Disruption of the Mechanism of Long Range Activation within the Human α-Globin Complex*

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The human α-globin complex lies at the tip of the short arm of chromosome 16. It comprises three functional globin genes (5′-2α-a1-3′), the expression of which is strictly dependent on a positive regulatory element located 40-kb upstream, HS-40. This DNase I-hypersensitive site is the only known regulatory element displaying strong erythroid-specific enhancer activity within the human α-globin complex. How this enhancer activity is shared among different erythroid genes present in the same cluster without affecting the ubiquitous genes present within and around the complex is poorly understood. To address this issue, we used hybrid murine erythroleukemia cells containing a single copy of human chromosome 16 and targeted the insertion of different sequences downstream of HS-40 by recombinase-mediated cassette exchange. We thus demonstrate that (i) HS-40-mediated erythroid-specific activation of the α-globin genes is impaired solely by the insertion of a promoter sequence and not a coding sequence, unless it is methylated, and that (ii) the degree of transcriptional repression observed seems to be related directly to the transcriptional rate of the inserted promoter. Taken together, these results emphasize the importance of promoter sequences as the main targets for the activation mechanism of the human α-globin genes by HS-40.

The human α-globin complex is located in the telomeric region of the short arm of chromosome 16. It comprises three active genes: the embryonic 2 gene and the two fetal-adult a2 and a1 genes, which are arranged from 5′ to 3′ in the order of their activation during development (5′-2α-a1-3′) (1). The complex is located in a GC-rich isochore within an early replicating and constitutively DNase I-sensitive chromatin domain in both erythroid and non-erythroid cells, although the α-globin genes are transcribed exclusively in erythroid cells (2–4). This transcriptional erythroid specificity is controlled by a single positive regulatory element, which corresponds to a DNase I-hypersensitive site located 40 kb upstream of the 2 gene and thus named HS-40 (5, 6). HS-40 is characterized by a high density of DNA binding sites for ubiquitous and erythroid-specific transcription factors (6–8) and confers a strong, erythroid-specific expression to the 2 and α-globin promoters in cell lines and transgenic mice (9–14). The human α-globin complex is also surrounded by several widely expressed genes, including the −14 gene, which is located 14-kb upstream of the 2 gene and is transcribed in the opposite orientation compared with the α-globin genes (15). Furthermore, the transcription of the −14 gene is independent of HS-40 despite the location of HS-40 in the fifth intron of the ubiquitous gene (5). HS-40 therefore appears to be involved in the highly selective and erythroid-specific transcriptional activation of the human α-globin genes. Nonetheless, the mechanism responsible for this selective activation remains unknown.

To characterize this mechanism, we investigated how the HS-40-mediated transcriptional activation of the human α-globin genes could be affected by the insertion of new sequences between HS-40 and the globin genes. In a previous study, we observed that the insertion of either an extra α-globin gene or a marker gene driven by a non-erythroid promoter in the vicinity of HS-40 led to a drastic reduction of HS-40-mediated activation of the resident α-globin genes, without necessarily leading to the activation of the newly inserted gene by HS-40. The results also demonstrated that the observed impairment of HS-40 activity was independent of the position, the orientation, and the erythroid or non-erythroid nature of the newly inserted gene (16).

To further investigate the mechanism underlying this phenomenon, we used the recombinase-mediated cassette exchange (RMCE)1 technique and a hybrid mouse erythroleukemia cell line (MEL) carrying a single copy of human chromosome 16. Through this study, we targeted the insertion of (i) the α-globin coding sequence alone, either methylated or unmethylated, (ii) the α-globin promoter alone, and (iii) the α-globin coding sequence driven by either the −14 gene promoter or an α-globin promoter devoid of its TATA-box, immediately downstream of HS-40. The results demonstrate that HS-40 activity is not impaired by the insertion of a coding sequence alone except when it is methylated but is impaired by the insertion of a sole promoter sequence. Moreover, our results indicate that this negative effect increases along with the transcriptional rate of the inserted promoter.

These results are discussed in term of the mechanism by which HS-40 activity can be captured by the different promoters present within the same human α-globin complex.

