Published in final edited form as:

Transgenic Res. 2012 June ; 21(3): 555–566. doi:10.1007/s11248-011-9556-0.

Prototypic chromatin insulator cHS4 protects retroviral transgene from silencing in *Schistosoma mansoni*

Sutas Suttiaprapa^{1,*}, Gabriel Rinaldi^{1,2,*}, and Paul J. Brindley¹

¹Department of Microbiology, Immunology & Tropical Medicine, School of Medicine and Health Sciences, The George Washington University, 2300 I Street NW, Washington, DC 20037

²Departamento de Genética, Facultad de Medicina 11800, Universidad de la República, (UDELAR), Montevideo, Uruguay

Abstract

Vesicular stomatitis virus glycoprotein (VSVG) pseudotyped murine leukemia virus (MLV) virions can transduce schistosomes, leading to chromosomal integration of reporter transgenes. To develop VSVG-MLV for functional genomics in schistosomes, the influence of the chicken β -globin cHS4 element, a prototypic chromatin insulator, on transgene expression was examined. Plasmid pLNHX encoding the MLV 5′- and 3′-Long Terminal Repeats (LTRs) flanking the neomycin phosphotransferase gene (*neo*) was modified to include, within the U3 region of the 3′-LTR, active components of cHS4 insulator, the 250 bp core fused to the 400 bp 3′-region. Cultured larvae of *Schistosoma mansoni* were transduced with virions from producer cells transfected with control or cHS4-bearing plasmids. Schistosomules transduced with cHS4 virions expressed two to 20 times higher levels of *neo* than controls, while carrying comparable numbers of integrated proviral transgenes. The findings not only demonstrated that cHS4 was active in schistosomes but also they represent the first report of activity of cHS4 in any Lophotrochozoan species, which has significant implications for evolutionary conservation of heterochromatin regulation. The findings advance prospects for transgenesis in functional genomics of the schistosome genome to discover intervention targets because they provide the means to enhance and extend transgene activity including for vector based RNA interference.

Keywords

Schistosome; pseudotyped retrovirus; cHS4 insulator; transgene

Introduction

Draft genome sequences have become available for two of the major species responsible for schistosomiasis, *Schistosoma japonicum* and *S. mansoni* (Berriman et al. 2009; *Schistosoma japonicum* Genome and Functional Analysis Consortium 2009). The schistosome genomes are 363–397 MB in size, and include ~13,000 protein encoding genes, of which the functions of only a small percentage have been characterized in depth (Han et al. 2009). Unlike for prokaryotic and unicellular eukaryotic pathogens, functional genomics toolkits are not well advanced for schistosomes, or indeed for the helminth parasites at large. We have shown that vesicular stomatitis virus glycoprotein pseudotyped murine leukemia virus (VSVG-MLV) virions can transduce different developmental stages of *S. mansoni* including

Correspondence: Paul J. Brindley, Department of Microbiology, Immunology & Tropical Medicine, George Washington University Medical Center, Ross Hall, 2300 I Street, Washington, D.C., 20037 USA; Fax +1 202 994 2913, paul.brindley@gwumc.edu.
*equal contribution

adult worms, schistosomules and eggs, leading to integration of reporter proviral transgenes into schistosome chromosomes (Kines et al. 2008; Kines et al. 2010; Rinaldi et al. 2011). Moreover, VSVG-MLV has recently shown utility in vector based RNA interference in adult schistosomes (Tchoubrieva et al. 2010) and for expression of oncogenes in larval blood flukes (Yang et al. 2010). Indeed, although other approaches are also in development for functional genomics of parasitic helminths (Dvorak et al. 2010; Lok 2009; Morales et al. 2007), pseudotyped MLV appears more well advanced at this time. The forward genetics strategy of insertional mutagenesis using integration proficient vectors such as VSVG-MLV represents a powerful approach to functional genomics (see Ivics et al. 2009).

There appear to be clear advantages to deployment of gammaretrovirus virions for functional genomics, including the infectious nature of retroviruses which facilitates transduction of the target organism, broad host and tissue ranges endowed by VSVG, and potential to produce virions at high titer (Amsterdam and Hopkins 2006; Mann et al. 2011; Mattison et al. 2010). However, there also are potential limitations; gammaretroviruses cannot transduce non-dividing cells well, transgene activity is influenced by positional effects within chromosomes, and attenuation of transgene expression can occur over time (e.g., Nabekura et al. 2006; Persons 2009). To advance the efficacy of VSVG-MLV for functional genomics of *S. mansoni*, we investigated activity of the cHS4 insulator (Chung et al. 1993) as a component of the proviral transgene for transduction of cultured schistosomules. cHS4, a nuclease hypersensitive region from the globin gene locus of the chicken is known as a barrier element that can protect against transgene silencing in mammalian and insect cells (Arumugam et al. 2009; Chung et al. 1993). A core objective of transgenesis as functional genomic tool is to achieve robust, sustained expression of the integrated transgenes. Chromatin insulators can suppress or inhibit transgene silencing not only for reporter genes such as *neo* or luciferase but also shRNA encoding cassettes for vector based RNA interference. We describe a new MLV construct, which includes a functional yet minimally sized, cHS4 element inserted within the retroviral 3'LTR and report that, after transduction of schistosomules, transgene expression of the neomycin phosphotransferase gene (*neo*) was protected from silencing.

Materials and Methods

Parasites

Biomphalaria glabrata snails infected with the NMRI (Puerto Rican) strain of *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD. Schistosomules were obtained from cercariae released from the snails, and cultured as described (Mann et al. 2010). In brief, cercariae were concentrated by centrifugation (800 g/10 min) and washed with schistosomule wash medium (Dulbecco's modified Eagle's medium supplemented with 200 units/ml of penicillin, 200 µg/ml of streptomycin, 500 ng/ml of amphotericin B and 10 mM HEPES). Cercarial tails were sheared off by 20 passes back and forth through a 22G emulsifying needle, after which schistosomule bodies were separated from tails by Percoll gradient centrifugation (Lazdins et al. 1982). Schistosomula were washed three times in wash medium and cultured at 37°C under 5% CO₂ in Basch's medium (Basch 1981) supplemented with washed human erythrocytes, one µl packed red cells per ml culture medium. Control and virion exposed schistosomules were examined, and images recorded, under bright light using a Zeiss Axio Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss).

