Characterization of the Human Artemis Promoter by Heterologous Gene Expression *In Vitro* and *In Vivo*

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Artemis is an endonucleolytic enzyme involved in nonhomologous double-strand break repair and V(D)J recombination. Deficiency of Artemis results in a B⁺ T⁺ radiosensitive severe combined immunodeficiency, which may potentially be treatable by Artemis gene transfer into hematopoietic stem cells. However, we recently found that overexpression of Artemis after lentiviral transduction resulted in global DNA damage and increased apoptosis. These results imply the necessity of effecting natural levels of Artemis expression, so we isolated a 1 kilobase DNA sequence upstream of the human Artemis gene to recover and characterize the Artemis promoter (APro). The sequence includes numerous potential transcription factor-binding sites, and several transcriptional start sites were mapped by 5' rapid amplification of cDNA ends. APro and deletion constructs conferred significant reporter gene expression *in vitro* that was markedly reduced in comparison to expression regulated by the human elongation factor 1- α promoter. *Ex vivo* lentiviral transduction of an APro-regulated green fluorescent protein (GFP) construct in mouse marrow supported GFP expression throughout hematopoietic lineages in primary transplant recipients and was sustained in secondary recipients. The human Artemis promoter thus provides sustained and moderate levels of gene expression that will be of significant utility for therapeutic gene transfer into hematopoietic stem cells.

Introduction

GENOME INTEGRITY is maintained by way of essential DNA double-strand break (DSB) repair mechanisms. Multicellular eukaryotic organisms resolve DSBs primarily through the canonical nonhomologous end-joining (NHEJ) pathway, which repairs genomic insults generated by both external-damaging agents and internal cellular processes. The NHEJ cascade begins when the Ku70/Ku80 heterodimer recognizes and binds a DNA DSB. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) then complexes with the DNA bound Ku to form the DNA-PK holoenzyme (Yaneva *et al.*, 1997; West *et al.*, 1998). The DNA-PK holoenzyme subsequently recruits and binds Artemis, which acquires endonucleolytic activity recognizing 5' and 3' overhangs (Ma *et al.*, 2002). After Artemis digests the DNA overhangs, a heteromultimer of XLF, XRCC4, and DNA Ligase IV is recruited to the synaptic complex to repair and seal the break (Grawunder *et al.*, 1997, 1998).

NHEJ also plays a fundamental role in the rearrangement of immunoglobulin (Ig) genes and T cell receptor (TCR) genes (van Gent *et al.*, 1996; Zhu *et al.*, 1996). This site-specific rearrangement process begins when the RAG-1 and RAG-2

complex is recruited to recombination signal sequences (RSS) flanking each V (variable), D (diversity), or J (joining) coding segment (Oettinger *et al.*, 1990). The RAG complex introduces a nick adjacent to each RSS; the resulting 3' hydroxyl group nucleophilically attacks the antiparallel DNA strand to form a hairpin structure at the coding ends (Roth *et al.*, 1992; McBlane *et al.*, 1995). The Artemis:DNA-PK complex endonucleolytically cleaves the coding end hairpin, and then the DSB is processed and repaired through the NHEJ pathway (Ma *et al.*, 2002).

Deficiency of Artemis disrupts both DNA DSB repair and V(D)J recombination, manifesting in humans as a radiation sensitive form of severe combined immunodeficiency characterized by the inability to rearrange Ig and TCR genes, ultimately resulting in the absence of both B and T lymphocytes. Artemis deficiency is a primary immunodeficiency (PI) designated SCID-A due to a founder mutation in Athabaskan-speaking Native Americans and significant incidence of the disease in this population. In general, PIs are treatable by allogeneic hematopoietic stem cell transplantation, with an attendant risk of infection, graft rejection, and graft-vs.-host disease associated with about 20% mortality worldwide. Accordingly, there is great interest in the

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development of more effective therapeutic approaches for this disease. Recent results from clinical trials have demonstrated the effectiveness of transplantation using autologous HSC after *ex vivo* genetic correction by retroviral transduction for two PIs, one caused by adenosine deaminase deficiency and the other caused by absence of the common gamma chain for several cytokine receptors (Aiuti *et al.*, 2009; Hacein-Bey-Abina *et al.*, 2010). Both of these studies reported long-term engraftment of corrected stem cells in the majority of patients, ultimately resulting in reconstitution of cellular and humoral immunity (Aiuti *et al.*, 2009; Hacein-Bey-Abina *et al.*, 2010). These results demonstrate the effectiveness of gene transfer for the treatment of PIs in general, potentially including SCID-A. However, we recently reported that overexpression of Artemis after lentiviral transduction is associated with cytotoxicity, inducing a halt in cell cycle progression, fragmentation of genomic DNA, and, ultimately, apoptosis (Multhaup *et al.*, 2010). These results highlight the importance of providing Artemis expression at a level that is nontoxic and yet sufficient to correct the T⁺B⁺ phenotype in preclinical studies and in clinical application to human SCID-A.