EXPERIMENTAL PROCEDURES

Cell Culture—All experiments were performed in the mouse erythroleukemia hybrid cell line L7585P3, which contains a single copy of

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¶ The abbreviations used are: RMCE, recombinase-mediated cassette exchange; HMBA, N,N′-hexamethylene bisacetamide; CMV, cytomegalovirus; nt, nucleotide(s); WT, wild type; LTR, long terminal repeat.
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FIG. 1. Characterization of hybrid MEL clones bearing the targeted insertion of the human α-globin coding sequence. A, schematic map of the human α-globin locus in parental cells (WT); cells harboring the HygroTK gene (HYTK →), and cells harboring the replacement of the HygroTK gene by the Dpa/α-globin gene either in the same orientation as the endogenous human α-globin genes (Dpa/α-globin →) or in the opposite orientation (Dpa/α-globin ←). The expected lengths of the SacI/BglII fragments revealed by Southern blot using the HS-40 probe are indicated under the map corresponding to each type of clone. B, Southern blot analyses of SacI/BglII-digested genomic DNA revealed by the HS-40 probe. Lane 1, WT parental hybrid MEL cells; lane 2, HYTK → clone; lanes 3–7, ganciclovir-resistant and hygromycin-B-resistant clones bearing the insertion of the α-globin coding sequence in either orientation. C, analysis of endogenous human and mouse α-globin gene expression in differentiated WT cells and targeted clones by RNase protection assay. The position and length of the human α-globin (αH) and mouse α-globin (αM) protected fragments are indicated on the right. D, quantitative analysis of the data presented in C. Results are expressed as ratios of human to mouse α-globin signals. The white portion of the bar represents the average deviation, and the n-value is shown on the figure.

normal human chromosome 16 (6, 17). Cells were cultured as described previously (6), and erythroid terminal differentiation was induced by growing the cells for 3 days in the presence of 5 mM N,N′-hexamethylene bisacetamide (HMBA; Sigma).

Exchange Plasmids—The following four exchange plasmids were derived from the previously described paα-globin gene plasmid (16).

To construct the Dpa/α-globin plasmid, the human α-globin promoter was deleted from the paα-globin gene plasmid as an XbaI/NcoI fragment, thus generating protruding 5′ and 3′ ends that were filled in by Klenow enzyme.

The paα/α-globin plasmid was obtained by deleting the human α-globin coding sequence from the paα-globin gene plasmid as an NcoI/BglII fragment and filling the protruding ends in by Klenow enzyme.

For the p-14α-globin plasmid, the 5′-14 gene minimal promoter for complete transcriptional activity was obtained by PCR reaction on LT55SF3 genomic DNA using the 5′ primer, GCCCTAGACGCGGCGGGAACCTGGTGCCCTC, and the 3′ primer, ACCTGAAACGACGCTATTGCT. The underlined sequences indicate a XbaI site that was added in order that the 611-bp long PCR product could be introduced into the paα-globin gene plasmid as an XbaI/NcoI fragment in place of the α-globin promoter (The NcoI site came from the pGEM-T PCR cloning vector; Promega).

The DTA/α-globin plasmid was obtained by mutating the TATA-box of the human α-globin promoter on the paα-globin gene plasmid in vitro using QuickChange® site-directed mutagenesis (Stratagene). For the purpose of this mutation, we used the 5′ primer, CGGCGGTCCCCCGCTCCACAAAGCAAGCTTCCCTGGCGCGCTCGCG, and the 3′ primer, CGCGAGCGGCCGGAACCTTGGCCTGGCGCGCCTCGCC, and the 3′ primer, CCACAAAGCAAGCTTCCCTGGCGCGCTCGCG, and the 3′ primer, CGCGAGCGGCCGGAACCTTGGCCTGGCGCGCCTCGCC. The underlined sequences indicate the insertion of a new HindIII restriction site, which was used to verify the mutation on the plasmid by enzymatic digestion prior to transfection.

For the Dpa/α-globin plasmid, the Dpa/α-globin plasmid was methylated in vitro using the SsI methylase (Roche Applied Science) according to the protocol supplied by the manufacturer. The Dpa/α-globin plasmid was then subjected to digestion by the methylation-sensitive restriction enzyme HpaII to clear the preparation of any incompletely methylated copies of the plasmid.