Plasmid constructs for cHS4-insulated murine leukemia virus gammaretrovirus

A 650 bp portion of full-length cHS4, comprising the 250 bp of core DNA sequence from the 5' end of cHS4 fused with 400bp from the 3' end of cHS4 was chemically synthesized

(GENEART AG, Regensburg, Germany). Plasmid pLNHX (Clontech, Mountain View, CA) was digested with *Xho*I to remove the *Drosophila* heat shock 70 gene promoter, to produce pLNHX Δ D70. The 650 bp portion of cHS4 was inserted into the *Xba*I site of the U3 region of the 3'LTR region of pLNHX Δ D70 to derive pLNHX_cHS4_650.

Transduction of schistosomes with pseudotyped virions

Production of VSVG-pseudotyped virions in packaging cells was undertaken as described (Kines et al. 2006). In brief, GP2–293 (modified HEK-293) packaging cells (Clontech) were transfected with pLNHX_ΔD70 or pLNHX_cHS4_650 along with a plasmid encoding VSVG, delivered in liposomes (Lipofectamine 2000, Invitrogen). Subsequently, virions in the culture supernatants were concentrated by high-speed centrifugation (Sorvall, SS–34 rotor) 50,000 g, 90 min, at 4°C. Pellets of concentrated virions were resuspended in modified Basch's medium at 4°C overnight, aliquoted and stored at –80°C. Viral titers were determined by real time PCR targeting viral genome and/or by functional assay using NIH-3T3 fibroblast cells cultured in the presence of G418/geneticin (Mann et al. 2011; Rinaldi et al. 2011).

Schistosomula ($2\text{--}5 \times 10^4$), at one day to seven days after transformation from cercariae, were cultured in 6 well plates containing one ml of virion preparation with an infectivity of 1×10^6 to 1×10^7 colony forming units (CFU)/ml, using similar CFUs of parental (from pLNHX_ΔD70) or cHS4 bearing (pLNHX_cHS4_650) virions in the presence of the cationic polymer polybrene. A control group of schistosomules treated with polybrene B in the absence of virions was included. After exposure to virions for 18 hours, culture media were replaced with virion-free medium supplemented with washed human erythrocytes. Thereafter, culture media were replaced every second day. Retrovirus-exposed schistosomules were harvested at various intervals after transduction, snap frozen and then stored at –80°C.

Transgene copy number

Genomic DNA (gDNA) was extracted from virion transduced and untreated, control schistosomules using a kit (E.Z.N.A. SQ Tissue DNA Kit, Omega Bio-tek), and gDNA concentrations determined by spectrophotometer (NanoDrop 1000). Quantitative PCRs were performed using TaqMan primers and probes specific for *neo* (forward primer, GGA GAG GCT ATT CGG CTA TGA C; reverse primer, CGG ACA GGT CGG TCT TGA C; probe, 5'-/56-FAM/CTG CTC TGA TGC CGC CGT GTT CCG/3IABIk_FQ/-3') and *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (*SmGAPDH*; GenBank M92359) (forward primer, TGT GAA AGA GAT CCA GCA AAC; reverse primer, GAT ATT ACC TGA GCT TTA TCA ATG G; probe, 5'-/56-FAM/AAG ACT CCA GTA GAC TCA ACG ACA T/3IABIk_FQ/-3'). Reactions were carried out using 100 ng of template gDNA in 20 μ l volumes along with Perfecta qPCR FastMix, UNG (Quanta Bioscience) and primer-probe sets. Quantitative PCRs were performed in triplicate, using 96-well plates (Bio-Rad), with a denaturation step at 95°C of 3 min followed by 40 cycles of 30 sec at 95°C and 30 sec at 55°C, in thermal cycler (iCycler, Bio-Rad) fitted with a real time detector (iQ5, Bio-Rad). The relative quantification assay $2^{-\Delta \Delta C_t}$ method (Livak and Schmittgen 2001), with *SmGAPDH* as the reference gene, was used to ascertain the relative transgene copy number of *neo* in gDNAs of schistosomules transduced with parental (from pLNHX_ΔD70) or cHS4 chromatin insulator bearing (pLNHX_cHS4_650) virions. Relative *neo* copy number in viral treated groups reflect the fold change of *neo* copy number normalized to the *SmGAPDH* reference gene and relative to the untreated control group (calibrator).

Expression of retroviral transgene

Total RNAs were extracted from pellets of frozen schistosomula using a kit (RNAqueous-4PCR, Ambion) and their concentrations estimated by spectrophotometer (NanoDrop 1000). cDNAs were synthesized from 500 ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad). End point PCR targeting the *neo* gene encoding neomycin phosphotransferase (forward primer, TGT GCT CGA CGT TGT CAC TGA A; reverse primer, ATG AAT CCA GAA AAG CGG CCA) was performed using the Promega GoTaq Green Master mix system, with 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 90 sec and final extension at 72 °C for 7 min. The *S. mansoni* actin 2 gene, GenBank U19945 (forward primer, CAG TGT TCC CTT CCA TCG TT, reverse primer, GGA CAG GGT GTT CTT CTG GA) was used as an endogenous schistosome gene control for integrity of the cDNA. Two negative control templates were included in the assays: first, RNA that was not reverse transcribed, and second, water was substituted for template cDNA. End-point PCR products were sized by electrophoresis in 1% agarose, after which images of the ethidium bromide-stained gel were obtained (Gel-Doc XR system, Bio-Rad). Quantitative PCRs for expression of *neo* and GAPDH were performed as above for determination of transgene copy number except that cDNA reverse transcribed from 500 ng of total RNA was employed as the PCR template. The relative quantification assay $2^{-\Delta \Delta C_t}$ method (Livak and Schmittgen 2001) using *SmGAPDH* as the reference gene was employed to estimate the relative expression of *neo* in schistosomules transduced with parental (from pLNHX_ΔD70) or cHS4 chromatin insulator bearing (pLNHX_cHS4_650) virions. Relative *neo* expressions in viral treated groups reflect the fold change of *neo* expression normalized to the *SmGAPDH* reference gene and relative to the untreated control group (calibrator).

