The role of EBV and genomic sequences in gene expression from extrachromosomal gene therapy vectors in mouse liver

Research Article

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Summary

A plasmid vector containing Epstein-Barr virus (EBV) sequences and the full genomic *SERPINA1* locus encoding the gene for ₁-antitrypsin is capable of providing long-term, high-level expression when transfected into mouse liver. It was unclear which viral and genomic sequences were required for efficient expression of this transgene *in vivo*. We tested here the requirement for EBV sequences for retention and expression of plasmid DNA in normal and replicating liver *in vivo*. The results showed that EBV sequences provided increased retention and expression of an extrachromosomal vector containing the full *SERPINA1* transgene, in addition to the expression provided by the full gene alone. We also minimized the *SERPINA1* sequence and determined which portions were necessary for persistent, high expression levels. Finally, we demonstrated that the *SERPINA1* sequence can act to enhance expression of a heterologous gene cloned within it. Expression from a factor IX minigene was increased ~50-fold when it was expressed from within the *SERPINA1* sequence, compared to a vector containing the factor IX minigene alone. The results presented here demonstrate that a significant amount of genomic sequence may be required for persistent, high levels of expression *in vivo* and that the persistence of plasmid DNA in dividing tissues and expression levels are enhanced by inclusion of EBV sequences on the vector.

I. Introduction

The ability to achieve persistent, regulated, high levels of transgene expression *in vivo* is often necessary for the success of a gene therapy vector. Unfortunately, with most gene therapy vectors used to date, expression is temporary, often falling to non-therapeutic or undetectable levels within a few weeks after treatment. For viral vectors, transience may be due to the immunogenicity of the vector, resulting in loss of transfected cells with a concurrent reduction in transgene expression. In the case of non-integrating vectors, viral or non-viral, transience can result from vector loss as the cells divide.

For both integrating and non-integrating systems, decreased transgene expression may also be attributable to DNA silencing. For example, when mouse hepatocytes were transfected *in vivo* with naked plasmid DNA encoding the AAT cDNA under control of the cytomegalovirus (CMV) promoter, day 1 expression levels of 500 µg/ml were observed. These levels fell to <10 µg/ml within 3 weeks after transfection (Zhang et al,

2000). Southern analysis of liver DNA showed that plasmid DNA was maintained extrachromosomally in the liver cells for at least 100 days, indicating that the decrease in expression was primarily a result of DNA silencing, rather than vector loss.

We observed similar results (Stoll et al, 2001) in experiments in which naked plasmid DNA was injected into mouse hepatocytes via hydrodynamic tail vein injection (Zhang et al, 1999). On the one hand, an extrachromosomal plasmid carrying the 19 kb genomic *SERPINA1* encoding human ₁-antitrypsin (AAT) resulted in expression levels of >300 µg/ml *in vivo* that persisted at these high levels for > 9 months. However, similar constructs carrying the AAT cDNA driven by the RSV promoter gave equivalent day 1 expression levels, but the expression dropped >100-fold within two weeks. Again, Southern analysis showed that plasmid DNA was maintained extrachromosomally in these relatively quiescent liver cells. In addition to the *SERPINA1* locus, the successful genomic AAT vector also possessed

sequences from Epstein-Barr virus (EBV) that can aid in extrachromosomal plasmid maintenance and expression.

Epstein-Barr virus (EBV) is a human herpes virus that is capable of maintaining its genome extrachromosomally in 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 (Yates et al, 1984, 1985; Reisman et al, 1985). Plasmids containing *EBNA1* and a truncated *ori*P 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 the cells, but can replicate efficiently only if the plasmid also contains a functional mammalian origin of replication, such as the 19 kb *SERPINA1* sequence (Krysan et al, 1989; Heinzel et al, 1991; Stoll et al, 2001). These same EBV components that provide replication and retention functions are also associated with transcriptional enhancer and anti-silencing activity (Reisman and Sugden, 1986; Kaneda et al, 2000). Furthermore, in addition to the replication function of the genomic *SERPINA1* sequence demonstrated in our previous experiments (Stoll et al, 2001), the full AAT gene was also able to provide more stable expression *in vivo* than its equivalent cDNA sequence, which may be subject to silencing.

Silencing of cDNA vectors may occur because the transgenes are often driven by viral promoters. It has been observed that many common viral promoters, such as those from cytomegalovirus (CMV), simian virus 40 (SV40), and Rous sarcoma virus (RSV) often exhibit markedly decreased activity in mammalian cells *in vivo* within a few weeks of transfection, a phenomenon that has been attributed to inhibition by various cytokines (Paillard, 1997). Gill (2001) recently demonstrated that the use of the cellular elongation factor 1 (EF1) and ubiquitin C (UbC) mammalian promoters gave increased persistence and ~10-fold higher expression levels of a luciferase reporter gene in lungs, compared to a control construct that expressed luciferase from the CMV promoter. Quantitative PCR analysis of plasmid vector in the lung tissue revealed that there were no significant differences in plasmid copy number in the CMV versus EF1 or UbC promoter vectors.

In addition to the reduced transgene silencing observed when mammalian promoters are used, genomic sequences may provide additional benefits that lead to increased transgene expression. Studies in transgenic mice have indicated that introns are essential for stable, high levels of transgene expression. In comparing transgenic mice generated with cDNA constructs versus full genomic sequences, the intronless constructs resulted in a lower frequency of transgenic mice expressing rat growth hormone (rGH), mouse metallothionein I (mMTI), or human -globin (hBG) reporter genes, as well as decreased expression levels in those mice that did have observable expression (Brinster et al, 1988). Similar results have been observed for AAT and -lactoglobulin expression constructs in mammary cells of transgenic mice (Whitelaw et al, 1991). It is possible that genomic introns

contain transcriptional enhancer sequences that may act on their own to increase transgene expression or may act in concert with upstream/promoter sequences. These intronic sequences may also act to help the transgene attain an open chromatin configuration, making it more accessible to transcription factors. This idea is supported by observations that deletion of intronic sequences makes transgenes more susceptible to chromosomal position effects *in vivo* than their full genomic counterparts (Webster et al, 1997).