To explore the potential of innate regulation using the endogenous human Artemis promoter (APro) region, a 1-kilobase (kb) sequence directly upstream of the human Artemis translational start site (TSS) was isolated by polymerase chain reaction (PCR) amplification, characterized, and shown to support green fluorescent protein (GFP) and luciferase reporter gene expression *in vitro*. 5' rapid amplification of cDNA ends (RACE) revealed evidence for multiple transcriptional start points, and several deletion constructs were found to promote reporter gene expression *in vitro*. Additionally, *ex vivo* transduction of mouse bone marrow with an APro-regulated GFP lentiviral vector resulted in GFP expression at a significantly reduced level in comparison with control mice transplanted with EF1 α -GFP transduced marrow. The human Artemis promoter supported GFP expression in all hematopoietic lineages that persisted in secondary transplant recipients. These results demonstrate that the human Artemis promoter provides moderate and yet reliable levels of expression in hematopoietic lineages, essential for regulated expression of potentially toxic gene products (such as Artemis) and reduced risk of genotoxicity associated with integrative gene transfer.

Materials and Methods

Isolation of the human APro region

A 1 kb sequence directly upstream of the human Artemis TSS was amplified from human genomic DNA (Roche) by PCR using forward primer 5' ACCGGTCAGAGAGCCGAAATCACGCC 3' and reverse primer 5' ACCGGTA GCGCCGCCGATCCCAGAGT 3' (flanked by *AgeI* restriction sites, underlined). Two rounds of PCR amplification were necessary to achieve a 1-kb product (5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 69°C, and 1.5 min at 72°C, with a final 10 min extension at 72°C). The product was gel extracted (Qiagen) and cloned into TOPO vector PCR2.1 to generate pCR2.1/APro. The resulting clone was sequenced and found to be identical to the published human chromosome 10 sequence 1 kb directly upstream of the human Artemis TSS (PubMed Reference: NT_077569).

Plasmid construction

APro regulated luciferase constructs. The full-length APro sequence was digested with *AgeI* and ligated into the *AgeI* site of pGL3Basic (Promega) directly upstream of the firefly luciferase gene to generate pGL3/APro. Several APro deletion mutants were generated from the full-length APro sequence by PCR amplification. Primers utilized to amplify truncated APro sequences are illustrated in Table 1. PCR products containing various portions of the APro sequence were digested and cloned into the *AgeI* site of pGL3Basic.

pCSII/AProGFP. A lentiviral vector plasmid designed for regulation of GFP by the full-length human APro was generated from pCSIIIEG (Agarwal *et al.*, 2006). The CMV promoter was excised from pCSIIIEG by *AgeI* digestion, and then the APro sequence was ligated into the *AgeI* site directly upstream of the GFP gene to generate pCSII/APro-GFP.

Mammalian cell culture and transfections

HEK 293T and murine NIH 3T3 tk⁻ cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic at 37°C and 5% CO₂. HEK 293T cells were transfected with 2 μ g of pGL3/APro or derivative deletion

TABLE 1. OLIGONUCLEOTIDE SEQUENCES USED DURING POLYMERASE CHAIN REACTION AMPLIFICATION

Reaction product	Forward primer 5'-3'	Reverse primer 5'-3'
-100 to ATG	<u>ACCGGTGCGAGGCAGCGCGGGCTTCCCGG</u>	<u>ACCGGTAGCGCCGCCGATCCCAGAGT</u>
-184 to ATG	<u>ACCGGTAGAACCCGACCGGATGCTCCT</u>	<u>ACCGGTAGCGCCGCCGATCCCAGAGT</u>
-374 to ATG	<u>ACCGGTGGCTGGGGCCACCTGCTCTGGG</u>	<u>ACCGGTAGCGCCGCCGATCCCAGAGT</u>
-500 to ATG	<u>ACCGGTGGTTAGAAATTGGGAACAGAAAG</u>	<u>ACCGGTAGCGCCGCCGATCCCAGAGT</u>
-1000 to -500	<u>ACCGGTACAGAGAGCCGAAATCACGCC</u>	<u>ACCGGTGTGGGATTACCGTGAGGGTTGTATG</u>
RT	No Forward Primer	<u>CCTTGGGATGCCGGCATGCATGGAT</u>
RACE	CGACTGGAGCACGAGGACACTGA	<u>GGATGCTTTGACTCTGCCCCCGGA</u>
	Gene Racer Forward Primer (Invitrogen, Carlsbad, CA)	

Forward and reverse oligonucleotide sequences, utilized to amplify various reaction products. Primers utilized to amplify human Artemis promoter constructs are flanked by an *AgeI* sequence (underlined). Reverse transcription (RT) and rapid amplification of 5' ends (RACE) indicate forward and reverse oligonucleotide sequences utilized during cDNA generation and 5' RACE, respectively, to identify human Artemis TSS.

