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Nucleic Acids Research, 1997, Vol. 25, No. 14 **2917–2922**

Analysis of enhancer function of the HS-40 core sequence of the human α-globin cluster

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Received December 3, 1996; Revised and Accepted May 28, 1997

ABSTRACT

HS-40 is the major regulatory element of the human α-globin locus, located 40 kb upstream of the ζ-globin gene. To test for potential interactions between HS-40 and the β- or the γ-globin gene promoters in stable transfection assays, the HS-40 core sequence was cloned upstream of either the β promoter or the γ promoter driving the neomycin phosphotransferase gene and enhancer activity was measured using a colony assay. In K562 or in MEL cells, enhancer activity of HS-40 was higher than that of the individual core sequences of the DNase I hypersensitive sites (HS) of the β-globin locus control region (LCR), and ~60% of the enhancer activity of a 2.5 kb μLCR, which contains the core elements of DNase I hypersensitive sites 1–4. In contrast to the synergistic interaction between the DNase I hypersensitive sites of β locus LCR, combination of HS-40 with these DNase I hypersensitive sites failed to display cooperativity in K562 cells and inhibited enhancer function in MEL cells. Inhibition of enhancer function was also observed when two copies of the HS-40 were arranged tandemly. We conclude that the core element of HS-40 (i) is a powerful enhancer of γ- and β-globin gene expression, (ii) in contrast to other classical enhancers, acts best as a single copy, (iii) does not cooperate with the regulatory elements of the β-globin locus control region.

INTRODUCTION

Effective gene replacement therapy for the human hemoglobinopathies will require consistent high level expression of the transferred globin gene within a significant population of erythroid precursor cells. Initial expression studies utilizing native β-globin gene promoter and enhancer elements resulted in inconsistent and very low level expression (1,2). In 1987, Grosveld *et al.* (3) described the human β locus control region (LCR) as an element composed of five DNase I hypersensitive sites (HS) located 6–20 kb upstream of the ε-globin gene. These authors also showed that the LCR activates the β-globin locus domain, it is a powerful globin gene enhancer, it directs erythroid lineage specific expression of linked genes and it protects the globin genes from position effect of the surrounding chromatin (3,4). The discovery of the β-globin LCR raised hopes that this element could provide consistent, high level expression in virally-mediated gene transfer. Although combinations of LCR elements are able to confer such expression in transgenic mouse models (3,4), consistent, near-endogenous levels of expression have been much more difficult to achieve in retroviral or other viral gene transfer systems (5–7). Indeed, the inclusion of LCR elements within globin expressing retroviruses does not appear to result in position-independent expression (5). The development of retroviral vectors containing the active elements of the globin LCR has also been complicated by difficulties in obtaining high titer producer cell lines and by genetic rearrangement of the viruses (8,9). While recent reports of improved LCR-containing retroviral vectors are encouraging, these vectors have yet to achieve adequate expression in pre-clinical models to justify human therapeutic trials (10).

An alternative approach to achieving consistent high-level expression from a transferred human β-globin gene is to incorporate regulatory elements from genes other than those of the β-globin locus which are also expressed at high levels in erythroid cells. One example of such a set of genes is the human α-globin genes. While the α-globin genes are expressed at levels equivalent to the human β-globin gene, their expression appears to be regulated, at least in part, by a different mechanism. In contrast to the β-globin locus, the major distant regulator of the α-globin locus is marked by a major DNase I hypersensitve site located ~40 kb 5′ of the embryonic α-globin gene (the ζ-globin gene) and it is designated as the HS-40 element (11). Natural deletion mutations suggest that the HS-40 element is required for normal expression of the α-globin genes (12,13). In contrast to the β-globin LCR, the HS-40 element does not confer position-independent expression of linked genes in transgenic mice, nor does it appear to be required for the organization of the chromatin structure of the α-globin locus (14,15). HS-40 also differs from the LCR in that it is contained within a single element of ~350 bp (16); in contrast, the functional activity of the β-globin LCR is contained within four elements which are widely separated within the β-globin LCR (3,17). The ability of HS-40 to direct high level expression in transgenic mice, its small size, and a mechanism of action apparently distinct from that of the β locus LCR make it a prime candidate to be a transcriptional enhancer useful in the construction of therapeutic β-globin gene transfer vectors.

