Insertion of CTCF-binding sites into a first-generation adenovirus vector reduces the innate inflammatory response and prolongs transgene expression

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We have made improvements to E1-deleted adenovirus (Ad) transducing vectors that both substantially reduce the innate inflammatory response provoked by the virus in BALB/c mouse ears and increase the duration of expression of the GFP transgene in BALB/c mouse liver. These improvements result from testing the hypothesis that induction of strong innate responses is primarily a result of the powerful enhancer contained within the strong CMV promoter activating expression of Ad genes retained within the vector. A DNA fragment containing four CTCF-binding sites, which was expected to act as a chromatin insulator, was introduced 5′, 3′, or both 5′ and 3′ of a CMV-GFP cassette in an attempt to reduce activation of Ad gene expression by the enhancer. The presence of this sequence in any of the configurations led to reduction of the innate immune response, as assayed by mouse ear swelling, to the low level induced by a virus deleted for the E1 region and carrying no introduced sequence. In addition, the duration of GFP expression in the liver more than doubled. The prolonged GFP expression indicates that GFP does not play the limiting role in shutting down vector expression. The CTCF-binding sequence introduced appears to act as a chromatin insulator in Ad DNA, but position-independence of the elements in reducing the innate immune response indicate unanticipated complexities in the mechanism by which Ad vectors induce innate immune responses.

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Introduction

First-generation adenovirus (FG Ad) vectors, deleted for the E1A, E1B, and E3 genes, have been widely used for transduction of humans and non-human animals. From some of the earliest studies in vivo, primarily in mice, it was clear that these simple vectors containing very strong foreign viral promoters/enhancers induce strong immune responses that limit the duration of transgene expression (Smith et al., 1993; Choi et al., 1993). In spite of the problems associated with FG Ad vectors, they remain the vector of choice for short-term in vivo studies in certain tissues, including liver and mammary gland, in mice. Improvements in FG Ad vectors would offer increased utility in such studies, with fewer complicating off-target effects.

There has been relatively modest study of the roles of vector components in induction of the innate immune responses. In contrast, the host innate response to Ad vectors has been studied in some detail, including the roles of toll-like receptors (Appledorn et al., 2006) and the complement pathway (Kiang et al., 2006). These studies directed modification of a FG Ad vector with improved function (Seregin et al., 2010).

Studies employing FG (Stilwell and Samulski, 2004) or both FG and helper-dependent (hd) Ad vectors (Muruve et al., 2004) have elucidated the pattern of expression of certain cytochemokines induced in mice. Early after transduction, expression of the cytochemokines examined is similar for hd and FG vectors. At longer times after transduction, cytochemokine expression induced by FG vectors remains at a high level while it falls in animals transduced with hd vectors (Muruve et al., 2004). The cytochemokines induced immediately after transduction include proteins of which synthesis and activities are inhibited by E1A proteins during infections with the wild-type virus: IL-6 (Janaswami et al., 1992; Takeda et al., 1994),
factors of which expression is activated in a type 1 interferon response (Reich et al., 1988), and likely other innate inflammatory mediators. In addition to playing an important role in the inflammatory response to FG Ad vectors, IFN-γ and TNF-α down-regulate the activity of the CMV promoter (Sung et al., 2001).

In immune-suppressed patients infected with wild-type Ads, there was a modest cytotoxic response with local infection but dramatically increased response in both level and scope when the infection was systemic (Haveman et al., 2010). This suggests that wild-type Ad is less efficient at countering cytotoxic expression in cells that are not normally targeted by the virus compared to cells that are normally infected. This is important in considering Ad vector function, as Ad vectors have been, in the great majority of tests, targeted to tissues that are not normally infected by the wild-type virus in immune competent individuals.

Transient, intensive immune suppression prior to and during transduction permits long-term transgene expression of FG Ad vectors (Yang et al., 1996a, 1996b; Kay et al., 1997; Jooss et al., 1996; Smith et al., 1996; Stein et al., 1998; Fontanellas et al., 2010). While immune suppression has potential for treatment of patients with FG Ad vectors, understanding of the processes by which FG Ad vectors induce innate and adaptive immune responses has the potential for improvement that may lead to prolonged transgene expression without the disadvantages associated with immune suppression or the difficulties in the preparation and purification of hd Ad vectors.

Because the host innate and adaptive immune responses are considerably more complex than the viral counter responses, examination of the roles of vector components that function to induce or inhibit immune responses offers promise for improvement of simple Ad vectors. The fact that Ad has developed mechanisms to counter the effects of both adaptive and innate immune responses (Reich et al., 1988; Janaswami et al., 1992; Takeda et al., 1994; Windheim et al., 2004; Lichtenstein et al., 2004; Horwitz, 2004) makes understanding the roles of vector components a potentially powerful tool for functional improvement of FG Ad vectors and a means to understand which factors act at rate-limiting steps in the immune responses to Ad vectors.