Bioinformatics

The amino acid sequences of human CCCTC-binding factor (CTCF; GenBank AAB07788), upstream transcription factor 1 (USF1; CAI15372) and vascular endothelial zinc finger 1 (VEZF1;NP_009077) which are known to interact with cHS4 and other chromatin insulators in other species (e.g., (Dickson et al. 2010; Heger et al. 2009; West et al. 2004)) were used as queries in Blastp searches <http://blast.ncbi.nlm.nih.gov/Blast.cgi> of the public databases, including the draft genome version 3.1 of *S. mansoni*, aiming to identify schistosome orthologues that might be capable of interacting with cHS4.

Results

Prototypic chromatin insulator cHS4 in U3 region of LTRs of MLV flanking *neo* transgene

Previously we demonstrated that pseudotyped MLV can transduce schistosomes, leading to integration of proviral transgenes in schistosome chromosomes (Kines et al. 2008). Earlier studies also had indicated that, in this situation, the 5'-LTR drove transcription of *neo* from the integrated provirus (Kines et al. 2006; Kines et al. 2008). It is notable that insertion of chromatin insulator sequences in the U3 region of the 3'-LTR of MLV results in two copies of the insulator in the proviral form of MLV (Rivella et al. 2000). However, the cHS4 element is 1.2 kb in length, sufficiently long to lead to reduced titers during production of the virions (Nielsen et al. 2009; Urbinati et al. 2009). Since shorter versions of cHS4 retain most or all of the insulator activity of the full length element (Aker et al. 2007; Arumugam et al. 2009), and have been included in retroviral and lentiviral vectors for mammalian cells (Aker et al. 2007; Arumugam et al. 2009), we employed a strategy that involved using part of the cHS4 element in a new construct for transduction of schistosomes. Our approach involved fusing the 250 bp core of cHS4 with the 3'-terminal 400 bp of the insulator, thereby halving the size of the insulator with the aim of retaining the ability to obtain high titers of virions (Aker et al. 2007; Arumugam et al. 2009; Li et al. 2009).

The retroviral vector pLNHX was modified to remove the D70 promoter (a *Drosophila* HSP70 gene promoter) resulting in a smaller plasmid termed, pLNHX_ΔD70 (insert size 5,301 bp). Thereafter, the shortened (650 bp) version of the 1.2 kb cHS4 insulator was inserted into the U3 region of the 3'-LTR of the retroviral insert of pLNHX, and this construct termed pLNHX_cHS4_650 (Fig 1). The integrity of the modifications was verified by nucleotide sequence analysis; the insert of pLNHX_cHS4_650 has been assigned GenBank number JN000001. Virions encoded by the inserts of pLNHX_ΔD70 and pLNHX_cHS4_650 were produced and titers determined; in general comparable titers were produced from both plasmids, the non-insulator and the insulator bearing forms. Titers in the range of 1 to 10×10^6 CFUs can be reliably produced from both plasmids (not shown). The inserts of these plasmids have been trimmed so that they include only one promoter driving one reporter gene, in this case the 5' LTR driving *neo* (Figure 1). Schistosomula were exposed to these virions. As with mammalian target cells, we anticipated that, once reaching cytoplasm of schistosome cells, viral reverse transcriptase would have reverse transcribed the RNA genomes to proviral cDNAs and that during reverse transcription, the cHS4 insulator at U3 of 3'-LTR would have been copied and transferred to the 5'-LTR (Hantzopoulos et al. 1989; Rivella et al. 2000). We also anticipated that insulated DNA would integrate into schistosome chromosomes through the enzymatic activity of retroviral integrase (Figure 1).

Equivalent transgene copy number in schistosomes transduced with insulated or control virions

Transgene copy number was investigated in schistosomes exposed to virions produced from the pLNHX_ΔD70 and pLNHX_cHS4_650 constructs. Schistosomules were collected three and 10 days after addition of virions. At this point, parasite populations were divided into two groups; gDNA was extracted from one, total RNA from the other. qPCR revealed the same or similar relative transgene copy number (i.e. *neo* copy number relative to the reference gene) in the gDNA from schistosomules exposed to either virion genotype: 23 and 24 copies in the day 3 groups, and 32 and 30 copies in the day 10 groups, of the pLNHX_ΔD70 and pLNHX_cHS4_650 virions, respectively (Figure 2, panel a). Analysis by qPCR of RNAs from the correspondent samples revealed that the relative expression of *neo* in the cHS4 encoded virion transduced worms was approximately double that seen in the schistosomes transduced with the control virus. This trend was evident at both three and 10 days after transduction with the pseudotyped virions (Figure 2b). The experiment was repeated, and similar findings were obtained on both occasions (not shown). Interestingly, whereas higher expression of *neo* was seen at both time points, there was nevertheless a substantial reduction of the level of expression at day 10 compared to day 3 (Figure 2b) (see below, also). Nonetheless, together these findings revealed that both virions, the parental version and the experimental form carrying the cHS4 chromatin insulator, were of comparable proficiency for transduction of schistosomules. We detected a similar density of proviral transgenes in the genomic DNA recovered from virion-exposed populations of schistosomules. More importantly, despite equivalent relative *neo* transgene copy number, they revealed substantially higher transgene expression from the cHS4-insulated proviral transgene than the control, non-insulated transgene.

