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Functional characterization of the sea urchin *sns* chromatin insulator in erythroid cells

Santina Acuto [a,*,1], Rosalba Di Marzo [a,1], Roberta Calzolari [a,1], Elena Baiamonte [b,1], Aurelio Maggio [a], Giovanni Spinelli [b]

[a] *Unità di Ricerca "P. Cutino", Ematologia II, A.O. "V. Cervello", via Trabucco n. 180-90146, Palermo, Italy*

[b] *Dipartimento di Biologia Cellulare e dello Sviluppo "Alberto Monroy", Università di Palermo, Parco d'Orleans II-90128 Palermo, Italy*

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Abstract

Chromatin insulators are regulatory elements that determine domains of genetic functions. We have previously described the characterization of a 265 bp insulator element, termed *sns*, localized at the 3′ end of the early histone H2A gene of the sea urchin *Paracentrotus lividus*. This sequence contains three *cis*-acting elements (Box A, Box B, and Box C+T) all needed for the enhancer-blocking activity in both sea urchin and human cells. The goal of this study was to further characterize the sea urchin *sns* insulator in the erythroid environment. We employed colony assays in human (K562) and mouse (MEL) erythroid cell lines. We tested the capability of *sns* to interfere with the communication between the 5′HS2 enhancer of the human β-globin LCR and the γ-globin promoter. We found that the *sns* sequence displays directional enhancer-blocking activity. By the use of antibodies against known DNA binding proteins, in electrophoretic mobility shift assays, we demonstrated the binding of the erythroid-specific GATA-1 and the ubiquitous Oct-1 and Sp1 transcription factors. These factors bind to Box A, Box B, and Box C+T, respectively, in both K562 and MEL nuclear extracts. These results may have significant implications for the conservation of insulator function in evolutionary distant organisms and may prove to be of practical benefit in gene transfer applications for erythroid disorders such as hemoglobinopathies and thalassemias.

Keywords: Chromatin insulator; Enhancer blocking; Erythroid transcription factor; Globin enhancer

Introduction

Insulators are genetic elements present in a wide range of eukaryotic organisms capable of attenuating the activity of enhancers or other regulative elements in a directional manner. They maintain transcription fidelity by interfering with the communication between enhancer and promoter only when interposed between the two [1,2]. Insulators, by restricting enhancer and silencer function, may impart functional independence to transcription units in the eukaryotic genome [3–10]. It has been suggested that some insulators, such as *gypsy* and c-*HS4*, with their bound proteins, may be tethered at the nuclear periphery forming loop domains [11,12]. In addition, insulators may act as barriers to the spreading of heterochromatin to euchromatic region [13–17]. Protection from chromosomal silencing requires the constitutive recruitment by the insulator binding proteins of histone modifying enzymes, such as H3 lysine 4 methyltransferase and histone acetylases, to counteract the propagation of condensed chromatin structures [18–20]. Because of the barrier function, an insulator placed in flanking location can buffer a transgene from extinction of expression and also block the passage of regulatory signals between the transgene and the chromatin domains at the genomic insertion site.

We have previously described the characterization of the sea urchin chromosomal insulator, termed *sns*, localized at the 3′ end of the α-H2A histone gene. When included in artificial constructs, *sns* displays directional enhancer-blocking activity in either orientation, both at early and late developmental

* Corresponding author. Fax: +39 091 6880828.
E-mail address: acutosanti@virgilio.it (S. Acuto).

[1] These authors contributed equally.

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stages [21,22]. In addition, if *sns* intervenes between two enhancers, only the enhancer located distally from the promoter with respect to the site of insertion is attenuated [22]. Recent evidence indicates that *sns* is part of a DNA fragment, defined as *sns* 5, needed for silencing the α-H2A histone gene at gastrula stage [23]. This result suggests that the sea urchin insulator, in the natural environment of the histone gene sequences, is involved in the mechanism of repression. Remarkably, *sns* shielded a promoter from the CMV enhancer in two different human cell lines using plasmid and retroviral vectors, suggesting that *sns* maintains the directional enhancer-blocking function in a human chromatin context [24].