constructs using the DNA calcium phosphate coprecipitation technique as described (Zufferey *et al.*, 1997). Briefly, 10 μ L of 2.5M CaCl_2 was added to 2 μ g of luciferase expression plasmid DNA to a final volume of 100 μ L for each well in a 6-well plate to be transfected. An equal volume of 2X BBS [0.5M BES, 150mM NaH_2PO_4 , 2.8M NaCl; pH 7.05] was added to the DNA-calcium mixture in a drop-wise fashion and thoroughly mixed. The CaCl_2 -DNA-BBS solution was added to each well containing 5×10^4 HEK 293T cells and incubated at 37°C with 3% CO_2 . Each culture was cotransfected with 0.25 μ g of pRL-CMV (Promega) as an internal control for transfection efficiency; assay results are expressed as firefly RLU/*Renilla* RLU. pKT2/Cal, a firefly luciferase construct regulated by the strong CAGS promoter (Wilber *et al.*, 2007), was utilized as a luciferase positive control, and the promoter-less pGL3Basic (Promega) was used as a luciferase background control.

BJAB, BLIN-1 K^+ , and BLIN-1 K^- cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and 1% antibiotic antimycotic at 37°C with 5% CO_2 and transfected in an Amaxa Nucleofector (Lonza). Cells were harvested, washed, and resuspended in Amaxa supplemented nucleofection buffer C. About 5×10^5 cells were mixed with 5 μ g of pGL3Basic/APro or derivative deletion construct plus 0.5 μ g of pRL-CMV and then electroporated at program setting X-001. Immediately after nucleofection, cells were transferred to prewarmed culture medium and incubated at 37°C with 3% CO_2 .

Forty-eight hours post-transfection, cells were collected and analyzed for firefly and renilla luciferase expression via Promega's Dual Luciferase Reporter Assay System. Briefly, cells were harvested by trypsinization, washed with 1X phosphate-buffered saline (PBS), and resuspended in passive lysis buffer (provided in the Dual Luciferase Reporter Assay System). After a 5-min incubation at room temperature, the lysate was vortexed vigorously and then cleared by centrifugation for 2 min at 13,200 g, collecting the supernatant. A 20 μ L aliquot of lysate supernatant was incubated with Promega firefly luciferase substrate, and then, subsequently, incubated with Stop and Glo *Renilla* luciferase substrate, assaying for luminescence in a Lumat LB 9507 luminometer. Student's *t*-test was utilized to statistically evaluate differences in luciferase expression levels between transfected cell populations.

5' RACE identification of transcriptional start points

5' RACE was carried out using RNA extracted with Trizol reagent (Invitrogen) and the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen), Version 2.0 kit and reagents, according to the manufacturer's instructions. Primers utilized for the 5' RACE protocol are listed in Table 1. RACE products were isolated by gel electrophoresis, extracted, cloned into TOPO vector PCR2.1, and sequenced. Vector NTI Software (Invitrogen) was used to align 5' RACE product sequences against the published human Chromosome 10 sequence (PubMed Reference: NT_077569).

Preparation and titering of lentiviral vectors

Vesicular stomatitis virus G (VSV-G) pseudotyped lentiviral vectors were generated as described (Zufferey *et al.*, 1997; Gori *et al.*, 2007). Briefly, 24 h pretransfection, 1.4×10^7 HEK 293T cells were seeded into poly-L-lysine coated 15-cm²

plates and cultured in DMEM supplemented with 1% penicillin streptomycin and 8% FBS at 37°C with 5% CO_2 . Lentiviral vector plasmid constructs were cotransfected with pANRF to provide lentiviral structural and enzymatic proteins and pMD.G to provide VSV-G envelope protein. Twelve hours post-transfection, the medium was replaced using DMEM supplemented with 4% FBS. Viral supernatants were collected 24, 36, and 48 h post-transfection, pooled, and then concentrated 100-fold by centrifugation at 23,000 g in a Sorvall RC5B centrifuge. Vector was resuspended in Iscove's modified DMEM, aliquoted, and stored at -80°C for future use. For quantification of vector titers, NIH 3T3 tk^- cells were transduced with varying amounts of vector in the presence of 8 μ g/mL polybrene. Forty-eight hours post-transfection, cells were harvested for flow cytometric analysis of GFP expression to determine the percentage of cells transduced. DNA was also extracted from the transduced cells and subjected to quantitative PCR as previously described, using a probe specific for the integrated lentiviral strong stop sequence or a probe for the GFP sequence as a measure of lentiviral vector titer (Gori *et al.*, 2007).