In this study, we hypothesized that the very strong enhancer contained within the CMV promoter plays the major role in the induction of innate immune responses to FG vectors through its effects on expression of Ad genes retained within the vector. We introduced four CTCF-binding sites in an attempt to provide chromatin insulator function to inhibit CMV enhancer-dependent activation of Ad genes and the presumed resultant innate immune responses. CTCF-binding of its DNA sites leads to a number of effects. The most widely studied of these is the insulation of gene expression from neighboring elements, particularly enhancers (Chung et al., 1993). CTCF also plays a number of other roles, including inhibition and activation of transcription and X chromosome inactivation. In its functions, CTCF interacts with a number of proteins, including cohesin, PARP1, YY1, and RNA polymerase II (reviewed in Gaszner and Felsenfeld, 2006; Wallace and Felsenfeld, 2007; Filippova, 2008; Zlatanova and Caiafa, 2009; Phillips and Corces 2009; Gause et al., 2008).

The introduction of CTCF binding sites at either or both sides of the transgene cassette led to significant reduction in the innate inflammatory response to the vector and improvements in the duration of transgene expression. Surprisingly, in contrast to the classical definition of an insulator, CTCF-binding sites acted in a position-independent manner to inhibit innate immune responses suggesting unexpected mechanisms in play in the context of Ad vectors.

Results

Construction of Ad vectors containing CTCF-binding sites

E1-deleted FG Ad vectors containing a CMV promoter (CMV)-GFP cassette in the leftward (anti-E1A) orientation (Fig. 1) were constructed in a series of viral backbones containing no deletions other than E1 or additionally deleted for the E3 or preterminal protein (pTP) genes (Table 1). The leftward orientation was chosen because results from the accompanying manuscript (Schaack et al.) indicate that this orientation leads to a greater innate immune response, as measured by ear swelling, than does the rightward orientation. The leftward orientation thus should provide a more stringent test of our primary hypothesis that the powerful foreign enhancers used in FG Ad vectors are the major contributor to induction of innate immune processes via activation of viral genes.

A DNA fragment containing four CTCF-binding sites (Fig. 2) was inserted 5′ of the CMV promoter/enhancer, 3′ of the GFP coding

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**Fig. 1.** Schematic structures of the left ends of the viruses encoding GFP. A: schematic structure of the full-length viral chromosome with primary transcripts indicated by arrows above the chromosome; early region transcripts are indicated below, and late region transcripts above, the viral chromosome. B–E: schematic structures of the left ends through the IVA2 gene of the vectors encoding GFP under the control of the CMV promoter (Table 1) used in this study. B: the left end vectors A, C, and G: viruses D and I: D: viruses B, E, and I: viruses F and J: pr: promoter; pA: polyadenylation site.
sequence, or in both the 5′ and 3′ positions in a variety of viral backbones (Table 1). As controls, vectors identical except that they did not contain introduced CTCF-binding sites were constructed. The vectors were used to test our hypothesis that the major contributor to the induction of the innate inflammatory response by FG Ad vectors containing very strong foreign enhancers is activation of expression of Ad genes retained in the vector by the enhancers. In particular, we hypothesized that activation of pIX expression, which does not occur in cells transduced by wild-type Ad5 until after the onset of viral DNA synthesis (Wilson et al., 1979; Crossland and Raskas, 1983), was likely raised by transcription read-through of the E1B transcription unit. The low level of 5′ half-chromatin insulator element (a generous gift of G. Westervelt) was inserted into the Ad chromosome on either or both sides of a CMV promoter and 3′ of the GFP coding sequence. 

CTCF-binding sites increase the duration of transgene expression

GFP-encoding vectors were injected into mice via the tail vein to target the liver. GFP expression was determined by fluorescence microscopy of liver sections. Vectors lacking CTCF-binding sites directed strong levels of GFP expression that were independent of the presence or absence of the E3 or preterminal protein genes on day 3 after injection, with little expression by day 7 (Fig. 4A and data not shown). All vectors containing CTCF-binding sites 5′, 3′, or 5′ and 3′ of the CMV-GFP cassette directed levels of GFP expression that were similar to expression directed by vectors lacking the CTCF-binding sites on day 1 (Fig. 4B and data not shown). GFP expression at 14 days was greater than at day 7 in livers transduced by vectors lacking CTCF-binding sites (Fig. 4B). Detectable GFP fluorescence was apparent 18 days after injection of CTCF-binding site-containing vectors, but was no longer apparent by day 21 (data not shown). Thus, the CTCF-binding sites led to increased duration of high-level GFP expression, again in a position-independent manner.

