Table 1) indicated that free plasmid DNA was still present in the liver seven months after injection. The colony numbers indicated that pEF-AAT, despite its higher levels of gene expression, was not present in greater quantities than the other two plasmids. The lower amount of pEF-AAT DNA present can be accounted for by the poorer delivery of large plasmids and the lower molar amount of DNA injected. These results reveal that the significant feature for expression levels is not copy number, but the nature of the sequences on the expression plasmid.

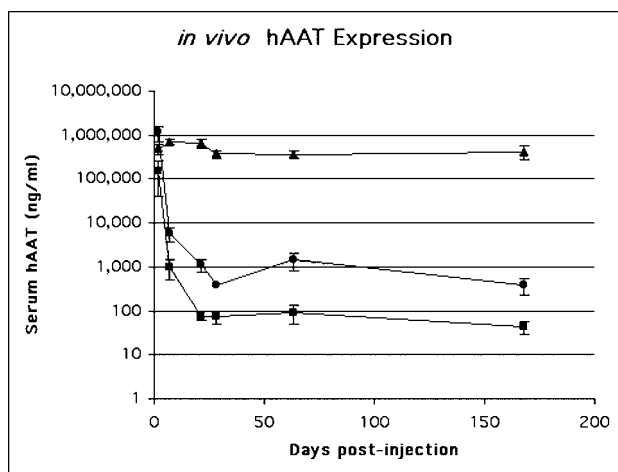


FIG. 3. AAT expression levels in mice. Groups of 3–5 mice were injected with 25 μ g of pEF-AAT (filled squares), pEF-cAAT (filled circles), or pcAAT (filled triangles). Blood was sampled periodically and serum AAT levels were determined by ELISA. Error bars represent the standard error from five, four, and three mice, respectively.

Mini-prep and restriction analyses of a sample of the colonies obtained confirmed that they represented the expected, unrearranged plasmids. This suggested that the bacterial colonies were derived from injected DNA still present in the mouse livers, not from later contamination. When the total liver DNA was subjected to digestion with *DpnI* before transformation, no colonies were obtained (Table 1). These results suggested that the extrachromosomal plasmid DNA present in the liver cells retained its bacterial adenine methylation pattern and therefore underwent little or no replication in the mammalian cell environment. This was expected, because little cell division occurs in undamaged liver [21].

The DNA extracted from the mouse livers was also analyzed by Southern blot after cutting with restriction enzymes that would release characteristic fragments from the plasmids. Using a probe from the ampicillin resistance gene common to all three plasmids, it was possible to quantify the amount of plasmid DNA remaining in the tissues. This analysis (Fig. 4) revealed that the amount of plasmid DNA was not greater for the pEF-AAT plasmid that showed greater gene expression. The relative amounts of DNA on the Southern blot correlated well with the bacterial colony numbers derived from the DNA (Table 1), given the decreased transformation efficiency of larger plasmids. The results are consistent with most or all of the plasmid DNA being present in an extrachromosomal state. This type of analysis cannot rule out the presence of integrated DNA, but makes it unlikely that a significant amount of integrated DNA could be present.

DISCUSSION

These results demonstrate that a plasmid containing human genomic *SERPINA1* and EBV components can

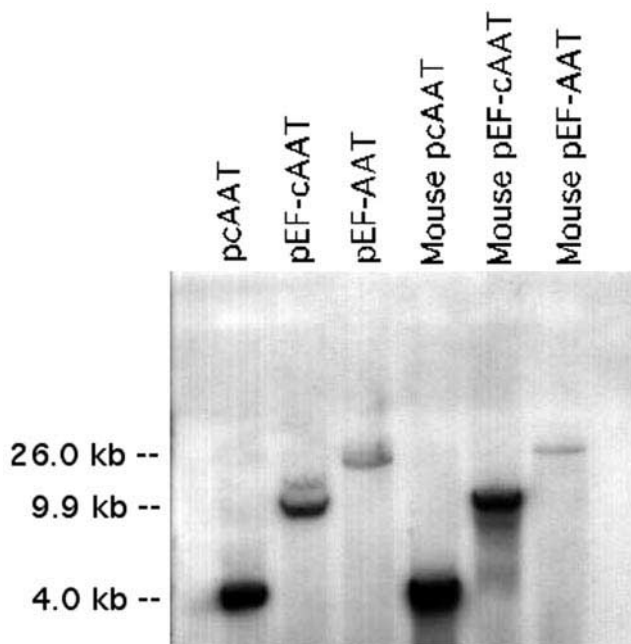


FIG. 4. DNA retention in liver. DNA was extracted from mouse livers harvested seven months after plasmid injection. DNA was digested with *EcoRV* (pcAAT and pEF-cAAT) or *BglIII* (pEF-AAT), which cut twice in the plasmids, and probed with a labeled fragment from the ampicillin resistance gene. Lanes 1–3 contain 500 pg of plasmid DNA, whereas lanes 4–6 contain 30 μ g of liver genomic DNA derived from mice injected with pcAAT, pEF-cAAT, and pEF-AAT, respectively.

