The EMBO Journal vol.11 no.11 pp.4095-4102, 1992

Erythroid-specific activity of the glycophorin B promoter requires GATA-1 mediated displacement of a repressor

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Communicated by M.Yaniv

We have performed a detailed analysis of the *cis*-acting sequences involved in the erythroid-specific expression of the human glycophorin B (GPB) promoter and found that this promoter could be divided into two regions. The proximal region, −1 to −60, contains a GATA binding sequence around −37 and an SP1 binding sequence around −50. This region is active in erythroid and non-erythroid cells. The distal region, −60 to −95, contains two overlapping protein binding sites around −75, one for hGATA-1 and one for ubiquitous proteins. This distal region completely represses the activity of the proximal promoter in non-erythroid cells and defines the −95 GPB construct as a GPB promoter that displays erythroid specificity. Using site directed mutagenesis, we show that the −37 GATA and the −50 SP1 binding sites are necessary for efficient activity of the −95 GPB construct. Mutations that impair the −75 GATA-1 binding result in extinction of the −95 GPB construct activity if the −75 ubiquitous binding site is not altered, or in loss of erythroid specificity if the −75 ubiquitous binding site is also mutated. Using a cotransfection assay, we found that hGATA-1 can efficiently activate transcription of the −95 GPB construct in non-erythroid cells. This transactivation is abolished by mutations that impair either the −37 GATA-1 or the −50 SP1 binding. Mutations that impair the −75 GATA-1 binding and still allow the −75 ubiquitous binding also abolish the transactivation of the −95 GPB construct, indicating that hGATA-1 can remove repression of the GPB promoter by displacement of the ubiquitous proteins. Finally, we show that the association of the hGATA-1 zinc fingers domain and either the acidic NH_2 domain (R1) or the internal domain (R2) of hGATA-1 can mediate the GPB promoter transactivation thereby indicating a functional redundancy of the hGATA-1 R1 and R2 domains.

Key words: *cis*-regulation/erythropoiesis/erythroid gene/transcription factor

Introduction

Regulation of the expression of hematopoietic-specific genes occurs in part at the transcription level and results from complex interactions between constitutive and hematopoietic restricted transcriptional factors. These interactions can result in either repression or activation of specific genes and are the primary events that will determine the different hematopoietic phenotypes. One approach to understanding these transcriptional mechanisms involves the identification of the *cis* and *trans* regulatory elements of genes encoding lineage-specific proteins.

Among the hematopoietic lineages, erythropoiesis is the object of intense research since many proteins specific to terminal erythroid differentiation are well characterized. Analysis of the sequences present in the regulatory regions of erythroid-specific genes has revealed three motifs, often associated. The first motif, GNTGAGTCA, overlaps an AP1 core sequence and can bind either AP1 or an erythroid-specific factor called NF-E2 (Mignotte *et al.*, 1989a; Ney *et al.*, 1990; Talbot *et al.*, 1990). The second motif, GGTGG, can bind either SP1 or the CCACC binding protein (Xiao *et al.*, 1987) and is found in close association with the third motif (for review see Philipsen *et al.*, 1990). This latter, WGATAR (W = A or T, R = A or G) or GATA motif, is found in the majority of defined regulatory elements of globin and other erythroid-specific genes (Evans *et al.*, 1988; Wall *et al.*, 1988; Martin *et al.*, 1989; Mignotte *et al.*, 1989a; Plumb *et al.*, 1989). Mutagenesis of GATA motifs present in erythroid promoters or enhancers has revealed that this sequence is necessary for activity of these regulatory regions both in transfection assays and in transgenic mice (Martin *et al.*, 1989; Mignotte *et al.*, 1989b; Talbot and Grosveld, 1991).

Molecular analysis of the *trans*-acting factors that can interact with this GATA motif has revealed a family of proteins now called the GATA family. Structural analysis of the different members of this family has shown a shared, highly conserved, zinc finger DNA-binding domain $Cys\text{-}X_2\text{-}Cys\text{-}X_{17}\text{-}Cys\text{-}X_2\text{-}Cys$ reminiscent of the DNA-binding domain of the steroid receptor gene family (Evans, 1988). The first cloned member of this family, GATA-1 (Evans and Felsenfeld, 1989; Tsai *et al.*, 1989) was previously thought to be erythroid-specific, but was later shown to be present also in megakaryocytes (Romeó *et al.*, 1990) and mast cells (Martin *et al.*, 1990). The second cloned member of the GATA family, GATA-3 (Yamamoto *et al.*, 1990; Joulin *et al.*, 1991; Ko *et al.*, 1991) is expressed in T lymphocytes. The third cloned member, GATA-2 (Yamamoto *et al.*, 1990; Lee *et al.*, 1991) seems to be widely expressed. Although the precise pattern of expression of the different GATA proteins during hematopoiesis is presently unknown, GATA-1 seems indispensable for terminal erythroid differentiation since GATA-1$^-$ embryo-derived stem (ES) cells cannot contribute to erythropoiesis in chimeric animals (Pevny *et al.*, 1991).

Transactivation by GATA-1 has been studied on a synthetic promoter containing GATA binding sites linked to a minimal human β-globin promoter (Martin and Orkin, 1991), on the chicken α-globin promoter (Evans and Felsenfeld, 1991) and on the mouse erythropoietin receptor

promoter (Chiba *et al.*, 1991; Zon *et al.*, 1991). Athough these studies clearly indicate that GATA-1 is a possible transactivator, they also suggest that interactions with other transcription factors are required to achieve efficient transactivation. Yet, no detailed analysis of these interactions has been performed. Finally, a structure–function analysis of mGATA-1 has revealed that an acidic, serine-rich amino-terminal domain of 66 residues is necessary for efficient transactivation (Martin and Orkin, 1991).