Unfortunately, the large size of most full genes often precludes their use in vectors. In order to obtain the expression advantages of intronic sequences, while still minimizing transgene size, heterologous introns and genomic minigenes have been developed. Palmiter (1991) found that including only select introns, specifically the first one, in the rGH gene resulted in transgenic frequencies and expression levels comparable to those achieved when the full rGH was used. Heterologous introns inserted between promoter and cDNA gave similar results (Palmiter et al, 1991). This strategy has been applied to the construction of expression vectors for therapeutically relevant genes. Miao (2000) constructed a human factor IX minigene, which included the ApoE hepatic locus control region (HCR), the hepatocyte-specific AAT promoter, and the human factor IX cDNA, with its intron A and 3' untranslated region (UTR). This 6.1 kb minigene was reported to provide therapeutic serum levels of factor IX (0.5 - 2 µg/ml) that were sustained for at least 225 days, whereas a 2.0 kb AAT promoter-factor IX cDNA construct gave transient expression that fell to <10 ng/ml within 2 weeks of treatment (Miao et al, 2000, 2001). While this minigene strategy may not be successful for all genes, it raises the possibility of creating a high-expressing transgene that is capable of stable expression *in vivo*, without the large sizes typical of most mammalian genes.

Another strategy to take advantage of the high expression capabilities of genomic sequences is to insert a cDNA sequence into the first exon of a full gene. The theory is that a well-expressed genomic sequence may provide an open chromatin configuration, transcriptional enhancers, and/or anti-silencing sequences that may act in *cis* to provide for stable expression of the heterologous cDNA. While this approach has been used successfully to achieve expression of diphtheria toxin (Palmiter et al, 1987) and rat transforming growth factor (Palmiter et al, 1991), it has not proven successful for all cDNAs and genomic sequences tested (Palmiter et al, 1991).

We have previously demonstrated that the 19 kb full genomic *SERPINA1* sequence is capable of giving stable, high levels of expression *in vivo* when introduced into mouse liver (Stoll et al, 2001). Unfortunately, 19 kb is still a rather large sequence. Reduction of the genomic size without loss of the good expression it provides would be beneficial. To achieve this result, we minimized the size of the AAT genomic sequence, both through reduction of flanking sequences and through generation of an AAT minigene that retains only its first intron and all flanking sequences. We explored the ability of this genomic sequence to stabilize and boost expression of a

heterologous gene, by inserting a human factor IX minigene (Miao et al, 2000) upstream of the first exon in *SERPINA1*. Here we report the successful reduction in size of the AAT gene, without loss of expression, as well as the successful stabilization of factor IX expression *in vivo* when expressed from within the AAT gene.

II. Materials and methods

A. Vector construction

Plasmids pEF, pEF-AAT, pEF-cAAT, and pcAAT have been described previously (Stoll et al, 2001). Removal of the EBV components (EBNA1 and family of repeats) from plasmid pEF-AAT was achieved by digestion with *Cla*I and vector religation, creating the plasmid pAAT.

Vectors pcAAT2 and pEFcAAT2 are identical to pcAAT and pEF-cAAT, respectively, except that the RSV promoter has been replaced with the endogenous, hepatocyte-specific AAT promoter. 471 bp containing the AAT promoter was PCR amplified from vector pF9 (Sclimenti et al, 2003) and cloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA), making the vector pTA-AATpro. From there it was subcloned as an *Mlu*I-*Hin*DIII fragment into the *Mlu*I-*Hin*DIII sites in the plasmid pcAAT, replacing the RSV promoter. This step created the vector pcAAT2. From that plasmid, the 2.1 kb AATpro-cDNA fragment was liberated as an *Xho*I fragment and cloned into the *Sal*I site in plasmid pEF, creating plasmid pEF-cAAT2.

Minimization of the AAT genomic sequence began with removal of some of the flanking sequences. From the 5' upstream sequence, 3.7 kb was removed by digesting pAAT with *Cla*I and *Mfe*I and religating the vector, creating the vector pAAT(-5'). From the 3' flanking sequence in pAAT, 970 bp was removed by deleting an *Afl*II-*Sal*I fragment, generating pAAT(-3'). This plasmid was further reduced by digestion with *Cla*I and *Mfe*I and religation, generating the double deletion plasmid pAAT , which is reduced by a total of 4.7 kb, compared to pAAT.

Cloning of the AAT minigene vector was performed as follows. The AAT cDNA was removed from pcAAT as a 1.3 kb *Bam*HI-*Pst*I fragment, and cloned into the *Bam*HI-*Pst*I sites of pBCSK+ (Stratagene, La Jolla, CA), creating the vector pBcAAT. An *Afl*II linker was cloned into its *Bgl*I-*Pst*I sites, generating vector pBcAATii. A 1529 bp fragment of AAT 3' sequence was PCR amplified and cloned into vector pCR2.1, creating pTA-AAT3', from which the AAT3' sequence was subcloned as an *Eco*RI fragment into the *Eco*RI sites of pBcAAT, creating pBcAAT-AAT3'. From this plasmid, a 622 bp *Ava*I-*Pst*I fragment was cloned into the *Ava*I-*Pst*I sites of pBcAATii, generating the vector pBcAATiii. Next, a 499 bp *Pst*I fragment from pBcAAT-AAT3' was cloned into pBcAATiii, creating plasmid pBcAATiv. A 552 bp *Stu*I-*Afl*II fragment from pBcAAT-AAT3' was cloned into pBcAATiv, generating pBcAATv. Next, a 771 bp *Bam*HI fragment from pAAT was cloned into the *Bam*HI site of pBcAATv, to create the plasmid pBcAATvi. The last step in creating the minigene was to clone the 2.9 kb minigene from pBcAATvi, as an *Afl*II-*Sna*BI fragment, into the *Afl*II-*Sna*BI sites of vector pAAT. The result is vector pAATmg, which lacks introns 2-4, resulting in a total reduction of 3.5 kb in gene size.

