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The E-Box of the Human Glycophorin B Promoter Is Involved in the Erythroid-Specific Expression of the GPB Gene

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Previous studies performed on the glycophorin B (GPB) expression demonstrated that this gene is expressed in erythroid cells only and that the ubiquitous factor Ku70 is involved in the process. Here, we investigated the contribution of the −70 E-box sequence toward the GPB promoter expression. We found that the E-box bound two factors, the USF1/USF2 protein and an unidentified ubiquitous protein which was named factor U. Site-directed mutagenesis performed on the −70 E-box showed that the USF factor had an activating effect in CAT assays. Conversely, mutation of the −70 E-box that impaired the binding of factor U led to a positive CAT activity in nonerythroid cells and thus to the loss of the erythroid-specific expression of the GPB gene. This indicates that, in addition to the Ku70 factor, the extinction of the GPB promoter expression in nonerythroid cells depends also on the repressing effect of the factor U. © 1999 Academic Press

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Regulation of the erythroid-specific gene expression has been widely explored and transcription of these genes appeared to reflect the interplay of multiple ubiquitous factors with a more limited array of tissue-restricted factors. The identification of nuclear DNA-binding factors that recognize *cis*-regulatory elements in promoter region and the determination of their role contributed to a better understanding of the mechanisms of the transcription regulation (1). Previous analyses performed on many erythroid promoters have shown that all of the erythroid-restricted genes are regulated by the transcription factor GATA1, more frequently in combination with CACCC/Sp1 or NFE2 factors (2, 3).

The human glycophorin A (GPA) and glycophorin B (GPB) are the major erythrocyte sialoglycoproteins that carry MN and Ss blood group antigens, respectively, and act as ligands for viruses, bacteria and parasites (4). Cell surface expression analysis indicated that GPA and GPB are restricted to cells or tissues of erythroid origin (5, 6). The molecular basis of erythroid-specific expression of the glycophorin genes has been investigated in some detail using the GPB promoter as a model (7, 8). These studies revealed that erythroid and ubiquitous regulating factors determined GPB expression. Deletion analysis and mutagenesis demonstrated that the −75 region, containing a WGATAR sequence that binds hGATA1 and an ubiquitous factor, is involved in the erythroid-specific expression of the GPB gene. We isolated the ubiquitous Ku 70 factor by the one-hybrid method and demonstrated that erythroid specificity of the GPB promoter expression could be attributed in part to the heterodimer Ku70/80 factor which binds the −75 WGATAR sequence in a specific manner. Whereas Ku suppresses the expression of the GPB gene in nonerythroid cells, hGATA1 relieves the suppressive effect of Ku on the GPB gene expression in erythroid cells (8).

The −75 region of the GPB gene contains an E-box sequence centered at position −70, overlapping with the −75 WGATAR sequence. It has been recently suggested that several bHLH proteins that bound E-box sequences (CANNTG) function as transcriptional regulatory factors of erythroid genes (9–11). Numerous functional roles were assigned to the E-box *cis*-acting element in erythroid genes, based on the different E-box binding factors and their involvement in differentiation, cell proliferation, and level of expression (repressor/activator) (11, 12). This report will focus on the role of the E-box binding factors of the GPB promoter and their relative effect toward the GPB promoter activity.

Abbreviations used: CAT, chloramphenicol acetyl transferase; bHLH, basic helix-loop-helix; CMV, cytomegalovirus; RSV-luc, Rous sarcoma virus luciferase.

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TABLE 1

Sequences Introduced in Reporter Plasmids for CAT Assays, and Oligonucleotides Used in EMSA

Plasmids	Mutations	Sequences (noncoding strand)
		−70 −75
		| |
pBL-95WT GPB	wild type E-box and WGATAR	5′CAT**CAGCTG**ATAGGC 3′
pBL-95M9 GPB°	mutated E-box** and mutated WGATAR#	5′CAT**CACGTG**ATAAGG 3′
pBL-95M29 GPB	mutated E-box	5′CAT**CGGCTG**ATAGGC 3′
pBL-95M33 GPB	mutated E-box**	5′CAT**CACGTG**ATAGGC 3′
Oligonucleotides		
		−70 −75
		| |
−75WT GPB	wild type E-box and WGATAR	5′ATCAT**CAGCTG**ATAGGCAGGGGAG 3′
−75M18 GPB	mutated E-box and WGATAR*	5′ATCAT**CAGAAG**ACTGGCAGGGGAG 3′
−75M22 GPB	mutated WGATAR	5′ATCAT**CAGCTG**AGAAGTAGGGGA 3′
−75M29 GPB	mutated E-box	5′ATCAT**CGGCTG**ATAGGCAGGGGAG 3′
−75M33 GPB	mutated E-box**	5′ATCAT**CACGTG**ATAGGCAGGGGAG 3′
MLP	wild type	**−55**
		|
		5′ACCCGGT**CACGTG**GCCTAC 3′
U3 R-EPO	wild type	**+37**
		|
		5′CCGCCGGACG**CAGCTG**ACCAGGCCCT 3′