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To assess the ability of HS-40, alone or in combination with regulatory elements of the LCR, to function as a transcriptional enhancer of expression from globin promoters we have used the colony assay of Moon and Ley (18). This assay has the advantage of allowing rapid assessment of enhancer function within a chromatin context. We have applied this assay to study the enhancer activity of the HS-40 element in a variety of experimental situations. We found that a single HS-40 core element displays enhancer activity nearly equivalent to that of an LCR cassette (nanoLCR) containing the core elements of HS2, HS3 and HS4 of the β-globin LCR. We also found that, in contrast to individual DNase I hypersensitive sites of the β locus LCR which increase globin gene expression in a synergistic fashion, HS-40 fails to cooperate with either the nanoLCR or with the individual LCR elements; constructs containing combinations of HS-40 with β locus LCR elements actually exhibit decreased enhancer activity. Similarly, a tandem arrangement of HS-40 elements results in decreased enhancer activity. These results provide additional evidence that the α-globin locus HS-40 is functionally distinct from the β-globin locus LCR and that it may be useful in the development of therapeutic globin gene vectors.

MATERIALS AND METHODS

Constructs

The original γ-neo and β-neo vectors were obtained from Dr T.Ley (Washington University, St Louis, MO) (18,19). The γ promoter consists of a 335 bp *Alu*I fragment extending from –299 to +36 with respect to the transcription initiation site of the $^{A}\gamma$-globin gene. The β promoter consists of a 421 bp *Acc*I–*Nco*I fragment extending from –375 to +46 with respect to the transcription initiation site of the β-globin gene. A 2.34 kb *Hin*dIII neomycin resistance (neoR) fragment was cloned downstream of the globin promoter as a reporter gene. The transcription cassette was excised from the original vectors with *Bam*HI, and subcloned to *Not*I site of pBluescript vector. The original HS-40 containing plasmid (pBS-αLCR) used in this study was obtained from Dr G.Atweh (Mount Sinai School of Medicine, New York, NY) (20). 255 bp HS-40 core was excised from pBS-αLCR with *Eco*RV and *Bam*HI, and cloned upstream of γ-neo and β-neo at the *Eco*RV site (HS-40–γ-neo and HS-40–β-neo). 5′ sequence of 255 bp HS-40 core is 5′-TCTGGAACCTATCAGGGAC-3′, and the 3′ end sequence of HS-40 core is 5′-CTCTCAGATAAACAGGAGGGGG-3′ (20). 2× HS-40–γ-neo and 2× HS-40–β-neo recombinants were generated by inserting another copy of 255 bp HS-40 core at upstream *Cla*I site of HS-40–γ-neo and HS-40–β-neo. HS-40 + HS1–γ-neo was generated by inserting 255 bp HS-40 at *Cla*I site upstream of the HS1–γ-neo which contains 316 bp HS1 (13104–13419) of the β-LCR. HS-40 + HS1–β-neo was similarly constructed. HS-40 + HS2–γ-neo was constructed by inserting HS-40 at *Sal*I site upstream of the HS2–γ-neo which contains 371 bp HS2 (8485–8855) of the β-LCR. HS-40 + HS2–β-neo was similarly cloned. The 1.1 kb nLCR cassette is generated by combining 282 bp of HS4 (942–1223), 426 bp of HS3 (4348–4773) and 371 bp HS2 (8485–8855) of the β-LCR together (21). nLCR–γ-neo and nLCR–β-neo were made by inserting nLCR cassette at the *Kpn*I and *Eco*RV sites upstream of the γ-neo or β-neo. nLCR–HS-40–γ-neo was constructed by inserting nLCR cassette at the *Cla*I site upstream of the HS-40–γ-neo. nLCR–HS-40–β-neo was similarly constructed. For cloning HS-40–γ-neo–HS-40 and HS-40–β-neo–HS-40, the HS-40 fragment was inserted at the *Sac*II site 3′ of the neomycin phosphotransferase gene of the HS-40–γ-neo or HS-40–β-neo. All HS-40 core derivatives were sequenced by the dideoxynucleotide method (22) to verify that the constructs were correctly made. Control plasmids μLCR–β-neo and μLCR–γ-neo were provided by Dr T.Ley (Washington University, St Louis). Plasmid DNA was prepared with PEG precipitation method to large quantity. DNA was linearized with *Sca*I and further purified for transfection experiments.