Lentiviral transduction and transplantation of mouse bone marrow

All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. CD45.2 and CD45.1 C57Bl/6 mice were obtained from the National Cancer Institute (Frederick, MD) and provided food and water *ad libitum*. Bone marrow was flushed from the long bones of the hind limbs of donor mice into DMEM supplemented with 10 U/mL heparin, 10% FBS, and 1% PenStrep antibiotic/antimycotic. Red blood cells were lysed using ammonium chloride hemolysis buffer [0.8% NH_4Cl with 0.1 mM EDTA] (StemCell Technologies). The remaining nucleated cells were washed with 1X PBS and then triturated into a single-cell suspension in transduction medium (complete StemPro-34 SFM media with supplement, 2 mM L-glutamine, 1% PenStrep [all from Invitrogen], 100 ng/mL murine IL-3, 100 ng/mL murine IL-6, 100 ng/mL murine TPO, 100 ng/mL murine SCF [all cytokines from R&D Systems], and 8 μ g/mL polybrene [Sigma-Aldrich]). The marrow was transduced twice, once immediately after marrow harvest and a second time 20 h after the first exposure. Transduced cells were harvested, washed, counted, and prepared as a single-cell suspension in Iscove's modified DMEM. Equal numbers of bone marrow cells were injected via lateral tail vein into lethally irradiated (800 rads, cesium source) congenic recipients. For secondary transplants, marrow samples were collected individually from primary recipients as just described, isolating the nucleated fraction and transplanting 5×10^6 cells into each of three secondary irradiated (800 rads) recipients.

Flow cytometry

Blood was collected via cheek puncture and assayed for GFP expression in specific hematopoietic compartments by flow cytometry. Whole blood was treated with ammonium chloride hemolysis buffer (0.8% NH_4Cl with 0.1 mM EDTA; StemCell Technologies), washed, and then pelleted leukocytes were resuspended in staining buffer [1X PBS plus 1% FBS, 0.002% sodium azide and fluorochrome-conjugated monoclonal antibodies for identification of hematopoietic cell type]. All antibodies utilized for flow cytometric analysis

were purchased from eBiosciences. GFP expression as well as immunophenotype analysis was conducted using monoclonal mouse antibodies against CD45.1, CD45.2, B220 (B lymphocytes), CD3ε (T lymphocytes), NK1.1 (Natural Killer cells), and Gr-1 (myeloid lineages) and assayed on an LSRII instrument. Data were collected using CellQuest Pro (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.) software. Isotype staining provided an internal control and was used to determine appropriate gating to distinguish between GFP-positive and GFP-negative cells. Mean fluorescence intensity (MFI) was determined using the FlowJo software statistics tool. Significance of differences between groups was determined via Student's *t*-test.

Results

Isolation and sequence of the human APro region

To identify the promoter of the human Artemis gene (APro), we examined the sequence of human chromosome 10p13 directly upstream of the human Artemis translational start site (TSS). A total of fourteen potential regulatory sites were identified within this region using the search engine TFSEARCH: Searching Transcription Factor Binding Sites (ver1.3) (Heinemeyer *et al.*, 1998) including several known hematopoietic transcription factor binding sites such as GATA-1, 2, 3 (−967, −880, −849, −175, and −16), AML1A (−32) and Lyf1 (−356) (Fig. 1). Additionally, a CAAT box at −811 and a TATA box at −595 were identified (Fig. 1): These motifs plus a potential 5' splice site (AAG|GTTAG) at base

pair −501 and several downstream 3' splice sites predicted the possibility of an intron in the 5' untranslated region of messages initiated at around −500bp or further upstream.

Considering the observed sequence characteristics and potential regulatory elements, we recovered the 1 kb segment of human chromosome 10 genomic DNA located directly upstream of the human Artemis TSS. The PCR product was cloned into TOPO vector PCR2.1, sequenced, and found to be identical to the published human genomic sequence (PubMed Reference: NT_077569).

Gene expression mediated by the human Artemis promoter

A reporter construct was assembled in which the 1-kb APro sequence was inserted immediately upstream of the firefly luciferase gene (pGL3Basic/APro). Due to its essential role in NHEJ, Artemis expression is not cell type specific. We therefore introduced pGL3Basic/APro into readily transfectable HEK 293T cells, where the APro sequence supported luciferase expression that was about 10⁵-fold lower than that of pKT2/Cal, a strong CAGS regulated firefly luciferase construct (Fig. 2A). Luciferase expression driven by a downstream 500 bp fragment (pGL3Basic/−500_TSS) was equal to or greater than that mediated by the full-length 1 kb APro sequence. In contrast, luciferase expression driven by an upstream 500 bp sequence (pGL3Basic/−1000−500_TSS) was indistinguishable from background (Fig. 2A). These results suggest that the 500 nt region immediately upstream of the TSS contains the human Artemis promoter.

FIG. 1. 5' upstream sequence of the human Artemis gene. The 1 kb genomic region located directly upstream of the human Artemis translational start site (TSS) was isolated by polymerase chain reaction amplification (nucleotide sequence shown). The region was analyzed using the online transcription factor binding site search engine TFSEARCH. Several potential transcriptional regulation sites were identified and are labeled according to their position relative to the human Artemis TSS. Transcription factor binding sites are in bold and are underlined. Canonical sequences are displayed directly above the APro sequence. The 5' termini of APro deletion constructs relative to the TSS are shown boxed in black. Transcriptional start sites within the APro region were identified by 5' rapid amplification of cDNA ends (RACE), clustered into four groups (see Figure 3), and the average start site location of each group is labeled and highlighted in light gray. A potential 5' splice site located at −501 is italicized.