Isolation of Clones Targeted by RMCE—106 hybrid MEL cells of a HYTK clone harboring targeted insertion of the previously described CMV-HygroTK cassette (16) were co-transfected using 20 μg of DAC-30 (Eurogentec), 1 μg of expression plasmid encoding the CRE recombinase (pCMV-CRE) (16), 1 μg of expression plasmid encoding the green fluorescent protein (pSV40-GFP) (16), and 2 μg of one of the exchange plasmids described above. 48 h after transfection, cells expressing the green fluorescent protein were sorted out by fluorescence-activated cell sorting and cloned in the presence of ganciclovir (10 μM). Isolated ganciclovir-resistant clones were then amplified and analyzed by Southern blot to verify the exchange of the CMV-HygroTK cassette for the newly inserted sequence.

RNase Protection Assay—Total cellular RNA was prepared using RNAplus® (Quantum Biotechnologies) according to the manufacturer’s instructions. RNase protection assays were performed as described previously (5) using 10 μg of total RNA and the following two labeled antisense RNA probes.

The human riboprobe (5) includes 231-nt complementary to the first intron and exon of both adult human α-globin genes and is expected to produce (i) a single protected fragment of 133 nt with normal α-globin mRNAs (αH), (ii) a single protected fragment of 75 nt with p-14α-globin...
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We have previously demonstrated that the insertion of a new human α-globin gene immediately downstream of HS-40 leads to a drastic decrease in HS-40-mediated transcriptional activation of the resident human α-globin genes, concomitant with an increase in activation of the inserted erythroid gene (16). To determine which part of the inserted gene is responsible for this competition over HS-40 activity, we decided to use the RMCE strategy (16) to target the insertion of different normal and mutated parts of an α-globin gene at the same unique site directly downstream of HS-40.

Endogenous Human α-Globin Expression Is Inhibited Only upon Insertion of a New Promoter Near HS-40—We inserted either the α-globin coding sequence alone (from the Δpα/α-globin plasmid) or only the promoter of the α-globin gene (from the pα/Δα-globin plasmid) by RMCE. The isolated clones were analyzed by Southern blot using an HS-40 probe for legitimate exchange of the CMV-HygroTK gene of the parental clone (HYTK clone) (16) for the cassettes of interest, as shown in Figs. 1, A and B, and 2, A and B. Five clones showed either the 2.2- or 1.3-kb fragment expected for the insertion of the Δpα/α-globin cassette in either transcriptional orientation, and six clones showed either the 1.5- or 2.1-kb fragment expected for the insertion of the pα/Δα-globin cassette in either orientation. The targeted clones and parental cells harboring no insertion (WT) were then grown for 4 days in the presence or absence of HMBA, a chemical inducer of erythroid differentiation. Equal amounts of total cellular RNA prepared from these cells were analyzed by RNase protection assay, using a mixture of probes allowing for the specific detection of human and mouse α-globin transcripts. We were unable to analyze the rate of transcription driven by the newly inserted α-globin promoter, as no additional sequence was added 3’ of the promoter in order to avoid any interference. Typical results are shown in Figs. 1C and 2C, and quantitative analyses obtained from three different experiments are presented in Figs. 1D and 2D.

The data indicate that the level of expression of the human endogenous α-globin genes does not significantly differ between the clones bearing the α-globin coding sequence and the parental cells harboring no insertion (Fig. 1, C and D, compare lanes 2–6 with lane 1). In contrast, the level of human α-globin mRNAs is markedly reduced in all of the clones bearing the α-globin promoter devoid of any coding sequence (Fig. 2, C and D, compare lanes 2–7 with lane 1). Indeed, the results demonstrate that the human endogenous α-globin expression in the presence of the α-globin promoter is only 45% of its level in WT cells. These data indicate that the inhibition of the human expected lengths of the EcoRI/XbaI fragments revealed by Southern blot using the HS-40 probe are indicated under the map corresponding to each type of clone. B, Southern blot analyses of EcoRI/XbaI-digested genomic DNA revealed by the HS-40 probe. Lane 1, WT cells; lane 2, HYTK clone; lanes 3–8, ganciclovir-resistant and hygromycin-sensitive clones bearing the insertion of the α-globin promoter in either orientation. C, analysis of endogenous human and mouse α-globin gene expression in differentiated WT cells and targeted clones by RNase protection assay. The position and length of each protected fragment are indicated on the right. D, quantitative analysis of the data presented in C. Results are expressed as the ratio of human to mouse α-globin signals. The white portion of the bar represents the average deviation, and the n-value is shown on the figure.