replicate and be retained long-term in dividing human and mouse tissue culture cells. Furthermore, in the absence of viral delivery methods, the EBV/AAT plasmid can provide long-term, therapeutically relevant expression levels of AAT *in vivo* when residing in quiescent liver cells. Mice were subjected to hydrodynamic tail vein injection with pEF-AAT, a plasmid containing the full genomic 19-kb *SERPINA1* in addition to *EBNA1* and its family of repeats binding sites from EBV. Serum levels of AAT protein were maintained at greater than 300 μ g/ml for at least nine months after a single 25 μ g injection of naked plasmid DNA. It has been demonstrated [6] that a conventional cDNA plasmid, when delivered to liver cells by the same hydrodynamic tail-vein injection procedure, persisted as extrachromosomal DNA for at least six months, but expressed AAT at only 2–5 μ g/ml. These lower expression results were obtained using a plasmid lacking EBV sequences and in which *SERPINA1* was present as a cDNA under the control of the viral cytomegalovirus (CMV) promoter. The ~100-fold greater *in vivo* expression mediated by our vector seems to be attributable to the genomic AAT sequences, possibly aided by the EBV sequences. What is the role of these elements in providing high-level, long-term gene expression?

EBV sequences provide a nuclear retention function that maintains extrachromosomal plasmid DNA in the

nucleus of dividing cells [12], but this retention function is apparently not relevant in these largely nondividing hepatic cells. All of the plasmids, with and without EBV sequences, persisted in liver. The EBV sequences may improve gene expression, as increases in gene expression of up to 100-fold for genes carried on EBV vectors have been observed in cultured cells and attributed primarily to *EBNA1-oriP* enhancer activity [22,23]. EBV vectors have also prolonged and increased gene expression *in vivo* when *oriP* and *EBNA1* were present on the plasmid [24–27].

In our studies, however, greatly elevated expression levels were sustained on an EBV vector only in the presence of the large human genomic *SERPINA1* fragment, thus proving that genomic sequences are required for long-term, high-level expression. This effect may be due to intron sequences and/or the endogenous *SERPINA1* promoters, enhancers, or other untranslated sequences that prevent silencing of gene expression. In many cases genomic sequences such as introns have given better gene expression than cDNAs in the context of chromosomal integration [28,29], and the same effect apparently occurs on extrachromosomal plasmids. For example, inclusion of chromosomal sequences comprising an intron, a 3' untranslated region, and the hepatic locus control region increased and stabilized therapeutic levels of factor IX expression from plasmid vectors *in vivo* [30].

AAT is normally maintained at high serum levels, in the mg/ml range. It is likely that the signals directing this expression reside in or near *SERPINA1* and are therefore present on our pEF-AAT vector. Support for this idea comes from studies in which genomic *SERPINA1* fragments identical to or smaller than the one present on our vector consistently gave rise to transgenic mice that expressed AAT at high levels, regardless of the integration site [31,32]. These observations are consistent with the location of signals and sequences that resist chromosomal position effects and silencing within or very near the genomic *SERPINA1*. The identity and mechanism of these sequence signals and whether they occur in other genes is not yet clear, but is under investigation. Given the strong positive effect of the genomic *SERPINA1* sequences, it is not clear whether the EBV sequences are making a significant contribution to maintenance of high-level gene expression in this context. This question will be addressed by constructing and testing genomic *SERPINA1* plasmids that lack EBV sequences. The molecular structure of the DNA components may also influence gene expression, as a recent study showed that linearized DNAs resulted in persistent and 10–100-fold higher levels of gene expression *in vivo* compared with their circular counterparts [33]. Whether or not inclusion of genomic and/or EBV-derived sequences influences the molecular structure and hence gene expression from our plasmids *in vivo* remains to be determined.