Tissue culture

Murine Erythroleukemia (MEL) cells and human K562 cells were maintained in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS) (GIBCO), heat-inactivated at 56°C for 30 min. Cells were cultured at 37°C in 5% CO_2 with 100% humidity.

Lipofection

DNA transfection was done by performing lipofection by a modified procedure of Dorman and Yang (23,24). Cells were washed twice with serum-free medium-RPMI 1640 for erythroid cell lines. For each erythroid cell transfection, 7 μg of linearized plasmid DNA and 50 μg of Lipofectin reagent (GIBCO) were each added to 1.5 ml of serum-free medium. The DNA and lipofectin solutions were mixed, vortexed, and added to 10^7 cells. Cells were incubated for 6 h at 37°C, at which time 7 ml of medium containing 20% FBS was added. Cells were collected 24 h later by centrifugation and added to 20 ml of fresh medium containing 10% FBS. Cells were grown for another 24 h and G418 (GIBCO) at 0.7 mg/ml was added.

Colony assays

Cells were grown under G418 selection and plated in different conditions. For K562 cells, 2×10^5 cells were plated per 10 cm Petri dish in semi-soft agar. For MEL cells, 10^6 cells were seeded in 96-well flat bottom plate and grown in suspension. Ten to 14 days later, visible colonies were quantitated in triplicate plates.

Chromatin structure analysis

Pools of transfected clones were selected and expanded in G418. DNase I sensitivity assays were performed as previously described (25). For Southern blotting, constructs were excised from genomic DNA using restriction enzyme sites present within the pbluescript SK backbone. These included *Sma*I or *Kpn*I located immediately 5′ of the construct and *Sma*I or *Dra*I located 3′ of the construct. The probe used in these experiments was a 900 bp *Eco*RI/*Hin*dIII fragment from the 3′ end of the neoR gene (26). The *Eco*RI site is within the gene and the *Hin*dIII site from an adjacent polylinker.

RESULTS

To characterize the enhancer function of the HS-40, we examined the activity of the 255 bp long HS-40 core sequence shown in Figure 1A. Enhancer activity was assessed using the colony assay of Moon and Ley (18,19). This assay measures enhancer activity by quantitating the neomycin-resistant colonies produced by

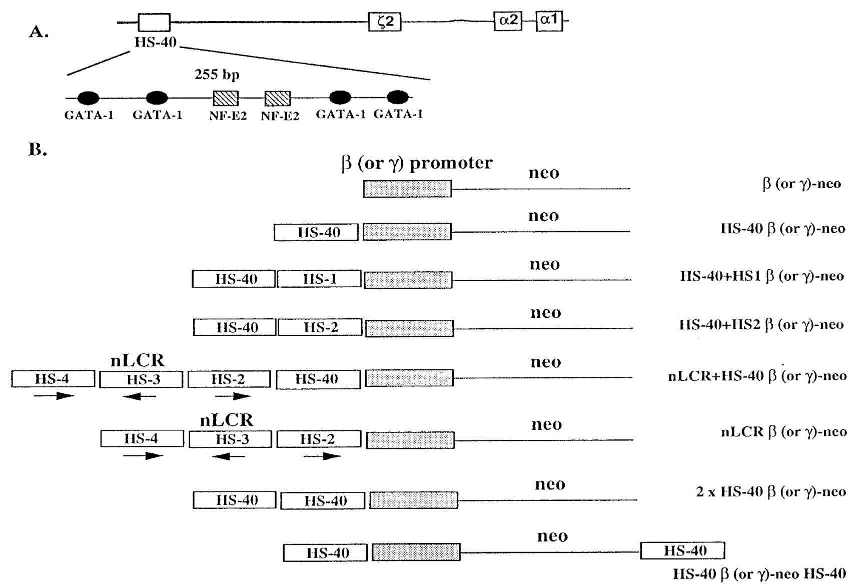

Figure 1. (**A**) Schematic representation of the human α-globin cluster and the transcriptional motifs present in the 255 bp HS-40 core element. (**B**) Diagrams of the HS-40–β-neo and HS-40–γ-neo constructs used for colony assays. Arrows indicate the genomic orientation (from 5′ to 3′) of the core sequences of DNase I the hypersensitive sites of the β locus LCR.