Vector pAAT-fIXmg was constructed as follows. A *Bgl*II linker was ligated into the *Sac*I site of pF9, generating pfIXmgi. Next, a 2.6 kb fragment of the AAT first intron was PCR amplified, digested with *Bam*HI and *Bgl*II, and cloned into the *Bgl*II site of pfIXmgi, creating pfIXmgii. The AAT promoter was released from pTA-AATpro as a *Sap*I-*Cla*I fragment and cloned into the *Sap*I-*Cla*I sites of pfIXmgii, generating the vector pfIXmgiii. Finally, the 7.9 kb fIXmg was released from plasmid pfIXmgiii by digestion with *Bgl*II and cloned into the *Bgl*II sites in plasmid pAAT, resulting in the final vector pAAT-fIXmg.

B. *In vivo* delivery and analysis.

C57BL/6 mice were injected over a period of 6 – 9 s with 25 µg of DNA in 1.8 ml of 0.9% NaCl, by hydrodynamic tail-vein injection (Liu et al, 1999; Zhang et al, 1999). 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 to measure serum AAT (Song et al, 1998; Yant et al, 2000) and/or serum fIX (Sclimenti et al, 2003). Some animals were subjected to partial, surgical hepatectomy (PH) (Chen et al, 2001) and then allowed to recover. Serum samples were obtained periodically by retro-orbital bleed and analyzed by ELISA. At 51 d post-PH, treated and control animals were sacrificed and the livers were removed for analysis of plasmid DNA. The liver tissue that was removed during PH surgery was also subjected to analysis. Liver tissue was diced and total DNA was prepared using the Blood and Cell Culture DNA Maxi kit (Qiagen, Valencia, CA). Southern blot analysis was performed as follows. DNA was digested with *Sca*I and separated on a 0.65% agarose gel. The gel was depurinated in 0.25 M HCl, denatured in 0.5 M NaOH, neutralized in 0.5M Tris-HCl (pH 7.0) and transferred to an S&S Nytran blotting membrane (Schleicher & Shuell, Keene, NH) in 20X SSC transfer buffer. The membrane was probed with a 484 bp fragment from the hygromycin resistance gene common to both plasmids, labeled with alkaline phosphatase enzyme, using the AlkPhos Direct DNA labeling kit (Amersham Pharmacia Biotech). Hybridization occurred at 55°C for 16 h in hybridization buffer provided with the kit. Membranes were washed according to protocol and incubated with CDP-Star Detection Reagent for 4 min. The membrane was then exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). Southern blot quantification was performed using Kodak 1D software.

III. Results

A. Role of EBV sequences in extrachromosomal plasmid expression *in vivo*

Our previous research showed that the plasmid pEF-AAT, bearing the 19 kb *SERPINA1* genomic locus encoding human ₁-antitrypsin on a vector with the EBV *EBNA1* and family of repeats retention sequences, was capable of maintaining long-term high expression levels after transfection into mouse liver (Stoll et al, 2001). We first wanted to determine what role the EBV components on this plasmid played in sustaining these high expression levels. *EBNA1* and the EBV family of repeats were removed, creating plasmid pAAT. Naked plasmid DNA of pEF-AAT and pAAT, along with pEF-cAAT and pcAAT control cDNA plasmids with and without EBV retention sequences (**Figure 1A**), were injected into the mouse liver via hydrodynamic tail-vein injection (Liu et al, 1999; Zhang et al, 2000). Serum AAT levels were determined by ELISA. The results are shown in **Figure 1B**. Both genomic vectors were able to provide expression levels ~1000-fold greater than the cDNA control vectors, which fell to 100 ng/ml within 3 weeks post-injection. While the non-EBV plasmid pAAT was still capable of providing stable, high-levels of expression of AAT (151 µg/ml at 6 months), the levels provided by pEF-AAT were ~4-fold

higher (585 µg/ml at 6 months). This result indicated that the EBV components provided some expression advantage to the plasmid pEF-AAT.

The liver is a relatively quiescent tissue, suggesting that cell division-induced vector loss should not be a significant problem. This conjecture is supported by observations that plasmid DNA is stably maintained extrachromosomally in liver cells (Zhang et al, 2000; Stoll et al, 2001). In order to determine if there was indeed a DNA retention advantage provided by the EBV sequences in pEF-AAT compared to pAAT, partial hepatectomy (PH) was performed on sample mice injected with each of these plasmids. Partial surgical hepatectomy involved removal of two-thirds of the liver, stimulating division and regeneration of the remaining liver tissue. Under these circumstances, extrachromosomal plasmid DNA is lost from the cells, unless it contains retention sequences such as those provided by *EBNA1* and the EBV family of repeats (Krysan et al, 1989). Following PH, serum levels of AAT were monitored, and the results are shown in **Figure 2A.** Liver genomic DNA was also analyzed in PH-mice before and after surgery and in control mice (**Figure 2B**).

The effects of partial hepatectomy on AAT expression and DNA retention are summarized in **Table 1**. In mice injected with the EBV vector pEF-AAT, partial hepatectomy resulted in a 1.7-fold decrease in total plasmid DNA in the liver and a 4.6-fold decrease in serum AAT levels. Mice injected with the non-EBV plasmid pAAT showed a 3.5-fold decrease in total liver plasmid DNA and a 7.0-fold decrease in serum AAT levels. These results suggested that the EBV sequences were playing a role in enhancing the retention and expression of plasmid DNA *in vivo* in the presence of cell division. When in non-

dividing tissue, the non-EBV pAAT plasmid appeared to be capable of maintaining stable, high-levels of expression. For this reason, and also to eliminate any effects on expression that the EBV sequences may have, all further experiments were conducted using a non-EBV extrachromosomal vector.