Note. The boldface oligonucleotides correspond to the normal or mutated E-boxes, and the underlined sequences to the juxtaposed normal or mutated WGATAR sequence. Plasmids pBL-95 (WT or mutated) correspond to the −95 to +43 region of the GPB gene. Positions of sequences refer to +1 = transcription start point. °presently described (2); ** binds USF only; * binds Ku70/80 only; # binds hGATA-1 only.

MATERIALS AND METHODS

Cell cultures and transfections. K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were transiently transfected by electroporation as previously described (8) and then incubated at 37°C for 24 h before harvest.

HeLa cells were cultured in Dulbecco's medium supplemented with 10% fetal calf serum. Cells were transiently transfected with the superfect reagent (Quiagen, Hilden, Germany). Twenty-four hours before transfection, 5×10^5 HeLa cells were plated in 60 mm diameter dishes with 5 ml of complete medium. 5 μg of test and reporter plasmids with 2 μg of RSV-luciferase construct were mixed with 20 μl of superfect reagent into 150 μl of medium without calf serum during 10 minutes for incubation. The mixture was diluted with 1 ml of complete medium and added on the cells during 4 h. The cells were then washed, and placed at 37°C in 5 ml of complete medium for 24 h before harvest.

Plasmids and CAT assays. Sequences containing the −95GPB promoter with or without mutation in the −70 E-box region were introduced in reporter plasmids for CAT assays as shown below (Table 1). The reporter vectors were constructed by cloning either the wild-type (WT) or the mutated −95 to +43 region of the GPB gene in front of the bacterial CAT gene on the pBLCAT3 vector (13).

The transfected cells were harvested, washed twice with PBS and resuspended in 100 μl of lysis buffer (250 mM Tris, 0.2% Triton X-100, 5 mM dithiothreitol and 10% glycerol pH 7.8). Cell extracts were assayed for both luciferase (14) and CAT activities (15). The luciferase activities were used as internal control values for normalization of transfection efficiency.

Protein preparation and electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared either from the non-hematopoietic cell line HeLa or from the erythroleukaemic cell line K562 as previously described (16) and according to Dignam's method (17). The protein concentration was measured by the Bio-Rad dye-binding assay (Bio-Rad, Hercules, CA) (18). In addition, mobility shift assays were performed using partially purified proteins as follows: crude total HeLa extracts were applied to a column of heparin agarose and eluted by a gradient of increasing KCl concentrations (19). The proteins eluted at 200 mM KCl concentration (fraction H0.2) were pooled, dialyzed against buffer D (50 mM Tris-HCl, pH 8.0, 50 mM KCl and 20% glycerol) and stored at −80°C.

Labeling of oligonucleotides, binding reactions and electrophoresis were performed as previously described (8). The gels were run for 2 h, dried and autoradiographed overnight. For competition experiments, DNA binding reactions were allowed to reach an equilibrium and a 250-fold excess of unlabeled specific competitor DNA was added to the binding reaction mixture. For supershift assays, the following rabbit polyclonal antibodies were used: sc-229 (specific of the USF1 subunit), sc-861 (specific of the USF2 subunit) (Santa Cruz Biotechnology). Antibodies were added lastly to the reaction mixture, which was then incubated at room temperature for 10 minutes before loading. Oligonucleotides used in EMSA are shown in Table 1.

