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# Enhancer dependent expression of the chicken β-hatching globin gene during erythroid differentiation

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The activity of the chicken $\beta^H$ globin gene promoter has been analysed in functional assays in both chicken and murine erythroleukaemia cells. Sequences between -251 and -146 bp, in the presence or absence of the chicken β globin locus enhancer, strongly repress transcription in erythroid cells before and after the induction of terminal differentiation. A 50 bp sequence (-98 to -146 bp), which contains adjacent cGATA-1 and NF1 protein binding sites *in vitro*, and which is bound by non-histone protein *in vivo*, is essential for full promoter activity. Mutagenesis studies indicate that both protein binding sites are required. During terminal differentiation, both the absence of repressor and the presence of the erythroid enhancer are required for maximal promoter activity, suggesting that the $\beta^A$, $\beta^\varepsilon$ and $\beta^H$ globin gene promoters compete for the enhancer during development. 

The developmental regulation of globin genes during erythropoiesis involves the interaction between promoter, enhancer and dominant control region (DCR) regulatory elements (1-23). These sequences occur in tissue-specific and developmentally regulated DNase1 hypersensitive sites (DHS) in erythroid nuclei, and bind regulatory transcription factors *in vivo* (5-7,9,24).

Proximal promoter sequences (between -1 and -100 bp), which include the conserved TATA, CCAAT and CACC motifs are required for promoter function (25-27). Distal promoter sequences modulate promoter activity, for example during the up-regulation of transcription during erythroid differentiation (1,2,4,6), and may mediate the interaction(s) between promoter and enhancer and/or DCR regulatory sequences (4,14, 16,17,20-22). These interactions appear to be mediated by changes in the levels of critical transcription factor(s) which bind to one or another regulatory sequence (4,6,14). Only one enhancer element has been identified in either the chicken β or α globin multigene domains (13,15,16,18), and although both the $\beta^A$- and $\beta^\varepsilon$-globin gene promoters are activated by the β-globin locus enhancer, there is promoter competition for the enhancer during development (14,17).

The chicken $\beta^H$- and $\beta^A$-globin gene are switched on in adult red blood cells at the same stage of embryogenesis (28). Although transcribed at 10-20% of the level of the $\beta^A$-globin gene (28), the $\beta^H$-globin gene promoter exhibits a strong nuclear DHS in the region between -70 and -200 bp (9). This includes a 50 bp sequence (-110 to -160 bp) which is bound by

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transcription factors *in vivo* (9), and contains adjacent *in vitro* binding sites for the NF1 protein and the erythroid cGATA-1 factor (also called EF1/Eryf1 (3,8,9,30)).

In this study, we have used functional assays to investigate the significance of the -110 to -160 bp $\beta^H$-globin gene promoter sequence and to determine whether the promoter is activated by the β-globin locus enhancer. Sequences between -146 and -251 bp repress promoter activity, but the -110 to -160 bp sequence which binds protein *in vivo* and *in vitro* is required for promoter activity. Maximal promoter activity was detected with the truncated promoter, in the presence of the chicken β-globin locus enhancer, and during terminal differentiation. This suggests that the $\beta^H$-globin gene promoter may also compete for the β-globin locus enhancer during development, and the enhancer is only fully functional during terminal differentiation.

## MATERIALS AND METHODS

Cell lines and cell culture:

Mouse (F41B2 (10)) and chicken (HD3 (29)) erythroleukaemia cells were cultured as described (9,10,29). HD3 cells were induced to differentiate by incubation in medium containing 10 mM Hepes (pH 8.0) and 20 mM H7 (37), at 42°C for 2 days (30).

Plasmids:

The promoter-less human growth hormone gene (pOGH) and pTKGH (pOGH driven by the Herpes simplex-2 thymidine kinase promoter) (31) were obtained from Biogenesis Ltd.