Glycophorin A (GPA) and B (GPB) are the major sialic acid-rich cell surface glycoproteins of human erythrocytes and are only expressed in the erythroid lineage. The glycophorin A and B genes have recently been cloned (for review see Cartron *et al.*, 1990) and analysis of their promoter structure (Vignal *et al.*, 1990) has shown numerous potential GATA binding sites. We have performed an analysis of the GPB promoter to determine the relative contribution of these GATA binding sites to the tissue specificity of the GPB gene expression and we describe a new function of GATA-1 in the activation of erythroid-specific genes.

Results

Analysis of the glycophorin B promoter by DNase I footprinting

A promoter fragment spanning nucleotides −140 to +43 relative to the transcription initiation site was analysed for the binding of nuclear factors by DNase I footprinting assays. Three footprints (E1, E2 and E3) were present only with erythroid nuclear extracts (KU812), whereas two footprints (U1 and U2) were obtained with erythroid or non-erythroid nuclear extracts (HeLa or KU812) (Figure 1A). The sequences covered by these footprints are shown in Figure 1B. The three erythroid-specific binding sites (−135, −75 and −37) contained the sequence 5′-TATC-3′, which matched the consensus core GATA sequence. One of the two ubiquitous footprints, U1, contained a CCCACC sequence often found in promoters or regulatory regions of erythroid-specific genes (Philipsen *et al.*, 1990).

Characterization of the trans-acting factors by mobility shift assays

To identify which proteins interacted with the footprinted sequences, we used mobility shift assays with oligonucleotides spanning each of the footprinted sequences. An oligonucleotide that covered footprint E1 (−37 region) efficiently bound to an erythroid factor (Figure 2A) and this binding was competed by a known GATA binding oligonucleotide (see Materials and methods) (Figure 2A), indicating that E1 was a GATA binding site. An identical result was obtained with an oligonucleotide that covered footprint E3 (not shown). An oligonucleotide that overlapped footprint U1 (−50 region) bound an ubiquitous factor (Figure 2A) and this binding could be competed by an SP1 binding oligonucleotide (see Materials and methods) (Figure 2A). The faster migrating complex observed with KU812 nuclear extracts was also obtained with other extracts from HeLa cells and corresponds to the intermediate complex previously described by Xiao *et al.* (1987). The U1 oligonucleotide did not bind the CCACC protein and did not compete the CCACC binding (data not shown), indicating that SP1, but not CCACC, may interact with the U1 sequence. An oligonucleotide that contained the E2 sequence (−75 region) bound two factors in non-erythroid nuclear extracts (Figure 2B) and three factors in erythroid nuclear extracts (Figure 2B). By competition assays with a known GATA binding oligonucleotide, we found that complex 3 contained hGATA-1 binding while complexes 1 and 2 were not competed (Figure 2B). These results indicated that the −75 region could bind either GATA-1 or ubiquitous proteins.

In order to characterize the binding sites of proteins 1, 2 and 3 more accurately, we performed a methylation interference assay using complexes obtained with KU812 or HeLa nuclear extracts. Complex 3 showed methylation interference that perfectly matched the hGATA-1 one (not shown). Complexes 1 and 2 showed similar methylation interference (Figure 3A) that overlapped the methylation interference obtained with the hGATA-1 complex (Figure 3B).

Deletion analysis of the GPB promoter

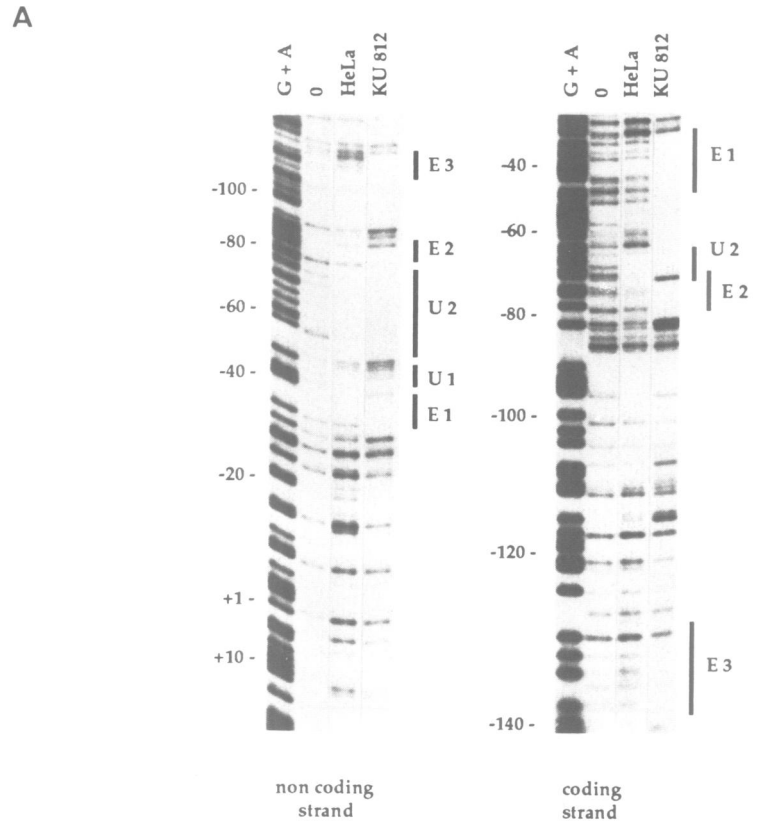
To delimit the sequences involved in the regulation of the GPB promoter, we first performed sequential 5′ deletions of this promoter and linked the remaining sequences to the chloramphenicol acetyl transferase (CAT) coding sequence. These constructs were introduced by transient transfection in erythroid (K562) or non-erythroid (HeLa) cells together with a plasmid expressing the firefly luciferase as an internal control for transfection efficiency. The −95 GPB construct showed the same erythroid specificity as the −150 construct used for the footprint analysis. Thus, we designed deletions starting from this −95 GPB construct. In K562 cells, CAT activity obtained with the −95 GPB construct was 9 ± 1-fold more than that obtained with pBLCAT3, the vector alone. In HeLa cells, the −95 GPB construct and pBLCAT3 showed similar activities. Using primer extension assays, we found that the −95 GPB–CAT construct was correctly initiated when transfected in K562 cells (not shown).