RESULTS

Analysis of the −70 GPB Sequence by Electrophoretic Mobility Shift Assays (EMSA)

As previously reported (7), EMSA performed with oligonucleotides encompassing the −75WT region of the GPB promoter revealed 3 different complexes (Fig. 1A, lanes 1–2). Complex 3 seen with the K562 nuclear extract corresponded to hGATA1 binding on the −75WGATAR region, and complex 2 seen both in K562 and HeLa nuclear extracts to the binding of the Ku70/80 heterodimer on the same sequence (8). Complex 1 present in erythroid and nonerythroid nuclear extracts was also previously identified as an E-box

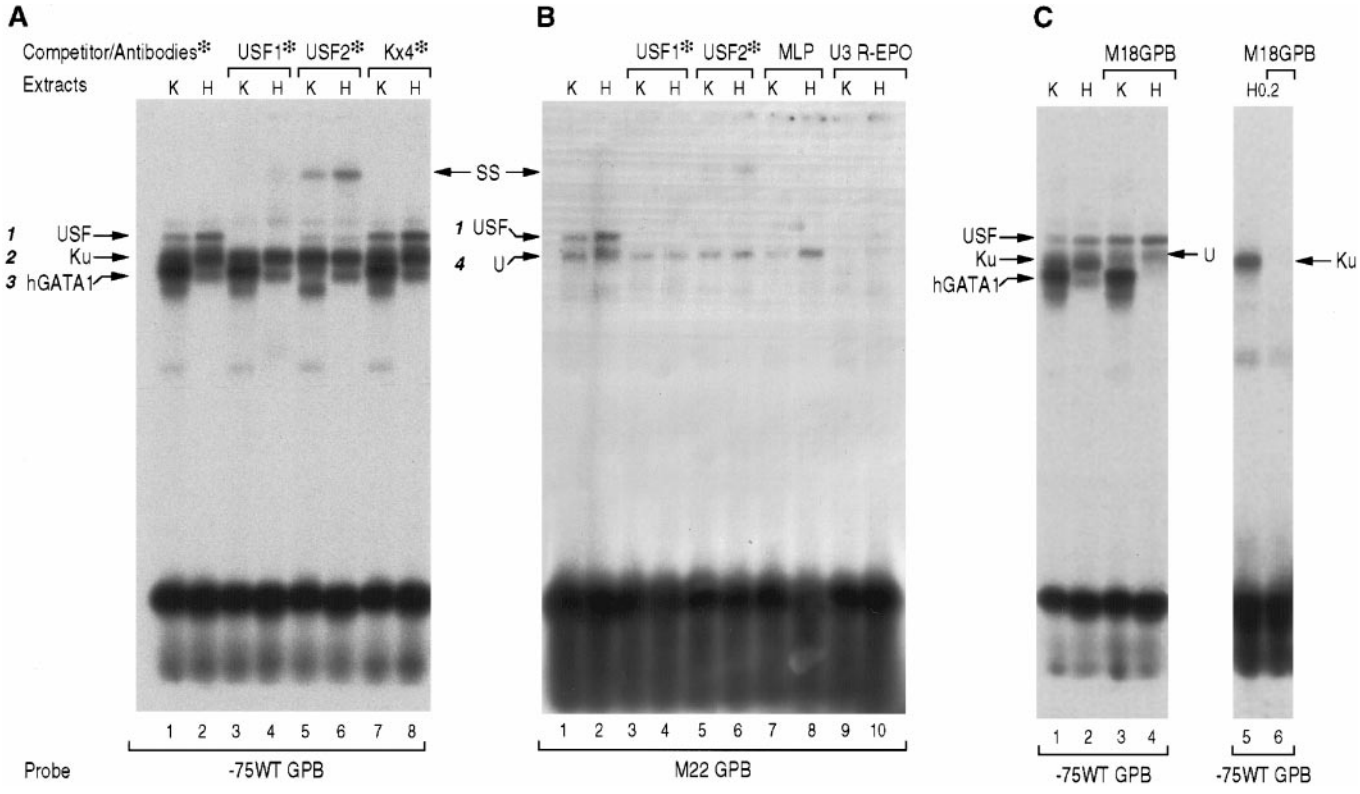

FIG. 1. Protein–DNA complexes detected by electrophoretic mobility shift assays. EMSA were performed with nuclear extracts prepared from erythroid K562 cells (K) and nonerythroid HeLa cells (H). [A] The −75WT GPB 5′ end-labeled oligonucleotide was used (lanes 1 to 8). Positions of the three retarded complexes are indicated. Antibodies directed against USF1 protein result in a complete disappearance of the complex (lanes 3–4). USF2 antibody results in a supershift (lanes 5–6). Complex formation was not affected by an irrelevant antibody (Kx4) directed against Kx protein (lanes 7–8). [B] EMSA performed with 5′ end-labeled mutated M22 GPB oligonucleotide show two distinct complexes indicated as complex 1 and 4. Antibody directed against USF1 factor resulted in a complete disappearance of the complex 1 (lanes 3–4), whereas the addition of USF2 antibody resulted in a supershift (lanes 5–6). A 250-fold excess of unlabeled MLP E-box containing oligonucleotide competed efficiently for complex 1 formation (lanes 7–8), when addition of a 250-fold excess of unlabeled U3-REPO E-box containing oligonucleotide competed for the complexes 1 and 4 formation (lanes 9, 10). [C] EMSA performed with 5′ end-labeled −75WT GPB oligonucleotide and K562 or HeLa nuclear extracts, or H0.2 HeLa extracts. A 250-fold excess of unlabeled M18 GPB oligonucleotide, that specifically recognized the Ku70/80 factor, competed for complex 2 formation without inhibition of complex 4 (lanes 1–6).