A 600 bp PvuII restriction fragment containing the chicken $\beta^H$ globin gene promoter (-547 bp to +43 bp)(9) was blunt end ligated into the HincII restriction site of pOGH (pBH-557). Promoter deletion constructs (pBH-344,-251,-146,-126 and -98) were obtained using the restriction sites shown in Figure 1A.

A 600 bp Sph1 restriction fragment containing the chicken β-globin locus enhancer (13,15,18), was cloned by blunt-end ligation into the Sma1 site of pIC20E (32), then excised by cleavage with EcoR1 and cloned into the EcoR1 site of the pBH-557 (pBH-557E), pBH-146 (pBH-146E) and pBH-128 (pBH-128E) promoter/hGH constructs.

The NF1 and cGATA-1 protein binding sites between -146 and -98 bp were mutated using the polymerase chain reaction (33). The NF1 (pBH-251M1) and cGATA-1 (pBH-251M2) mutations in the pBH-251 promoter deletion construct are shown in Figure 1C.

Transfections:

F4-1BD cells were transfected by the calcium phosphate precipitation technique (34). 20 μg of test plasmid, 10 μg of reference plasmid pHSVβGAL plamid (35), and 10 μg of carrier pIC20H plasmid (32) were transfected into $10^6$ cells in 10 ml. After 16 hrs , 10 ml of fresh medium was added, and cells and medium harvested after a further 48 hrs.

HD3 cells were transfected by the DEAE-dextran procedure (13). 10 μg of test plasmid and 10 μg of reference pHSVβGAL plasmid were transfected into $2.4X10^6$ cells in 1 ml of DMEM containing 0.8 mg/ml DEAE-dextran and the DNA. After incubation at 37°C for 1.5 hrs, cells were washed and resuspended in 10 ml of medium. After 24 hrs at 37°C, the cells were split into two flasks, and cells in one flask induced to differentiate at 42°C in the presence of the protein kinase inhibitor H7 (37). Medium and cells of control and 42°C/H7 cells were harvested after 48 hrs.

Growth hormone assay:

hGH protein in the medium (100 μl) of transfected cells was assayed using the Allegro dual growth hormone specific monoclonal antibody system as recomended by the suppliers (Biogenesis Ltd.). hGH gene expression was calculated first by correcting hGH levels for transfection efficiency using β-galactosidase activity assayed in cell lysates (35). Background expression (cells transfected with pOGH) was then subtracted and hGH gene expression expressed relative to that obtained in parallel transfections with pTKGH.

## RESULTS

The sequence of the chicken $\beta^H$-globin gene promoter (-251 to +43 bp) encompassing the nuclear DHS and the previously characterised major protein binding sites is summarised in