Fig. 2. Characterization of clones bearing the targeted insertion of the human α-globin promoter alone. A, a schematic map of the human α-globin locus in WT cells, HYTK clone (HYTK →), and cells harboring the replacement of the HygroTK gene by the pα/Δα-globin construct in the same orientation as the endogenous human α genes (pα/Δα-globin ←) or in the opposite orientation (pα/Δα-globin →). The hybrid mRNAs (p-14/α), and (iii) two protected fragments of 97 and 34 nt with TATA-box-mutated α-globin mRNAs (ΔTATA/α).

The mouse α-globin riboprobe includes 180 nt complementary to the 3’ end of the first exon of the mouse α-globin genes and is expected to give a protected fragment of 75 nt (αM) with mouse α-globin mRNAs. Radioactive signals corresponding to each specific protected fragment were quantified using a GS-525 Molecular Imager (Bio-Rad) and the Molecular Analyst software (Bio-Rad).
Fig. 3. Characterization of clones bearing the targeted insertion of the methylated human α-globin coding sequence. A, schematic map of the human α-globin locus in WT cells, HYTK clone, and cells harboring the replacement of the HygroTK gene by the MΔpaα-globin construct in either the same orientation as the endogenous human α-globin genes (MΔpaα-globin →) or in the opposite orientation (MΔpaα-globin ←). The expected lengths of the PstI fragments revealed by Southern blot, using the HS-40 and human H9251-globin probes, are indicated under the map corresponding to each type of clone. B, Southern blot analyses of PstI/H11006 HpaII-digested genomic DNA revealed by the human H9251-globin probe in order to check for the insertion of the methylated human α-globin coding sequence. Lanes 1 and 2, WT cells; lanes 3 and 4, HYTK → clone; lane 5-10, ganciclovir-resistant and hygromycin-sensitive clones bearing the insertion of the methylated human α-globin coding sequence. C, Southern blot analyses of PstI/MaeII-digested genomic DNA revealed by the HS-40 probe in order to check for the propagation of methylation toward HS-40. Lanes 1 and 2, HYTK → clone; lanes 3-8, ganciclovir-resistant and hygromycin-sensitive clones bearing the insertion of the methylated α-globin coding sequence. D, analysis of endogenous human and mouse α-globin gene expression in differentiated and undifferentiated WT cells and targeted clones by RNase protection assay. The position and length of each protected fragment are indicated on the right. E, quantitative analysis of the data presented in D. Results are expressed as the ratio of human to mouse α-globin signals with the value of two different experiments shown for each clone.
endogenous α-globin gene expression observed when a new gene is inserted near HS-40 is due to the specific insertion of a promoter sequence.