binding factor (8), and corresponds to the binding of an heterodimer of USF1/USF2 factors (Fig. 1A, lanes 3–6).

The −75 WGATAR and the −70 E-box sites contained in the −95 GPB promoter (nt −95 to +43) are overlapping. To analyse only regulating factors that bind the −70 E-box sequence we performed site directed mutagenesis of the −75 WGATAR motif so that Ku and hGATA1 binding were impaired (M22 GPB mutant, Table 1). EMSA performed with the M22 GPB oligonucleotide using erythroid (K562) and nonerythroid (HeLa) nuclear extracts, showed two different complexes, one of which migrated as complex 1 and the other as complex 2 (Fig. 1B, lanes 1–2). Addition of a 250-fold excess of cold E-box oligonucleotide corresponding to the MLP (CA**CG**TG) (20) or to the U3 sequence of the R-EPO promoter (CA**GC**TG) (21) showed that complex 1 formation was abolished with the two different E-box oligonucleotides (Fig. 1B, lanes 7–10). However, the formation of the second complex was only efficiently competed by addition of a 250-fold excess of the unlabeled U3-REPO oligonucleotide containing the CA**GC**TG sequence (Fig. 1B, lanes 9 and 10). Addition of antibodies directed against USF1 or USF2 resulted successively in a complete disappearance or a supershift of complex 1 (Fig. 1A, lanes 3–6), without affecting the second complex formation in the two different cell lines (Fig. 1B, lanes 3–6). All the results suggested that a new complex, called complex 4, migrating at the same level as complex 2, did not result from another heterodimeric form of the USF factor (22).

To ascertain that the formation of complex 4 did not correspond to Ku or did not arise from an artefactual binding site created by the mutagenesis performed to obtain the M22 GPB mutant, further experiments were carried out with partially purified H0.2 extracts of total HeLa cell lysates (8,19). Using these extracts and radiolabeled −75 WT GPB oligonucleotide in EMSA analysis, only complex 2 formation corresponding to the heterodimeric form of the Ku protein was shown, as

determined by supershift analysis (8). No complex formation was observed with the radiolabeled M22 GPB oligonucleotide and the H0.2 fraction (data not shown), indicating that complex 4 did not contain the Ku70/80 factor.

To determine if complex 4 comigrated with complex 2 in EMSA performed with the −75 WT GPB oligonucleotide, the M18 GPB oligonucleotide (Table 1), that specifically recognized the Ku70/80 heterodimer, was used in competition experiments. Addition of a 250 fold excess of cold M18 GPB oligonucleotide resulted in a partial inhibition of bandshift at the complex 2/complex 4 level using the K562 and HeLa nuclear extracts (Fig. 1C, lanes 1–4), and a complete inhibition of bandshift using the H0.2 HeLa fraction (Fig. 1C, lanes 5–6). These results indicate that complex 4 may be detected with the −75WT GPB oligonucleotide after displacement of complex 2 and confirm the comigration of the two complexes, one corresponding to the Ku70/80 antigen (complex 2), and the second corresponding to an unidentified ubiquitous protein provisionally called factor U (responsible for complex 4 formation, detected with the M22 GPB oligonucleotide).