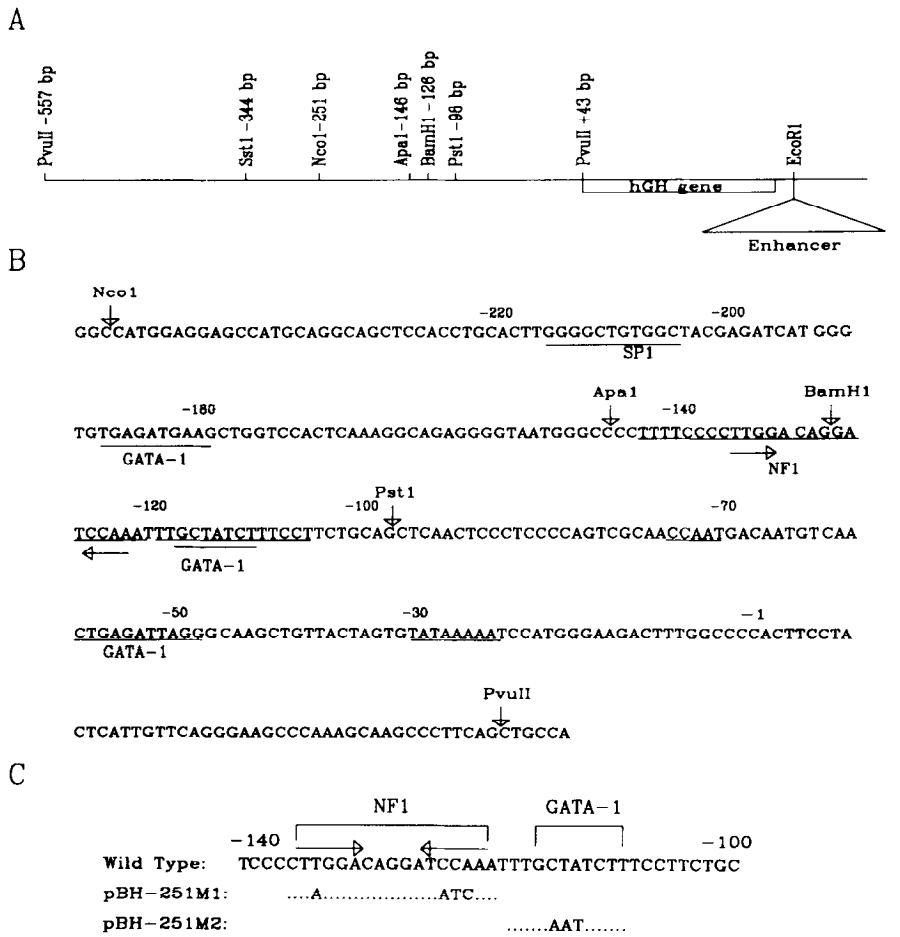


Figure 1.
A). Restriction map of the 600 bp chicken $\beta^H$-globin gene promoter (9) linked to the hGH gene (pOGH (31)) and showing the 3' EcoR1 restriction site into which the chicken β locus enhancer was cloned. Promoter deletion constructs were generated using the restriction sites as shown.
B). The sequence of the chicken $\beta^H$-globin gene promoter between the 3' PvuII site (+43 bp) and the 5' Nco1 site (-251 bp) (9). Protein binding sites ((8,9,11) and data not shown), conserved consensus sequences and appropriate restriction sites are shown and include the inverted repeat (---→ ←---) NF1 binding site (9,11). Nucleotide numbering is with respect to the transcription initiation site (+1).
C). Site-directed mutagenesis was employed to introduce 3-4 bp changes into the adjacent NF1 (pBH-251M1) or cGATA-1 (pBH-251M2) protein binding sites between -100 and -140 bp. The mutations introduced into the pBH-251 construct are shown and compared to the wild-type sequence.

Figure 1B. *In vitro* protein binding studies suggest that the protein binding to the site between -200 and -220bp (footprint G in (8)) also binds the GC-rich repeat sequence of the mouse α1 globin gene promoter (36) and to an Sp1 binding site (data not shown), and is therefore Sp1 or the SP1-like factor αIRF (36).

In initial transfection experiments S1 nuclease protection assays were used to assay transcription from transfected constructs in erythroid cells. Although protected bands indicative of correct initiation of transcription were observed, these were weak and irreproducible (data not shown). It was therefore necessary to use a very much more sensitive technique to assay the

activity of promoter sequences. The $\beta^H$ globin gene promoter (-557 bp to +43, see Figure 1A and (9)) was therefore linked to the human growth hormone (hGH) reporter gene (pBH-557), and its ability to drive hGH gene expression in transiently transfected mouse (MEL) or chicken (HD3) erythroleukaemia cells was determined using a highly sensitive radioimmunoassay. In all cases, hGH gene expression is expressed relative to that obtained using the pTKGH construct.