The vector pEF-cAAT, containing EBV sequences and AAT cDNA expressed from the viral RSV promoter, did not

express AAT at serum levels greater than those observed for the non-EBV vector, pcAAT. The AAT cDNA achieved levels of approximately 1 $\mu\text{g/ml}$, more than two orders of magnitude lower than the genomic AAT plasmid. These results are unlikely to be due to mutation or truncation of the pcAAT plasmid, because the experiments were repeated several times with consistent results, the plasmids were found to be intact after rescue from the liver, and the values agree with other studies with similar plasmids [6]. The cDNA with the EBV sequences did poorly, expressing levels of ~ 100 ng/ml of AAT, indicating that the EBV sequences were not effective in improving expression of this cDNA construct, whereas we have found that they are effective in conjunction with a factor IX mini-gene that has genomic rather than viral-derived control sequences (unpublished data). The lower expression of pEF-cAAT relative to pcAAT may be explained by the size difference between these two plasmids. pEF-cAAT is twice as large as pcAAT, so when 25 μg of each plasmid were injected into mice, twice as many pcAAT molecules were delivered. Day one serum AAT levels were 7.5-fold higher in the pcAAT-injected mice than in pEF-cAAT injected animals.

The expression levels observed using a plasmid containing EBV sequences and the full-length genomic *SERPINA1* sequence are more than two orders of magnitude greater than those observed from conventional plasmids delivered in a similar manner [6] and place the expression level in a range that would be curative for the AAT deficiency disorder in humans. We observed levels of AAT of > 300 $\mu\text{g/ml}$, approaching the normal levels of ~ 800 $\mu\text{g/ml}$ for humans. The pEF-AAT plasmid is clearly providing key functional elements missing from simple cDNA plasmids and achieved extraordinary levels of AAT that exceed most previously reported values. A gutless adenovirus vector, a type of vector noted for high expression, achieved levels of 50 $\mu\text{g/ml}$ [17], well below the values we are seeing without the viral concerns. Higher levels were seen with high doses of gutless adenoviral vectors [34]. AAV vectors often give excellent levels of gene expression. When used to deliver AAT cDNA under the control of the CMV promoter, a dose of 1.4×10^{13} particles injected intramuscularly provided ~ 400 $\mu\text{g/ml}$ of AAT in the same C57BL/6 mice that we used [35]. The AAV result is similar to what we are seeing with an easily prepared plasmid construct that is cheaply available in unlimited quantities. Our dose of a single 25 μg injection, equivalent for pEF-AAT to $\sim 10^{12}$ plasmid DNA molecules, is actually less than the number of AAV particles used above [35].

These experiments also demonstrate that mammalian whole gene sequences are capable of acting as origins of replication for extrachromosomal plasmids. We hypothesized that this would prove true, based on previous findings that large random fragments of human DNA of ~ 16 kb or larger are able to mediate extrachromosomal replication [13]. The 19-kb *SERPINA1* genomic fragment is capable of mediating replication of the extrachromosomal plas-

mid pEF-AAT in both human and rodent cell lines. This lack of species specificity for replication is typical of replication mediated by genomic, as opposed to viral, DNA [14]. Extrachromosomal replication of pEF-AAT also occurred in a mouse hepatocyte cell line that expressed *SERPINA1*, though the degree of replication was reduced. These results are consistent with previous findings for extrachromosomal replication, in which transcription inhibits replication to varying degrees [19]. Extrachromosomal replication mediated by genomic DNA is under strict cell cycle control and occurs in only dividing cells [18]. The normal liver cells that were the delivery site in this study do not undergo frequent cell division [21], so the ability of the plasmid to replicate would not in itself confer an advantage in this setting. However, EBV sequences do provide for long-term expression of genes carried on vectors in dividing tissue culture cells [20,36–39], suggesting that EBV vectors may be useful for gene therapy in dividing cells [15,40]. Thus, the potential of large genomic plasmids such as pEF-AAT to replicate could be useful in the context of gene therapy target tissues that are dividing, such as stem cells or tumor cells [15].

Our results demonstrate the feasibility of using naked plasmid DNA as a gene therapy vector. While tail-vein injections are currently limited to mice, continued research may lead to similar delivery methods in higher mammals, including humans. The type of EBV/human genomic plasmid used here may also provide improved expression levels and persistence for other tissues and disease targets. The most appealing aspect of naked DNA gene therapy vectors is the absence of a viral delivery vector, thereby reducing potential immunogenicity, toxicity, or integration problems associated with such vectors. With an effective delivery method, naked plasmid DNA that possesses sequences to ensure high-level, long-term gene expression, such as the EBV and/or genomic sequences described here, may be an ideal vector for gene therapy.