Mutagenesis of the −70 E-Box

In order to evaluate the functional significance of the two E-box binding proteins evidenced in EMSA (USF and factor U), we performed site directed mutagenesis looking for a sequence that selectively impaired binding of one or the other E-box protein without destroying the WGATAR overlapping sequence. Oligonucleotides containing wild type or mutated E-box sequences were tested *in vitro* and EMSA profiles were compared to the −75WT GPB oligonucleotide in erythroid and nonerythroid cell lines (Fig. 2). With the M33 GPB oligonucleotide, carrying the mutation changing CAGCTG→CACGTG (see Table 1), complex 1 was obtained, but complex 4 formation was impaired, as determined after displacement of complex 2 by a 250-fold excess of M18 oligonucleotide (Fig. 2, lanes 3–6). Instead, a band of slightly slower mobility was detected, which corresponds to a complex of an heterodimeric USF isoform, since addition of antibody directed against USF1 to the EMSA mixture resulted in a supershift (Fig. 2, lanes 7–8). The M29 GPB oligonucleotide, obtained by changing CAGCTG→CGGCTG (Table 1) impaired the binding of all E-box binding proteins. Complex 1 was absent, and no complex 4 formation was observed after displacement of complex 2 with a 250-fold excess of M18 oligonucleotide (Fig. 2, lanes 9–12). We did not succeed to obtain an mutated E-box GPB oligonucleotide that only allowed the complex 4 formation without affecting the hGATA-1/Ku70 binding on the overlapping WGATAR sequence. More than fifteen mutagenesis, performed on each nucleotide of the E-box, were tested. Most of them destroyed the binding of USF and U proteins, some created binding sites for new protein(s) in addition to the binding of one or the other E-box binding factor. Others mutations reduced equally the binding of the two factors.

Effect of E-Box Point Mutations on the Activity of the GPB Promoter

Next, we performed CAT assays in K562 and HeLa cells transiently transfected with pBL-95 GPB mutant constructs, carrying the M29 and M33 oligonucleotide sequences. The CAT activity in K562 cells was estimated in regard to the pBL-95WT GPB construct (100 ± 10%). Previous studies have demonstrated that the GPB promoter is completely inactive in nonerythroid cells. In HeLa cells, pBL-95WT GPB construct exhibited a CAT activity equal to the background level of the pBLCAT3 plasmid, that was estimated to 8 ± 2% (7). The CAT activities represent the average of at least three normalized experiments.

The CAT activity obtained with the pBL-95M29 GPB construct, for which binding of the two E-box proteins was impaired, was 3 fold lower compared to the pBL-95M33 GPB activity in K562 cells, and was about half of the pBL-95M33 GPB level in HeLa cells, in which the CAT activities of the GPB constructs are much lower (Fig. 3). This result demonstrated the activating role of USF factor on GPB promoter expression, since the only difference between the two constructs was the removal of USF binding for the pBL-95M29 GPB plasmid.

The pBL-95M33 GPB construct, that only allowed USF binding (no complex 4 formation in EMSA) exhibited a 3 fold higher activity than the pBL-95WT GPB in K562 cells (Fig. 3). The upregulation of the reporter gene could be interpreted as being caused by the missing of a negative factor (U protein) and/or by the activating effect of USF. A 3 fold increase was also obtained with the pBL-95M33 GPB construct transfected in HeLa cells, that revealed GPB expression in nonerythroid cells when factor U binding was impaired. This last result proves that factor U acts as a repressor of the GPB promoter expression, at least in HeLa cells.

Finally, the CAT activity exhibited by the pBL-95M29 GPB construct was closed to the CAT activity of the WT plasmid (1.3 fold and 1.6 fold the CAT activity of the pBL-95WT GPB construct in K562 and HeLa cells, respectively). Thus, the removal of both the activating (USF) and the repressing (factor U) factors binding had almost no consistent effect on the activity of the GPB promoter, at least in our experimental conditions.

Effect of E-Box and/or WGATAR Point Mutations on the Activity of the GPB Promoter in Nonerythroid Cells

To ascertain the E-box involvement in the erythroid specific expression of the GPB, we compared the CAT