## B. Minimization of AAT sequences required for expression *in vivo*

While the 19 kb *SERPINA1* sequence was not prohibitively large for use in extrachromosomal gene therapy vectors, it was still a cumbersome size. It was likely that some of the 8.5 kb of flanking sequences were not necessary for regulation or enhancement of gene expression. In order to address this question and possibly minimize the functional size of this genomic sequence, we began by deleting segments of flanking sequence and analyzing the *in vivo* expression of these deleted vectors. Using convenient restriction enzyme sites, 3.7 kb of 5' flanking sequence were removed from pAAT to generate pAAT(-5'), 921 bp were removed from the 3' flanking sequence to create pAAT(-3'), and both deletions were generated in the same plasmid to produce plasmid pAAT . These plasmids, along with the full gene vector pAAT and a cDNA vector pcAAT, were injected hydrodynamically into the tail-vein of mice and serum AAT levels were monitored by ELISA. The results are shown in **Figure 3**. All genomic vectors were able to provide high expression levels of AAT, >190 µg/ml at two months post-injection, though vectors pAAT(-5') and pAAT gave ~2-fold higher expression levels than pAAT and pAAT(-3').
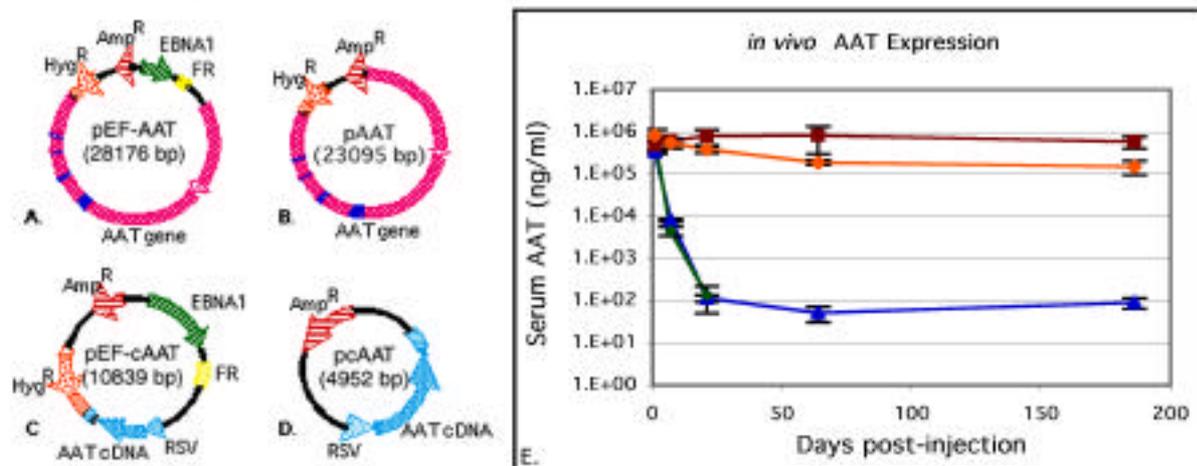
Figure 1. Effect on AAT expression of removal of EBV sequences. (**A**) pEF-AAT contains the 19 kb AAT genomic region on a plasmid with the EBV family of repeats (FR) and *EBNA1* gene, (**B**) pAAT contains the 19 kb AAT genomic region (encoded by *SERPINA1*) in a non-EBV backbone, (**C**) pEF-cAAT contains the AAT cDNA driven by an RSV promoter, on a plasmid containing the EBV FR and *EBNA1* retention sequences, (**D**) pcAAT contains the AAT cDNA driven by an RSV promoter in a non-EBV backbone. (**E**) Groups of 5 mice were injected with 25 µg of pEF-AAT (■), pAAT (♦), pEF-cAAT (●), or pcAAT (▲). Blood was sampled periodically, and serum AAT levels were determined by ELISA. Error bars represent the standard error from five mice.
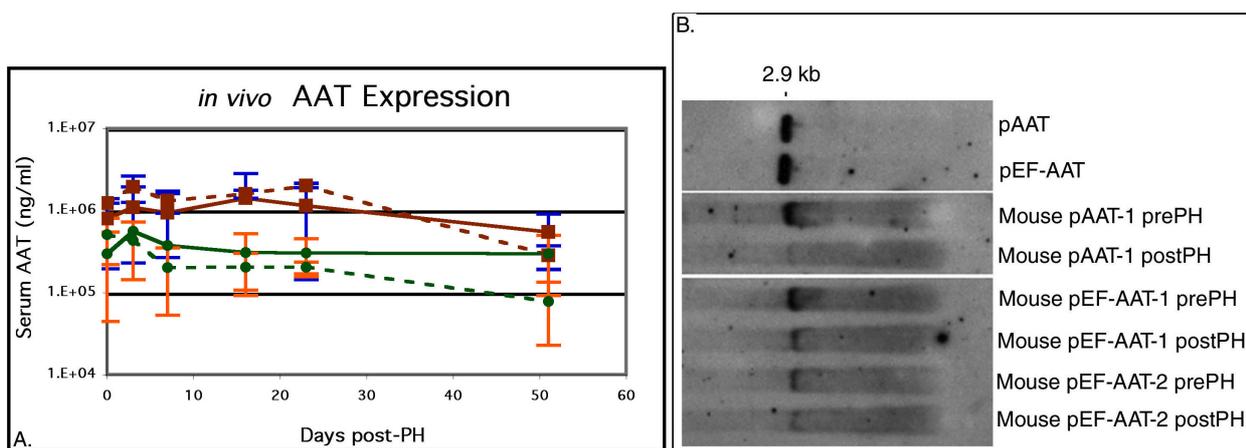
Figure 2. Effect of partial hepatectomy (PH) on plasmid DNA and AAT expression *in vivo*. (**A**) AAT expression, as determined by ELISA on day of and following PH, in mice injected with pEF-AAT (■) or pAAT (●). Solid lines are control (no PH) mice; dashed lines underwent PH on Day 0. (**B**) DNA was extracted from mouse livers harvested during PH and at 51 d post-PH. DNA was digested and probed with a labeled fragment from the hygromycin resistance gene.
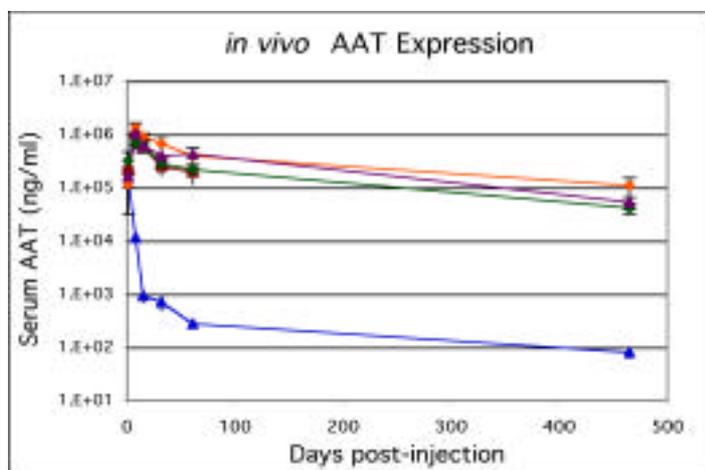
Figure 3. Effect on AAT expression of removal of flanking sequences in *SERPINA1*. Groups of five mice were injected with 25 µg of pAAT (■), pAAT(-5') (●), pAAT(-3') (♦), pAAT (▲), or pcAAT (▲). Blood was periodically sampled, and serum AAT levels were determined by ELISA. Error bars represent the standard error from five mice. Plasmids are described in the text.