Quantitative rtPCR analysis of Ad gene expression in mouse liver

Livers were transduced via tail vein injection with a variety of viruses. Three days after injection, mice were killed and livers harvested. Liver sections from mice transduced with the GFP-encoding vectors again showed similar fluorescence between mice injected with vectors containing or not containing CTCF-binding sites (data not shown). Total RNA was tested in duplicate for vector-encoded RNA expression by quantitative rtPCR for a variety of Ad genes as well as the GFP gene (Fig. 5) using primers listed in Materials and methods. Data were normalized to 18S rRNA. For the viruses encoding GFP, data were further normalized to GFP expression. pIX RNA expression varied in surprising fashion (Fig. 5). pIX RNA was relatively highly expressed in livers transduced by each of the CMV-GFP vectors and was only modestly lower in the presence of the CTCF-binding sites between the CMV and pIX promoters (virus D, Table 1). pIX RNA expression was significantly higher in cells transduced with the vector containing CTCF-binding sites placed 3′ of the GFP coding sequence (virus E, Table 1). Given that ear swelling was at the same low level in the two vectors containing the introduced CTCF-binding sites and was much higher in the absence of CTCF-binding sites, pIX RNA expression levels correlate poorly with the level of the innate inflammatory response. Further, because of the similarity in GFP RNA levels between livers transduced with virus C lacking CTCF-binding sites and virus D with CTCF-binding sites between the CMV and pIX promoters, this evidence raises the question of whether the CTCF-binding sites act as classically-defined chromatin insulators in Ad5 chromatin. Expression of pIX RNA was apparent at a very low level in cells transduced with the empty vector (virus K, Table 1) and at a relatively low level in cells transduced by the non-inflammatory virus L (Table 1) deleted for the E1A 289R-coding sequence and the pTP gene. The signal in cells transduced by virus L likely represents E1B RNA, as the pIX gene is nested within the 3′ end of the E1B transcription unit. The low level of pIX expression in cells transduced with virus K may result from the loss of inhibition of the pIX promoter that occurs by transcription read-through from the E1B promoter (Vales and Darnell, 1989).

Expression of L1 RNA, which normally occurs at a low level prior to Ad DNA replication in cells infected with wild-type Ad5 (Shaw and Ziff, 1980) and of which promoter (the major late promoter) lies between the 3′ ends of the pIX and E2B coding sequences (Fig. 5), was apparent in cells transduced with each of the 3 GFP vectors. Surprisingly, L1 expression was 60-fold higher in the vector with the CTCF-binding sites 5′ of the GFP gene (virus D) and 30-fold higher in the vector with no introduced CTCF-binding sites (virus C) than in livers transduced by vector E with the CTCF-binding sites inserted 3′ of the GFP coding sequence.

Table 1. Genotypes of the viruses used in this study. ‘Promoter’ indicates the promoter used to drive GFP expression.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genotype</th>
<th>Promoter</th>
<th>CTCF-binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>E1A−E1B−pTP−GFP+</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>B</td>
<td>E1A−E1B−pTP−GFP+ 3′ ins</td>
<td>CMV</td>
<td>3′ of GFP coding sequence</td>
</tr>
<tr>
<td>C</td>
<td>E1A−E1B−GFP+</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>D</td>
<td>E1A−E1B−GFP 5′ ins</td>
<td>CMV</td>
<td>5′ of CMV promoter</td>
</tr>
<tr>
<td>E</td>
<td>E1A−E1B−GFP+ 3′ ins</td>
<td>CMV</td>
<td>3′ of GFP coding sequence</td>
</tr>
<tr>
<td>F</td>
<td>E1A−E1B−GFP 5′ 3′ ins</td>
<td>CMV</td>
<td>5′ and 3′ of CMV-GFP</td>
</tr>
<tr>
<td>G</td>
<td>E1A−E1B−E3−GFP+</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>H</td>
<td>E1A−E1B−E3−GFP+ 5′ ins</td>
<td>CMV</td>
<td>5′ of CMV promoter</td>
</tr>
<tr>
<td>I</td>
<td>E1A−E1B−E3−GFP+ 3′ ins</td>
<td>CMV</td>
<td>3′ of GFP coding sequence</td>
</tr>
<tr>
<td>J</td>
<td>E1A−E1B−E3−GFP+ 5′ 3′ ins</td>
<td>CMV</td>
<td>5′ and 3′ of CMV-GFP</td>
</tr>
<tr>
<td>K</td>
<td>E1A−E1B−</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>L</td>
<td>E1A−E1B−pTP−</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>M</td>
<td>E1A−E1B−LacZ+</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>N</td>
<td>E3 (Ad5dl309)</td>
<td>CMV</td>
<td>none</td>
</tr>
<tr>
<td>O</td>
<td>E1A−E1B−E3−GFP+</td>
<td>CMV</td>
<td>none</td>
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<tr>
<td>P</td>
<td>E1A−E1B−E3−LacZ+</td>
<td>CMV</td>
<td>none</td>
</tr>
</tbody>
</table>