**Insertion of a Methylated Coding Sequence Impairs the Human α-Globin Expression**—As we had found that the α-globin coding sequence inserted near HS-40 has no effect on the activation of the α-globin genes during erythroid differentiation, we wondered whether methylation could turn this sequence into an interfering one. We therefore targeted the insertion of an in vitro methylated α-globin coding sequence. Southern blot analyses were performed to identify correctly targeted Δpαα-globinMeth clones using the PstI restriction enzyme and an α-globin probe (Fig. 3, A and B). All of the Δpαα-globinMeth clones, as well as the WT and HYTK controls, showed the 1.5-kb fragment corresponding to the human resident α-globin genes. The four Δpαα-globinMeth clones showed an extra 0.9-kb signal as expected for the insertion of the Δpαα-globinMeth cassette in either orientation (Fig. 3B and data not shown). In parallel, the methylation state of the inserted sequence was analyzed by using the methylation-sensitive restriction enzymes HpaII, MaelII, and Hhal (Fig. 3B and data not shown). The results clearly indicate that following treatment with an enzyme such as HpaII, the 1.5-kb signal corresponding to the nonmethylated human resident α-globin genes disappears in all Δpαα-globinMeth clones and controls (Fig. 3B, compare each odd lane with its following even lane), whereas each of the Δpαα-globinMeth clones conserves the 0.9-kb fragment, proving that the HpaII enzyme is unable to digest the Δpαα-globinMeth cassette as was expected for a methylated sequence (Fig. 3B, lanes 6, 8, and 10). We then digested the Δpαα-globinMeth clone genomic DNA using the methylation-insensitive isoschizomer of HpaII, MspI (data not shown). In that case, the 0.9-kb fragment disappeared in each of the Δpαα-globinMeth clones, confirming that the HpaII/MspI sites (i) are present in the Δpαα-globinMeth cassette and (ii) are methylated. To test the spreading of methylation from the methylated cassette to adjacent genomic DNA, we analyzed the DNA methylation status from the inserted cassette up to the core region of HS-40 by Southern blot, using the HS-40 probe and the methylation-sensitive restriction enzymes HpaII, MaelII, and Hhal (Fig. 3C and data not shown). Fig. 3C presents typical results obtained following digestion by PstI either alone (Fig. 3C, compare lane 1 with each odd lane) or in combination with MaelII, which recognizes a site within HS-40 core (compare lane 2 with each even lane). All of the results showed an identical pattern for the control and Δpαα-globinMeth clones, indicating that there is no spreading of methylation from the newly inserted sequence and that neither HS-40 nor the endogenous α-globin genes located downstream are damaged by DNA methylation. To determine the effect of insertion of the Δpαα-globinMeth cassette on the endogenous human α-globin gene expression, we then analyzed the level of transcription of the human α-globin genes in the Δpαα-globinMeth clones. Equal amounts of total cellular RNA from the methylated clones and parental cells harboring no insertion were analyzed by RNase protection assay before and after induction with HMBA (Fig. 3D). Quantitative results obtained from two different experiments are shown in Fig. 3E.

Surprisingly, the data indicate that the human endogenous α gene expression in clones bearing the methylated insertion corresponds to only 60% of the normal human α-globin gene expression in WT cells (Fig. 3D, compare lane 1 with lanes 3, 5, 7, and 9, and Fig. 3E, compare lanes 2–5 with lane 1), although, as previously described, the insertion of the same unmethylated sequence does not lead to any change in the globin gene expression.

These results demonstrate that the decrease in activation of the human α-globin genes in the Δpαα-globinMeth clones is not due to a spreading of methylation, which would inhibit HS-40, but to a modification of the chromatin status resulting from the insertion of the methylated sequence between HS-40 and the α-globin genes.

**Insertion of an Ubiquitous Promoter Leads to Partial Inhibition of the Endogenous α-Globin Expression**—The human α-globin complex is surrounded by several widely expressed genes, including the −14 gene, the promoter of which is naturally located between HS-40 and the human α-globin genes. As we have shown that promoters are able to trap HS-40 activity, we wondered whether the resident −14 gene competes with the downstream α-globin genes. Therefore, we tested the effect of the insertion of an additional −14 promoter on the human α-globin gene activation. For this experiment, we inserted the −14 promoter driving the α-globin coding sequence so that we could accurately quantify the effect of the −14 promoter itself because (i) we have shown that the α-globin coding sequence alone has no influence on the expression of the human endogenous α-globin genes, and (ii) it enables us to make a comparison with the previous insertions by RNase protection assay using the same human α-globin probe. Clones were analyzed by Southern blot using the HS-40 probe, and five clones showed either the 1.5- or 2.9-kb fragment expected for correct insertions in either orientation (Fig. 4, A and B). Equal amounts of total cellular RNA, prepared from the characterized clones and WT cells before or after induction with HMBA, were analyzed by RNase protection assay. Only the results following HMBA treatment are presented, as the expression of the p14/α-globin construct is identical before and after induction (data not shown). The transcription of the inserted gene is observed as a protected signal of 97 nt (Fig. 4C); quantitative results obtained from three different experiments are shown in Fig. 4D.