Table 1: Effect of partial hepatectomy (PH) on retention of plasmid DNA in liver and AAT expression *in vivo*

			AAT expression analysis[a]			Southern Analysis[b]	
			Decrease in AAT expression	Avg Decrease in expression per group	Fold decrease in expression in PH vs non-PH groups	Decrease in plasmid DNA in liver	Avg Decrease in plasmid DNA in liver per group
pEF-AAT	Mouse 1	PH	3.5	4.6	3.3	1.78	1.7
	Mouse 2		5.6			1.67	
	Mouse 3	No PH	1.3	1.4		n/a	n/a
	Mouse 4		1.5			n/a	
pAAT	Mouse 1	PH	7.8	7.0	7.0	nd	3.5
	Mouse 2		6.1			3.48	
	Mouse 3	No PH	0.8	1.0		n/a	n/a
	Mouse 4		1.1			n/a	

[a]AAT expression was determined by ELISA, as reported in **Figure 2A**.
[b]Plasmid DNA presence in liver was determined by southern blot, as shown in **Figure 2B**. Quantification of band intensities was performed using Kodak 1D software. Mice are labeled 1-4 as they are labeled in **Figure 2B**.

Control cDNA vectors pcAAT and pEF-cAAT (**Figure 1A**) were both expressed from the Rous sarcoma virus (RSV) promoter. It was possible that the AAT genomic sequence was not necessary at all, and that the high expression was simply the result of the strength of the AAT promoter. To address this possibility, the RSV promoter was replaced with the AAT promoter in plasmids pcAAT and pEF-cAAT, generating the vectors pcAAT2 and pEF-cAAT2, respectively (**Figure 4A**). Following liver transfection via tail-vein injection, *in vivo* expression was monitored by ELISA (**Figure 4B**). The AAT promoter was able to provide some transient stability to cDNA expression *in vivo*. While the RSV-cDNA vectors fell to nearly undetectable levels within 2 weeks

post-injection, the AAT promoter-cDNA vectors did not fall to these levels until ~5 weeks post-injection. However, there was no significant difference in expression levels from the two promoters. Ultimately, expression was lost from all four cDNA vectors, whereas the genomic sequence in pAAT maintained expression at >280 µg/ml for the duration of the experiment.

Miao (2000) created a well-expressed human factor IX minigene that retained only a portion of the first intron of that gene. We wondered if construction of a similar minigene for AAT would be equally successful. We created the plasmid pAATmg, which contained the full 5' and 3' flanking sequences of pAAT, the AAT promoter, and all exons, but retained only the 6.1 kb first intron (**Figure 4A**). Serum AAT levels were monitored by ELISA following tail-vein injection of plasmid DNA into mice, with the results shown in **Figure 4C**. The 15.5 kb AAT minigene was not capable of providing stable, high expression levels of AAT *in vivo*. While day 1 levels of expression were within 3-fold of each other, expression from pAATmg dropped ~100-fold below that of pAAT within 3 weeks post-injection, and continued to drop, approaching cDNA expression levels by day 85.

C. Expression of a heterologous gene from within SERPINA1

Our unsuccessful attempt to create an AAT minigene indicated that not all genes could be easily minimized to a size convenient for use in gene therapy vectors, transgenic mice, or numerous other applications, while still maintaining high expression. Due to the high expression levels of AAT *in vivo* in these and previous experiments (Stoll et al, 2001), we hypothesized that the full-genomic *SERPINA1* might contain sequences that could enhance expression of a heterologous gene cloned within it. To demonstrate this idea, a human factor IX minigene (Miao et al, 2000) was cloned into the first exon of the AAT gene. Miao (2000) reported high levels of expression for this factor IX minigene that were maintained *in vivo* for 225 days. However, Sclimenti (2003) observed only transiently high expression levels for this minigene that stabilized at 100 ng/ml after 2-3 months post-injection. We therefore chose this factor IX minigene (fIXmg), rather than a factor IX cDNA, to clone within *SERPINA1* for expression studies, creating the vector pAAT-fIXmg (**Figure 5A**). Plasmid DNA was transfected into mouse liver by hydrodynamic tail-vein injection, and serum fIX levels were monitored by ELISA. In addition to pAAT-fIXmg, we also tested expression from control plasmids pfIX and pDY-fIX, which contained the fIXmg on a plasmid with EBV retention sequences (Sclimenti et al, 2003). Results are shown in **Figure 5B**. As observed by Sclimenti (2003), the factor IX minigene alone plasmid, pfIX, had high day 1 levels (13 µg/ml) that fell to <100 ng/ml by 5 months post-injection. In contrast, pDY-fIX maintained expression ~6-fold higher after 1 year, and pAAT-fIXmg expression of fIX was even higher at 1.9 µg/ml at 1 year post-injection. These results indicated that there were sequences within *SERPINA1* that were able to

act in *cis* to increase expression of nearby or embedded heterologous genes.