Prototypic insulator cHS4 protects retroviral transgene from silencing in schistosomes

Next, we investigated the phenomenon of higher levels of expression of *neo* in schistosomules transduced with insulated proviral transgenes, over longer periods of time - at 5, 10 and 20 days after virus transduction. Total RNA extracted from schistosomula transduced with insulated and parental virions were reverse transcribed and used as template for PCR. Both endpoint RT-PCR and qRT-PCR were employed to visualize and quantify expression of the *neo* transgene. In analysis by endpoint PCR, ethidium-stained products

showed stronger signals for *neo* transcripts in the cHS4 insulated virions compared to the control virions, at each of interval after exposure. No differences were evident in expression of actin, a control target for RNA integrity. As expected, no *neo* signal was observed in control schistosomules not exposed to virions (Figure 3a). In like fashion, analysis by qRT-PCR confirmed higher expression of *neo* in schistosomes exposed to insulated compared to non-insulated virions; the ratio of *neo* expression in the pLNHX_cHS4_650 compared to pLNHX_ΔD70 virions was 3.2, 6.9 and 19.6 at 5 days, 10 days and 20 days after transduction, respectively (Figure 3b). Although the relative expression of *neo* was substantially greater at each time point in schistosomes transduced with insulated compared with control virions, transgene expression globally diminished in all the transgenic parasites over time. Thus, cHS4 inhibited transgene silencing but did not completely block it. By light microscopy, no differences were apparent in the viability or appearance of schistosomules among the non-virus exposed control schistosomules, or the virion exposed groups (Figure 3c); the representative micrographs in Figure 3 show schistosomules at 10 days after exposure to the virions. The experiment was repeated, with similar gene expression being observed on both occasions (not shown).

Potential cHS4 binding proteins of schistosomes

Bioinformatic searches were carried for putative orthologues of the binding proteins CTCF, USF1 and VEZF1, revealing schistosome matches to each: CTCF, match accession numbers XP_002575256, XP_002578242, XP_002575460; USF1, accessions XP_002579915, XP_002578293, XP_002571520; and VEZF1, accessions XP_002577022, XP_002575460, XP_002575256 from *S. mansoni* with the E-values < 1E-4 and 28–44 % similarities (Table S1).

Discussion

We have recently transduced the human blood fluke *S. mansoni* with a pseudotyped gammaretrovirus, murine leukemia virus (see Mann et al. 2011). Our aim is to use this approach for functional genomics of schistosomes, which are major neglected tropical disease pathogens of humanity and for which draft genome sequences have recently been reported (Berriman et al. 2009; Hotez et al. 2008; *Schistosoma japonicum* Genome and Functional Analysis Consortium, 2009). The nature of the retroviral developmental cycle, in particular the integration of the proviral form into host chromosomes and target site preferences for insertion into host chromosomes are desirable attributes that make these vectors attractive for functional genomics and transgenesis (Roth et al. 2011; Wang et al. 2007). However, gammaretroviral and lentiviral vectors have drawbacks as reagents for functional genomic and gene therapy approaches, including transgene silencing due to position effects, oncogene activity, and silencing of the transgene cargo by proviral 5'-LTRs. Interest in retroviral vectors also is tempered, particularly in clinical settings, by issues of genotoxicity involving the activation of cellular oncogenes flanking sites of vector integration (Bohne and Cathomen 2008; Hacein-Bey-Abina et al. 2003; Li et al. 2009; Ott et al. 2006).

The cHS4 element, a well characterized vertebrate chromatin insulator from the control region of the chicken β -globin locus, contains a DNase-I hypersensitive site (cHS4) and constitutes the boundary of the β -globin locus (Chung et al. 1993; Reitman and Felsenfeld 1990). cHS4 exhibits classical insulator activities, including the ability to block the interactions of globin gene promoters and enhancers in cell lines, with the ability to protect expression cassettes in *Drosophila*, transformed cell lines, and transgenic mammals from position effects (Chung et al. 1993; Emery et al. 2000). The cHS4 insulator has been incorporated into retroviral and lentiviral gene therapy vectors aiming to improve performance and relieve other impediments (Desprat and Bouhassira 2009; Emery et al.

2002; Emery et al. 2000; Li and Emery 2008; Li et al. 2009). With retroviral vectors, constructs have been assembled by flanking the vector with the cHS4 element using a double-copy arrangement, wherein cHS4 elements were cloned into the 3'-LTR of the producer plasmid. From this arrangement, they are copied into the 5'-LTR during reverse transcription of the retroviral RNA genome and proviral integration, resulting in a proviral insertion where the 5'-LTR and internal transgene sequences are insulated from host genomic sequences on both sides of the proviral transgene and the 3'-LTR is insulated from genomic sequences downstream of the insertion (Emery et al. 2002; Emery et al. 2000; Hantzopoulos et al. 1989; Li and Emery 2008; Li et al. 2009).

Shorter versions of cHS4 have been developed which retain the insulator activity of the full length element (Aker et al. 2007; Arumugam et al. 2009). Here we modified the MLV vector pLNHX by insertion of a shortened version of cHS4, comprising 250 bp of the 5' core region of cHS4 fused to 400 bp of the 3' region of cHS4. The 650 bp element was inserted into the U3 domain of retroviral 3'-LTR, resulting in replication and transfer to the 5'-LTR bp during reverse transcription (Emery et al. 2000; Hantzopoulos et al. 1989). Relative copy numbers of the transgene were found to be comparable in insulated and control virus transduced schistosomes. By contrast, reporter *neo* transgene expression observed in schistosomules transduced with insulated virions was from two to 20 fold higher in parasites carrying the cHS4 chromatin insulator, flanking both sides of the proviral transgene. Therefore the stronger *neo* expression detected in the schistosomes transduced by the insulated retrovirus can be explained by a stronger promoter activity (because the transgene was insulated) rather than a higher genome density of integrated proviral transgenes. The increase of *neo* expression in schistosomes transduced with insulated virions may also result from the reduction of position effects which allowed for homogeneity of transgene expression from integrated provirus. In any event, this ratio is similar to the 2–10 fold increase in transgene expression in cHS4 insulated virus compared to un-insulated counterparts seen with short forms of cHS4 in mammalian cells (Arumugam et al. 2007; Emery et al. 2002). On the other hand, there appeared to be down regulation of *neo* expression from both insulated and non-insulated transgenes in the worms over the 20 days during which the transgenic schistosomules were observed. Clearly, however, the cHS4 insulator slowed this process. The general decline in transgene expression may relate to developmental regulation of the promoter, in this case the 5'-LTR of the transgene retrovirus. Alternatively, other transcriptional silencing mechanisms, not efficiently protected against by cHS4, might be at play.