The results of this deletion analysis are summarized in Figure 4. In erythroid cells, sequential removal of sequences resulted in a continuous loss of activity of the GPB promoter, the −37 mutant being completely inactive. In HeLa cells, the −95 GPB construct was completely inactive, whereas constructs −76 and −51 were active and correctly initiated. These results indicated that the −95 GPB construct contained the *cis*-acting sequences necessary for erythroid-specific activity and that a sequence located between −95 and −75 impairs the activity of this construct in HeLa cells.

Effect of point mutations on the activity of the GPB promoter

The results obtained with the GPB promoter deletion analysis indicated that the −95 construct was the minimal construct that displayed erythroid specificity. We subsequently performed site directed mutagenesis on three protein binding sites of the −95 GPB construct, E_1 (−37), U_1 (−50) and E_2 (−75), to investigate their relative contribution to the erythroid activity of the −95 GPB construct.

Point mutations that impaired GATA-1 binding around −37 or SP1 binding around −50 resulted in the complete inactivity of these −95 GPB mutants (Figure 5, mutant 1 or 2) both in K562 and HeLa cells. The −75 region was analysed using three mutants. One mutation (Figure 5,



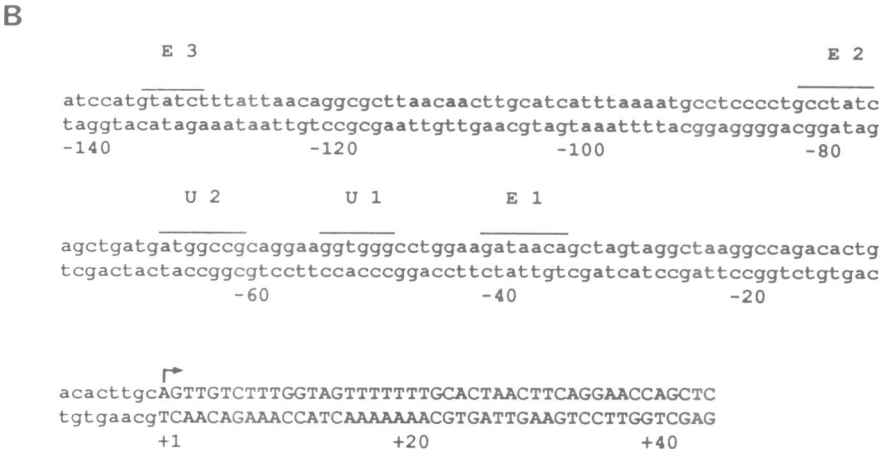


Fig. 1. DNase I footprinting analysis of the GPB promoter. **A**. The −166 to +43 promoter fragment was ^{32}P-labelled either on the coding strand or on the non-coding strand, then incubated with HeLa or KU812 nuclear extracts and treated with DNase I. G + A is a Maxam and Gilbert depurination of the same fragment and O is the DNase I digestion in the absence of nuclear extract. Regions protected from DNase I digestion are indicated by lines. E designates erythroid-specific protection while U designates non-erythroid protection. **B**. Footprints observed wiith HeLa (U) or KU812 (U + E) extracts are summarized on the sequence of the GPB promoter. +1 indicates the CAP site.

mutant 3) allowed the binding of the ubiquitous proteins *in vitro*, but impaired the GATA-1 binding on the −75 sequence (Figure 2C, lane M3). This mutation resulted in a complete extinction of activity of this mutated promoter in erythroid cells. A second mutation (Figure 5, mutant 4) impaired protein binding on the −75 sequence *in vitro* (Figure 2C, lane M4). This mutant was active both in erythroid and non-erythroid cells. A third mutation (Figure 5, mutant 5) allowed the binding of GATA-1 *in vitro*, but impaired the ubiquitous proteins binding (Figure 2C, lane M5). This mutant was as active as the −95 parental construct in K562 cells and also showed activity in HeLa cells. Thus, a repressor site and a GATA binding site overlap around −75 and are involved in the erythroid specificity of the −95 GPB construct.