As the pBH-557 construct is relatively inactive in both cell types (Figure 2), a number of promoter deletion constructs were therefore also assayed. Maximal promoter activity in both MEL or HD3 cells is detected when sequences upstream of -146 bp are deleted (pBH-146)(Figure 2). As pBH-557, pBH-344 and pBH-251 have equally low activities compared to pBH-146, the negative regulatory element is localised between -251 and -146 bp. Further deletion of 5' promoter sequences (pH-128 or pBH-98 constructs) progressively reduces promoter activity, suggesting that sequences between -146 bp and -98 bp, which are bound by

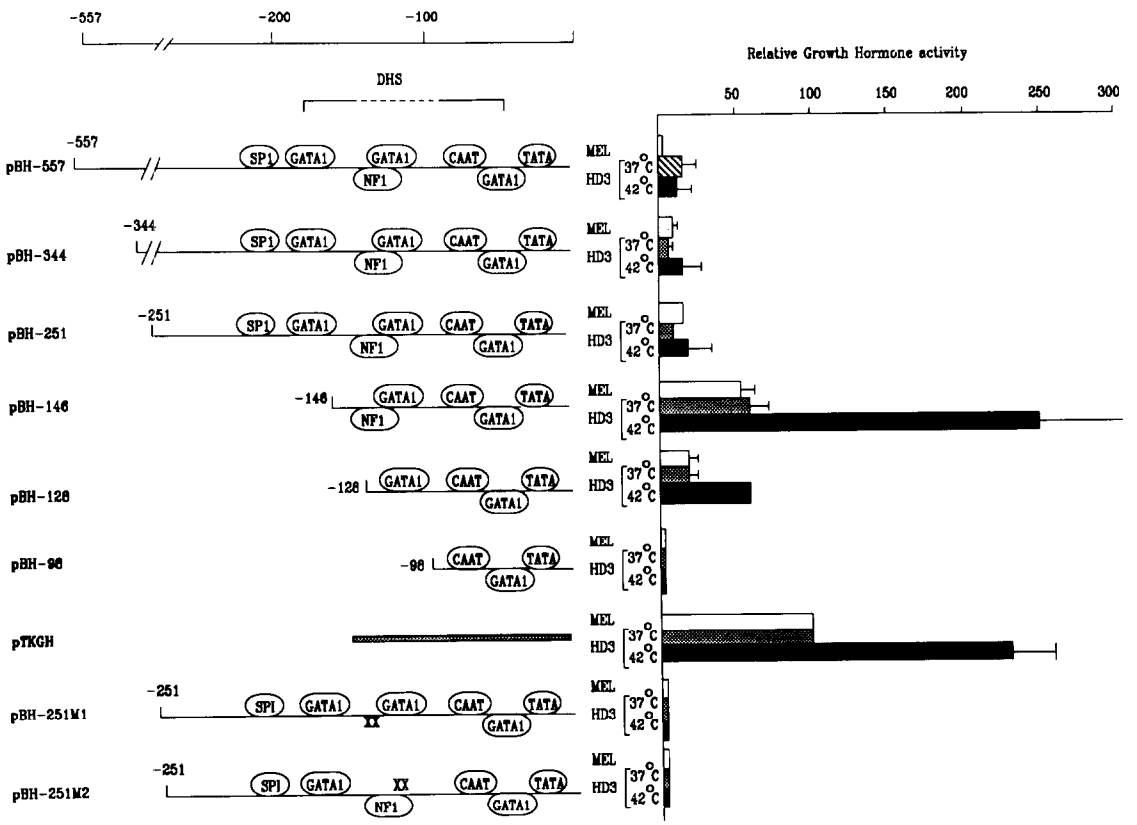


Figure 2.
Functional analysis of the chicken $\beta^H$-globin gene promoter/hGH chimaeric constructs in MEL or HD3 cells.
The various promoter deletion constructs, their major protein binding sites and the location of the nuclear DHS are schematically represented. pBH-251M1 and pBH-251M2 are the NF1 and cGATA-1 mutations respectively.Constructs were transfected into either uninduced MEL cells (MEL) or into HD3 cells,half of which were maintained at 37°C (HD3-37°C) and the other half induced to differentiate (HD3-42°C) for two days. hGH gene expression is expressed relative to that obtained in parallel transfections with pTKGH (pTKGH=100) in uninduced MEL or HD3 cells. Values represent the means obtained in at least two separate transfection experiments, and the S.E.M. is shown in experiments repeated more than twice.

transcription factors *in vitro* and *in vivo* (9,11), are required for maximal promoter activity. The introduction of 3-4 bp changes into either the NF1 or cGATA-1 protein binding sites to alter the recognition binding sites of the pBH-251 construct (pBH-251M1 and pBH-251M2 respectively, Figure 1C) reduce promoter activity to below detectable levels in both cell types (Figure 2), indicating that both protein binding sites are required.