CTCF-binding sites reduce ear swelling induced by Ad vectors

Swelling assays after subdermal injection of the viruses into mouse ears were used as a sensitive, indirect test of induction of total innate immune responses (Moorhead et al., 1999; Schaack et al., 2004; Schaack, 2005). Vectors lacking the CTCF-binding sites induced statistically identical high levels of swelling independent of the E3 and pTP genes (Fig. 3). In contrast, all of the vectors containing one or two sets of 4 CTCF-binding sites, regardless of their positions, induced statistically identical levels of swelling that were equal to the low level of swelling induced by a control virus deleted for the E1 region but containing no expression cassette. Surprisingly, the suppression of ear swelling by the CTCF-binding sites occurred even when the sites were downstream of the GFP coding sequence. Thus, the effect of the CTCF-binding sites on the overall innate immune response is position-independent.
sequence. This evidence suggests that major late promoter activity also correlates poorly with the intensity of the innate immune response.

E2A RNA was expressed at moderate to low levels in cells transduced with each of the viruses except for the empty vector (virus K), where no expression was apparent, and the virus encoding the E1A 12S mRNA (virus L), where a somewhat higher level was apparent. The E1A 243R protein likely activates the E2A promoter through its effects on the transcription factor E2F, which plays an important role in E2 transcription (Bagchi et al., 1990). There was no apparent expression of either E2B, encoding the essential replication proteins DNA pol and pTP, or IVa2, which lies within the 3′ end of the E2B transcription unit. However, the pTP gene, which lies in the E2B transcription region, has an effect on ear swelling (Schaack et al., 2004), suggesting that pTP is expressed, at least at a low level, in mouse ears. E3 expression was similar after transduction by the vectors with no CTCF-binding sites (virus C) or containing CTCF-binding sites 5′ of the CMV promoter/enhancer (virus D) but was significantly lower when the CTCF-binding sites were 3′ of the GFP coding sequence (virus E). Finally, E4 expression was very low in cells transduced by the empty vector (virus K) and was expressed at higher, similar, but still modest levels in livers transduced by the other viruses tested. Thus, E4 expression appears to be relatively independent of the introduced CTCF-binding sites.

Placement of CTCF-binding sites between the CMV enhancer and the pIX promoter was expected to significantly reduce the innate response and increase duration of transgene expression. In contrast, placement of the CTCF-binding sites downstream of the GFP coding sequence was expected to have little or no effect. The finding that the chromatin insulator had position-independent effects on the innate inflammatory response and the duration of transgene expression therefore further questions whether the CTCF-binding sites are acting as chromatin insulators.

CTCF binds to the CTCF-binding sites in the adenovirus vectors

The factor CTCF binds to chromatin insulators and plays an important role in chromatin insulator function (Chung et al., 1993). Because CTCF-binding sequences do not appear to be acting as
classical insulators in Ad vectors, we sought to determine if CTCF binds the CTCF-binding sites introduced into the vectors. ChIP assays were performed from transduced livers using polyclonal antiserum recognizing human CTCF expected to cross react with murine CTCF. CTCF-binding sites on both sides of the GFP cassette were immunoprecipitated (Fig. 6), indicating that CTCF binds to the CTCF-binding sequences in the context of Ad vector chromatin.

**Ad vector containing CTCF-binding sites induces a neutralizing immune response**

To test for the dependence of induction of a neutralizing response on the CTCF-binding sites, a super-transduction protocol was used. An unmodified FG vector encoding LacZ (virus M, Table 1) was injected via the tail vein 28 days after injection of buffer, the virus encoding GFP with CTCF-binding sites 5′ of the gene (virus E), or the virus encoding GFP with no CTCF-binding sites (virus C). Staining of liver sections with X-gal was negative after supertransduction, suggesting that the presence of the CTCF-binding sites did not affect the neutralizing response induced by the vector (Fig. 7).

**Hematoxylin and eosin staining of liver sections did not demonstrate a cell infiltrate regardless of whether transduction of the liver was primary (injection of the LacZ-encoding vector after mock injection) or secondary when no transduction by the LacZ-encoding virus was evident (Fig. 7).** The absence of an apparent cell infiltrate in response to liver transduction in BALB/c mice has been observed previously (Schaack et al., 2004).
CMV promoter-dependent changes in Ad chromatin

The CMV promoter causes absence of local chromatin structure in CMV DNA (Nitzsche et al., 2008). To test whether the presence of the CMV promoter causes global changes in Ad vector chromatin structure, we used a “dot” formation assay after transduction of MEFs (Chen et al., 2007; Walkiewicz et al., 2009). In this assay, antibodies against Ad protein VII, which is the major Ad chromatin protein, are used to determine whether protein VII is highly localized through binding to Ad DNA to yield foci or dots of protein VII signal, or not bound to Ad DNA, in which case diffuse nuclear staining is apparent (Chen et al., 2007; Walkiewicz et al., 2009). In the presence of E1A protein, which activates expression of all of the Ad early region genes, dots are lost as a function of time between 4 and 16 hr after infection, even when Ad DNA cannot be replicated (Chen et al., 2007).