Because of the potential use of *sns* in gene transfer experiments in human cells, we carried out functional tests and DNA binding studies in erythroid cell lines. Here, we show, by colony assays, that *sns* maintains its insulator activity in erythroid milieu, in that it directionally blocks the human erythroid-specific enhancer 5′HS2 of β-globin LCR in the interaction with the γ-globin promoter in human (K562) and mouse (MEL) cell lines. In addition, by electrophoretic mobility shift assays (EMSA), we show that the three *cis*-acting elements, essential for the enhancer-blocking activity [22], bind specific nuclear proteins, both erythroid and ubiquitous transcription factors.

Materials and methods

Plasmid constructions

The DNA construct p^{A}γNEO that was used for the assembly of the plasmids for the enhancer-blocking assays and displayed in Fig. 1 was previously described [25]. To generate plasmid A, we cloned, upstream to the 1.3 Kb ^{A}γ promoter, the 435 bp *Eco*RI–*Eco*RI fragment, containing the sequence essential for

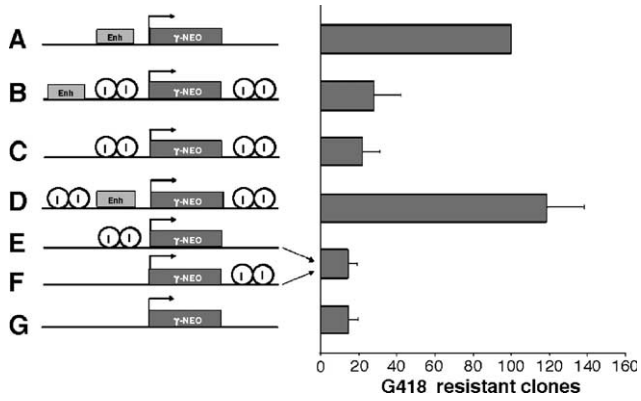

Fig. 1. Enhancer-blocking assay. The human erythroleukemic cell line K562 was stable transfected by electroporation with the constructs shown on the left. G418-resistant clones were selected and counted 2 weeks later. The relative numbers of G418-resistant clones for each construct are shown on the right. The data shown are the results of six experiments using three different plasmid preparations. The sea urchin insulator is indicated by I. Enh corresponds to the human β-globin LCR 435 bp 5′ DNAse I hypersensitive site 2 essential for the enhancer activity. The arrow at the 5′ end of the gene labeled NEO indicates the 1.3 kb ^{A}γ promoter of the G418 resistance gene. The average of the absolute numbers of G418-resistant clones for the construct A in the six experiments was 136, and it was arbitrarily set to 100.

the enhancer activity of 5′ hypersensitive site-2 (5′HS2) of the human β-globin LCR. The enhancer-less plasmids (E and F of Fig. 1) contain two tandem copies of the sea urchin insulator *sns* that were cloned, respectively, upstream the ^{A}γ promoter and downstream the NEO coding sequences. In plasmid C, the two *sns* sequences were placed flanking the enhancer-less transcription unit. Construction of plasmids B and D was carried out in two steps. We first inserted the 435 bp *Eco*RI 5′HS2 fragment in the *Eco*RI site upstream of the two *sns* copies of plasmid pEGFP-2x *sns* [24]. The 5′HS2-2x *sns* cassette was then excised with *Bgl*II–*Bam*HI double digestion, filled in with the Klenow DNA polymerase I, and ligated upstream of the ^{A}γNEO gene of construct F. All DNA constructs were sequenced to determine the orientation of the inserts.

Transfection and colony assay

Enhancer-blocking assays were performed as described by Chung et al. with minor modifications [26]. Briefly, 3×10^{6} mid-log phase human K562 and mouse MEL erythroleukemia cells were harvested and washed with phosphate-buffered saline (PBS) before resuspension in 0.5 ml of cold PBS. 3 μg of linearized DNA was added to the cells. After 10 min on ice, the cells were electroporated using a Bio-Rad gene pulser at 200 V, 960 μF and stored on ice for 15 min. The transfected cells were transferred to 35 ml of medium (RPMI for K562 and DMEM for MEL) containing 10% heat-inactivated fetal calf serum and aliquoted in four 96-well plates. *Neomycin*-resistant clones were selected in 800 μg/ml G418 (Gibco-BRL) and counted after 12–14 days.