Effect of the repressor binding sequence on heterologous promoters

We next investigated whether the potential repressor site deduced from the previous experiments could act on heterologous promoters. We cloned both a 30 bp GPB DNA fragment that contained the GATA and the repressor binding

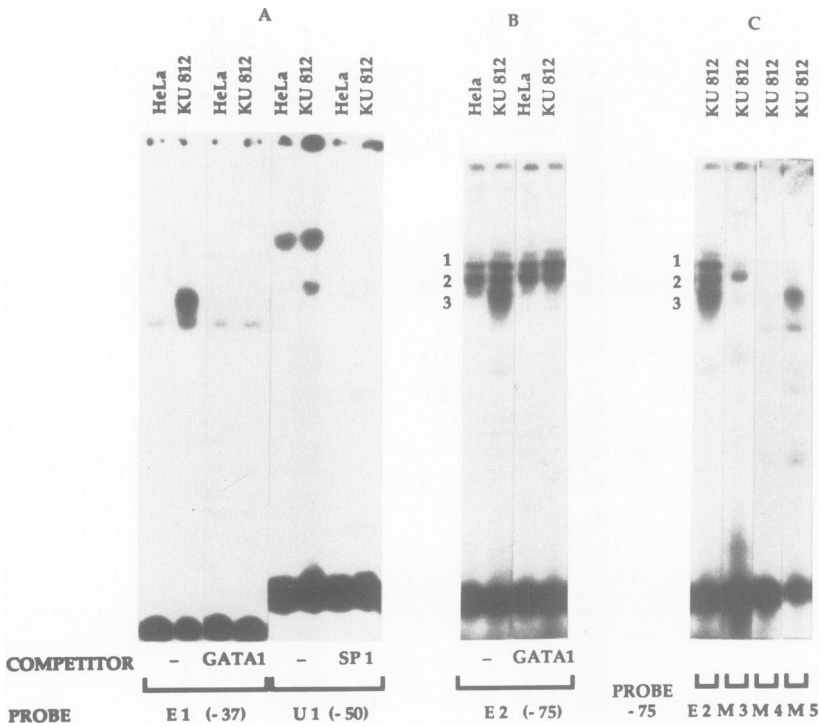


Fig. 2. Gel mobility shift assays using various oligonucleotides and nuclear extracts from HeLa or KU812 cells. **A**. An oligonucleotide spanning the footprint E1 (nucleotides −51 to −27) was 5′end-labelled and incubated with HeLa or KU812 nuclear extracts in the absence or in the presence of an excess (100×) of cold PBGD oligonucleotide known to bind hGATA-1 [−70 binding site of the human PBGD erythroid-specific promoter (see Materials and methods)]. A second 5′ end-labelled oligonucleotide spanning the footprint U1 (nucleotides −66 to −38) was incubated with HeLa or KU812 nuclear extracts in the absence or in the presence of an excess (100×) of cold oligonucleotide that was known to bind SP1 only (see Materials and methods). **B**. An oligonucleotide spanning the footprint E2 (nucleotides −87 to −65) was 5′ end-labelled and incubated with HeLa or KU812 nuclear extracts in the absence or in the presence of an excess (100×) of cold −70 PBGD GATA binding oligonucleotide. 1, 2 and 3 indicate the three complexes obtained. **C**. An oligonucleotide spanning the footprint E2 (nucleotides −87 to −65) and three oligonucleotides containing mutations of this E2 region (M3, M4 and M5; see Materials and methods) were 5′ end-labelled and incubated with KU812 nuclear extract. 1, 2 and 3 indicate the three complexes obtained with oligonucleotide E2.

sites (E2) or a mutated version of this DNA fragment that only contained the repressor binding site (M3) upstream from two different promoters: the −112 porphobilinogen deaminase (PBGD) promoter, which has been shown to display erythroid specificity (Frampton *et al.*, 1990), and the −105 thymidine kinase (tk) promoter (Luckow and Schütz, 1987), which is active in many cell types. The constructs we obtained were fused to the CAT coding sequence and the resulting plasmids were transfected into erythroid (K562) or non-erythroid (HeLa) cells. As shown in Figure 6, the repressor binding site diminished the activities of both promoters in erythroid cells and had no effect on the tk promoter in non-erythroid cells. These results indicated that the repressor sequence alone displayed dominance on heterologous promoters in erythroid cells and that hGATA-1 could remove this repression when a GATA binding site is contained in the repressor sequence.

Determination of the cis-acting sequences required for efficient transactivation of the GPB promoter by hGATA-1

To investigate the ability of hGATA-1 to affect GPB promoter activity in non-erythroid cells, we used reporter plasmids containing the −95 construct, with or without mutated sites, upstream from the coding sequence for the bacterial CAT gene. These plasmids were transfected into HeLa cells together with a plasmid containing the hGATA-1 cDNA expressed from the SV40 enhancer/promoter. In control experiments, the expression vector contained an hGATA-1 cDNA that was deleted for the zinc finger domain. As shown in Figure 7, the −95 GPB promoter was 20-fold more active when cotransfected with hGATA-1 than when transfected with the expression vector alone or the mutated hGATA-1 cDNA. We found that mutations at the −37 GATA binding site (mutant 1) or at the −50 SP1 binding site (mutant 2) completely impaired hGATA-1 transactivation. The three −75 mutants were also cotransfected with hGATA-1. Mutant 3, which contained at around −75 the repressor binding site, but not the GATA binding site, was not transactivated by hGATA-1; while mutant 4, which did not contain either the repressor or the GATA binding site, was transactivated by hGATA-1 (Figure 7, mutants 3 and 4). This transactivation, which was 2-fold weaker than the wild-type −95 GPB construct transactivation, indicated that the −75 GATA binding site was also important by itself. Mutant 5, which could only bind hGATA-1 around −75, was transactivated as efficiently as the wild-type −95 GPB construct (Figure 7, mutant 5). The results indicate that the repressor must be displaced for efficient activity of the −95 GPB construct and that the SP1 binding site is necessary for hGATA-1 mediated transactivation.