As expected (Chen et al., 2007; Walkiewicz et al., 2009), the control, phenotypically wild-type d309 (virus N), exhibited significant loss of localized protein VII at 16 hr (Fig. 8). In contrast, for virus O, in which the E1A promoter directs GFP expression, the localization of protein VII at 4 and 16 hr is indistinguishable, suggesting that the presence of the relatively weak E1A promoter driving transgene expression does not lead to significant chromatin rearrangement. The pTP deletion mutant virus encoding GFP (virus A), which does not have introduced CTCF-binding sites, exhibited significant loss of dots, suggesting that the presence of the CMV promoter leads to global rearrangement of Ad chromatin and that protein VII relocation is not dependent on vector DNA replication because deletion of the pTP gene results in replication incompetence (Schaack et al., 1996).

Chromatin rearrangement in cells transduced by viruses C (no CTCF-binding sites inserted), D (CTCF-binding sites 5′ of the CMV enhancer) and E (CTCF-binding sites 3′ of the CMV enhancer) correlate well with the quantitative rPCR results. There is little loss of protein VII dots in cells transduced by virus E (Fig. 8), which exhibited very little expression of L1 and early region genes in mouse liver (Fig. 5). In contrast, there is significant loss of localized protein VII signal in cells transduced by virus C and cells transduced by virus D (Fig. 8). Both of these vectors exhibited relatively high-level expression of L1 and early region gene RNA expression in mouse liver (Fig. 5).

Discussion

In this study, we have demonstrated that introduction of a DNA fragment containing 4 CTCF-binding sites into the Ad vector chromosome significantly reduces the innate inflammatory response induced by the vector and prolongs transgene expression in mouse liver. The CTCF-binding sites were introduced to test our hypothesis that Ad genes retained in the vector are transcriptionally activated by the powerful enhancer contained within the CMV promoter and that the enhancer is indirectly responsible for induction of the majority of the innate immune response induced by the vector through increased expression of Ad genes. In particular, we expected that pIX expression, which normally occurs only after the onset of viral DNA replication (Crossland and Raskas, 1983), would have a significant effect on induction of the innate inflammatory response. We hypothesized that pIX induces inflammatory effects that normally are blocked by early region proteins, including E1A and E1B (Schaack et al., 2004), of which expression precedes pIX in infections by the wild-type virus. However, our results demonstrate that the mechanism by which the innate inflammatory response is mediated correlates poorly with the level of pIX RNA as well as with RNA levels from any of the individual Ad genes tested at 72 hr after transduction.

Chromatin insulators have been used in FG Ad vectors previously, in particular in attempts to block the effects on tissue-specific promoters of transcription from the inverted terminal repeat (Steinwaerder and Lieber, 2000; Cheng et al., 2004; Ren et al., 2006; Rohmer et al., 2008; Danielsson et al., 2008). The introduction of poly A sites rather than chromatin insulators had similar effects, raising the possibility that the mechanism involved differs from simple insulation of the promoters. In addition, chromatin insulators have been used to block enhancer effects in a two-gene conditionally replicating anti-tumor vector (Martin-Duque et al., 2004) and to block epigenetic effects from prokaryotic stuffer DNA (Ross et al., 2009).

A search of the Ad5 genome demonstrated neither any strong matches for the consensus CTCF-binding site (Essien et al., 2009; Kim et al., 2007) nor clustering of potentially weaker binding sites (data not shown). Thus, it appears unlikely that our results are affected by naturally occurring CTCF-binding sequences.

Fig. 8. Loss of protein VII from Ad chromosomes in vitro. Ad5d309, a control virus phenotypically wild-type in tissue culture, and other viruses as indicated were used to transduce MEFs. Four and 16 hr after transduction, cells were fixed and stained with antibody against protein VII and counterstained with DAPI to identify nuclei. Dots are positive for localized protein VII staining due to binding to the Ad chromosome.
CTCF binding sites and chromatin insulation in Ad vectors

Our results raise the possibility that the CTCF-binding sites do not act as chromatin insulators in Ad5 chromatin because pIX RNA is expressed at similar levels in livers transduced by virus C (Table 1) lacking introduced CTCF-binding sites and virus D with CTCF-binding sites introduced between the CMV enhancer and the pIX promoter (Fig. 5). However, there are complicating factors that result from the strong inflammatory response provoked by the vector lacking introduced CTCF-binding sites. It is likely that many, if not all, of the Ad promoters are responsive, either negatively or positively, to inflammatory stimuli. For example, TNF-α upregulates the E3 promoter (Deryckere et al., 1995). The CMV promoter is inhibited by IFN-γ and TNF-α (Qin et al., 1997), and the pIX promoter may be inhibited by inflammatory mediators.