Nuclear extracts and EMSA analysis

Total nuclear extracts were prepared from several cell lines as previously described [27]. Protein concentration was determined by Bradford's method. EMSA were performed by incubating 1 ng of ^{32}P-labeled Box A, Box B oligonucleotides, and Box C+T fragment obtained by PCR as described [22], with 5 to 10 μg of total nuclear extracts on ice for 20 min and then fractionated on 5% polyacrylamide gel in 0.5× TBE. For competition experiments, nuclear extracts were preincubated with 20 or 40 ng of unlabeled homologous or heterologous oligonucleotides. Competition-supershift experiments with antibody anti-GATA-1, anti-Sp1, anti-Oct-1, and anti-CTCF (Santa Cruz-Biotechnology) were performed by preincubating the nuclear extracts on ice for 1 h with 2 μg of each antibody.

List of oligonucleotides

Only the sense strand is reported.

- Box A: 5′ gatccaaacctcaacacctcaacggcccttatcagggccaccaa 3′
- Box B: 5′ gatcagtctctgtaattcataatagtctctgtaattcataat 3′
- GATA: 5′ atccacactatctcaatgct 3′
- OCT: 5′ aactcaatgcaaatatggct 3′
- Sp1: 5′ attcgatcggggcggggcgag 3′
- EKLF: 5′ gtggagcccacaccctagggtt 3′

Results

The sea urchin insulator sns attenuates the globin enhancer in erythroid cell lines in a directional manner

By colony assay [26], we tested whether the sea urchin *sns* insulator displays enhancer blocker activity in erythroid environment. The DNA constructs depicted in Fig. 1 were derived from the previously described pγNEO plasmid [25] which contains the *neomycin* (G418)-resistant gene transcribed by the 1.3 Kb human ^{A}γ-globin promoter. These constructs were transfected by electroporation in human (K562) and mouse (MEL) erythroleukemia cell lines, and G418-resistant clones were counted after 14 days of selection. Six independent experiments using three different plasmid preparations for each construct were carried out. The results from K562 are shown in Fig. 1; similar results were obtained from experiments performed in MEL cell line (data not shown). Twenty six individual resistant clones were analyzed by Southern blot to determine the integrated copy number. From one to three copies were detected in each clone (data not shown). The number of G418-resistant clones obtained from each construct was compared to that of the enhancer-containing construct (5′HS2-γNEO, construct A) arbitrarily set to 100. The average of the absolute numbers of G418-resistant clones for the construct A in the different experiments was 136. To address if *sns* element displays enhancer-blocking activity, we constructed plasmid B in which two copies of the *sns* insulator element were placed between the enhancer and the ^{A}γ-globin promoter. Two additional copies of *sns* were cloned downstream to the NEO coding region to block enhancer function of a tandem integrant. The results of the enhancer-blocking assay upon transfection of construct B indicated a 3.5-fold decrease in the G418-resistant clones ($P < 0.001$), suggesting that *sns* shielded the γNEO reporter gene from the enhancer activity of the human erythroid-specific LCR when interposed between the two elements.

The enhancer-less construct (construct C), in which γNEO gene was flanked by two copies of sns sequence element, generated a number of clones similar to that of construct B ($P < 0.001$), implying that the enhancer-blocking effect of *sns* observed for the construct B is almost total. A construct containing 400 bp plasmid fragment interposed between 5′HS2 enhancer and ^{A}γ-globin promoter was used as control. The number of G418-resistant clones obtained from this construct was comparable to that of the un-insulated 5′HS2-γNEO plasmid (data not shown). When the 5′HS2-γNEO transcription unit was flanked by two copies of the sea urchin insulator (construct D), a slight increase of the *neomycin*-resistant clones was observed ($P < 0.01$). To explore if *sns* has enhancer or silencer activity per se, we generated constructs E and F where two copies of insulator element were cloned upstream and downstream to the reporter gene respectively. The number of G418-resistant clones generated by these two constructs was similar to that obtained from γNEO plasmid construct G. From this result, it is evident that no enhancer or silencer activity can be assigned to *sns* element. Altogether, these results demonstrate that the sea urchin insulator *sns* blocks a tissue-specific enhancer from the interaction with the promoter in a directional manner.