It has been determined that cHS4 is active in a spectrum of metazoans, including vertebrate and insect species (Chung et al. 1993; Emery et al. 2000; Rincon-Arano et al. 2007; Sekkali et al. 2008; Taboit-Dameron et al. 1999). Moreover, this chromatin insulator has been employed in gammaretrovirus, lentivirus, *piggyBac* transposon, and phiC31 integration system vectors (Aker et al. 2007; Arumugam et al. 2007; Emery et al. 2000; Evans-Galea et al. 2007; Jakobsson et al. 2004; Nishiumi et al. 2009; Rivella et al. 2000; Sarkar et al. 2006). Functional analyses of cHS4 have identified CTCF and USF-1/2 motifs in the proximal 250 bp of cHS4, termed the 'core' region that includes five protein binding sites revealed by DNase I foot printing, which provide enhancer blocking activity and reduce position effects (Bell et al. 1999; Chung et al. 1997; Recillas-Targa et al. 2002). The CTCF binding site footprint II (FII) is necessary and sufficient for enhancer blocking. The binding of USF (upstream stimulatory factor) proteins to footprint FIV is a essential component of its barrier activity (West et al. 2004). USF recruits histone modification complexes and is critical for maintenance of a chromatin barrier (Huang et al. 2007; West et al. 2004). The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation (Mutskov et al. 2002). Protection from DNA methylation is separable from other insulator activities and is mapped to three transcription factor

binding sites occupied by the zinc finger protein VEZF1, a novel chromatin barrier protein and a candidate factor for the protection of promoters from DNA methylation (Dickson et al. 2010). The present study provides the first report of the cHS4 insulator protecting transgene expression in schistosomes; indeed we unaware of investigations of cHS4 in any Lophotrochozoan species. This is notable since the Lophotrochozoan assemblage of phyla includes about half of known metazoan species. Although the insulator activity of cHS4 on transgene activity has been demonstrated to relate to chromatin modification and enhancer blocking, the mechanism by which cHS4 protects transgene silencing in schistosomes remains to be determined. Database searches indicated the existence of homologues of insulator proteins CTCF, USF and VEZF1 in *S. mansoni* which may support the activity of cHS4 in schistosome chromatin. It would also be of interest to locate schistosome, or other Lophotrochozoan, insulators and to compare their performance with that of cHS4 in these transgenic parasites.

In conclusion, we determined that the prototypic chromatin insulator cHS4 protected transgene expression in schistosomes from silencing. We consider this to be a noteworthy advance in development of vectors that can deliver sustained transgene expression which will enhance the prospects for functional genomics approaches for schistosomes utilizing VSVG-MLV transgenesis. The insulated MLV encoding *neo* can be tested for neomycin/G418 selection of transgenic schistosomes and for germ line transgenesis, given that *neo* is active in *C. elegans* and facilitates the selection of transgenic worms on G418 (Giordano-Santini and Dupuy 2011; Giordano-Santini et al. 2010). More specifically, this short form of cHS4 can be included in gain-of-function vectors to protect expression of reporter genes in schistosome including *neo* and *Luciferase*, into loss-of-function vector-RNAi constructs to ensure expression of transgene hairpin RNAs (Ayuk et al. 2011; Tchoubrieva et al. 2010), and in vectors targeting the schistosome germ line (see Mann et al. 2011). This addition to the nascent functional genomics toolkit for schistosomes will facilitate identification of new interventions targeting this neglected tropical disease pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Schistosome-infected snails were supplied by Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD USA) under National Institutes of Health (NIH) National Institute of Allergy and Infectious Disease (NIAID) contract HHSN2722010000051. We thank Victoria Mann and Tunika Okatcha for discussions and review of the manuscript. These studies were supported by NIH-NIAID award R01AI072773 (the content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID or the NIH).

References

- Aker M, Tubb J, Groth AC, Bukovsky AA, Bell AC, Felsenfeld G, Kiem HP, Stamatoyannopoulos G, Emery DW. Extended core sequences from the cHS4 insulator are necessary for protecting retroviral vectors from silencing position effects. *Hum Gene Ther*. 2007; 18:333–343. [PubMed: 17411365]
- Amsterdam A, Hopkins N. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet*. 2006; 22:473–478. [PubMed: 16844256]
- Arumugam PI, Scholes J, Perelman N, Xia P, Yee JK, Malik P. Improved human beta-globin expression from self-inactivating lentiviral vectors carrying the chicken hypersensitive site-4 (cHS4) insulator element. *Mol Ther*. 2007; 15:1863–1871. [PubMed: 17622240]
- Arumugam PI, Urbinati F, Velu CS, Higashimoto T, Grimes HL, Malik P. The 3' region of the chicken hypersensitive site-4 insulator has properties similar to its core and is required for full insulator activity. *PLoS ONE*. 2009; 4:e6995. [PubMed: 19746166]