The pIX promoter is simple, with binding sites for Sp1 and RBP as well as a TATA box (Babiss and Vales, 1991). RBP, when bound first to the promoter in vitro, represses transcription, although it does not repress transcription when Sp1, TFIIA, and TFIID are allowed to bind first (Olave et al., 1998). It is possible that RBP acts to inhibit transcription of the pIX gene while or after the strong innate immune response develops, leading to reduced pIX RNA synthesis in the absence of CTCF-binding sites in the Ad chromosome.

This argument suggests that the proper comparison for determination of chromatin insulator effects of the CTCF-binding site fragment is virus E, with CTCF-binding sites 3′ of the GFP coding sequence, and virus D, with CTCF-binding sites 5′ of the CMV enhancer. These viruses induce statistically identical levels of ear swelling so it is reasonable to assume that inflammation-dependent effects on transcription from the pIX promoter are similar between livers transduced by the two vectors. The chromatin insulator effect of the four CTCF-binding sites would thus be the ratio of pIX expression directed by the virus with CTCF-binding sites 3′ of the CMV coding sequence: pIX expression directed by the virus with CTCF binding sites between the CMV enhancer and the pIX promoter, or approximately 4-fold. The relatively modest effect of the chromatin insulator may result from the fact that Ad DNA is bound by protein VII (Chatterjee et al., 1986) rather than histones prior to transcriptional activation of a major part of the viral chromosome.

The role of pIX in induction of the innate immune response

pIX is normally expressed only after the onset of viral DNA replication (Crossland and Raskas, 1983). However, as demonstrated by Nelson and Kay (1997) using vectors of which DNA was modified by methylation at XhoI sites, FG Ad vector DNA is not replicated or is, at most, replicated to a very low level after transduction of mouse liver. Thus, it appears that pIX expression in livers transduced by FG Ad vectors bypasses the normal transcriptional control.

pIX expression is normally inhibited by transcription readthrough from the E1B promoter (Vales and Darnell, 1989) in a complex manner: E1B transcription is activated by transcription read through from the E1A promoter (Falck-Pedersen et al., 1985; Maxfield and Spector, 1997; Shen and Spector, 2003) as well as by E1A protein (Jones and Shenk, 1979). Deletion of the E1A and E1B promoters and coding sequences thus likely leads to slightly more readily activated pIX expression in the absence of DNA replication as seen in liver transduced by the control virus K. The higher levels of pIX expression apparent in liver transduced with viruses containing the CMV promoter therefore likely result from effects of the CMV enhancer.

In infections by wild-type Ad5, pIX is expressed only after early region genes, including E1A and E1B, which act to block host innate immune responses, have been expressed for several hours. This raises the possibility that pIX is inflammatory and that early region proteins act to block inflammatory responses induced by pIX.

Nakai et al. (2007) demonstrated that an Ad vector containing the CMV promoter driving transgene expression in the rightward orientation led to splicing of the transgene RNA in-frame into the pIX coding sequence. When the weaker but still very strong EF1α promoter was used in place of the CMV promoter, expression of the pIX fusion protein occurred at a significantly lower level and transgene expression was prolonged. In the rightward orientation, transcription from the CMV or EF1α promoter may inhibit expression from the pIX promoter by transcription read through, as the E1B promoter does. The conclusion from this study was that pIX expression might play an important role in the induction of immune responses that limit Ad vector function.

In contrast to the conclusions of Nakai et al. (2007) and our expectations, our results suggest that pIX does not play the limiting role in the innate immune response to Ad vectors. Neither the similar, high levels of pIX RNA expression induced by the unmodified vector and the vector with CTCF-binding sites between the CMV enhancer and the pIX promoter nor the significantly higher level of pIX RNA expression induced when the CTCF-binding sites were placed 3′ of the GFP gene correlates with the innate inflammatory response induced or the duration of GFP expression.

Evidence that the CMV enhancer affects the limiting role in transgene expression

Our results demonstrate that the duration of GFP expression differs dependent on the presence or absence of the CTCF-binding sites when the CMV promoter is used. The mechanism by which the effects of the CTCF-binding sites, particularly CTCF-binding sites 3′ of the GFP coding sequence, occur is not clear. However, the reduction in the innate inflammatory response and the prolonged transgene expression in livers transduced by vectors that have CTCF-binding sites inserted offers support for our hypothesis that the very strong enhancers in the foreign promoters typically used in FG Ad vectors are primarily responsible for limiting the duration of transgene expression.

