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The repressor which binds the –75 GATA motif of the GPB promoter contains Ku70 as the DNA binding subunit

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ABSTRACT

Glycophorin B (GPB) is an abundant cell surface glycoprotein which is only expressed in human erythroid cells. Previous functional analysis demonstrated that the repression of the GPB promoter is determined by the binding of a ubiquitous factor which recognizes a GATA motif centered at position –75. In erythroid cells this ubiquitous factor is displaced by the binding of the erythroid-specific factor hGATA1. Here, we have iden-tified the Ku70 protein as a candidate GPB repressor DNA binding subunit through the screening of a human HeLa expression library using the –75 GATA sequence as bait (one-hybrid method). Electrophoretic mobility shift assays demonstrated that the ubiquitous factor that binds the –75 GATA sequence was the Ku70–Ku80 (Ku) heterodimer. Co-transfection experiments demonstrated that overexpression of Ku70 in the K562 erythroleukeamic cell line resulted in transcriptional repression of the chloramphenicol acetyltransferase reporter gene when placed under the control of the wild-type GPB promoter. Conversely, no repression was observed when a mutation that abolished the binding of Ku was introduced in the GPB promoter construct. Altogether, these results indicate that Ku binds *in vivo* to the –75 WGATAR motif and is involved in negative regulation of the GPB promoter. These findings suggest that, besides its role in many functions, Ku is also involved in transcriptional regulation of erythroid genes.

INTRODUCTION

Several *cis*-acting sequences are involved in the transcriptional activity of erythroid genes, but a combination of three motifs, CCACC, the SP1 binding site and WGATAR, are the core elements of their regulatory sequences (1,2). Ubiquitous transcription factors bind to CCACC or SP1 sequences, whereas the transcription factor GATA-1, which is restricted to erythroid/megakaryocytic cells and to Sertoli cells of the testis, binds to the WGATAR motif (3–5). In addition to their presence within promoters, the WGATAR motifs are found in enhancer elements of erythroid genes and in the locus control region (LCR) of the human β-globin genes cluster.

The human erythrocyte glycophorins A, B and E (GPA, GPB and GPE) are typical examples of erythroid-specific membrane proteins which are encoded by a small gene family composed of three tandemly organized genes located on chromosome 4q28–q32 and which evolved by successive gene duplication events (reviewed in 6,7). GPA and GPB are the major red cell surface glycoproteins (85 and 10% of the PAS-positive material, respectively) that carry the MN and Ss blood group antigens, respectively, and act as ligands for viruses, bacteria and parasites, whereas GPE is poorly expressed. Recent studies have shown that the level of cell surface expression of GPA, GPB and GPE on erythroid cells is predominantly regulated post-transcriptionally by mRNA stability (8).

The molecular basis of erythroid-specific expression of the glycophorin genes has been investigated in some detail using the GPB promoter as a model (9). These studies, based on DNase I footprinting, mobility shift assays, deletion analysis and hGATA-1 transactivation of wild-type and mutant constructs measured by chloramphenicol acetyltransferase (CAT) assays in erythroid and non-erythroid cells, have shown that the –95 GPB promoter (–95 to +43 GPB fragment) could be subdivided into two main regions. (i) A proximal region (–60 to –1) which contains a binding site for hGATA-1 around position –37, and an SP1 binding site around position –50. These sites were shown to be necessary for the initiation of transcription. (ii) A distal region (–95 to –60) which contains a WGATAR sequence around position –75 that binds hGATA-1 and an unidentified ubiquitous protein(s) and an E-box on position –70.

Mutagenesis performed on the –95 GPB construct, which only allowed the binding of ubiquitous proteins on the –75 sequence, resulted in a complete repression of the proximal promoter activity in erythroid and non-erythroid cells. These findings demonstrated that the GPB promoter is under dominant negative regulation, due to the ubiquitous protein(s) that binds to the

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WGATAR –75 sequence. In erythroid cells, hGATA1 binding could remove repression of the GPB promoter by displacement.

This report will focus on the cloning of the ubiquitous protein(s) which binds to the –75 GPB sequence with the aim of identifying the protein(s) repressing the GPB promoter activity.