HD3 cells are transformed with the avian erythroblastosis virus containing the v-erbA oncogene and a temperature sensitive v-erbB oncogene (29). When grown at 37°C, very low levels of globin gene expression are observed. However, when the HD3 cells are shifted to 42°C (to inactivate the temperature sensitive V-erbB (29)) in the presence of the protein kinase C inhibitor H7 (37) (to inactivate V-erbA (30)) 60% of the cells become positive for benzidine staining and Northern blot analysis has confirmed that the α and β-globin genes, including the $\beta^H$-gene, are now expressed (30). Furthermore, Choi and Engel (14) have shown that the enhancer downstream of the βH-globin gene is active in HD3 cells which have been induced to differentiate at 42°C.

When the various promoter constructs were assayed in HD3 cells which had been induced to differentiate (Figure 2), only the pBH-146 and pBH-128 promoter deletion constructs exhibit a significant (3-4 fold) increase in promoter activity compared to that in uninduced HD3 cells. However, as a 2.4 fold increase was also observed with the non-erythroid pTKGH positive control, it suggests that the majority of the observed increases in promoter activity during HD3 cell terminal differentiation are non-specific.

As the developmental regulation of the β-globin multigene family is mediated by the promoter competition for enhancer elements (13-17,19,20), the chicken β-globin locus enhancer (13,15,18) was cloned immediately downstream of the hGH gene (Figure 1A) in the pBH-557, pBH-146 and pBH-128 constructs, and the effect on promoter activity assayed. The enhancer does not significantly derepress the activity of the pBH-547 construct in HD3 cells, and has a small (1-2 fold) effect on promoter activity of the pBH-146 bp and pBH-128 bp constructs (Figure 3) in either HD3 or MEL cells. However, during HD3 cell terminal differentiation (Figure 4), it is clear that the presence of the enhancer and the absence of the negative regulatory element yields a 4-5 fold stimulation of transcription in addition to the 3-4 fold effects observed with the same promoter constructs in the absence of the enhancer. Thus, the enhancer is having its maximal effect during terminal differentiation, but neither the enhancer nor differentiation can compensate for the repressor activity of the upstream (-146 bp to -251 bp) sequences.

## DISCUSSION

The lower transcription rate of the endogenous $\beta^H$-globin gene compared to the $\beta^A$-globin gene in embryonic chick red blood cells (28) could be due to (i) an intrinsically weak promoter, (ii) a weak interaction between promoter and enhancer/DCR regulatory elements, or (iii) repression by a negative regulatory element. The studies reported suggest that the $\beta^H$-globin gene promoter may be repressed by a negative regulatory element and, compared to the $\beta^A$- and $\beta^\varepsilon$-globin genes (13-15,18), responds relatively weakly to the β-globin locus enhancer.