MEL or K562 cells transfected with HS-40 (or LCR) containing constructs. Since only a threshold level of neomycin phosphotransferase is required for colony survival, any interaction between enhancer and promoter resulting in production of the minimal amount of the enzyme required for cell survival, results in formation of a viable colony. The number of colonies so formed provides a measure of enhancer strength (18,19).

The γ-neo recombinant contains a neomycin phosphotransferase gene linked to a γ gene promoter extending 299 bp upstream of the transcription initiation site and containing +36 bp of the 5′ untranslated region (GeneBank Humhbb.gb_pr 39133–39431). The β gene promoter of the β-neo recombinant extends 375 bp upstream of the transcription initiation site and contains +46 bp of the untranslated region (61784–62154). The sequences of the γ and β gene promoters contain all the *cis* elements required for optimal γ or β gene transcription (27,28). We included two control plasmids in our assays, μLCR–β-neo and μLCR–γ-neo, both containing a 2.5 kb LCR cassette consisting of sequences of the DNase I hypersensitive sites 1, 2, 3 and 4. The enhancer activity of the 2.5 kb μLCR was given the arbitrary value of 100% and all of our data were expressed as a percentage of the number of colonies produced by the μLCR-containing constructs.

The interaction between HS-40 and the β-globin gene promoter was investigated by transfecting the recombinant genes into the mouse erythroleukemia cell line MEL585. These cells are committed to the adult stage of erythropoiesis and produce only adult murine globins. The interaction between HS-40 and the γ-globin promoter was examined in the human erythroleukemia cell line K562. These cells express high level of γ-globin, small amount of ε-globin and no adult (β)-globin.

Interactions of HS-40 with the β- and γ-globin promoters

To characterize the interaction of HS-40 with the β- or γ-globin promoter, we inserted the 255 bp HS-40 core sequence upstream of the β- or γ-globin promoter (Fig. 1B). In MEL 585 cells, the number of colonies produced by a control β-neo construct which did not contain an enhancer was 0.4% ± 0.3 of the number of colonies produced by the μLCR–β-neo construct (Fig. 2A). When the HS-40 core sequence was added, the number of colonies increased 130-fold to 52.5 ± 9.8% of μLCR–β-neo (Fig. 2B). In K562 cells the number of colonies produced by the $^{A}\gamma$-neo construct was 5.2 ± 2.8% of the colonies of the μLCR–$^{A}\gamma$-neo construct (Fig. 3A). Addition of the HS-40 core to the $^{A}\gamma$-neo construct increased the number of colonies 11-fold, to 57.2 ± 6.1% of the μLCR–$^{A}\gamma$-neo (Fig. 3B). These results showed that HS-40 is a strong enhancer of β- and γ-globin gene expression.

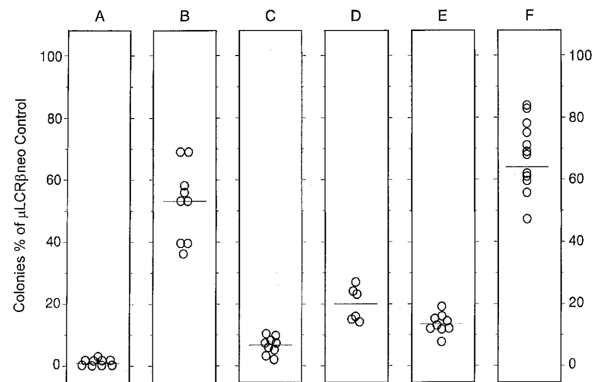

Figure 2. Interactions of HS-40 with the β gene promoter and regulatory elements of the β locus LCR. The β-neo-containing constructs were transfected into MEL cells. The number of colonies produced by the transfected MEL cells is expressed as percent of the number of colonies produced by the control μLCR–β-neo construct. (**A**) β-neo construct with no enhancer. (**B**) HS-40–β-neo. (**C**) HS-40–HS1–β-neo. (**D**) HS-40–HS2–β-neo. (**E**) HS-40–nLCR–β-neo. (**F**) nLCR–β-neo. The nanoLCR is composed of the core sequences of HS2, HS3 and HS4. Notice that HS-40 displays as high enhancer activity as the nLCR [compare (B) to (F)]. Also notice that the HS-40 fails to cooperate with HS1, HS2 or the nLCR.