MATERIALS AND METHODS

Strains, media and microbiological technique

For the one-hybrid screen, the *Saccharomyces cerevisiae* strain YM954 was used (MAT**a**, *ade2, his3, leu2, lys2, trp1, ura3, gal4, gal80*) (10). Standard yeast genetic methods and media were used, as described in Sherman *et al.* (11). *Saccharomyces cerevisiae* was transformed after lithium acetate treatment as described by Gietz *et al.* (12). Yeast plasmid recovery was performed as described by Ausubel *et al.* (13). A human HeLa cDNA library constructed on the yeast shuttle vector pGADGH LEU2 (Clontech, CA) provided the expression of proteins fused to the Gal4 transcription activation domain.

Cell cultures, transfections and CAT assays

K562 cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum. The cells were transfected by electroporation at 200 V, 960 µF in 180 µl phosphate-buffered saline containing 10 mM HEPES pH 7.4, using 10^7 cellules per assay, with 5 µg of pCDNA3 plasmid together with 10 µg of the CAT reporter gene vector and 2 µg of RSV-luciferase construct. The luciferase activities were used as internal control values for the normalization of transfection efficiency. After transfection, cells were grown for 24 h before harvesting. Cells were lysed in 100 µl of 250 mM Tris, 0.2% Triton X-100, 5 mM dithiotreitol and 10% glycerol pH 7.8, and assayed for both luciferase (14) and CAT activities (9,15). The results were ascertained by repeating the experiments three times using two different DNA preparations. Sequences introduced in reporter plasmids for CAT assays contained the –75 GPB wild-type sequence 5′-CAGCTGATAGGC-3′ (lower strand) or the M9 oligonucleotide 5′-CACGTGATAAGG-3′ (lower strand).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts prepared either from the non-haematopoietic cell line HeLa or from the erythroleukaemic cell line K562. Nuclear extracts were purified from exponentially growing cells as previously described (16), according to Dignam's method (17). In addition, mobility shift assays were performed using partially purified proteins as follows. Crude HeLa extracts were applied to a column of heparin–agarose and eluted by a gradient of increasing KCl concentration (18). The proteins eluted at 200 mM KCl concentration were pooled, dialyzed against buffer D (50 mM Tris–HCl pH 8.0, 50 mM KCl and 20% glycerol) and stored at –80°C. The following oligonucleotides were used: the –75 GPB wild-type sequence, 5′-ATCATCAGCTGATAGGCAGGGGAG-3′ (lower strand); a –75 GPB mutated sequence (D13 oligonucleotide) in which the central GATA box was replaced by GACA, 5′-ATCATCAGCTGACAGGCAGGGGAG-3′ (lower strand); the HS3 FP1 sequence of the β-globin gene (19), 5′-GGAACCTCTGATAGACACATCTGGCACACC-3′ (lower strand); the adenovirus major late promoter (MLP) oligonucleotide containing an E-box, 5′-GTAGGC*CACGTG*ACCGGGT-3′ (20); the –75 mutated sequence M9, 5′-ATCATCACGTGATAAGGAGGGGAG-3′ (lower strand); the –37 GPB hGATA-1 oligonucleotide, 5′-TGGGCCTGGAAGATAACAGCTAGTA-3′ (upper strand). All binding reactions were carried out in 10 µl containing binding buffer (5 mM Tris–HCl pH 8.0, 25 mM NaCl, 0.5 mM dithiotreitol, 0.5 mM EDTA and 1% Ficoll) in the presence of 0.3 µg of poly(dI·dC) competitor DNA with 5 µg of protein. Oligonucleotides were 5′-labeled using the T4 DNA kinase and [γ-^{32}P]ATP (110 TBq/mmol). Approximately 15 000 c.p.m. of probe (~0.2 ng) were used in each binding mixture. Samples were incubated for 10 min at room temperature before loading onto a 4% polyacrylamide gel in 0.25× TBE buffer (22 mM Tris pH 8.3, 22 mM boric acid and 0.5 mM EDTA) and electrophoresed at 10 V/cm at room temperature. The gels were run for 2 h, dried and autoradiographed overnight.

For competition experiments, DNA binding reactions were allowed to reach equilibrium and a 250-fold excess of unlabeled specific competitor DNA was added to the binding reaction mixture. To determine the presence of the Ku protein within the retarded complexes, supershift assays were performed. The following anti-Ku monoclonal antibodies (21) were used: clone 162 (anti Ku70–Ku80 heterodimer); clone 111 (specific to the Ku80 subunit); clone S5C11 (specific to the Ku70 subunit). All these antibodies were either generous gifts from W. H. Reeves (University of North Carolina, Chapel Hill, NC) or purchased from NeoMarkers (Fremont, CA). Anti-USF1 and anti-USF2 polyclonal antibodies were donated by Dr M. Raymondjean (INSERM U129, Paris, France). Antibodies were added to the EMSA mixture for 10 min before loading (15 µg of monoclonal antibodies or 5 µl of polyclonal antiserum).