The sea urchin sns insulator binds erythroid-specific and ubiquitous transcription factors

We have previously described three *cis*-acting sequence elements, Box A, Box B, and Box C+T, that are each required for the enhancer-blocking function of *sns* in sea urchin embryos and are binding sites for specific protein complexes [22,23]. Recent evidence indicates that all three sequence elements are needed for the enhancer-blocking function of *sns* in transfected human H1299 cell line (unpublished observation). Therefore, we addressed the question of whether the three boxes bind erythroid nuclear factors. Nuclear extracts were prepared from erythroid MEL and K562 cell lines and used in electrophoretic mobility shift assays (EMSA). The specificity of protein–DNA interaction was assessed by competition in EMSA by using excess of consensus binding sites for known transcription factors and preincubation of nuclear extracts with specific antibodies. Assisted computer analysis revealed that the three *sns* *cis*-acting sequence elements contain putative binding sites for both erythroid-specific and ubiquitous transcription factors, and we used the consensus sequence for these factors in competition experiments. The results of the EMSA experiments using ^{32}P-labeled Box A oligonucleotide are shown in Fig. 2. The Box A formed a major retarded DNA complex in both erythroid nuclear extracts that was competed out by 20- to 40-fold excess of unlabeled Box A (lanes 2 and 10 of Fig. 2). The heterologous DNA binding sites Box B did not affect nuclear proteins Box A interaction (lanes 3 and 11 of Fig. 2) and neither did the EKLF and Oct transcription factor binding sites (lanes 4, 6, 12, and 15 of Fig. 2). Specific competition was instead observed with the consensus binding site for the erythroid-specific GATA family transcription factors (lanes 5 and 13 of Fig. 2). The specificity of interaction of GATA-1 transcription factor was further demonstrated by the effect of the preincubation of the nuclear extracts with the antibody anti-GATA-1. We observed in fact supershift with the MEL extracts (lane 8 of Fig. 2) and competition with the human K562 proteins (lane 14 of Fig. 2).

As for the Box A, one major and specific retarded DNA–protein complex was observed when the Box B binding site was challenged with both the MEL and K562 nuclear extracts (Figs. 3A and B). The Box B is a direct repeat of AT-rich sequence [22] and contains a putative binding site for the Oct-1 transcription factor. As expected, full inhibition of the most retarded complex was observed when the nuclear extracts were preincubated with molar excess of Box B (lanes 2 and 8 in Fig. 3) and Oct-1 binding sites (lanes 5 and 10 in Fig. 3). A slight competition was also observed with GATA oligonucleotide in MEL extracts, but K562 (lanes 4 and 9 in Fig. 3).

The specificity of the complex and the nature of the bound proteins were also ascertained by monitoring the effect of the preincubation of the MEL and K562 nuclear extracts with Oct-1 and GATA-1 antisera. The antiserum against GATA-1 did not

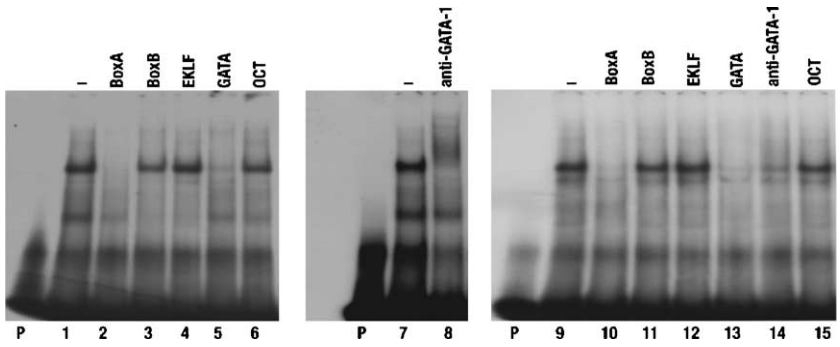

Fig. 2. EMSA analysis of the Box A sequence. Radiolabeled probe A was incubated with 10 μg of MEL (lanes 1–8) or 5 μg of K562 (lanes 9–15) nuclear extracts. The DNA–protein complexes in the absence of competitors as resolved by polyacrylamide gel electrophoresis are shown in lanes 1, 7, and 9. In competition experiments, nuclear extracts were preincubated with 40-fold excess (MEL extracts) or 20-fold excess (K562 extracts) unlabeled homologous or heterologous oligonucleotides. The oligonucleotides containing consensus motifs for the transcription factor recognition sites are indicated above the lanes. Only the GATA oligonucleotide (lanes 5 and 13) appeared to compete as efficiently as the homologous Box A sequence (lanes 2 and 10). Preincubation of the nuclear extracts with anti-GATA-1 antibody produced a supershifted complex with the MEL nuclear extracts (lane 8) and inhibition with the K562 nuclear extracts (lane 14). P denotes the free Box A probe.

affect the protein–DNA interaction (not shown). Thus, we believe that the slight competition observed with the GATA binding site (lane 4 of Fig. 3) might be due to other GATA family factors. By contrast, anti-Oct-1 antibody completely inhibited the formation of the most retarded complex in both cell lines (lanes 12 and 14 in Fig. 3). In conclusion, the results of the EMSA shown in Fig. 3 unequivocally identified the Oct-1 transcription factor as one of the Box B binding proteins.