The data indicate that the p14/α-globin gene expression is very weak (Fig. 4D). Indeed, it represents only 10% of the human resident α-globin gene expression in WT cells (Fig. 4C, compare lanes 2–6 with lane 1, and Fig. 4D, compare lane 1 with each odd lane), therefore corresponding to a level of expression six times less than those of the entire α gene inserted in the same place (16). The data also show that following induction of the cells in differentiation, the mean expression of the human endogenous α genes in p14/α-globin clones represents only 56% of their expression in WT cells (Fig. 4C, compare lanes 2–6 with lane 1, and Fig. 4D, compare lane 1 with each even lane). These results demonstrate that the ubiquitous −14 promoter can interfere with HS-40-mediated erythroid activation of the human α-globin genes.

**Insertion of a Human α-Globin Gene Devoid of Its TATA-box Leads to Partial Inhibition of Endogenous α-Globin Gene Activation**—We have shown that the insertion of the α-globin (16), LTR (16), or CMV (16) promoter has a much more drastic effect on HS-40-mediated activation of the human α-globin genes than the insertion of the −14 promoter. One of the major differences existing between the latter and the other promoters is the absence of a TATA-box in the −14 promoter. We have thus attempted to examine the putative role of the TATA-box in the mechanism of HS-40-mediated transcriptional activation of the human α-globin genes by inserting an α-globin gene with a mutated TATA-box. The ΔTATA/α-globin cassette was constructed by replacing the six bases, ATAAAC, forming the α-globin TATA-box by AAGCTT so that we would not modify the spacing of the promoter features (see “Experimental Procedures”). ΔTATA/α-globin clones were analyzed by Southern blot using the HS-40 probe, and six clones showed either the 2.8- or 1.3-kb fragment expected for the insertion of the cassette in either orientation (Fig. 5, A and B). Equal amounts of
FIG. 4. Characterization of clones bearing the targeted insertion of the ubiquitous −14 promoter. A, schematic map of the human α-globin locus in WT cells, HYTK clone, and cells harboring the p-14/α-globin construct either in the same orientation as the endogenous human α-globin genes (p-14/α-globin −→) or in the opposite orientation (p-14/α-globin ←→). The expected length of the EcoRI/XbaI fragments revealed by Southern blot using the HS-40 probe, are indicated under the map corresponding to each type of clone. B, Southern blot analyses of EcoRI/XbaI-digested genomic DNA revealed by the HS-40 probe. Lane 1, WT cells; lane 2, HYTK → clone; lanes 3–7, ganciclovir-resistant and hygromycin-sensitive clones harboring the insertion of the −14 promoter in either orientation. C, analysis of p-14/α-globin and endogenous human and mouse α-globin gene expression in differentiated WT cells and targeted clones by RNase protection assay. The position and length of each protected fragment are indicated on the figure. D, quantitative analysis of the data presented in C. Results are expressed as ratios of human to mouse α-globin signals (striped bars) or p-14/α-globin to mouse α-globin signals (stippled bars). The white portion of the bar represents the average deviation, and the n-value is shown on the figure.

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contrary to what we were expecting according to the literature (18, 19), we observed that the α-globin gene devoid of its TATA-box is still expressed and fairly up-regulated following addition of HMBA. Indeed, before induction with HMBA, the ΔTATA/α-globin gene is expressed as efficiently as the α-globin gene driven by its normal promoter when inserted at the same place (Ref. 16 and data not shown), and following induction of the cells in differentiation, the mutated gene expression increases strongly to reach a mean value of 24% of the expression of both human endogenous α-globin genes, which corresponds to 41% of the normal α-globin gene expression when inserted in the same place (16). These results emphasize the fact that the ΔTATA/α-globin gene still responds to HS-40-mediated activation (Fig. 5, C and D). Moreover, the results indicate that, following induction of the ΔTATA/α-globin clones with HMBA, the human resident α genes are expressed to a mean rate of 50% of their normal expression in WT cells, which is very similar to the data obtained with the p-14/α-globin clones.

Unfortunately, we were unable to compare the level of human endogenous α-globin expression between the ΔTATA/α-globin clones and WT cells before induction in differentiation, because this level is not significantly above the background level in either type of cells (data not shown). These results overall demonstrate that the TATA-box plays a partial role in HS-40-mediated activation of the human α-globin genes.