FIG. 2. *In vitro* deletion analysis of the human APro region. (A) HEK 293T cells and (B) BJAB lymphoid cells were transfected with reporter constructs consisting of firefly luciferase cDNA regulated by APro, an APro deletion construct, the CAGS promoter (pKT2/Cal), or a promoterless control. Each cell population was cotransfected with a CMV regulated *Renilla* luciferase encoding plasmid to control for transfection efficiency. Luciferase expression was assayed and plotted as the ratio of firefly RLU/*Renilla* RLU as described in Materials and Methods.

To map those regions within the downstream 500 nt of the APro sequence that are important for gene expression, deletion constructs were generated based on the clustered location of TFSEARCH-identified transcription factor binding sites. The region from -374 to the Artemis TSS, including USF and Lyf1 binding sites (pGL3Basic/ -374_{TSS}), was found to mediate the same level of luciferase expression as compared with the full-length construct ($p = 0.41$) but only half the amount of luciferase expression conferred by the -500_{TSS} construct ($p < 0.01$) (Fig. 2A). However, the region from -184 to the Artemis TSS (pGL3Basic/ -184_{TSS}), including a GATA-1 binding site, supported luciferase expression at a level similar to that of the full-length APro ($p = 0.08$) and -500_{TSS} sequences ($p = 1.0$) (Fig. 2A). Finally, a fragment extending from nt -100 to the human Artemis TSS, containing two major transcription factor binding site clusters (AML1A, GATA-1, STAT-1, Elk-1, and NRF-2), and the upstream APro construct -1000 to -500 supported minimal luciferase activities at levels slightly above background ($p < 0.008$ and $p < 0.03$, respectively) (Fig. 2A). These results define a functional promoter region in HEK 293 cells extending as little as 184 bp upstream of the TSS of the human Artemis gene.

Artemis plays a key role in lymphocyte development during the receptor V(D)J recombination stage. To assess the expression profile of APro deletion constructs in lymphoid cell lines, Jurkat cells (an immortalized human T cell

line) and BJAB cells (a human EBV-negative Burkitt-like B cell lymphoma) were electroporated and luciferase expression was assayed 2 days later. Surprisingly, Jurkat cells expressed a high level of luciferase after transfection with pT2/Cal but yielded only background levels of luciferase expression in all deletion construct transfection reactions (data not shown), suggesting that Artemis expression in mature T cells may be very moderate to null. On transfection into BJAB cells, the full-length APro sequence and deletion constructs -374 and -184 displayed luciferase expression patterns similar to those seen in HEK 293T cells, that is, the observed expression level was substantially less than that mediated by the CAGGS promoter, yet significantly above background (Fig. 2B). Interestingly, the upstream APro construct -1000 to -500 , containing the TFSearch-identified TATA box, generated the highest level of luciferase expression in BJAB cells (Fig. 2B), in contrast to the background levels observed in 293T cells transfected with this construct ($p = 0.05$) (Fig. 2A). This upstream region mediated luciferase expression at levels indistinguishable from the full-length APro construct ($p = 0.3$) as well as the -500 and -184 to TSS regions ($p = 0.08$ and $p = 0.1$, respectively). These results may not be surprising, considering the numerous lymphoid specific transcription factor-binding sites located in this upstream region, suggesting the potential for two or more regions of the APro sequence that regulate Artemis gene expression.

5' RACE identifies multiple transcriptional start points

To determine the position of transcript initiation, 5' rapid amplification of cDNA ends was carried out as described in Materials and Methods using human liver RNA and total cellular RNA extracted from BLIN-1 K⁺, BLIN-1 K⁻, BJAB, and Jurkat cell lines. BLIN-1 is a cell line derived from a human B cell lymphoma; BLIN-1 K⁻ is characterized as retaining the ability to continuously undergo V(D)J recombination, whereas BLIN-1 K⁺ is characterized as kappa light chain positive, therefore having completed V(D)J recombination. These cell lines are of interest not only due to their lymphocytic lineage but also because of the anticipated requirement of Artemis for V(D)J recombination in BLIN-1 K⁻. BLIN-1 K⁺ serves as an internal control for the cessation of V(D)J recombination.

Several 5' RACE products were identified that grouped into four clusters (Fig. 3). The most 3' cluster from -18 to -36 (Group 1) was the only group amplified from BLIN-1 K⁻ RNA, but was also amplified from BLIN-1 K⁺ and liver RNA. Group 2 consisted of isolated 5' RACE products dispersed between -46 and -79, generated only from BLIN-1 K⁺ and liver RNA. A single start site was identified in the BJAB lymphoma line, located at base pair -108 (Group 3), which was not observed in any of the other samples. Finally, a far upstream group of 5' RACE products from -376 to

-421 (Group 4) was amplified only from liver RNA. No 5' RACE products were identified using RNA extracted from Jurkat cells.