IV. Discussion

We previously reported (Stoll et al, 2001) that the plasmid pEF-AAT, containing the 19 kb *SERPINA1* genomic locus encoding human ₁-antitrypsin and the *EBNA1* and family of repeat sequences from Epstein-Barr virus,
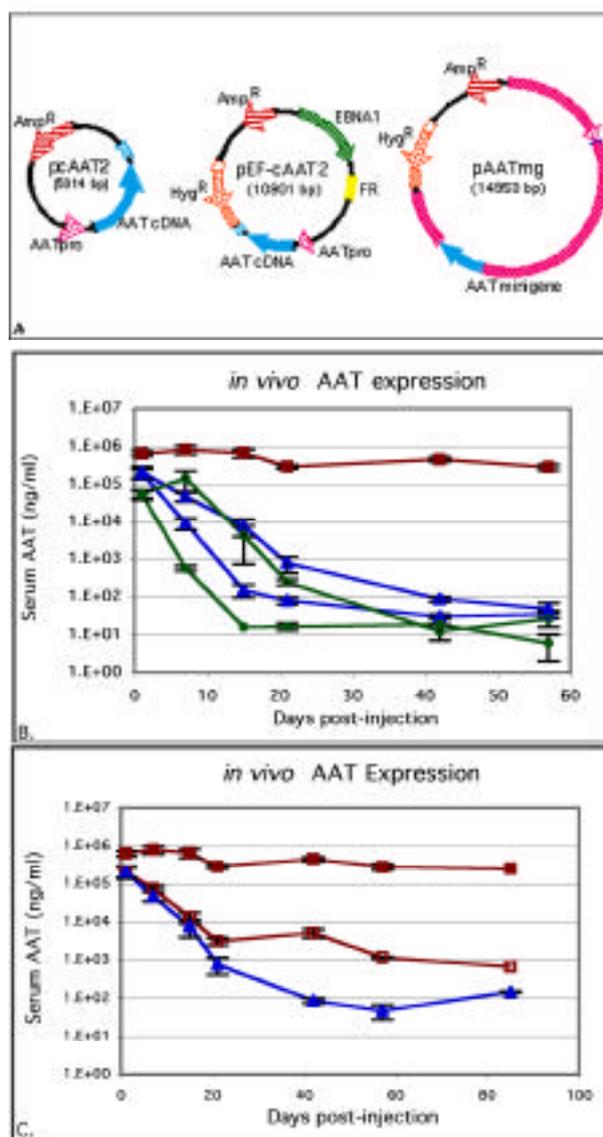
Figure 4. *SERPINA1* sequence requirements for AAT expression *in vivo*. (**A**) Plasmids. pcAAT2 and pEF-cAAT2 contain AAT cDNA driven by the mammalian hepatocyte-specific AAT promoter, without and with EBV sequences, respectively. pAATmg contains the AAT minigene, which retains all flanking sequences, exons, and the first intron of *SERPINA1*.. (**B**) AAT expression levels in mice injected with 25 µg of pAAT (■; **Figure 1B**), pEF-cAAT (●; **Figure1A**), pEF-cAAT2 (○), pcAAT (▲; **Figure 1D**), or pcAAT2 (△). (**C**) AAT expression levels in mice injected with 25 µg of pAAT (■), pAATmg (□), or pcAAT2 (△). (**B & C**) Blood was sampled periodically, and serum AAT was determined by ELISA. Error bars represent the standard error from five mice.
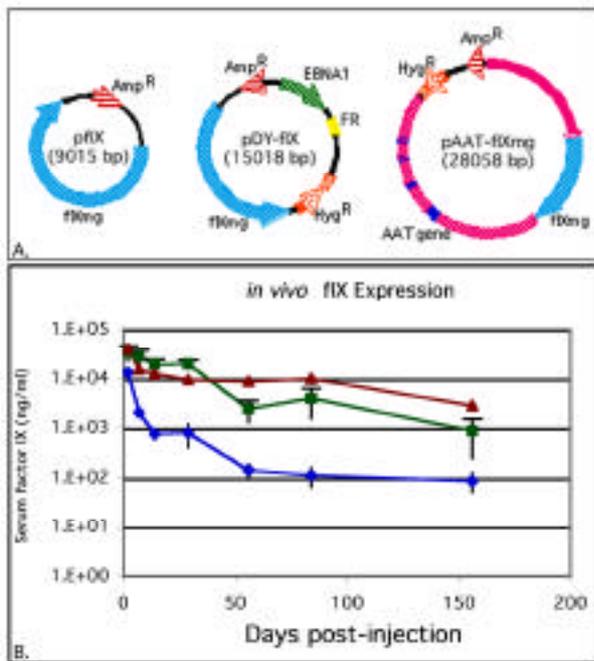
Figure 5. Effect of genomic sequence on expression of a heterologous fIX minigene. (**A**) Plasmid maps. (**B**) Factor IX expression levels in mice. Groups of 5 mice were injected with 25 µg of pfIX (◆), pDY-fIX (■), or pAAT-fIXmg (▲). Blood was sampled periodically, and serum fIX levels were determined by ELISA. Error bars represent the standard error.

was able to provide long-term high-level expression of AAT *in vivo*. The AAT genomic sequence was capable of acting as a mammalian origin of replication for this extrachromosomal plasmid (Stoll et al, 2001), and the EBV sequences can act to retain the plasmid in cells. However, these features were not likely to be relevant *in vivo* because these experiments were conducted in non-dividing liver cells.