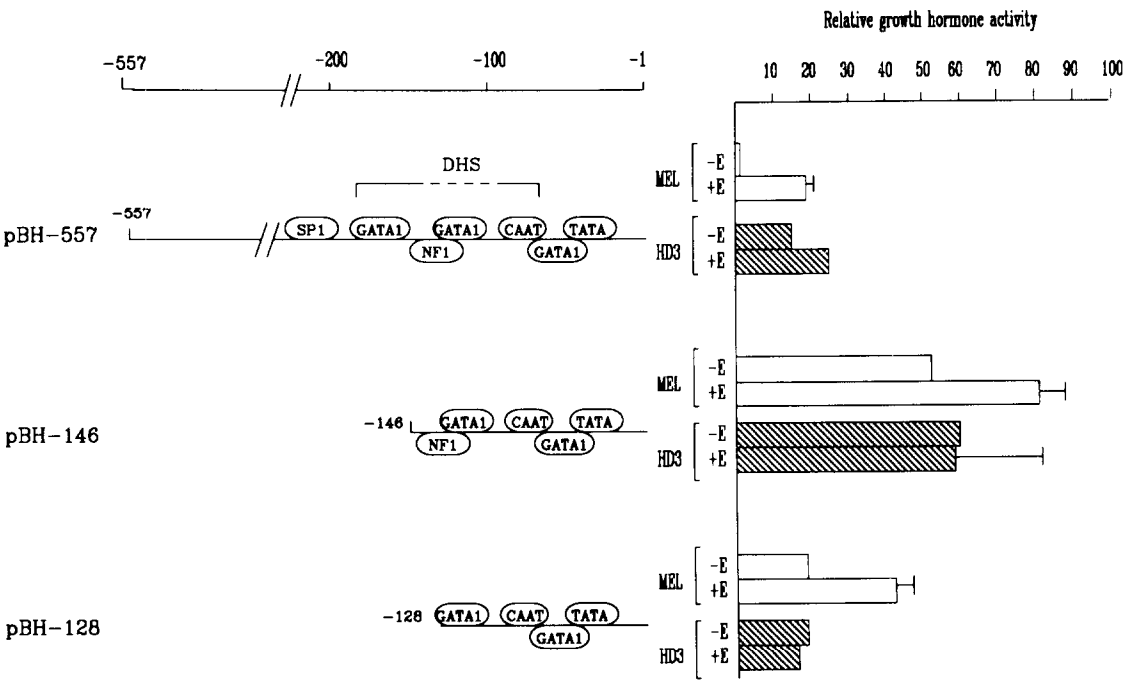


Figure 3.
The effects of the β globin locus enhancer on the activity of the $\beta^H$-globin gene promoter. The activities of the pBH-557, pBH-146 and pBH-128 promoter deletion constructs in the presence (+E) or absence (-E) of the chicken β globin locus enhancer was compared in uninduced MEL and HD3 cells. hGH gene expression is expressed relative to that obtained with pTKGH in paralell transfections (pTKGH=100), and values represent the mean of at least two separate experiments, and S.E.M. values shown where more than two experiments were performed. The major protein binding sites in the promoter constructs and the location of the nuclear DHS are schematically represented.

The functional analyses obtained with the $\beta^H$-globin gene promoter are similar to those reported for the chicken α-globin promoters (16). In the absence of the enhancer neither the $\alpha^D$- (1.5 kbp) or $\alpha^A$- (2 kbp) globin gene promoters show detectable transcription in 9 day chick erythrocytes. However, promoter truncation to within 200-250 bp of the cap site resulted in a functional $\alpha^D$-promoter (but not $\alpha^A$-promoter). Significant promoter activation by the α-globin locus enhancer was obtained with the truncated promoters, although again the $\alpha^D$-promoter/enhancer construct was up to 10 times more active. Similarly, the $\beta^H$-promoter (600 bp) is very weak in functional assays, is activated by the deletion of upstream sequences, and the β-globin locus enhancer has its maximal effect on the truncated $\beta^H$ promoter. Interestingly, similar results were obtained in both MEL and HD3 cells, indicating that the regulatory mechanism(s) is conserved. However, the β-globin locus enhancer has little effect on the promoter in uninduced MEL and HD3 cells. Significant activation of the $\beta^H$-promoter by the enhancer is only observed when the HD3 cells are induced to differentiate, and is consistent with the reported differentiation-dependant enhancer activation of the $\beta^A$-globin gene promoter in HD3 cells (13).