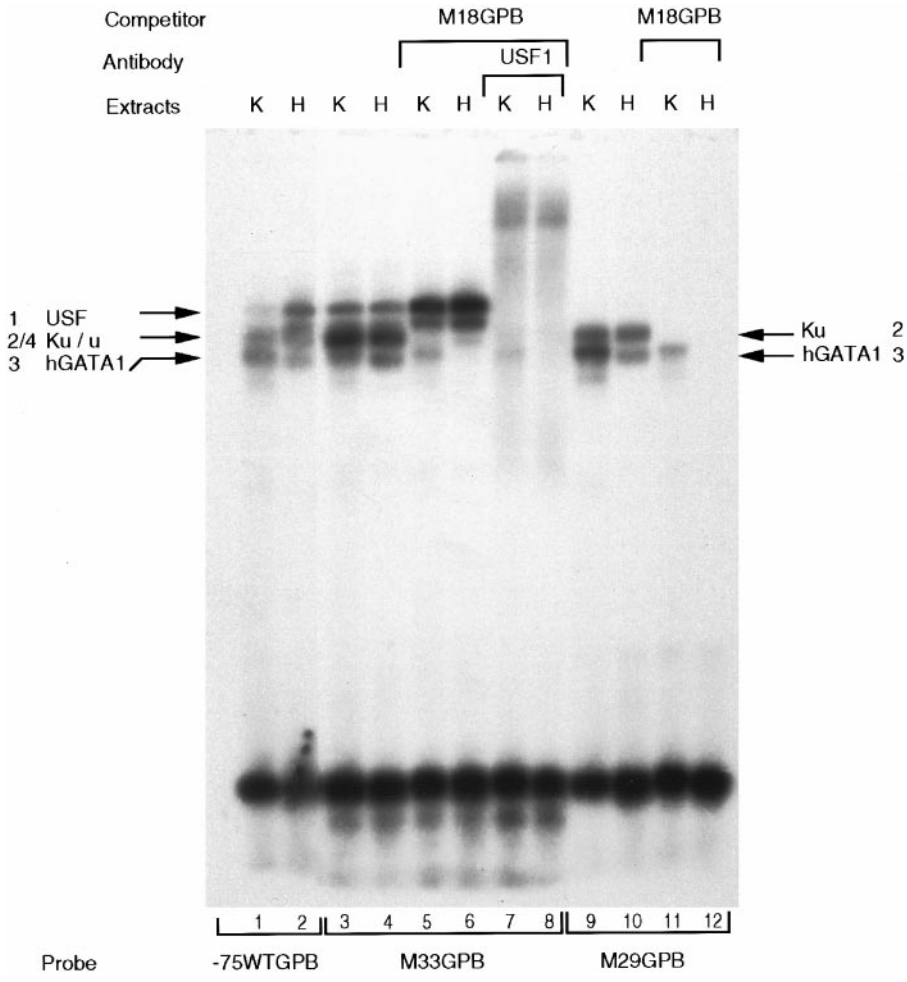

FIG. 2. Mutagenesis on the −70 E-box of the GPB promoter. EMSAs were performed with labeled mutated GPB oligonucleotides and nuclear extracts from K562 (K) and HeLa (H) cells. The M33 GPB oligonucleotide showed the only binding of the USF factor on the −70 E-box (lanes 3–4). The complex 4 formation was impaired, as demonstrated by the addition of a 250-fold excess of unlabeled M18 GPB oligonucleotide. Complex indicated by an asterisk corresponded to an heterodimeric isoform of USF observed only with the M33 GPB probe (lanes 5–6). Antibody directed against the USF1 factor resulted in a complete disappearance of the complex 1 and the complex indicated by an asterisk (lanes 7–8). EMSA performed with the M29 GPB probe showed the only formation of complexes 2 and 3 (lanes 9–10). Addition of a 250-fold excess of unlabeled M18 GPB oligonucleotide competed with complex 2 formation (lanes 11–12).

activities of the pBL-95M33 and the pBL-95M9 GPB constructs previously described (7, 8) after transfection in HeLa cells. The M9 GPB oligonucleotide bound the USF factor on the −70 E-box sequence and hGATA-1 on the −75 WGATAR sequence, but was negative for the Ku and U factors binding. The results shown in Fig. 4 indicate that a CAT activity of 24% was generated by the pBL-95M33 GPB plasmid, for which the binding of the factor U only was impaired. However, the CAT activity reached 41% with the pBL-95M9 GPB plasmid, for which the binding of both Ku and U factors was impaired, a value well above that of the M33 construct, suggesting that Ku and U proteins have about an equal and additive repressing effect on GPB expression in nonerythroid cells.

DISCUSSION

We have demonstrated in this report that the −70 E-box sequence (CAGCTG) of the human GPB promoter binds an unidentified protein named factor U in competition to the USF heterodimeric complex.