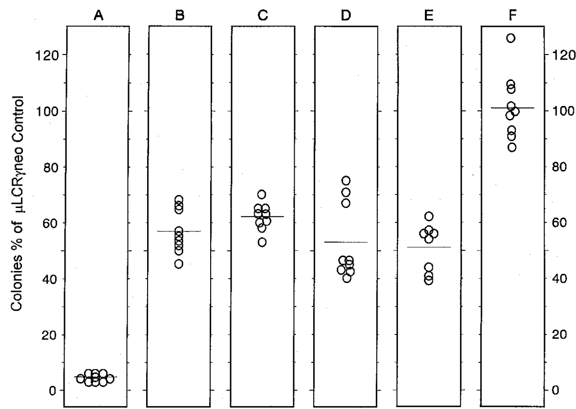

Figure 3. Interaction of HS-40 with the γ gene promoter and regulatory elements of the β locus LCR. The γ-neo containing constructs were transfected in K562 cells. The number of colonies produced are expressed as percent of the control µLCR γ-neo. (**A**) γ-neo construct without enhancer. (**B**) HS-40–γ-neo. (**C**) HS-40–HS1–γ-neo. (**D**) HS-40–HS2–γ-neo. (**E**) HS-40–nLCR–γ-neo. (**F**) nLCR–γ-neo. Notice that the number of colonies produced by HS-40–γ-neo is >50% of those produced by µLCR–γ-neo and that there is no cooperation between HS-40 and the DNase I hypersensitive sites of the β LCR [compare (B) with (C), (D) and (E)]. Also notice that in contrast to the results obtained with the β gene promoter-containing HS-40 constructs (Fig. 2), the LCR elements do not inhibit the interactions between HS-40 and the γ gene promoter.

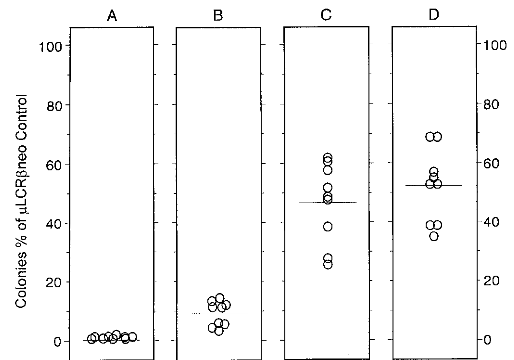

Figure 4. Tandem arrangements decrease enhancer activity of the HS-40 core in MEL cells. (**A**) β-neo construct without enhancer. (**B**) Tandemly arranged HS-40s in the HS-40–HS-40–β-neo construct. (**C**) Two HS-40 bracketing the β-neo gene in the HS-40–β-neo–HS-40 construct. (**D**) HS-40–β-neo control. Notice that the tandem arrangement of the HS-40 elements strikingly inhibits enhancer function while the placement of two HS-40 sequences, one 5′ and the other 3′ to the β-neo gene does not decrease enhancer activity.

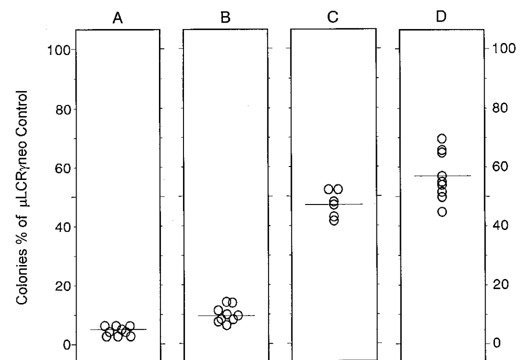

Figure 5. Tandem arrangements decrease the enhancer activity of HS-40 core in K562 cells. (**A**) γ-neo construct without enhancer. (**B**) HS-40–HS-40–γ-neo. (**C**) HS-40–γ-neo–HS-40. (**D**) HS-40–γ-neo control.