- Ayuk MA, Suttiaprapa S, Rinaldi G, Mann VH, Lee CM, Brindley PJ. *Schistosoma mansoni* U6 gene promoter-driven short hairpin RNA induces RNA interference in human fibrosarcoma cells and schistosomules. *Int J Parasitol.* 2011; 41:783–789.10.1016/j.ijpara.2011.02.004 [PubMed: 21447344]
- Basch PF. Cultivation of *Schistosoma mansoni* in vitro. II. production of infertile eggs by worm pairs cultured from cercariae. *J Parasitol.* 1981; 67:186–190. [PubMed: 7241278]
- Bell AC, West AG, Felsenfeld G. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell.* 1999; 98:387–396. [PubMed: 10458613]
- Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, Mashiyama ST, Al-Lazikani B, Andrade LF, Ashton PD, Aslett MA, Bartholomeu DC, Blandin G, Caffrey CR, Coghlan A, Coulson R, Day TA, Delcher A, DeMarco R, Djikeng A, Eyre T, Gamble JA, Ghedin E, Gu Y, Hertz-Fowler C, Hirai H, Hirai Y, Houston R, Ivens A, Johnston DA, Lacerda D, Macedo CD, McVeigh P, Ning Z, Oliveira G, Overington JP, Parkhill J, Pertea M, Pierce RJ, Protasio AV, Quail MA, Rajandream MA, Rogers J, Sajid M, Salzberg SL, Stanke M, Tivey AR, White O, Williams DL, Wortman J, Wu W, Zamanian M, Zerlotini A, Fraser-Liggett CM, Barrell BG, El-Sayed NM. The genome of the blood fluke *Schistosoma mansoni*. *Nature.* 2009; 460:352–358. [PubMed: 19606141]
- Bohne J, Cathomen T. Genotoxicity in gene therapy: an account of vector integration and designer nucleases. *Curr Opin Mol Ther.* 2008; 10:214–223. [PubMed: 18535928]
- Chung JH, Bell AC, Felsenfeld G. Characterization of the chicken beta-globin insulator. *Proc Natl Acad Sci U S A.* 1997; 94:575–580. [PubMed: 9012826]
- Chung JH, Whiteley M, Felsenfeld G. A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell.* 1993; 74:505–514. [PubMed: 8348617]
- Desprat R, Bouhassira EE. Gene specificity of suppression of transgene-mediated insertional transcriptional activation by the chicken HS4 insulator. *PLoS ONE.* 2009; 4:e5956. [PubMed: 19536296]
- Dickson J, Gowher H, Strogantsev R, Gaszner M, Hair A, Felsenfeld G, West AG. VEZF1 elements mediate protection from DNA methylation. *PLoS Genet.* 2010; 6:e1000804. [PubMed: 20062523]
- Dvorak J, Beckmann S, Lim KC, Engel JC, Grevelding CG, McKerrow JH, Caffrey CR. Biolistic transformation of *Schistosoma mansoni*: Studies with modified reporter-gene constructs containing regulatory regions of protease genes. *Mol Biochem Parasitol.* 2010; 170:37–40. [PubMed: 19914302]
- Emery DW, Yannaki E, Tubb J, Nishino T, Li Q, Stamatoyannopoulos G. Development of virus vectors for gene therapy of beta chain hemoglobinopathies: flanking with a chromatin insulator reduces gamma-globin gene silencing in vivo. *Blood.* 2002; 100:2012–2019. [PubMed: 12200360]
- Emery DW, Yannaki E, Tubb J, Stamatoyannopoulos G. A chromatin insulator protects retrovirus vectors from chromosomal position effects. *Proc Natl Acad Sci U S A.* 2000; 97:9150–9155. [PubMed: 10908661]
- Evans-Galea MV, Wielgosz MM, Hanawa H, Srivastava DK, Nienhuis AW. Suppression of clonal dominance in cultured human lymphoid cells by addition of the cHS4 insulator to a lentiviral vector. *Mol Ther.* 2007; 15:801–809. [PubMed: 17299406]
- Giordano-Santini R, Dupuy D. Selectable genetic markers for nematode transgenesis. *Cell Mol Life Sci.* 2011; 68:1917–1927. [PubMed: 21431833]
- Giordano-Santini R, Milstein S, Svrikapa N, Tu D, Johnsen R, Baillie D, Vidal M, Dupuy D. An antibiotic selection marker for nematode transgenesis. *Nat Methods.* 2010; 7:721–723. [PubMed: 20729841]
- Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med.* 2003; 348:255–256. [PubMed: 12529469]
- Han ZG, Brindley PJ, Wang SY, Chen Z. *Schistosoma* genomics: new perspectives on schistosome biology and host-parasite interaction. *Annu Rev Genomics Hum Genet.* 2009; 10:211–240. [PubMed: 19630560]