MATERIALS AND METHODS

Plasmids. Plasmids pEF and pEF-AL (formerly called pDY⁻ and pDYAL, respectively) have been described [14,18]. The nomenclature “EF” was formulated for clarity, to signify *EBNA1* (E) and the family of repeat (F) *EBNA1* binding sites. We constructed pEF-AAT by cloning a 19-kb *Sall* fragment from phage clone α NN (a gift from Mark Dyaico, Stratagene) carrying the complete human *SERPINA1* into pEF. A 2.0-kb *XhoI* fragment from pRSV.hAAT.bpA [33], known as pCAAT here (Fig. 1D), carrying the AAT cDNA under control of the Rous Sarcoma Virus promoter was cloned into pEF to form pEF-cAAT (Fig. 1E).

Cell lines. Hepa1a is a mouse hepatoma cell line; 293 is a human embryonic kidney cell line [41]; and C127 is a mouse mammary cell line. We grew 293 and C127 cell lines in Dulbecco's modified Eagle's medium and Hepa1a cells in minimum essential medium. Both media contained 9% fetal bovine serum, 1% penicillin, and 1% streptomycin. All cells were grown at 37°C in a 5% CO₂ incubator.

Southern blot. We split 293 cells into 60-mm dishes containing fresh medium and grew them to 70–80% confluency. Cells were then transfected with 5 μ g of DNA by the Lipofectamine method (Life Technologies,

Gaithersburg, MD) in Optimem I medium, according to the manufacturer's protocol. Twenty-four hours after transfection, cells were trypsinized and transferred to 100-mm dishes in fresh medium. Forty-eight hours after transfection, selection was begun in medium containing 250 μ g/ml hygromycin. Cells were grown under selection for 35 d. Plasmid DNA was then isolated by the Hirt procedure [42]. This DNA was digested with *MluI* and *MboI* and separated on a 0.7% agarose gel. The gel was deproteinized in 0.33 M HCl, and DNA was transferred to a Zeta-Probe GT (BioRad, Hercules, CA) membrane with 0.5 M NaOH transfer buffer. The membrane was then probed with a 2.8-kb fragment from pEF, labeled with ³²P by random primer extension. Hybridization occurred at 65°C for 16–20 h in a solution of 7% SDS, 1 \times SSC, and 100 μ g/ml salmon sperm DNA. The membranes were washed in solutions of 0.33 \times to 1 \times SSC, 0.5% SDS, 1 mM EDTA at 60°C. Membranes were then wrapped in Saran Wrap and exposed to film.

Bacterial colony formation. We split 293, C127, and Hepa1a cells into 100-mm dishes containing fresh medium and grew them to 70–80% confluency. Cells were then transfected with 5.5 μ g of DNA by the Lipofectamine Plus (Life Technologies) method in Optimem I medium, according to the manufacturer's protocol. Twenty-four hours after transfection, medium was changed to that containing hygromycin. For 293, the hygromycin concentration was 250 μ g/ml, whereas for C127 and Hepa1a, the hygromycin concentration was 600 μ g/ml. Cells were grown under selection for 24–39 d. Plasmid DNA was then isolated by Hirt extraction [42]. DNA was digested with *DpnI* or mock digested without enzyme. A fraction of the digestion mixture was transformed by electroporation into *E. coli* strain DH10B, and bacterial colony formation was assayed on agar plates containing 50 μ g/ml ampicillin and 50 μ g/ml methicillin (Sigma, St. Louis, MO).

In vivo delivery and analysis. C57BL/6 mice were injected over a period of 6–9 s with 25 μ g of DNA in 1.8 ml of 0.9% NaCl, by hydrodynamic tail-vein injection [4,5]. Serum samples were periodically obtained by retro-orbital bleed. All animal procedures were performed under the guidelines set forth by Stanford University and the National Institutes of Health. The samples were assayed by a polyclonal capture ELISA assay [35,43]. Some animals were sacrificed and livers removed for analysis of plasmid DNA. Liver tissue was diced and total DNA prepared using the Blood and Cell Culture DNA Maxi kit (Qiagen, Valencia, CA). DNA was digested with *EcoRV* or *BglII* and separated on a 0.65% agarose gel. The gel was deproteinized in 0.25 M HCl, denatured in 0.5 M NaOH, and transferred to a Zeta-Probe GT (BioRad) blotting membrane in 10 \times SSC transfer buffer. The membrane was probed with a 692-bp fragment from the ampicillin resistance gene common to all the plasmids, labeled with ³²P by random primer extension. Hybridization occurred at 65°C for 5 h in Rapid-Hyb Buffer (Amersham Pharmacia). Membranes were washed in 20–40 mM Na₂HPO₄, 1–5% SDS at 65°C. Southern blotting was carried out as described above. Other aliquots of liver DNA were transformed into *E. coli* strain DH10B and evaluated for colony formation as described above.

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