The transcriptional start sites identified by 5' RACE are consistent with the luciferase expression profile observed on APro deletion analysis. The full-length pGL3Basic/APro and deletion constructs pGL3Basic/APro-500_TSS and pGL3Basic/APro-184_TSS that exhibited the highest levels of luciferase activity in 293 cells (Fig. 2) correspond with transcription start points identified in liver RNA located at base pairs -420 and -80, respectively (Fig. 3). Moreover, 5' RACE products were not recovered using RNA extracted from Jurkat cells, consistent with the lack of expression observed on transfection of APro regulated constructs. The absence of Groups 1 and 2 5' RACE products from BJAB cells is consistent with the higher level of luciferase expression observed after transfection of the upstream -1000 to -500 APro deletion construct, even though we did not observe 5' RACE products from this region containing a TFSearch-identified TATA box.

The human Artemis promoter supports moderate but sustained levels of gene expression in vivo

Our motivation in characterizing the human Artemis promoter is to incorporate it into vectors to achieve moderate

FIG. 3. Transcriptional start sites in the APro region mapped by 5' RACE. 5' RACE was performed on RNA isolated from (A) BLIN K⁻ cells, (B) BLIN K⁺ cells, (C) BJAB cells, and (D) human liver. Each 5' RACE product is displayed as a hatched bar positioned along the axis labeled with numbers representing nucleotide position upstream of the human Artemis TSS. The number of stacked hatched bars represents the number of 5' RACE products identified at that particular nucleotide position: 3, 2, and 1 stacked bars represent 3, 2, and 1 5' RACE products, respectively. 5' RACE products are grouped into four regions and are boxed in light gray.

expression levels *in vivo*. We, thus, generated a lentiviral vector with the GFP coding sequence positioned immediately downstream of the full-length APro sequence (CSII/AProGFP; Fig. 4). Considering the sequence motifs contained in this region and expression profiles generated from deletion analysis, we determined that the full 1 kb APro sequence would be most appropriate to include in our vector design. Since CSII has a self-inactivating vector design, GFP expression in transduced cells is entirely dependent on expression driven by the APro sequence. Vectors were generated, and vector titer was quantified by flow cytometry as described in Materials and Methods. Flow cytometric analysis of NIH 3T3 cells transduced with CSII/AProGFP demonstrated that the CSII/AProGFP vector achieved a similar gene transfer frequency as the control CSIIIEG vector, in which GFP is regulated by the strong EF1 α promoter (Fig. 4). However, the CSII/AProGFP transduced population exhibited an MFI of 21.5, 10-fold lower than populations transduced with CSIIIEG, which demonstrated an MFI of 216 (Fig. 4). The APro sequence thus confers a low level of expression compared with stronger promoters such as CAGGS or EF1 α after transfection (Fig. 2A) or after lentiviral transduction (Fig. 4), respectively.

To test for APro mediated gene expression *in vivo*, whole bone marrow was harvested from C57BL/6 CD45.1 animals and transduced overnight with either CSII/AProGFP or the control vector CSIIIEG at a multiplicity of infection of 10. The transduced marrow was transplanted into lethally irradiated CD45.2 recipient animals, which were subsequently assayed monthly for donor engraftment and GFP expression by flow cytometry. Animals from both groups exhibited high levels of engraftment ($\sim 90\%$) with GFP expression persisting up to 4 months. Animals transplanted

with CSII/AProGFP-transduced marrow expressed GFP in about 33% of the donor leukocyte compartment, whereas animals transplanted with CSIIIEG-transduced marrow maintained GFP expression in about 11% of the leukocyte compartment 4 weeks post-transplant (Fig. 5). Interestingly, at 16 weeks post-transplantation we found that CSII/AProGFP transduced cells in the peripheral blood exhibited a diminished level of GFP expression (average MFI = 24.5) in comparison with the CSIIIEG control (average MFI = 1458) ($p < 0.001$) (Fig. 5), similar to the shift in MFI observed in transduced 3T3 cells.

Secondary transplantation was carried out by harvesting marrow from each primary recipient 4 months post-transplant and infusing each marrow sample into three recipient animals preconditioned with 800 rads, cesium source. GFP expression was sustained in the secondary transplant recipients receiving either CSII/AProGFP or CSIIIEG transduced marrow, demonstrating the capability of these lentiviral vectors to maintain gene expression after transduction into primitive hematopoietic stem cells engrafted in the primary recipients (Fig. 5). Since our intention is ultimately to use the human Artemis promoter to regulate gene expression for correction of SCID-A, we assayed for APro mediated expression in lymphocyte populations engrafted in secondary recipients. GFP expression was observed in both lymphoid and myeloid compartments, including B cells (B220⁺NK1.1⁻), T cells (CD3e⁺), and myeloid cells (CD11b) in animals that received marrow transduced with CSII/AProGFP (Fig. 5). These results verify that the Artemis promoter provides a moderate level of expression in differentiated lymphoid cells after lentiviral transduction into long-term repopulating hematopoietic stem cells.