The third *cis*-sequence analyzed was the pyrimidine-rich Box C+T. This sequence interacted with several proteins forming different complexes. The EMSA pattern of Fig. 4 refers to the MEL nuclear extracts. Identical pattern was obtained also with the K562 extracts (not shown). In sea urchin, specific binding occurred with the fourteen CT (GA in the other strand) repeats of the Box C+T [22]. These CT/GA repeats did not interact with the erythroid nuclear proteins as molar excess of the 7-mer GAGA oligonucleotide did not affect the DNA–protein complexes (lane 3 of Fig. 4). This result was confirmed by using a shorter C+T oligo, lacking the CT/GA repeat, which reproduced a specific pattern of retarded DNA–protein complexes identical to that of the full C+T DNA fragment (not shown). Of the several DNA binding sites used in the competition assays, only those for CTCF and Sp1 transcription factors affected the two slower migrating complexes (lanes 2 and 4 of Fig. 4). Because of this observation, we analyzed the specificity of competition by probing the nuclear extracts with CTCF and Sp1 antisera. As it is shown in Fig. 4 (lanes 10 and 12), only the antibody anti-Sp1 efficiently competed the retarded complexes.

Discussion

The results presented in this paper demonstrate that the sea urchin chromosomal insulator *sns* interferes with the interaction of the human β-globin 5′HS2 with the γ-globin promoter in stable transfected erythroid cells. Attenuation of enhancer function occurs only when *sns* is interposed between the two regulatory elements, indicating that the sea urchin sequence behaves as a positional enhancer blocker also with tissue-specific transcription sequence elements in an erythroid milieu.

It is of some interest that we reproducibly observe 1.2-fold increase of G418-resistant clones number upon transfection of a construct that contains the 5′HS2-γNEO reporter flanked by two copies of the sea urchin *sns* element (Fig. 1, construct D).

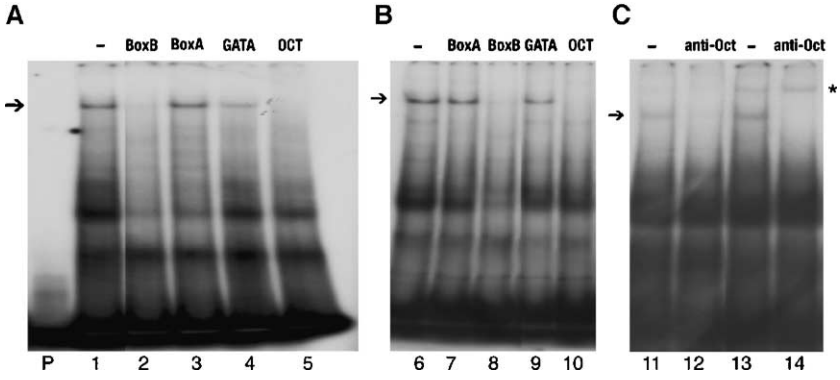

Fig. 3. EMSA analysis of the Box B sequence. Conditions are those described in Fig. 2. (A) 10 μg of MEL nuclear extracts. (B) 5 μg of K562 nuclear extracts. (C) Nuclear extracts from MEL (lanes 11–12) and K562 (lanes 13–14) preincubated with anti-Oct-1 antibody (lanes 12 and 14). The DNA–protein complexes in the absence of competitors are shown in lanes 1, 6, 11, and 13. Oligonucleotides competitors are reported above the lanes. The retarded band specifically competed by both Oct-1 binding site (lanes 5 and 10) and anti-Oct-1 antibody (lanes 12 and 14) is indicated by an arrow. Asterisk refers to a retarded band shown in panel C that appeared with this nuclear extracts preparation. P denotes the free Box B probe.