- Hantzopoulos PA, Sullenger BA, Ungers G, Gilboa E. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc Natl Acad Sci U S A*. 1989; 86:3519–3523. [PubMed: 2542934]
- Heger P, Marin B, Schierenberg E. Loss of the insulator protein CTCF during nematode evolution. *BMC Mol Biol*. 2009; 10:84. [PubMed: 19712444]
- Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation*. 2008; 118:1311–1321. [PubMed: 18382743]
- Huang S, Li X, Yusufzai TM, Qiu Y, Felsenfeld G. USF1 recruits histone modification complexes and is critical for maintenance of a chromatin barrier. *Mol Cell Biol*. 2007; 27:7991–8002. [PubMed: 17846119]
- Ivics Z, Li MA, Mates L, Boeke JD, Nagy A, Bradley A, Izsvak Z. Transposon-mediated genome manipulation in vertebrates. *Nat Methods*. 2009; 6:415–422. [PubMed: 19478801]
- Jakobsson J, Rosenqvist N, Thompson L, Barraud P, Lundberg C. Dynamics of transgene expression in a neural stem cell line transduced with lentiviral vectors incorporating the cHS4 insulator. *Exp Cell Res*. 2004; 298:611–623. [PubMed: 15265707]
- Kines KJ, Mann VH, Morales ME, Shelby BD, Kalinna BH, Gobert GN, Chirgwin SR, Brindley PJ. Transduction of *Schistosoma mansoni* by vesicular stomatitis virus glycoprotein-pseudotyped Moloney murine leukemia retrovirus. *Exp Parasitol*. 2006; 112:209–220. [PubMed: 16530185]
- Kines KJ, Morales ME, Mann VH, Gobert GN, Brindley PJ. Integration of reporter transgenes into *Schistosoma mansoni* chromosomes mediated by pseudotyped murine leukemia virus. *FASEB J*. 2008; 22:2936–2948. [PubMed: 18403630]
- Kines KJ, Rinaldi G, Okatcha TI, Morales ME, Mann VH, Tort JF, Brindley PJ. Electroporation facilitates introduction of reporter transgenes and virions into schistosome eggs. *PLoS Negl Trop Dis*. 2010; 4:e593.10.1371/journal.pntd.0000593 [PubMed: 20126309]
- Lazdins JK, Stein MJ, David JR, Sher A. *Schistosoma mansoni*: rapid isolation and purification of schistosomula of different developmental stages by centrifugation on discontinuous density gradients of Percoll. *Exp Parasitol*. 1982; 53:39–44. [PubMed: 6276213]
- Li CL, Emery DW. The cHS4 chromatin insulator reduces gammaretroviral vector silencing by epigenetic modifications of integrated provirus. *Gene Ther*. 2008; 15:49–53. [PubMed: 17989708]
- Li CL, Xiong D, Stamatoyannopoulos G, Emery DW. Genomic and functional assays demonstrate reduced gammaretroviral vector genotoxicity associated with use of the cHS4 chromatin insulator. *Mol Ther*. 2009; 17:716–724. [PubMed: 19240697]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Lok JB. Transgenesis in parasitic nematodes: building a better array. *Trends Parasitol*. 2009; 25:345–347. [PubMed: 19617000]
- Mann VH, Morales ME, Rinaldi G, Brindley PJ. Culture for genetic manipulation of developmental stages of *Schistosoma mansoni*. *Parasitology*. 2010; 137:451–462. [PubMed: 19765348]
- Mann VH, Suttiaprapa S, Rinaldi G, Brindley PJ. Establishing transgenic schistosomes. *PLoS Negl Trop Dis*. 2011 (in press).
- Mattison J, Kool J, Uren AG, de Ridder J, Wessels L, Jonkers J, Bignell GR, Butler A, Rust AG, Brosch M, Wilson CH, van der Weyden L, Largaespada DA, Stratton MR, Futreal PA, van Lohuizen M, Berns A, Collier LS, Hubbard T, Adams DJ. Novel candidate cancer genes identified by a large-scale cross-species comparative oncogenomics approach. *Cancer Res*. 2010; 70:883–895. [PubMed: 20103622]
- Morales ME, Mann VH, Kines KJ, Gobert GN, Fraser MJ Jr, Kalinna BH, Correnti JM, Pearce EJ, Brindley PJ. piggyBac transposon mediated transgenesis of the human blood fluke, *Schistosoma mansoni*. *FASEB J*. 2007; 21:3479–3489. [PubMed: 17586730]
- Mutskov VJ, Farrell CM, Wade PA, Wolffe AP, Felsenfeld G. The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation. *Genes Dev*. 2002; 16:1540–1554. [PubMed: 12080092]

- Nabekura T, Otsu M, Nagasawa T, Nakauchi H, Onodera M. Potent vaccine therapy with dendritic cells genetically modified by the gene-silencing-resistant retroviral vector GCDNsap. *Mol Ther*. 2006; 13:301–309. [PubMed: 16311073]
- Nielsen TT, Jakobsson J, Rosenqvist N, Lundberg C. Incorporating double copies of a chromatin insulator into lentiviral vectors results in less viral integrants. *BMC Biotechnol*. 2009; 9:13. [PubMed: 19239708]
- Nishiumi F, Sone T, Kishine H, Thyagarajan B, Kogure T, Miyawaki A, Chesnut JD, Imamoto F. Simultaneous single cell stable expression of 2–4 cDNAs in HeLaS3 using psiC31 integrase system. *Cell Struct Funct*. 2009; 34:47–59. [PubMed: 19305101]
- Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, Glimm H, Kuhlcke K, Schilz A, Kunkel H, Naundorf S, Brinkmann A, Deichmann A, Fischer M, Ball C, Pilz I, Dunbar C, Du Y, Jenkins NA, Copeland NG, Luthi U, Hassan M, Thrasher AJ, Hoelzer D, von Kalle C, Seger R, Grez M. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med*. 2006; 12:401–409. [PubMed: 16582916]
- Persons DA. Hematopoietic stem cell gene transfer for the treatment of hemoglobin disorders. *Hematology Am Soc Hematol Educ Program*. 2009:690–697. [PubMed: 20008255]
- Recillas-Targa F, Pikaart MJ, Burgess-Beusse B, Bell AC, Litt MD, West AG, Gaszner M, Felsenfeld G. Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. *Proc Natl Acad Sci U S A*. 2002; 99:6883–6888. [PubMed: 12011446]
- Reitman M, Felsenfeld G. Developmental regulation of topoisomerase II sites and DNase I-hypersensitive sites in the chicken beta-globin locus. *Mol Cell Biol*. 1990; 10:2774–2786. [PubMed: 2160585]
- Rinaldi G, Suttiaprapa S, Brindley PJ. Quantitative retrotransposon anchored PCR confirms transduction efficiency of transgenes in adult *Schistosoma mansoni*. *Mol Biochem Parasitol*. 2011; 177:70–76. [PubMed: 21251928]
- Rincon-Arango H, Furlan-Magaril M, Recillas-Targa F. Protection against telomeric position effects by the chicken cHS4 beta-globin insulator. *Proc Natl Acad Sci U S A*. 2007; 104:14044–14049. [PubMed: 17715059]
- Rivella S, Callegari JA, May C, Tan CW, Sadelain M. The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites. *J Virol*. 2000; 74:4679–4687. [PubMed: 10775605]
- Roth SL, Malani N, Bushman FD. Gammaretroviral integration into nucleosomal target DNA in vivo. *J Virol*. 2011; 85:7393–40. [PubMed: 21561906]
- Sarkar A, Atapattu A, Belikoff EJ, Heinrich JC, Li X, Horn C, Wimmer EA, Scott MJ. Insulated piggyBac vectors for insect transgenesis. *BMC Biotechnol*. 2006; 6:27. [PubMed: 16776846]
- Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium. The *Schistosoma japonicum* genome reveals features of host-parasite interplay. *Nature*. 2009; 460:345–351. [PubMed: 19606140]
- Sekkali B, Tran HT, Crabbe E, De Beule C, Van Roy F, Vleminckx K. Chicken beta-globin insulator overcomes variegation of transgenes in *Xenopus* embryos. *FASEB J*. 2008; 22:2534–2540. [PubMed: 18359926]
- Taboit-Dameron F, Malassagne B, Viglietta C, Puissant C, Leroux-Coyau M, Chereau C, Attal J, Weill B, Houdebine LM. Association of the 5'HS4 sequence of the chicken beta-globin locus control region with human EF1 alpha gene promoter induces ubiquitous and high expression of human CD55 and CD59 cDNAs in transgenic rabbits. *Transgenic Res*. 1999; 8:223–235. [PubMed: 10478492]
- Tchoubrieva EB, Ong PC, Pike RN, Brindley PJ, Kalinna BH. Vector-based RNA interference of cathepsin B1 in *Schistosoma mansoni*. *Cell Mol Life Sci*. 2010; 67:3739–3748. [PubMed: 20339897]
- Urbinati F, Arumugam P, Higashimoto T, Perumbeti A, Mitts K, Xia P, Malik P. Mechanism of reduction in titers from lentivirus vectors carrying large inserts in the 3'LTR. *Mol Ther*. 2009; 17:1527–1536. [PubMed: 19384292]