Localization of hGATA-1 protein domains that are sufficient to transactivate the GPB promoter

To map the hGATA-1 domains responsible for transcriptional activation, we constructed deletion mutants of the

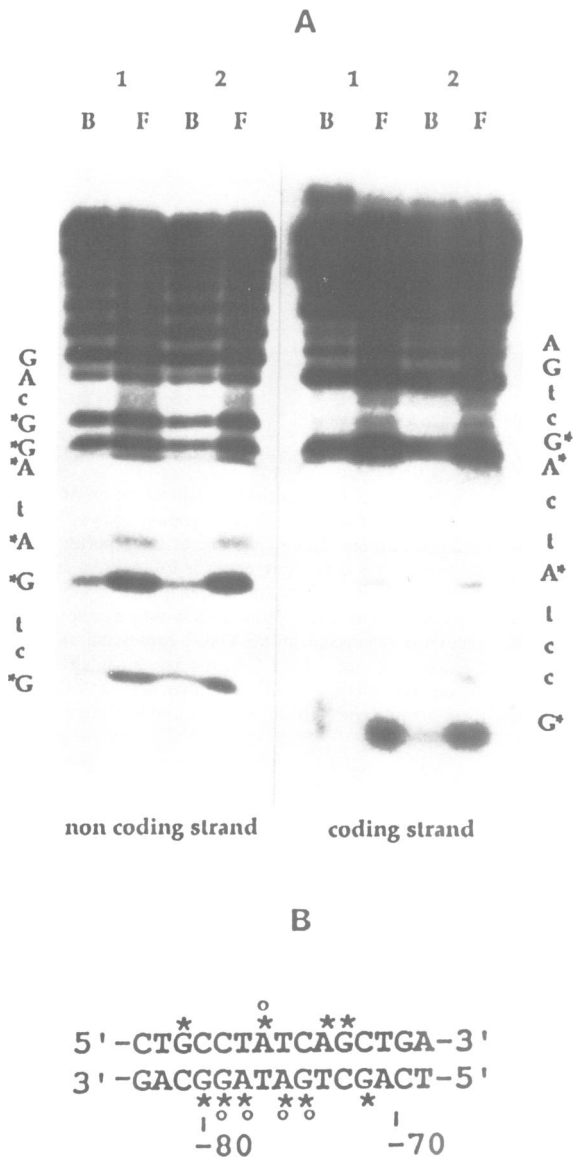


Fig. 3. Methylation interference experiments on both strands of the −87 to −65 oligonucleotide. **A.** Retarded complexes 1 and 2 (see Figure 2B) as well as the free DNA were excised and treated as described in Materials and methods. The sequence of both strands is indicated. F, free DNA; B, bound DNA. **B.** Summary of the methylation interferences obtained on the −87 to +65 oligonucleotide using complexes 1 or 2 (*) or using complex 3 (o) which corresponded to hGATA-1.

wild-type hGATA-1 cDNA in the pECE expression vector (Figure 8) and cotransfected them with the −95 GPB promoter into HeLa cells. These mutants were designed to test each of the three domains delimited by structural study of hGATA-1 (Trainor *et al.*, 1990). Transactivation was measured by CAT activity and GATA-1 products were assayed for DNA binding by gel shift assays using a 5′ end-labelled GATA binding site (not shown).

We found that the association of the hGATA-1 zinc fingers domain and either the acidic NH_2-terminal (domain R1) or the internal R2 domain of hGATA-1 was sufficient for efficient transactivation of the −95 GPB promoter (Figure 8, constructs Δ 1−84, 329−413 and Δ 79−188, 329−413). The zinc fingers domain alone was unable to transactivate

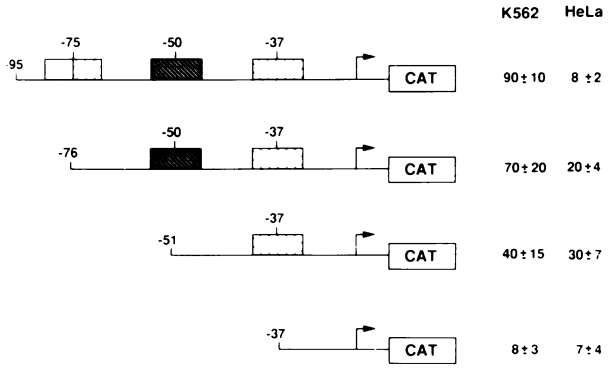


Fig. 4. Effect of 5′ deletions on the expression of the GPB promoter transfected in K562 or HeLa cells. Transfections of HeLa and K562 cells were performed using the constructs indicated and the level of CAT activity was measured 24 h later. The CAT activity obtained with pBLCAT3, the vector alone, transfected in K562 or HeLa cells is normalized to 10. These CAT activities are the average of three experiments with different preparations of plasmids. ▨ indicates a GATA binding site, ▩ the SP1 binding site and ▥ the −75 binding site.

the −95 GPB promoter although this construct was expressed and translocated to the nucleus (not shown).