FIG. 4. APro mediates low levels of green fluorescent protein (GFP) expression after lentiviral transduction *in vitro*. Mouse 3T3 cells were transduced with increasing amounts (0.3, 1, or 3 μ L) of either (A) an EF1 α regulated GFP lentiviral vector (CSIIIEG) or (B) an APro regulated lentiviral vector (APro-GFP) and analyzed by flow cytometry for GFP expression. Dot plot analysis reveals a similar transduction frequency for each vector; however, CSIIIEG transduced cells expressed GFP at a mean fluorescence intensity (MFI) significantly higher than APro-GFP transduced cell populations. (C) Histograms displaying the MFI exhibited by 3T3 cells transduced with 1000 μ L of either CSIIIEG or APro-GFP lentiviral supernatant reveal that cells transduced with CSIIIEG exhibited an MFI of 216, whereas cells transduced with APro-GFP exhibited a much lower MFI of 21.5. CMV, cytomegalovirus early promoter/enhancer region; U3/U5/R, unique 3'/unique 5'/repeat regions of the HIV long terminal repeat; WPRE, woodchuck post-transcriptional regulatory element.

FIG. 5. APro mediated GFP expression *in vivo*. Donor CD45.1 C57BL/6 bone marrow was transduced with either (A) an EF1 α regulated GFP lentiviral vector (CSIIIEG) or (B) an APro regulated lentiviral vector (APro-GFP) and transplanted into recipient CD45.2 C57BL/6 animals preconditioned with 800 rads. GFP expression was monitored by flow cytometry over a 16-week time period and is indicated for individual animals, represented by open circles, with mean values represented as solid black bars, both in units of percentage GFP positive cells in peripheral blood donor lymphocytes. (C) Four months post-primary transplant, animals were sacrificed and total bone marrow was infused into secondary C57BL/6 recipients. Four months post-secondary transplant, GFP expression mediated by the APro-GFP vector was observed to persist in secondary transplant recipients. (D) GFP expression mediated by the CSIIIEG vector and by the APro-GFP vector was observed in both myeloid and lymphoid lineages. Subpopulations are graphed as percentages of the total GFP⁺ transduced donor (CD45.1) population.

Discussion

We isolated a 1-kb region of human genomic DNA directly upstream of the Artemis TSS by PCR amplification (APro). An *in silico* transcription factor binding site analysis of this sequence uncovered several transcription factor binding sites, leading us to further characterize the genomic region for promoter activity. 5' RACE analysis indicated multiple transcriptional start points in RNA extracted from liver and lymphoid cell lines, and deletion analysis revealed divergent expression patterns between 293T cells and the BJAB Burkett lymphoma-like cell line. Transfection studies *in vitro* revealed the potential for this sequence to regulate gene expression in multiple cell types; additionally, APro mediated expression at a level that was substantially lower than that mediated by the strong EF1 α promoter. Further, GFP expression regulated by APro was observed *in vivo* in mice transplanted with marrow that had been transduced

with an APro-regulated lentiviral vector. Finally, expression was sustained in secondary transplant recipients in both myeloid and lymphoid lineages, establishing the effectiveness of APro to serve as a proficient promoter for gene expression within the hematopoietic system.

The human APro region identified through this study exhibits several characteristics similar to those observed for promoters of other NHEJ proteins. The downstream promoter region extending from -184 to the human Artemis TSS contains neither a TATA box nor a CCAAT box, motifs also absent in the promoters for DNA-PKcs (Connelly *et al.*, 1998), Ku70 (Takiguchi *et al.*, 1996) and other housekeeping genes. Lack of a distinct TATA box within this region of the human APro sequence is associated with considerable variability in the location of transcriptional start sites, as determined by 5' RACE, analogous to variability in transcriptional initiation sites observed for other NHEJ messages such as DNA-PKcs, in which at least 6 transcriptional

start sites were observed (Connelly *et al.*, 1998). We also tested full-length APro and deletion constructs transfected into 293T cells for the effect of irradiation or exposure to the radiomimetic chemicals bleomycin and etoposide, but induced expression upon DNA damage was not observed (data not shown). Similarly, neither transcription nor translation of DNA-PKcs are induced upon DNA damage in either human or mouse cells (Lee *et al.*, 1997; Connelly *et al.*, 1998). These observations indicate the similarity of human Artemis transcriptional regulation with that of other NHEJ components.