DISCUSSION

In a previous study using hybrid MEL cells, which contain a single copy of human chromosome 16, we demonstrated that insertion of a new gene immediately downstream of HS-40 leads to a drastic decrease in HS-40-mediated activation of the endogenous human α-globin genes (16). The observation that the inserted α-globin gene is activated at the expense of the human endogenous α genes suggests a phenomenon of competition as part of the mechanism of long range activation of the α-globin genes by HS-40. In this report, we therefore inserted different normal or mutated parts of a human α-globin gene, at the same site immediately downstream of HS-40, to determine which of the inserted gene features are responsible for the phenomenon of competition.

We observed that insertion of the human α-globin coding sequence devoid of a promoter had no effect on endogenous human α-globin expression, whereas insertion of the α promoter alone led to a level of endogenous human α-globin expression of only 45% of its level in WT cells. These results
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Nonetheless, the α-globin promoter alone induces a less drastic effect than the entire α gene inserted at the same site (16). Although, we could not quantify the transcription rate driven by the α promoter alone (see “Results”), we can hypothesize that its weaker negative effect is because of a less efficient transcription rate in the absence of a coding sequence. Indeed, a transcriptional positive regulatory element has been described downstream of the α-globin gene cap site (20) that is not included in the pα/Δα-globin construct. This element seems to be necessary for the α promoter to achieve maximal transcription level (20), but it has no activity by itself as shown by the neutrality of the α-globin coding sequence when inserted alone (Fig. 1D).

Even though the α-globin coding sequence alone has no effect, the insertion of its in vitro methylated form induced a mild decrease in human endogenous α-globin expression. Having demonstrated that methylation does not spread from the coding sequence toward adjacent sequences, such as HS-40 or the endogenous α genes, this observation suggests that epigenetic modifications such as DNA methylation interfere with the mechanism of α-globin activation by HS-40 and that the integrity of the 40 kb that lies between HS-40 and the α genes is essential. Nonetheless, it remains possible that insertion of methylated DNA in such a close proximity to HS-40 induces more subtle changes in HS-40 tertiary structure and/or factor binding pattern.

The data obtained from insertion of both the coding sequence and the promoter alone, along with our previous results (16), indicate that HS-40 may be able to recognize any promoter present in its vicinity regardless of its capacity to activate transcription from this promoter. Because the promoter of the ubiquitous −14 gene naturally lies between HS-40 and the globin genes, we decided to insert the −14 promoter driving the α-globin coding sequence directly downstream of HS-40 to test its effect on the endogenous human α-globin expression. We thus showed that the −14 promoter, as the other non-erythroid promoters, CMV and LTR (16), can trap HS-40 activity without being activated itself and induces partial inhibition of the human endogenous α gene expression, underlining the fact that the −14 gene might not be totally neutral regarding HS-40 activation of the globin genes in the natural context of the intact locus.

Although our results illustrate the fact that HS-40 may recognize any promoter in its surroundings, they also suggest that TATA-box-containing promoters (LTR, CMV, α-globin) may compete more efficiently with the endogenous human α genes for activation by HS-40 than TATA-box-less promoters such as the −14 promoter. Indeed, the residual level of endog-

and cells harboring the replacement of the HygroTK gene by the ΔTATA/α-globin construct either in the same orientation as the endogenous human α-globin genes (ΔTATA/α-globin →) or in the opposite orientation (ΔTATA/α-globin ←). The expected lengths of the SacI/BglIII fragments revealed by the HS-40 probe are indicated under the map corresponding to each type of clone. B, Southern blot analyses of SacI/BglIII-digested genomic DNA revealed by the HS-40 probe. Lane 1, HYTK → clone; lanes 2–6, ganciclovir-resistant and hygromycin-sensitive clones bearing the insertion of the human α-globin gene devoid of its TATA-box in either orientation. C, analysis of ΔTATA/α-globin and endogenous human and mouse α-globin gene expression in differentiated WT cells and targeted clones by RNase protection assay. The position and length of each protected fragment are indicated in lane 1. D, quantitative analysis of the data presented in C. Results from two different experiments are shown for each clone and are expressed as the ratio of human to mouse α-globin signals (striped bars) or ΔTATA/α-globin to mouse α-globin signals (stippled bars).