USF is a helix loop helix ubiquitous transcription factor, that was identified because of its involvement in the transactivation of the adenovirus major late promoter (23, 24). E-box sequences have already been shown to regulate the expression of erythroid-specific genes such as globin (9, 10). Here, we report that USF interacted *in vitro* and *ex vivo* with the −70 E-box of the GPB promoter depending for its binding of the E-box sequence. Qyang *et al.* (25) showed that the USF

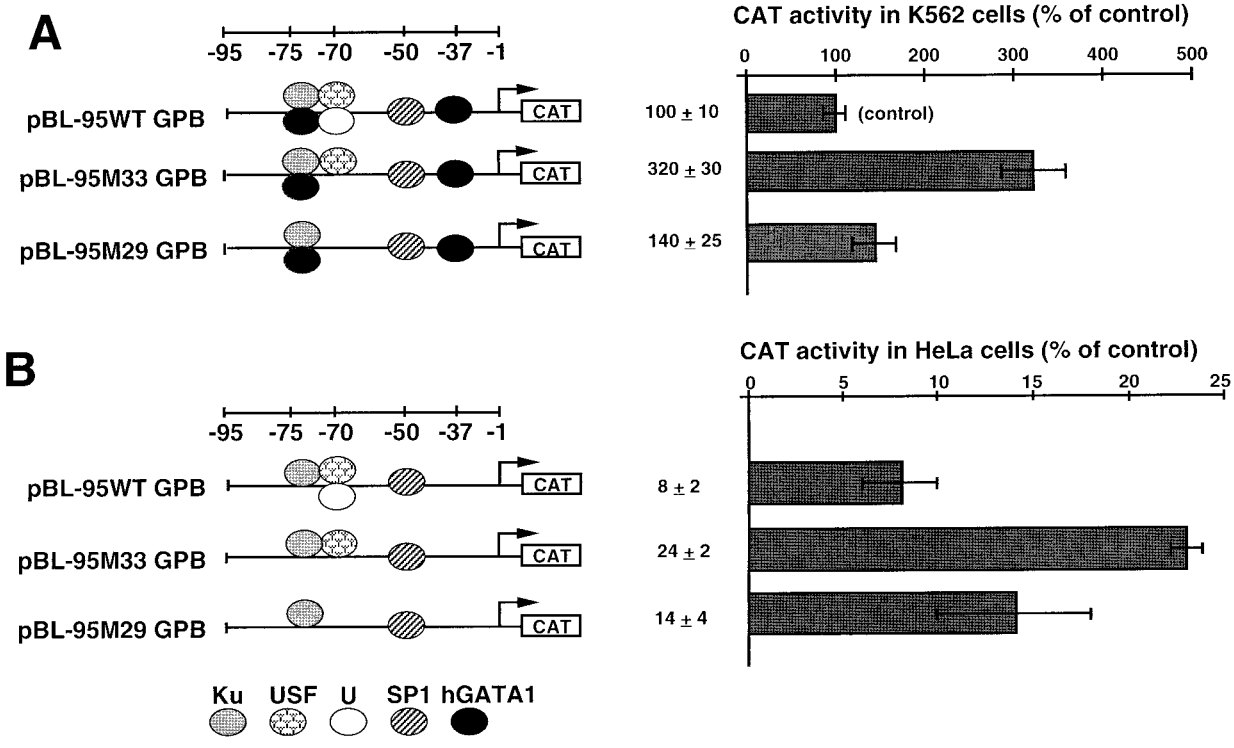

FIG. 3. Role of the E-box binding proteins in erythroid and nonerythroid cells. Left panels: schematic representation of reporter plasmids used for transfection. Right panels: CAT assays performed in cells transiently transfected with the pBL-95WT GPB or pBL-95mutated GPB constructs. [A] Effect of point mutation on the −70 E-box GPB sequence in K562 cells. The pBL-95WT GPB exhibited a 100 ± 10% CAT activity used as a reference. [B] The same mutations described in A were analysed in B in the nonerythroid HeLa cells. The pBL-95WT GPB construct exhibited a CAT activity of 8 ± 2%, equal to the background.

factor is transcriptionally active in HeLa cells. By site directed mutagenesis of the E-box (this paper) and by overexpression of the USF protein (our unpublished results) we found that USF is an activator of the pBL-95WT GPB promoter in HeLa cells at least. As it has been previously demonstrated that the GPB promoter is not functional in nonerythroid cells (7), we assumed that the enhancer activity of USF on the GPB promoter