Our 5' RACE results and the *in vitro* deletion mapping presented in this study suggest the potential for multiple regulatory regions that comprise the endogenous Artemis promoter. This is consistent with Artemis' involvement in several distinct cellular functions such as NHEJ (Ma *et al.*, 2002), V(D)J recombination (Ma *et al.*, 2002), and apoptosis (Britton *et al.*, 2009). It is tempting to speculate that Artemis expression may be spatially and/or temporally regulated per required function, similar to several other proteins demonstrated to be differentially regulated through transcript initiation at multiple promoter sites. For example, the murine α -amylase gene exhibits tissue specific expression regulated by two separate promoters (Schibler *et al.*, 1983). S1 nuclease mapping revealed that liver tissues yielded one minor α -amylase transcript, whereas the parotid gland yielded two α -amylase transcripts including the minor transcript plus one additional major transcript; additionally, synthesis of each transcript was found to initiate directly downstream of TATA boxes identified by sequence analysis (Schibler *et al.*, 1983). Our data show two potential promoter regions regulating human Artemis expression: one containing a well-defined TATA box with the ability to regulate expression in B-lymphoid cells and the downstream promoter region being less defined in that it does not contain a TATA box, yet regulates expression in several other cell types. It will be of interest to study the relationship between Artemis function and tissue type expression and address the possibility that one region of the APro sequence is necessary for expression during V(D)J recombination, whereas the other region confers constitutive expression in support of NHEJ.

Gene transfer is emerging as a promising approach for treatment of genetic disorders. However, recently observed adverse events have called to attention the importance of regulating expression of therapeutic genes. Specifically, two independent studies have reported correction of X-linked SCID by *ex vivo* transduction of CD34⁺ hematopoietic stem cells using a retroviral vector expressing the common cytokine-receptor gamma chain (common γ chain) (Gaspar *et al.*, 2004; Hacein-Bey-Abina *et al.*, 2010). Long-term engraftment of corrected stem cells was observed in the majority of patients, ultimately resulting in reconstitution of a functional lymphocyte compartment. To date, however, five out of twenty patients treated for X-linked SCID by gene transfer have developed T cell outgrowth resulting in leukemia, from which one child has died (Howe *et al.*, 2008; Hacein-Bey-Abina *et al.*, 2008). Although insertional activation of the LMO2 oncogene was reported in four of the leukemic cases, overexpression of the common γ chain induces cellular proliferation and thus may have contributed to the T lymphocyte clonal outgrowth (Howe *et al.*,

2008; Amorosi *et al.*, 2009). More tightly regulated expression of the common γ chain may thus reduce the risk of oncogenesis resulting from common γ chain overexpression.

Achieving transgenic expression of human Artemis for the correction of SCID-A will be challenging considering previous results regarding Artemis overexpression. Recently, two independent groups reported correction of a murine model of SCID-A by lentiviral vector-mediated gene transfer (Mostoslavsky *et al.*, 2006; Benjelloun *et al.*, 2008). In both studies, SCID-A animals receiving HSC transduced with a lentiviral vector encoding a human Artemis cDNA regulated by the PGK promoter exhibited repopulation of both B and T lymphocyte compartments. However, Mostoslavsky *et al.* reported the inability of either CMV or EF1 α regulated human Artemis lentiviral vectors to restore B and T cells in RAG-1 deficient animals receiving SCID-A HSCs transduced with either vector (Mostoslavsky *et al.*, 2006; Benjelloun *et al.*, 2008). Considering the endonucleolytic nature of Artemis, these results lead us to consider whether Artemis overexpression might be inherently toxic. We subsequently characterized the effect of Artemis overexpression and found it to be associated with cytotoxicity, ultimately resulting in a halt in cell cycle progression, fragmentation of genomic DNA, and apoptosis (Multhaup *et al.*, 2010). These results emphasize the importance of providing Artemis expression at a level that is nontoxic and yet sufficient to correct the T⁺B⁺ phenotype in preclinical studies and in clinical application to human SCID-A.

The endogenous human Artemis promoter provides expression both *in vitro* and *in vivo* at levels significantly lower than those of well-characterized strong promoters such as CMV and EF1 α . Further, when studied *in vivo*, the Artemis promoter regulated expression in several lymphoid cell populations, demonstrating the ability of Artemis promoter to mediate expression in important hematopoietic compartments after gene transfer into hematopoietic stem cells. The APro sequence thus has great potential as a regulator of therapeutic gene expression, including expression of the human Artemis gene for *in vivo* correction of human SCID-A. With this in mind, we have recently generated lentiviral vectors employing innate regulation of human Artemis cDNA using its own endogenous promoter sequence and have achieved successful *ex vivo* gene transfer resulting in functional lymphocyte repopulation of a murine model of SCID-A (M. Multhaup *et al.*, manuscript in preparation). Overall, these results demonstrate that providing innate Artemis expression via *ex vivo* lentiviral transduction into hematopoietic stem cells may serve as a clinically relevant and feasible treatment of human SCID-A. As an element that supports moderate but reliable levels of expression, the human APro also has the potential for reduced genotoxicity associated with integration of other therapeutic genes.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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