FIG. 5. Characterization of clones bearing the targeted insertion of the human α-globin gene devoid of its TATA-box. A, schematic map of the human α-globin locus in WT cells, HYTK clone, emphasize the central role of the promoter sequence in the competition mechanism among genes of a single complex for activation by HS-40.
enous human α expression compared with WT cells is 11–12% for the insertion of the LTR, CMV, and α-globin promoters (16), whereas it is 56% for the insertion of the −14 promoter. It is known that some enhancers, when shared between a TATA-box-containing and a TATA-box-less gene, preferentially activate the TATA-box-containing gene (21). We thus decided to test the putative role of the TATA-box in the mechanism of HS-40 activity and demonstrated that insertion of an α-globin gene devoid of its TATA-box decreases the human endogenous α gene expression down to 50% of its level in WT cells; the insertion of a normal α gene leaves only 11% of the endogenous human α gene expression (16).

It has been shown that no transcription is detected from a mouse α-globin gene devoid of its TATA-box in in vitro transcription assays (19), and mutations in the TATA-box of the β-globin promoter have also proved to drastically down-regulate transcription of the β-globin gene in transient transfection experiments (18). In the current study, however, we observed that the TATA-mutated α gene is still transcribed and fairly efficiently activated during erythroid differentiation, although it is expressed approximately half as much as the normal α gene inserted at the same position (48% of a single endogenous α gene expression in WT cells for the TATA-mutated α gene compared with 112% for the normal inserted α gene). These results seem to emphasize the fact that the TATA-box is an essential feature for enabling a promoter to trap HS-40 activity efficiently. Nonetheless, because the −14 and TATA-mutated α promoters both lack a TATA-box and show a weaker expression, it is difficult to argue whether the most important factor for a promoter to efficiently trap HS-40 activity is the presence of a TATA-box or a high level of transcription.

However, when compiling the results obtained from the constructs that share the same α-globin coding sequence and that differ only in their promoters, we observed a clear correlation between the rate of mRNAs produced by the inserted constructs and the decrease in human endogenous α-globin mRNAs (Fig. 6). Thus, the importance of the transcription machinery composition and/or efficiency in the competition mechanism that regulates the human α-globin gene activation by HS-40 is emphasized. This observation is in agreement with our previous results obtained with the LTR and CMV promoters (16). Indeed, insertion of these promoters known to drive high transcription rates (independently of any activation by HS-40) led to strong impairment of HS-40-mediated activation of the endogenous human α-globin genes (16).

More generally, the results presented in this and previous (16) reports highlight the mechanism that allows a promoter to trap HS-40 activity at the expense of the human α-globin genes. Indeed, it appears that HS-40 activity can be trapped (i) regardless of the orientation and downstream or upstream position of the inserted gene (16), (ii) regardless of the erythroid or non-erythroid nature of the inserted gene (Ref. 16 and data presented in this report), but (iii) according to the transcription rate initiated by the promoter (herein) and (iv) with a maximum efficiency when no epigenetic modification alters the chromatin lying between HS-40 and the human α genes (herein). Nonetheless, all of these observations were made in a MEL cell line. So, although the natural chromosomal environment is respected, it would be interesting to verify our hypothesis in a more physiological model such as whole mice.

A competition phenomenon is also known to occur within the human and mouse β-globin loci, which are very homologous to the human α-globin complex. A “looping” model, suggesting that distal dominant positive elements can physically interact with one target gene at a time by looping out the intervening DNA, has been described to explain the mechanism of competition within the mouse β-globin complex (23, 24). This model could also be applied to the human α-globin locus. Indeed, HS-40 could interact preferentially with the closest promoter via protein/protein interactions, forming a loop of DNA, which would be impaired by methylation of the intervening sequence. These interactions could lead either to an activation process for an erythroid promoter or to a sterile interaction with a non-erythroid promoter. With HS-40 being a unique element, each interaction would be at the expense of the other genes present in the same complex. The stronger the interaction with one gene, the less the others would be expressed, thus explaining the phenomenon of competition. Our results would complete this model further by emphasizing the role played by the rate of transcription in the strength of interaction between HS-40 and any particular promoter.

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**Insertion of New Promoters into the Human α-Globin Complex**