Interactions of the HS-40 with DNase I hypersensitive sites of the β locus LCR

Additive or synergistic effects between HS-40 and elements of the β-globin LCR would be useful in the design of globin gene therapeutic vectors. We therefore examined whether combinations of the HS-40 core with the core elements of the DNase I hypersensitive sites of the β locus LCR, or the addition of HS-40 to a 1.1 kb nanoLCR cassette, would further increase globin gene expression. The 1.1 kb nLCR cassette contains the core elements of the HS2, HS3, HS4 of the β locus LCR (21). The HS-40 core-containing recombinants HS-40 + HS1 β (or γ)-neo, HS-40 + HS2 β (or γ)-neo, and nLCR + HS-40–β- (or γ)-neo shown in Figure 1B were constructed.

Figure 2 shows the effects of combinations of these regulatory elements on the β gene promoter following transfection of MEL cells. While the number of colonies produced by the HS-40–β-neo construct was 52.5% of µLCR–β-neo, the combination of HS-40 with HS1 decreased the number of colonies to 6.3% ± 1.9 of µLCR–β neo (Fig. 2C). The combination of HS-40 with HS2 decreased the number of colonies to 20% ± 4.3 of the µLCR–β-neo (Fig. 2D), while the combination of HS-40 with nLCR decreased the number of colonies to 13.3% ± 2.9 of µLCR–β-neo (Fig. 2E). The control construct nLCR–β-neo alone produced 64% ± 8.9 of the number of colonies of the µLCR (Fig. 2F).

The effect of combinations of HS-40 with elements of the LCR on γ gene promoter was tested following transfection of K562 cells. The combination of HS-40 with HS-1 produced a number of colonies (62% ± 4.3 of µLCR–γ-neo, Fig. 3C) which was similar to those produced by HS-40–γ-neo (Fig. 2B). Similarly, there was no significant difference in the number of colonies produced by the HS-40–γ-neo construct and the combination of HS-40 with HS-2 (compare Fig. 3D and B) or the combination of HS-40 with the nLCR (Fig. 3E). Thus, HS-40 appears to interact differently with the β or the γ gene promoter in the presence of elements of the β locus LCR. The presence of LCR elements apparently inhibit the interaction between HS-40 and the β gene promoter in an adult erythroid transcriptional milieu while they do not affect the interaction between the HS-40 and the γ promoter in a fetal-like erythroid transcriptional environment.

Tandem arrangements of HS-40

To test whether multiple copies of the HS-40 increase enhancer activity, two copies of the HS-40 core were inserted upstream of the β or γ promoter (Fig. 1B), and the constructs were tested in MEL and K562 cells. The number of colonies produced by the construct HS-40 + HS-40–β-neo was only 10% ± 4 of the µLCR–β-neo (Fig. 4B) while the number produced by HS-40 + HS-40–γ-neo was 9.6% ± 2.4 of µLCR–γ-neo (Fig. 5B). These results suggest that tandem arrangements of the HS-40 core sequence may result in chromatin conformational changes which affect its function as an enhancer.

To investigate whether the decrease in HS-40 enhancer activity was due to the tandem arrangement of two HS-40 core sequences, the constructs HS-40–β-neo–HS-40 and HS-40–γ-neo–HS-40 (Fig. 1B) were used in colony assays. The number of colonies

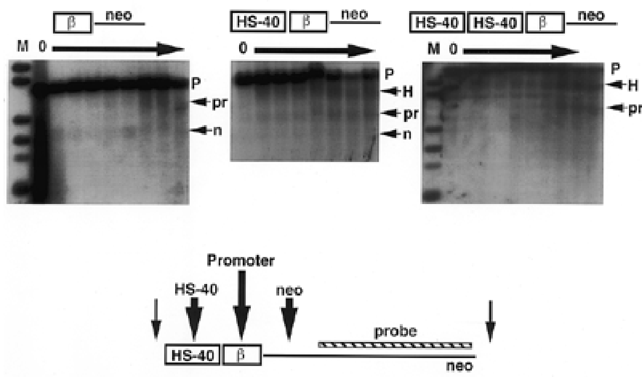

Figure 6. Chromatin structure of constructs transfected in MEL cells. DNase I hypersensitive site assays were performed on pools of G418 clones containing the indicated constructs. Parental band (P) and HSs associated with β-globin promoter (Pr), HS-40 (H) and the neoR (n) gene are indicated for each construct. The arrows above each blot indicate increasing DNase I concentration. The schematic diagram shows the location of HSs for the HS-40/β-globin promoter/neoR gene construct (large arrows) and the probe used in Southern blots. The small arrows indicate cut sites for restriction enzymes used to excise the construct from genomic DNA (see Materials and Methods). M, molecular weight markers (2.3, 2.0, 1.4, 1.1, 0.9 and 0.6 kb).