The defined activities of CTCF bound to its sites in chromatin include transcriptional activation, transcriptional repression, transcriptional silencing, and chromatin insulation (Nikolaev et al., 2009; Phillips and Corces, 2009). CTCF-DNA complexes include a variety of additional proteins (Zlatanova and Caiafa, 2009) that contribute to the effects induced. While the nature of the CTCF complex bound to Ad DNA is not clear, the demonstration that CTCF binds the Ad DNA fragments that contain the introduced CTCF-binding sequences suggests that it plays a key role in the effects that we observed, including reduction of the innate immune response and prolonged transgene expression. The position independence of the effects of the CTCF-binding sites suggests that they affect the innate immune response to the vector through a novel mechanism.

Shut off of GFP expression

Examination of GFP expression (Fig. 4) permits a rough analysis of the kinetics of shut off of GFP expression. The half-life of GFP is approximately 26 hr in mouse LA-9 cells (Corish and Tyler-Smith, 1999) and the half-life of GFP mRNA in HEK293T cells is approximately 7 hr (Sacchettia et al., 2001). GFP expression at 7 days after transduction appears to be less than 10% of that at 3 days after transduction with virus G (Fig. 4), which lacks introduced CTCF-binding sites. Assuming that the half-lives of GFP and GFP mRNA are similar in liver and in the tissue culture cells in which they were tested, GFP synthesis would have ceased on or about day 4 to permit decay of fluorescence to the level apparent on day 7 (Fig. 4). Further, assuming that the shut off of GFP expression occurs at the level of transcription and not translation, GFP mRNA synthesis would have to be shut off on days 3–4. In contrast, GFP synthesis would have to have
shut down on or about day 11 and GFP mRNA synthesis shut off on
days 10–11 to yield the fluorescence signal apparent on day 14 in liver
transduced by the vectors containing CTCF-binding sequences (Fig. 4
and data not shown). Therefore, we estimate the effect of the inserted
CTCF-binding sites appears to be approximately 3-fold in terms of
duration of transgene expression.

Validation of use of the mouse ear-swelling model for vector design

The correlation between ear swelling and effectiveness of the
vectors in liver offers validation for the use of the mouse ear-swelling
model as a sensitive and relatively rapid indicator of the total innate
immune response induced by the vector. The ear-swelling model is
dependent on the overall innate response and independent of the
identification of specific cytochemokines induced. It has allowed us to
identify virus mutants and vectors that span a wide range of abilities
to induce the overall innate response as well as some of the Ad genes
that affect the innate response (Moorhead et al., 1999; Schaack et al.
2004; Schaack et al., accompanying manuscript; this study). Determina-
tion of the cytochemokines induced by these viruses will provide
baselines for modestly inflammatory and highly inflammatory viruses
that will aid in determination of which cytochemokines play limiting
roles in the immune responses to Ad vectors.

Conclusion

This analysis leaves questions regarding the activity of the CTCF-
binding sites: 1) Why is L1 expression 60-fold lower in liver
transduced by virus E, with CTCF-binding sites 3’ of the GFP coding
sequence, than in liver transduced by virus D, with CTCF-binding sites
between the CMV enhancer and the pIX gene? 2) Why is early region
gene expression, in particular E3 expression, so different in livers
transduced by virus E, with CTCF-binding sites 3’ of the GFP coding
and virus D, with CTCF binding sites 5’ of the CMV enhancer? 3) Why
is early region gene expression so similar in livers transduced by the
highly inflammatory virus C, lacking introduced CTCF-binding sites,
and the modestly inflammatory virus D, containing CTCF-binding sites
5’ of the CMV enhancer? 4) Most importantly, what is the mechanism
(s) by which CTCF-binding sites reduce the innate inflammatory
response induced by the Ad vectors and prolong transgene
expression?

FG Ad vectors remain the gene-delivery vectors of choice for
studies of metabolic processes in mouse liver and mammary gland,
among other tissues. The results that we have presented here provide
improvement of these vectors for transduction in vivo with prolonged
time windows in which analyses can be done. In addition, the reduced
inflammatory response should lead to fewer off-target effects. Finally,
addressing questions that have been raised by this study offers
promise for further improvements to Ad vectors.

Materials and methods

Cell lines

HEK293 cells (Graham et al., 1977) and HEK293-pTP cells (Schaack
et al, 1995; Schaack et al., unpublished), which constitutively express
Ad preterminal protein, were grown in DMEM containing high
glucose supplemented with 10% bovine calf serum.

Construction and growth of viruses

Viruses (Table 1 and Fig. 1) were constructed using the method of
He et al. (1998) as previously modified (Oricalky and Schaack, 2001).
Viruses were grown in either HEK 293 cells or HEK 293-pTP cells. At
full CPE, infected cells were collected, frozen and thawed 3× to release
the virus, cellular debris was pelleted, and the virus-containing
supernatant was purified and concentrated by step- and isopycnic-
gradient centrifugation. Viral particle titers were determined by
OD260, with one OD260 = 10^12 particles/ml.