Discussion

Our studies on the human GPB promoter enlighten several features related to hGATA-1 mediated activation of erythroid-specific genes. Analysis of the *cis*-acting sequences involved in the GPB promoter activity delineated two regions within this promoter. The proximal region contains a binding site for hGATA-1 around −37 and an SP1 binding site around −50. These SP1 and GATA motifs are regularly seen in close association with erythroid-specific regulatory regions (Philipsen *et al.*, 1990) and this indicates possible interactions between hGATA-1 and SP1. In the GPB promoter, this association alone is active both in erythroid and non-erythroid cells. The nature and the position of the −37 sequence may explain the activity of this proximal region in HeLa cells. Indeed, the −37 sequence, AAGATAACA, is also a potential binding site for TFIID (for review see Saltzman and Weinmann, 1989). As no hGATA-1 protein is present in HeLa cells, the activity of the proximal region could be the result of an SP1−TFIID interaction already shown to promote accurate initiation of transcription *in vivo* (Jones *et al.*, 1985; Pugh and Tjian, 1990). Using crude nuclear extracts, we could not detect a TFIID binding on this −37 sequence (Figure 2) but experiments using TFIID cDNA indicated a low affinity binding of TFIID on this −37 sequence (data not shown). These results are similar to the ones obtained in the study of the chicken β-globin TATA region (Fong and Emerson, 1992) and indicate a possible function of GATA proteins in the establishment of a stable transcriptional preinitiation complex.

Contrary to the proximal region (−1 to −60), the −95 GPB construct displayed activity only in erythroid cells. Analysis of the *cis*-acting elements involved in this selective expression revealed at −75 the presence of a sequence that can bind either ubiquitous proteins and/or hGATA-1. As methylation interference assays indicate that the *cis*-acting sequence recognized by the ubiquitous proteins overlaps the

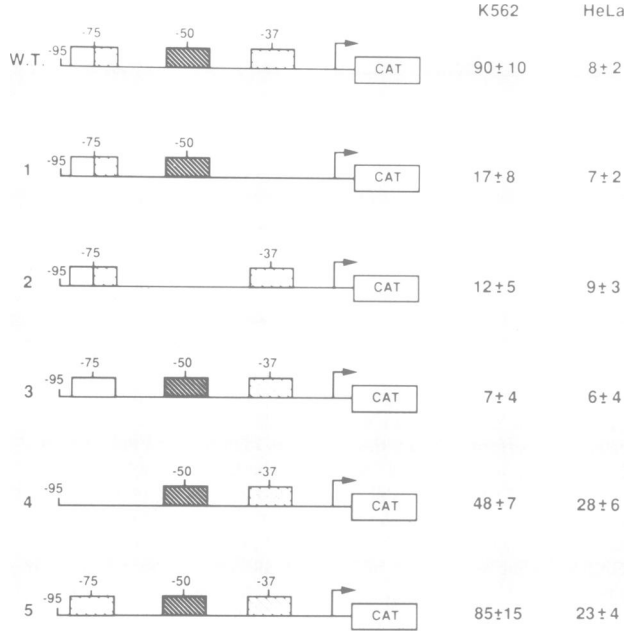


Fig. 5. Effect of point mutations on −37, −50 and −75 binding sites of the −95 GPB construct. Using site directed mutagenesis, the −37 GATA binding site (mutant 1) or the −50 SP1 binding site (mutant 2) of the GPB promoter were destroyed. Mutants 3, 4 and 5 are only mutated on the −75 binding sites. Mutant 3 was obtained by point mutations that only allowed the ubiquitous binding, mutant 4 could neither bind the ubiquitous protein(s) nor hGATA-1 and mutant 5 could only bind hGATA-1. Each mutated promoter was fused to the CAT coding sequence and transfected into HeLa or K562 cells. CAT activity was measured 24 h after transfection and normalization of CAT activities is the same as in Figure 4. ▨ indicates a GATA binding site, ▩ an SP1 binding site, ▧ the −75 binding site and □ a binding site only for the ubiquitous protein.

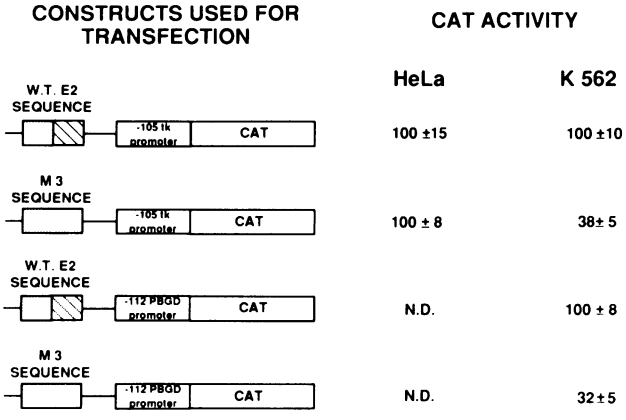


Fig. 6. Effect of the repressor binding site on heterologous promoters. The hybrid promoters shown contained either the wild-type −75 sequence (E2) or a mutated −75 sequence (M3) that could only bind the repressor. These promoters were fused to the CAT coding sequence and transfected into K562 or HeLa cells. The numbers are a mean of three experiments. Activities were quantified as follows: 100 corresponds to 50- (−105 tk promoter in HeLa cells), 20- (−105 tk promoter in K562 cells) or 10-fold (−112 PBGD promoter in K562 cells) activation above the activity of the vector alone. ▧ indicates possible binding sites for the ubiquitous and GATA proteins, □ a binding site only for the ubiquitous protein.