The $\beta^H$-globin gene promoter nuclear DHS contains a 50 bp sequence which binds protein *in vivo* (9) and can bind both NF1 and cGATA-1 *in vitro* (8,9,11). Deletion or mutation of these sequences reduces promoter activity suggesting that in this context cGATA-1 and NF1

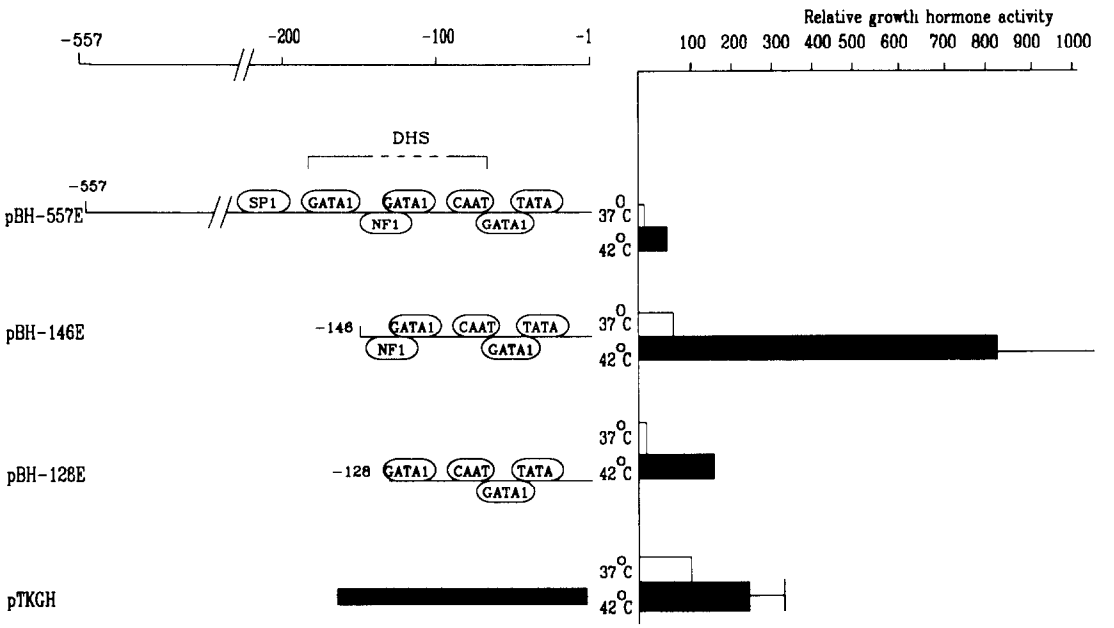


Figure 4.
Enhancer activation of the $\beta^H$-globin gene promoter during HD3 cell differentiation.
The activities of the pBH-557E, pBH-146E, and pBH-126E promoter deletion constructs containing the β globin locus enhancer at the 3' end of the hGH gene was compared in uninduced HD3 cells (37°C) and and HD3 cells induced to differentiate (42°C). Values are expressed relative to pTKGH in uninduced HD3 cells (pTKGH=100) in parallel transfections, and S.E.M. values shown where experiments were repeated more than twice. The major protein binding sites and the location of the nuclear DHS are schematically illustrated.

are transcriptional activators. In contrast, the NF1 binding site in the chicken $\beta^A$-globin gene promoter nuclear DHS (5,6,9,11) acts as a repressing sequence, probably beacause of the competitive binding of the NF1 and CACC factors to overlapping binding sites.

In conclusion, we have presented evidence which may explain the comparatively low levels of $\beta^H$-globin gene expression observed *in vivo*. The presence of promoter repressor sequences, and a comparatively weak response to the β-globin enhancer evidently combine to down-regulate promoter activity. However, the $\beta^H$ promoter does respond to the β-globin enhancer during terminal differentiation suggesting that at least three of the β-globin genes compete for enhancer activity during development. The negative regulatory element is currently being characterised in more detail.

## ACKNOWLEDGMENT

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