- Wang D, Jao LE, Zheng N, Dolan K, Ivey J, Zonies S, Wu X, Wu K, Yang H, Meng Q, Zhu Z, Zhang B, Lin S, Burgess SM. Efficient genome-wide mutagenesis of zebrafish genes by retroviral insertions. *Proc Natl Acad Sci U S A*. 2007; 104:12428–12433. [PubMed: 17640903]
- West AG, Huang S, Gaszner M, Litt MD, Felsenfeld G. Recruitment of histone modifications by USF proteins at a vertebrate barrier element. *Mol Cell*. 2004; 16:453–463. [PubMed: 15525517]
- Yang S, Brindley PJ, Zeng Q, Li Y, Zhou J, Liu Y, Liu B, Cai L, Zeng T, Wei Q, Lan L, McManus DP. Transduction of *Schistosoma japonicum* schistosomules with vesicular stomatitis virus glycoprotein pseudotyped murine leukemia retrovirus and expression of reporter human telomerase reverse transcriptase in the transgenic schistosomes. *Mol Biochem Parasitol*. 2010; 174:109–116. [PubMed: 20692298]

Figure 1.

Schematic overview of insulator vector and predicted outcome of virion transduction of schistosomes. The pLNHXΔD70 plasmid was constructed by removing *Drosophila* heat shock protein 70 promoter from pLNHX. The 650 bp active component of the chicken cHS4 insulator, 250 bp of core DNA sequence fused with 3' 400bp of cHS4 insulator was chemically synthesized as a linear DNA fragment, which was subsequently inserted into the U3 domain of the 3'-LTR of pLNHXΔD70 to derive plasmid pLNHXcHS4_650 (top panel). Replication incompetent VSVG-pseudotyped virions were produced in packaging cells. Schistosomules of *Schistosoma mansoni* were cultured with virions from pLNHXΔD70 and pLNHXcHS4_650 constructs (center). After transducing the schistosome surface (Kines et al. 2006), it is predicted that the gammaretroviral RNA genome is reverse transcribed to non-integrated proviral cDNA; and during this process, the 650bp of cHS4 insulator is copied and transferred from 3'-LTR to 5'-LTR to generate the viral construct containing insulator at both sides of LTR (Rivella et al. 2000). The proviral genome, carrying the neo transgene flanked by LTRs bearing the cHS4 chromatin insulator, approaches the nucleus at cell division, and integrates into a schistosome chromosome catalyzed by retroviral integrase.

Figure 2.

Determination of relative transgene copy number and expression in schistosomules transduced with insulated and non-insulated gammaretrovirus virions by quantitative real time PCR. **A.** Relative transgene copy number determined in genomic DNAs from schistosomules at three and 10 days after transduction by pseudotyped gammaretroviral virions; pLNHX_ΔD70 virions (green bars), pLNHX_cHS4_650 virions (red bars). **B.** Expression of transgene *neo* detected in schistosomule groups shown in Panel A. Ratios between the relative transgene copy numbers and transgene expression detected in schistosomules transfected with the insulated and non-insulated virions are shown in Panels A and B, respectively

Figure 3.

Expression of *neo* transgene protected from silencing in schistosomules transduced with cHS4 insulated gammaretrovirus virions. **A.** Analysis by end-point RT-PCR of *neo* expression in schistosomules five, 10 and 20 days after exposure to pseudotyped virions where expression levels of *neo* and *actin* (control for integrity of RNA) were visualized in ethidium-stained agarose gel; control, RNA from age-matched schistosomules not exposed to various; ΔD70, schistosomules exposed to pLNHX_ΔD70 virions; cHS4_650, schistosomules exposed to pLNHX_cHS4_650 virions, in which the chromatin insulator cHS4 fragment flanks the *neo* transgene. **B.** Analysis by quantitative RT-PCR of the *neo* expression in schistosomules exposed to pLNHX_ΔD70 virions (green bars) or to pLNHX_cHS4_650 virions (red bars) for five, 10 and 20 days as indicated in Panel A. Ratio between the relative transgene expression detected in schistosomules transfected with the insulated and non-insulated virions is shown on the top of each panel. (Note that different Y-axis scales were used at each time point.) **C.** Micrographs of schistosomules not exposed to virions (control) or exposed 10 days previously to pLNHX_ΔD70 (ΔD70) or pLNHX_cHS4_650 (cHS4_650) virions. Bars = 100 μm