hGATA-1 binding site, we speculate that hGATA-1 and ubiquitous proteins binding are mutually exclusive. Point mutations that only allow ubiquitous proteins binding on the −75 sequence revealed that these proteins could completely

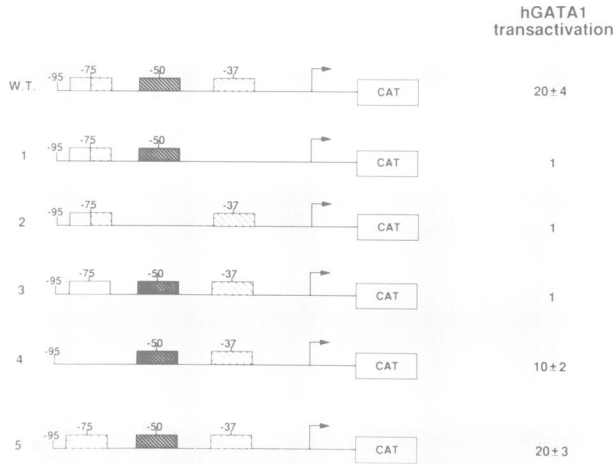


Fig. 7. *Cis*-acting sequences involved in the transactivation of the human GPB promoter by hGATA-1. Transactivation analysis was performed by transfecting into HeLa cells one of the reporter plasmids shown with pECE plasmids expressing either hGATA-1 or, as a control, with hGATA-1$^-$ zinc finger (pGΔA) or pECE alone. After normalization of transfections, the transactivation was monitored by ratio of CAT activities obtained with hGATA-1 expressing vector and pGΔA or pECE alone. ▨ indicates a GATA binding site, ▩ the SP1 binding site, ▧ possible binding site for the ubiquitous and GATA proteins and □ a binding site only for the ubiquitous protein.

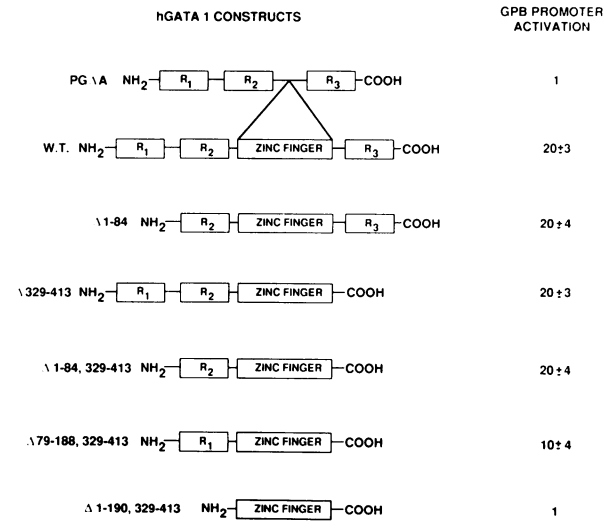


Fig. 8. Transcriptional activation by deletion mutants of hGATA-1. The reporter plasmid was the −95 GPB construct and hGATA-1 mutants are designated by the amino acids deleted. Level of transactivation reflects the mean of two independent experiments for each construct. R1, R2, R3 and zinc finger corresponded to the hGATA-1 protein domains previously characterized by sequence analysis (Trainor *et al.*, 1990).

repress the activity of the proximal promoter both in erythroid and non-erythroid cells. This indicates that the GPB promoter activity is under dominant negative regulation. This type of transcriptional regulation is very well documented in bacterial and phage systems where binding of repressors and RNA polymerase are mutually exclusive competitive events (Ptashne, 1988). In eukaryotes, the large T antigen of simian virus uses a similar strategy for binding to three sites overlapping with the early promoter and thus preventing other transcription factor interaction (Hansen *et al.*, 1981). This type of regulation has also been described for hematopoietic genes such as rat platelet 4 gene (Ravid *et al.*, 1991)

or chicken lysosyme gene (Baniahmad *et al.*, 1987). Among erythroid-specific genes, the human ϵ globin gene has been shown to be negatively regulated during development (Xao *et al.*, 1989; Raich *et al.*, 1990) and mutations in non-deletional hereditary persistence of fetal haemoglobin (HPFH) also indicate a possible repression of the activity of the human γ promoters during development (Superti-Furga *et al.*, 1988). Our studies on the GPB promoter show that its negative regulation can be removed if hGATA-1 binds to this −75 sequence, possibly by a displacement of the ubiquitous protein(s).

Using a cotransfection assay, we showed that the −95 GPB promoter is efficiently transactivated by hGATA-1. We also showed that the −50 SP1 binding site is necessary both for correct transactivation of the GPB promoter by hGATA-1 and for efficient activity of the GPB promoter transfected in erythroid cells. These results differ from those obtained with the chicken α globin gene promoter (Evans and Felsenfeld, 1991) where mutations in the SP1 binding site impair all activity of this promoter in erythroid cells, but allow a small although significant residual transactivation by cGATA-1. This discrepancy can be related to the structure of the chicken α globin gene promoter where a TATA box and a SP1 binding site are found downstream from the GATA binding sites. Mutations on the SP1 binding site result in a promoter composed of GATA binding sites and a canonical TATA box. This type of promoter has been shown to be transactivated by mGATA-1 but is completely inactive when transfected in erythroid cells (Martin and Orkin, 1991). Together with recent data on the erythropoietin receptor gene (Zon *et al.*, 1991), our results indicate that non-globin erythroid-specific genes, which very often harbour no canonical TATA box (Beaupain *et al.*, 1990), may have a regulation different from that of the globin genes.