The plasmid with the CMV major immediate early (CMV)
promoter-GFP gene introduced in the more inflammatory anti-E1A
orientation (see accompanying paper Fig. 1) with the SV40 poly A site
introduced downstream of the promoter was used for all new
constructs. A DNA fragment containing 4 CTCF-binding sites (Fig. 2)
was introduced downstream of the poly A site and/or upstream of the
CMV promoter.

Vectors containing a single set of CTCF-binding sites stably
maintained the GFP cassette as demonstrated by plaque assays after
repeated rounds of growth. In contrast, the GFP cassette was unstable
in vectors containing chromatin insulators flanking the gene. Deletion
of the GFP cassette appears to occur through recombination between
the chromatin insulators, with retention of a single chromatin
insulator element, as demonstrated by PCR and restriction digestion
analyses of plaque-purified, GFP-negative vectors (data not shown),
raising the possibility that a small chromosomal loop forms between
the 2 sets of 4 CTCF-binding sites after binding of CTCF. After four
rounds of growth of the vectors containing the pTP gene and chromatin
insulators flanking the CMV-GFP cassette (viruses D and
E, Table 1) to produce high titer stocks, examination of approximately
50 plaques showed no apparent loss of GFP expression in HEK 293
cells infected with viruses F and J (Table 1). However, after a second
set of rounds of growth, approximately 20% of the plaques from
viruses containing CTCF sites 5’ of the CMV promoter and 3’T of the
GFP coding sequence showed loss of GFP expression. Data from vectors
containing two chromatin insulators are presented from first rounds
of growth only.

A vector deleted for the pTP gene and containing the GFP cassette
flanked by 2 sets of CTCF-binding sites showed significant loss of GFP
expression as determined by plaque assays after the first set of rounds
of growth and was not used in these studies.

Injections of mice and analysis of swelling

All procedures involving animals followed the guidelines of, and
were approved by, the University of Colorado Institutional Animal
Care and Use Committee. Female, 6-week-old BALB/c mice were
anesthetized by intraperitoneal injection of avertin prior to the
measurement of ears and virus injection.

Virus stocks were diluted in PBS. 2 × 10^10 particles in 10 μl were
injected subdermally into both ears of each mouse tested. Ear
thickness was measured immediately prior to and every 24 hr
through 72 hr after injection using a Mitutoyo model 7326 engineer’s
micrometer. Ear measurements are reported in units of 10^-4 in. Ears
that developed a hematoma were excluded from analysis. Data
represent the average values from 6 or more ears after subtraction of
swelling induced by injection of buffer alone, ± the standard error of
the mean. Swelling induced by injection of ears with buffer alone
averaged 4.80 ± 0.24 units for all mice analyzed. Statistical signifi-
cance was determined using the 2-tailed t test.

For liver transduction, 10^10 purified particles diluted to 200 μl in
PBS were injected into the tail vein. Mice were killed at various times
as noted after transduction. For analysis of GFP expression, livers were
harvested and fixed overnight in formalin (Fisher Scientific) or in 4%
paraformaldehyde (Fisher Scientific) for 3 hr (no differences in GFP
fluorescence were apparent between the fixation processes; data not
shown), then cryoprotected by overnight incubation at 4°C in PBS
containing 30% w/v sucrose. Liver blocks were then frozen and
sectioned using a Leica CM 1850 cryotome. Sections affixed to slides
were examined for GFP fluorescence and images collected using a
Nikon TE2000-E UV microscope. For analysis of supertransduction,
adjacent sections were stained with X-gal followed by hematoxylin
and eosin and examined microscopically.
Quantitative real-time PCR

Three days after transduction of mouse livers, total RNA was prepared from livers using Trizol (Invitrogen) extraction. cDNA was synthesized using oligo dT as primer. qRT-PCR was performed using the following primers: E2A, GGTAGGCTGCTCCCCCCAA and ACTCTGCGGTCGCAA; E2B, GGACAGGCTTTCGAC and CATGCTTTAGTGCTGGT; E3A, GACAAGGCGATGAAA and AGATAGCCTTCCCCATG; E4, CCATGAGCTCGACCCATC and GCTGCTGCTCAAACCTCTT; GFI, GAGGCGGCGACGATGGA and CCAGAGCCGAAGTGATCC. SYBR green was used to quantify PCR products.

Chromosomal immunoprecipitation

Binding of CTCF to the insulator sequences introduced into the vectors was determined 3 days after transduction of mouse liver via tail vein injection of 1011 virus particles. DNA isolated by Trizol vectors was determined 3 days after transduction of mouse liver via tail vein injection of 1011 virus particles. DNA isolated by Trizol

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