In order to determine whether the EBV sequences were providing any advantage *in vivo* in expression or retention of the vector, we constructed the genomic/non-EBV vector pAAT. Vectors with and without EBV sequences both provided stable, high expression levels *in vivo* (>150 µg/ml), but the pEF-AAT vector with EBV sequences was ~4-fold higher (**Figure 1B**). Following partial hepatectomy in representative mice, we observed an average 3.3-fold decrease in AAT expression in pEF-AAT-injected mice and a 7.0-fold decrease in serum AAT in pAAT-injected mice (**Figure 2A**; **Table 1**). Southern analysis revealed that partial hepatectomy-induced hepatocyte replication resulted in loss of plasmid DNA in both the presence and absence of EBV retention sequences. However, pEF-AAT mice retained twice as much plasmid DNA as did pAAT mice, which directly correlated with the observed serum AAT levels in these two different groups of mice. These results indicated that the EBV sequences were providing increased retention to these extrachromosomal vectors in dividing tissue *in vivo*.

The ~4-fold higher expression levels in pEF-AAT mice vs. pAAT mice pre-PH suggested that the EBV

sequences were also providing expression enhancement activity. Enhancer activity has previously been attributed to the action of these EBV sequences (Kenney et al, 1998; Langle-Rouault et al, 1998; Sclimenti et al, 2003). This enhancer function appears to rely on the basal expression ability of the transgene construct. The EBV sequences provided a ~4-fold boost in expression from the full AAT gene, but were unable to prolong or increase expression from AAT cDNA constructs. Even when the viral RSV promoter driving the cDNA was replaced with the human AAT promoter, expression was only minimally prolonged, with or without EBV sequences (**Figure 4B**). This result suggests that either the AAT promoter alone was not able to maintain persistent expression *in vivo*, or that these cDNA constructs lacked structural or regulatory sequences that contribute to the persistence of expression *in vivo*, as was observed for the full-genomic construct pAAT.

Using the pAAT vector, we attempted to minimize the size of the *SERPINA1* locus required for high expression levels *in vivo*. We observed an ~2-fold increase in expression when 3.7 kb of 5' upstream sequence was deleted from the vector, either alone or in combination with 921 bp deleted from the 3' end (**Figure 3**). It is possible that the 5' flanking sequence may contain a repressor sequence that results in enhanced expression when it is removed from the *SERPINA1* genomic sequence. However, expression levels were still remarkably high and stable for all four of these genomic vectors, indicating that a reduced genomic sequence of 14.4 kb was sufficient for attaining good expression of AAT *in vivo*.

Further minimization of the genomic sequence, through creation of an AAT minigene deleted of all but the first intron, resulted in an ~100-fold decrease in expression *in vivo* (**Figure 4C**). However, an alternative AAT minigene, retaining all introns except the first one and flanked by -lactoglobulin sequences, has been shown to express AAT at high levels in mice transgenic for the construct (Whitelaw et al, 1991; Clark et al, 1993). Considering these two different findings together suggests that there are sequences within the last three exons that are necessary for efficient AAT expression *in vivo*, though the nature of these expression modulation sequences is unclear. It is possible that one or more of the deleted introns contain transcriptional enhancers. Alternatively, the first intron may contain transcriptional repressor sequences, which are naturally modulated by sequences in the downstream exons, removal of which in our minigene resulted in suppression of expression. Also, it may be that the additional exons are required to allow stabilization and/or proper processing of the mRNA. While several labs have observed high expression levels from minigenes containing only a first intron (Palmiter et al, 1991; Miao et al, 2000), our results suggest that not all genes are amenable to such simple minimization. Creation of genomic minigenes may require analysis of multiple intron combinations to identify a minigene that provides a suitable expression profile *in vivo*.

While *SERPINA1* is 19 kb long, thus prompting our attempts to minimize the amount of sequence sufficient for high expression levels *in vivo*, it is still a relatively small

gene. Some therapeutically relevant genes are much larger, such as factor VIII (~185 kb), cystic fibrosis transmembrane regulator (~230 kb), and dystrophin (~2.4 Mb). Unfortunately, cDNA constructs often provide only transient expression *in vivo*. It has been observed that a cDNA sequence cloned within another full-length gene can benefit from the genomic sequence in *cis*, allowing for stable expression of the cDNA (Palmiter et al, 1987; Palmiter et al, 1991). To test this idea for the AAT gene, we cloned the human factor IX minigene (Miao et al, 2000) into *SERPINA1* between the promoter and the initiation codon. We observed an ~50-fold increase in fIX expression when the fIX minigene was placed within the AAT gene, compared to a vector containing only the minigene (**Figure 5B**). The AAT gene enhanced expression of the fIX minigene to levels ~9-fold higher than EBV sequences enhanced fIX expression (pDY-fIX; **Figure 5B**). It therefore appears that the AAT genomic sequence is capable of increasing and maintaining expression of a heterologous minigene. It seems likely that these AAT sequences will be capable of exerting a similarly beneficial effect on other cDNA or minigene constructs. Since AAT is expressed only in the liver, it is possible that this effect will be limited to expression from that tissue. However, if the benefit is the result of a tissue-independent enhancer activity or a structurally open chromatin configuration of the genomic sequence, it may be extendable to other tissues, provided that a suitable promoter drives the heterologous cDNA.