Mutational analysis of the hGATA-1 protein indicates a possible functional redundancy of the R1 and R2 domains of hGATA-1 for efficient transactivation of the −95 GPB promoter. These two domains are thought to be related by duplication (Trainor *et al.*, 1990) and our results strengthen the existence of multiple activation domains within GATA-1 (Martin and Orkin, 1991), a property already found in other transcriptional activators (Mitchell and Tjian, 1989). It will indeed be interesting to determine whether the R1 and the R2 domains of GATA-1 can interact with similar proteins to allow efficient activation of erythroid-specific genes or if these two domains act by two different mechanisms.

Materials and methods

Oligonucleotides

The oligonucleotides used for competition in the gel shift assays contained the following sequences (coding strand): −70 porphobilinogen deaminase (PBGD) GATA binding site (Mignotte *et al.*, 1989a) 5′-ATGGGCCTTATC-TCTTTACC-3′; SP1 binding site 5′-CGCAGAGGGGCGGGCCTGG-3′. The oligonucleotides used for site directed mutagenesis contained the following mutations (coding strand: mutant 1, −37 GATAA → CTTAA; mutant 2, −50 GGTGGG → GCACGG; mutant 3, −75 CTATC → CTCGC mutant 4, −75 CTATCA → TTCGCT; mutant 5, −75 CCCCTGCCTATCA → GACATGCTTATCT.

DNase I footprinting and gel shift assay

Nuclear extracts were prepared by the method of Dignam *et al.* (1983) from exponentially growing cells: HeLa as a non-hematopoietic cell line and KU812 (Kishi, 1985) as a human erythroleukaemic cell line. After cotransfection in HeLa cells, micronuclear cell extracts were prepared as described by Schreiber *et al.* (1989). DNase I footprinting of the GPB promoter was performed as previously described by Mignotte *et al.* (1989a) using an amplified fragment that spans nucleotides −166 to +43 relative to the cap site. Gel shift assays were performed using the 5′ end-labelled oligonucleotides indicated.

Methylation interference

Methylation interference was performed as described previously by Mignotte *et al.* (1989a).

Construction of reporter plasmids

GPB−CAT parental vector (−95) was constructed by fusion of a GPB promoter sequence (−95 to +43) to the CAT bacterial gene followed by the t intron and polyadenylation signals from SV40 using pBLCAT3 vector (Luckow and Schütz, 1987). For deletion, various restriction enzymes that cut the −95 to +43 region of the GPB promoter were used and each deleted promoter was fused to the CAT gene. For site directed mutagenesis, the −95 to +43 region was subcloned into phage M13mp18 and used as template for mutagenesis using the Amersham 'Oligonucleotide Directed *in vitro* Mutagenesis System'. Oligonucleotides used for mutagenesis were used in band-shift assays to check their impaired binding. All the GPB mutants were then fused to the CAT coding sequence and the resulting constructs were sequenced before use. The hybrid promoters were constructed by cloning a *Hin*dIII−*Hae*III DNA fragment that spans the wild-type or the mutant 3 −75 binding sites upstream from the −105 tk promoter of the −112 PBGD erythroid promoter. The resulting promoters were sequenced and fused to the CAT gene.

Construction of expression vectors

Plasmids that constitutively express hGATA-1 and hGATA-1 mutants were constructed using the pECE vector (Ellis *et al.*, 1986) where the inserted cDNAs are transcriptionally directed by the SV40 promoter and enhancer. An *Eco*RI DNA fragment containing 120 bp of the 5′ untranslated region, the complete coding region as well as 60 bp of 3′ untranslated region of hGATA-1 was inserted at the unique *Eco*RI site of the pECE vector and its orientation relative to the SV40 promoter/enhancer was checked by a *Bam*HI digestion. pGΔA, the hGATA-1 mutant that contained no zinc finger, was obtained by an *Apa*I digestion of hGATA-1 and ligation. This results in a conserved open reading frame through hGATA-1 but a deletion of the two zinc fingers. Mutagenesis of hGATA-1 was performed after subcloning of hGATA-1 into phage M13mp18 and site directed mutagenesis. All the hGATA-1 mutants were then subcloned into the pECE vector and sequenced again.

Transfection of K562 and HeLa cells

Transfection of K562 and HeLa cells was performed by electroporation using 10 μg of reporter gene plasmid and 2 μg of the RSV luciferase plasmid. Transactivation was studied by electroporation of HeLa cells with the reporter genes, the indicated hGATA-1 construct and the RSV−luciferase plasmid. Typically, we used 4 μg of SV40−hGATA-1 construct together with 10 μg of GPB−CAT and 2 μg of RSV−luciferase plasmids. Twenty-four hours after transfection, the cells were harvested and CAT activity was assayed as described (Gorman *et al.*, 1982) using amounts of extract containing identical luciferase activity. Transactivation was quantified by comparison of the CAT activities obtained by cotransfection of the reporter plasmid either with hGATA-1 constructs or with the pECE vector or pGΔA vector as mock transactivators.

Acknowledgements

We thank members of P.-H.R.'s laboratory for constant support, V.Mignotte for providing the pGΔA construct and J.F.Eleouet for the −112 PBGD construct. We thank members of Dr Yaniv's laboratory and N.Blumenfeld for critical reading of the manuscript. V.L. is supported by the Ministère de la Recherche et de la Technologie. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique and the Association pour la Recherche contre le Cancer.

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Received on June 9, 1992; revised on July 29, 1992