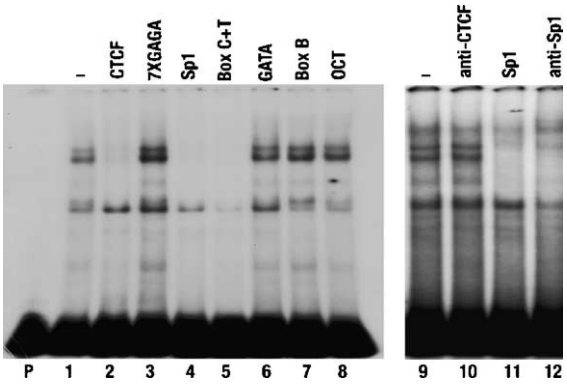

Fig. 4. EMSA analysis of the Box C+T sequence. 5 μg of MEL nuclear extracts was incubated with labeled Box C+T probe in the absence (lanes 1 and 9) or in the presence of 20-fold excess of oligonucleotides competitors. Oligonucleotides competitors for transcription factors anti-CTCF and anti-Sp1 antibodies are reported above the lanes. CTCF and Sp1 binding sites competed the retarded complexes (lanes 2, 4, and 11). The antibody against Sp1 inhibited the specific protein–DNA interaction (lane 12). P denotes the free Box C+T probe.

This observation may have two meanings; first, it may demonstrate that the sea urchin insulator has a neutral role on the transcriptional activation of the γNEO gene by the erythroid-specific enhancer. In addition, *sns* might protect the transgene from position effects of the surrounding chromatin at the integration site, suggesting a possible barrier function for the *sns* element in erythroid cells. These findings are in agreement with our recent observations that the sea urchin *sns* 5 fragment, which includes *sns*, involved in the silencing of the H2A histone gene in sea urchin [23], is capable of reducing the influence of the chromatin environment on an integrated retroviral transgene (unpublished results). The observation that only a slight rise in the number of *neomycin*-resistant clones is generated by construct D is in line with the evidence that the chicken β-globin 5′HS4 (cHS4) insulator also does not improve gene expression and position effect protection when it is in the presence of a strong enhancer [28,29]. This is also consistent with the demonstration that transcription factors may contribute in the establishment of an active domain that is independent of the influence of flanking chromatin [30].

The activity of chromosomal insulators depends on the binding of nuclear proteins. We have shown here that, in vitro, the *sns* insulator binds transcription factors whose binding sites are located in the previously identified sequence elements needed for the directional enhancer-blocking activity in sea urchin and human cells [22]. The use of antiserum against transcription factors whose binding sites inhibited the formation of specific retarded complexes indisputably allowed the identification of GATA-1, Oct-1, and Sp1 activators as the binding proteins, respectively, to the Box A, Box B, and Box C+T. Of the three transcription factors, GATA-1 is erythroid-specific. Chromatin immunoprecipitation analysis is in progress to confirm the binding of these transcription factors to *sns* sequences in vivo. The binding of transcription factors to a genomic insulator is not surprising. The prototypic vertebrate insulator c-HS4 binds two known transcription factors, CTCF, required for the directional enhancer-blocking activity [28], and USF, involved in the barrier function [18]. In addition, some reports have demonstrated that a variety of transcription activation domains tethered to tandem DNA sites can reconstitute bona fide insulator in yeast and mammals [30,31]. It remains to be established how transcription factors bound to the *sns* insulator can block the interaction between the β-globin LCR and γ-globin promoter and at the same time have a neutral role on the expression of the reporter transgene. In conclusion, our results have significant implications for the evolutionary conservation of insulator function from sea urchin to man.

Silencing and position effect are considered significant obstacles to obtain a consistent level of transgene expression in viral gene therapy [32]. Furthermore, recent studies have shown insertional activation of an adjacent oncogene upon integration in the genome of retroviral vectors in a gene therapy protocol against an immune disease [33]. The design and incorporation of effective chromatin insulator sequences in the next generation of gene therapy vectors should be helpful in reducing these unwanted side-effects. The relatively small size of the sea urchin insulator *sns*, the apparent absence of enhancer and cell specificity, as well as the capability to bind tissue and ubiquitous transcription factors may prove to be of practical benefit for the development of vectors in gene transfer application for erythroid disorders such as hemoglobinopathies and thalassemias.

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