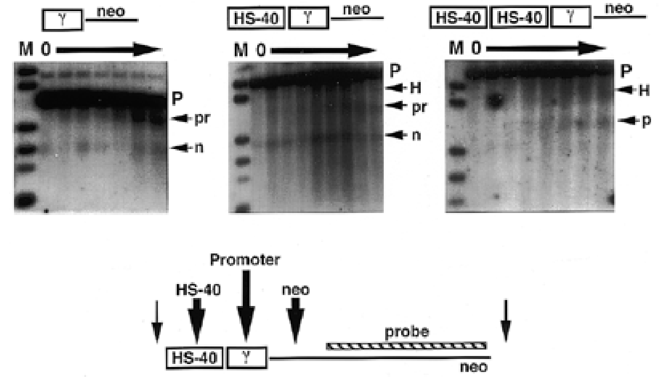

Figure 7. Chromatin structure of constructs in transfected K562 cells. DNase I hypersensitive site assays were performed on pools of G418 clones containing the indicated constructs. Parental band (P) and HSs associated with γ-globin promoter (Pr), HS-40 (H) and the neoR (n) gene are indicated for each construct. The arrows above each blot indicate increasing DNase I concentration. The schematic diagram shows the location of HSs for the HS-40/γ-globin promoter/neoR gene construct (large arrows) and the probe used in Southern blots. The small arrows indicate cut sites for restriction enzymes used to excise the construct from genomic DNA (see Materials and Methods). M, molecular weight markers (2.3, 2.0, 1.4, 1.1, 0.9 and 0.6 kb).

produced by HS-40–β-neo–HS-40 was 47% ± 12.0 of the μLCR–β-neo (Fig. 4C), i.e., similar to the number obtained with the HS-40–β-neo construct. The number of colonies produced by HS-40–γ-neo–HS-40 was 47.3% ± 3.6 (Fig. 5C), i.e., was close to the number produced by the HS-40–γ-neo.

Chromatin structure of integrated HS-40 constructs

To further characterize the function of HS-40 constructs we performed DNase I sensitivity assays to monitor the formation of DNase I hypersensitive sites within the stably transfected recombinants. We were particularly interested to see whether the double HS-40 constructs would exhibit an altered chromatin structure. Pools of G418-resistant clones for β- (or γ)-neo, HS-40–β- (or γ)-neo, and HS-40/HS-40–β- (or γ)-neo constructs were tested. The location of the observed DNase I hypersensitive sites and the Southern blot probes used are shown schematically in Figures 6 and 7. In all constructs containing HS-40, a DNase I hypersensitive site is created in the position of this element. Similarly, all constructs containing a β- or γ-globin promoter form DNase I hypersensitive sites over approximately –100 to –200 bp of the β or γ promoters. A third DNase I hypersensitive site located at approximately +350 bp of the neo^R coding sequence is also formed. Notice, in Figures 6 and 7, that that this DNase hypersensitive site is seen in all but the double HS-40 constructs. These results suggest that the tandem arrangement of two HS-40 elements disrupts the formation of the chromatin structure which is associated with efficient β-neo or γ-neo transcription.

DISCUSSION

Despite ongoing development, use of the elements of the β-globin LCR has yet to yield a therapeutically useful β-globin gene transfer vector. One approach to the further development of such vectors is the utilization of alternate regulatory elements which are able to drive high levels of expression of the genes of the erythroid lineage. The HS-40, the major regulatory element of the α-globin locus is one such candidate. Ren *et al.* (29) have used the HS-40 element in the production of a γ-globin expressing retroviral vector. Incorporation of HS-40 into the LTRs of this vector resulted in genetically stable, high-titre viruses which, in a limited number of MEL cell clones, produced γ-globin mRNA levels equivalent to the endogenous β-major globin gene (29). We have utilized the neomycin resistance colony forming assay of Moon and Ley to test a variety of β- and γ-globin promoter constructs containing HS-40 alone and in combination with other enhancers. This colony assay allows the rapid screening of constructs in a chromatin context and uses erythroleukemia cells which have not been induced by reagents promoting erythroid differentiation and therefore retain their proliferative potential. The use of uninduced cells represents an important difference from conventional assays measuring globin gene expression at the globin mRNA or the globin protein levels in transgenic mice or in induced erythroleukemia cells. The latter assays are done in terminally differentiated erythroid cells that have lost their proliferative capacity and most likely have different transcriptional environments compared to the uninduced erythroleukemia cells.