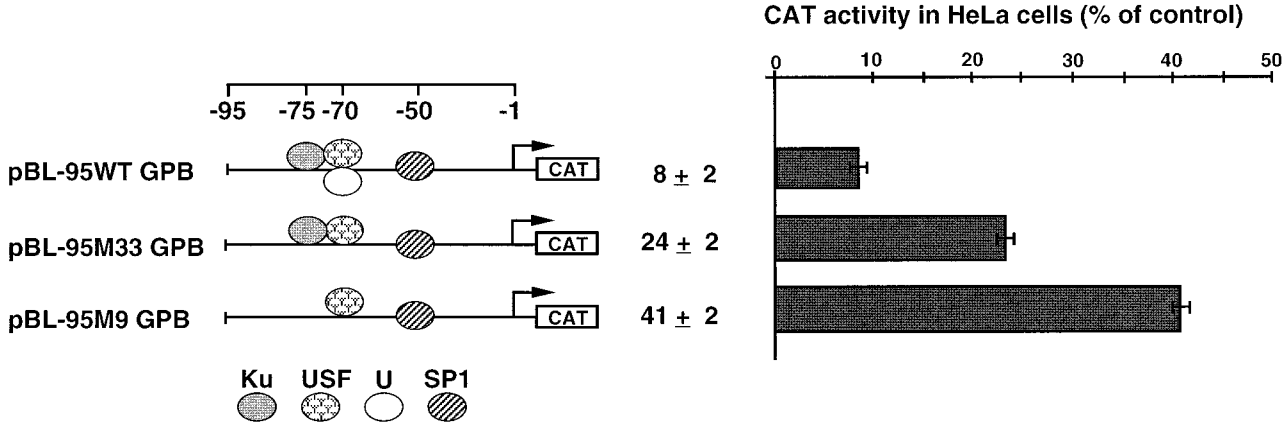

FIG. 4. Role of the factor U in nonerythroid cells. Left panels: schematic representation of reporter plasmids used for transfection. Right panels: CAT assays performed in HeLa cells transiently transfected with the pBL-95WT GPB and the pBL-95M33 GPB (this report), as well as the previously described pBL-95M9 GPB constructs (8). The 100% CAT value used as control is as in Fig. 3. The pBL-95WT GPB plasmid gave a CAT activity identical to background (8 ± 2%) in HeLa cells. The pBL-95M33 GPB construct, which corresponded to the only absence of the factor U binding, reached a value 3-fold upper that of the wild type (24 ± 2%). The pBL-95M9 GPB construct, for which the binding of Ku and the factor U was impaired, had a CAT activity of 41 ± 2%, about 5-fold the CAT activity of the pBL-95WT GPB plasmid.

should be prevented in these cells, perhaps by a competitive effect with the factor U or by a suppressive activity of another factor.

The second ubiquitous factor called U that binds the −70 E-box of the GPB promoter remains unidentified. Competition assays using oligonucleotide containing E-box sequence showed that complex 4 formed by factor U depends on the specific sequence CA**GC**TG, as its DNA binding was abolished when the sequence was changed to CA**CG**TG (Fig. 1B). Site directed mutagenesis suggests that the factor U acts as a repressor in erythroid as well as in nonerythroid cells. Moreover, results obtained in nonerythroid cells with the pBL-95M33 GPB construct clearly indicate that the factor U is required to prevent the nonerythroid expression of the GPB gene. We have previously demonstrated that the erythroid-specific expression of the GPB protein was transcriptionally regulated (8). First, we determined that the binding of the Ku70 factor to the GPB promoter led to a repression of GPB transcription in nonerythroid cells. We assume here that in addition to Ku, another protein, the E-box binding factor U, prevents GPB expression in nonerythroid cells. Only the combination of the two factors results in a complete extinction of the GPB gene expression. This is supported by results obtained with a previously described pBL-95M3 GPB mutant which bound the USF protein, Ku and factor U (our unpublished results), but not hGATA-1 (as shown in (7)). This mutant exhibited a background CAT activity in nonerythroid cells as the wild type.

In the erythroid cell, the displacement of the Ku repressor by hGATA-1 on the WGATAR sequence allows GPB gene transcription (7). However, the relative contribution of the E-box and of the binding of the factor U and USF towards the GPB promoter expression in the erythroid context remains unclear. The mutagenesis that abolishes the binding of the two E-box binding factor has almost no effect on the activity of the GPB promoter in erythroid cells. When EMSAs were carried out with the −70 GPB probe using K562 nuclear extracts obtained after hemin induction of differentiation, we found an increased binding of the USF factor correlated with a decreased binding of the factor U (our unpublished results), suggesting that the role of these proteins might consist to maintain and/or modulate the level of GPB promoter expression during erythroid differentiation, but this needs further investigation. Additionally, molecular identification of factor U will be necessary and we will take advantage of using wild type and mutated −70 E-box GPB sequences for differential screening in the yeast by the one hybrid method.

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