While we have demonstrated that some minimization of *SERPINA1* genomic size did not affect its *in vivo* expression, there was a limit to the amount and choice of sequences that could be removed without affecting expression. For example, the last three introns of *SERPINA1* appeared to be much more important than the first intron alone, as evidenced by the difference in *in vivo* expression from these two different minigene constructs (**Figure 4C**) (Whitelaw et al, 1991), though our pAATmg construct actually retained 1.8 kb more genomic sequence. A factor IX minigene containing only the first intron has been shown to provide adequate expression levels *in vivo* (Miao et al, 2000). It is therefore important to note that the sequences required for efficient expression *in vivo* are likely to differ from gene to gene. These results demonstrate the value of utilizing genomic sequences for *in vivo* expression of a transgene in a gene therapy system.

Acknowledgments

References

Brinster, RL, Allen, JM, Behringer, RR, Gelinas, RE and Palmiter, RD (**1988**) Introns increase transcriptional efficiency in transgenic mice. **Proc Natl Acad Sci U S A** 85, 836-840.

Chen, ZY, Yant, SR, He, CY, Meuse, L, Shen, S and Kay, MA (**2001**) Linear DNAs concatemerize in vivo and result in sustained transgene expression in mouse liver. **Mol Ther** 3, 403-410.

Clark, AJ, Archibald, AL, McClenaghan, M, Simons, JP, Wallace, R and Whitelaw, CB (**1993**) Enhancing the efficiency of transgene expression. **Philos Trans R Soc Lond B Biol Sci** 339, 225-232.

Gill, DR, Smyth, SE, Goddard, CA, Pringle, IA, Higgins, CF, Colledge, WH and Hyde, SC (**2001**) Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. **Gene Ther** 8, 1539-1546.

Heinzel, SS, Krysan, PJ, Tran, CT and Calos, MP (**1991**) Autonomous DNA replication in human cells is affected by the size and the source of the DNA. **Mol Cell Biol** 11, 2263-2272.

Kaneda, Y, Saeki, Y, Nakabayashi, M, Zhou, WZ, Kaneda, MW and Morishita, R (**2000**) Enhancement of transgene expression by cotransfection of oriP plasmid with EBNA-1 expression vector. **Hum Gene Ther** 11, 471-479.

Kenney, S, Ge, JQ, Westphal, EM and Olsen, J (**1998**) Gene therapy strategies for treating Epstein-Barr virus-associated lymphomas: comparison of two different Epstein-Barr virus-based vectors. **Hum Gene Ther** 9, 1131-1141.

Krysan, PJ, Haase, SB and Calos, MP (**1989**) Isolation of human sequences that replicate autonomously in human cells. **Mol Cell Biol** 9, 1026-1033.

Langle-Rouault, F, Patzel, V, Benavente, A, Taillez, M, Silvestre, N, Bompard, A, Sczakiel, G, Jacobs, E and Rittner, K (**1998**) Up to 100-fold increase of apparent gene expression in the presence of Epstein-Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids. **J Virol** 72, 6181-6185.

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

Miao, CH, Ohashi, K, Patijn, GA, Meuse, L, Ye, X, Thompson, AR and Kay, MA (**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.

Miao, CH, Thompson, AR, Loeb, K and Ye, X (**2001**) Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. **Mol Ther** 3, 947-957.

Paillard, F (**1997**) Promoter attenuation in gene therapy: causes and remedies. **Hum Gene Ther** 8, 2009-2010.

Palmiter, RD, Behringer, RR, Quaife, CJ, Maxwell, F, Maxwell, IH and Brinster, RL (**1987**) Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. **Cell** 50, 435-443.

Palmiter, RD, Sandgren, EP, Avarbock, MR, Allen, DD and Brinster, RL (**1991**) Heterologous introns can enhance expression of transgenes in mice. **Proc Natl Acad Sci U S A** 88, 478-482.

Reisman, D and Sugden, B (**1986**) trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. **Mol Cell Biol** 6, 3838-3846.

Reisman, D, Yates, J and Sugden, B (**1985**) A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. **Mol Cell Biol** 5, 1822-1832.

Sclimenti, CR, Neviaser, AS, Baba, EJ, Meuse, L, Kay, MA and Calos, MP (**2003**) Epstein-Barr Virus Vectors Provide Prolonged Robust Factor IX Expression in Mice. **Biotechnol Prog** 19, 144-151.

Song, S, Morgan, M, Ellis, T, Poirier, A, Chesnut, K, Wang, J, Brantly, M, Muzyczka, N, Byrne, BJ, Atkinson, M and Flotte, TR (**1998**) Sustained secretion of human alpha-1-

antitrypsin from murine muscle transduced with adeno-associated virus vectors. **Proc Natl Acad Sci U S A** 95, 14384-14388.

Stoll, SM, Sclimenti, CR, Baba, EJ, Meuse, L, Kay, MA and Calos, MP (**2001**) Epstein-Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice. **Mol Ther** 4, 122-129.

Webster, J, Donofrio, G, Wallace, R, Clark, AJ and Whitelaw, CB (**1997**) Intronic sequences modulate the sensitivity of beta-lactoglobulin transgenes to position effects. **Gene** 193, 239-243.

Whitelaw, CB, Archibald, AL, Harris, S, McClenaghan, M, Simons, JP and Clark, AJ (**1991**) Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic mice. **Transgenic Res** 1, 3-13.

Yant, SR, Meuse, L, Chiu, W, Ivics, Z, Izsvak, Z and Kay, MA (**2000**) Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. **Nat Genet** 25, 35-41.

Yates, J, Warren, N, Reisman, D and Sugden, B (**1984**) A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. **Proc Natl Acad Sci U S A** 81, 3806-3810.

Yates, JL, Warren, N and Sugden, B (**1985**) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. **Nature** 313, 812-815.

Zhang, G, Budker, V and Wolff, JA (**1999**) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. **Hum Gene Ther** 10, 1735-1737.

Zhang, G, Song, YK and Liu, D (**2000**) Long-term expression of human alpha1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using a hydrodynamics-based procedure. **Gene Ther** 7, 1344-1349.