Our data support previous results (20,30) suggesting that HS-40 is a regulatory element able to interact with both homologous and heterologous promoters. In contrast to the core elements of the individual DNase I hypersensitive sites of the β locus LCR which show minimal enhancer activity in the colony assay of Moon and Ley (21), the HS-40 displays 50–60% of the activity of control constructs containing a 2.5 kb μLCR cassette. It is presently assumed that in addition to their other functions, DNase I hypersensitive sites 2, 3 and 4 of the β locus LCR also function as globin gene enhancers when they are stably integrated into the genomes of erythroleukemia lines or in transgenic mice. Our results suggest that as assessed by the colony assay, HS-40 displays higher enhancer activity compared to the individual core elements of the β locus LCR.

Previous studies in transgenic mice and in transfected MEL cells have shown that the enhancer activity of the β locus control region represents the cooperative interaction between DNase I hypersensitive sites 1–4 (3,31). We therefore examined whether combinations of HS-40 with DNase I hypersensitive sites of the

LCR further increase the enhancer activity of HS-40. We found that the HS-40 fails to cooperate with the core elements of the DNase I hypersensitive sites of the LCR. Instead of increasing enhancer activity, the presence of HS1, or of HS2, or of a 1.1 kb cassette containing the core sequences of HS2, HS3 and HS4, inhibited the enhancer activity of HS-40 in transfected MEL cells. There was no cooperation (but also no inhibition) of enhancer activity between HS-40 and the core element of the LCR in γ gene promoter constructs tested in K562 cells. The lack of cooperation between HS-40 and the elements of the LCR adds to the growing evidence that HS-40 is a regulatory element distinctively different from the β locus LCR. Thus, although HS-40 is responsible for the erythroid lineage-specific expression of α-globin genes, it does not protect the α-globin transgenes from the effects of the position of integration. Furthermore, HS-40 driven α-globin gene expression is not developmentally stable in transgenic mice, being 2–4-fold lower in adult transgenic animals compared to transgenic embryos (14,32,33). Craddock *et al.* (34) have further observed that, in contrast to the β-globin locus LCR, HS-40 exerts no discernible effect on long-range chromatin structure, and in addition, does not influence the formation of DNase I hypersensitive sites at the α-globin gene promoter.

It is established that tandem arrangements of enhancer sequences increase the activity of classical enhancers (35–38). To test whether HS-40 behaves similarly to classical enhancers, constructs containing tandem arrangements of the 255 bp HS-40 core sequence were produced and tested in the colony assay of Moon and Ley. Surprisingly, we found that in both K562 and MEL cells, tandem arrangements of the HS-40 core decreased the HS-40 enhancer activity significantly. The apparent interpretation of the results is that the tandem arrangement of the HS-40 core sequence result in chromatin conformational changes which affect its interaction with the globin gene promoter. The reason for this negative effect remains obscure. Inhibition of enhancer function requires a tandem arrangement of the HS-40 core sequences because placement of two HS-40 cores, one at the 5′ and the other of the 3′ end of the γ-neo or β-neo constructs does not result in inhibition of the HS-40 enhancer function. These results suggest that the presence of two HS-40 elements disrupts the formation of a chromatin structure associated with efficient transcription of the γ-neo or β-neo genes resulting in the decreased colony formation. Our studies of the chromatin structure of the HS-40 constructs support this conclusion as a specific DNase I hypersenstive site, located within the proximal neoR gene, is lost in cells containing the tandem HS-40 element.

ACKNOWLEDGEMENTS

This work was supported by NIH grants HL53750 and DK45365 to G.S.; and R01 HL52243 to C.H.L. We thank Dr Q.Li for his advice in the production of the recombinants used in this study, Ms Betty Nakamoto for her help with the illustrations and Ms S.Brenner for assistance in the preparation of the manuscript.

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