Plasmids

The CYC1–HIS3 fusion gene used for the one-hybrid screen has been described previously (22). The 5′ *cis*-acting sequence of the human GPB gene (corresponding to the –75 region) was cloned as three oriented copies (23) 200 bp upstream from the TATA box, owing to the unique *Xho*I cloning site. The resulting plasmid was linearized with *Nco*I (within the URA3 marker) and used to transform the YM954 strain. A control hybrid experiment was performed under the same experimental conditions using the M1 promoter region from the human aldolase A gene as bait (24). Stable uracil prototroph transformants were selected and correct integration events were verified by Southern blot analysis. In a second set of experiments, two different CYC1–HIS3 fusion genes, cloned on the pH39-670 yeast replicative plasmid TRP1 (D.Thomas, unpublished results) were used. They contained three oriented copies of either the –75 region of the *GPB* gene or the HS3 FP1 region of the human β-globin gene which were both cloned 200 bp upstream from the TATA box. The oligonucleotides used for the described cloning experiments were: GPB D13 oligonucleotide (above) and the HS3 FP1 oligonucleotide of the human β-globin gene (19).

The plasmids that provided expression of either the Ku70 or Ku80 factor under the control of the CMV promoter region in K562 cells were constructed using the pcDNA3 vector (Invitrogen, Leek, The Netherlands). The complete coding regions of the corresponding cDNAs were inserted downstream from the CMV promoter region between the unique *Bam*HI and *Xho*I cloning sites. For the CAT assays, the reporter vectors were constructed

by cloning either the wild-type or the mutated (M9 mutant) –95 to +43 region of the GPB gene in front of the bacterial CAT gene on the pBLCAT3 vector (25).

RESULTS

Characterization of proteins recognizing the –75 GPB region in a one-hybrid assay

To identify proteins that recognize the –75 region of the GPB promoter, we used the one-hybrid method, a strategy that successfully allowed the cloning in yeast of several DNA binding factors (22,23,26). We constructed a reporter sequence that contains three oriented copies of a 24 bp sequence corresponding to the –75 GPB region. The oligonucleotides were designed to match the naturally occurring GPB promoter region except that a cytosine was introduced in place of a thymine at position –76 (D13 oligonucleotide; see Materials and Methods). This mutation was shown to increase the binding of ubiquitous factors, without affecting the migration profiles in mobility shift assays (not shown). This oligonucleotide was inserted 200 bp upstream of the TATA box of the yeast CYC1 promoter (deleted for its own upstream activating sequences) and placed upstream of the *HIS3* gene. This construct was integrated into the genome of a *leu2*, *his3*, *gal4*, *gal80* yeast strain (YM954) at the URA3 locus. The resulting strain displayed a leaky histidine prototroph phenotype which was suppressed by the addition of 25 mM aminotriazole (AT), a specific inhibitor of the *HIS3* encoded product, to the medium. This strain was transformed by a human HeLa cell cDNA library that provided the expression of proteins fused to the Gal4 transcription activation domain. Transformants were selected directly for growth in the absence of both leucine and histidine and in the presence of 25 mM AT. From the screening of ~8×10^6 transformants, 72 colonies able to grow in the presence of 25 mM AT were obtained. Plasmid DNA was recovered from these colonies and used to retransform the parent strain to eliminate false positives. Among the 72 original plasmids, only 27 were found to lead to leucine and histidine prototroph transformants capable of growing in the presence of 25 mM AT. Restriction mapping as well as sequence analyses showed that these plasmids corresponded to eight different human cDNAs fused in-frame to the Gal4 activation domain. In order to assess the DNA binding specificity of the cloned factors, plasmids representing each of the eight different classes were introduced into two control yeast strains that differ by the CYC1–HIS3 fusion gene integrated at the URA3 locus. In the first one (YML0), the CYC1 promoter, deleted for its own UAS sequences, did not contain any additional sequence. In the second strain (YMM3), the CYC1 activating sequences were replaced by a fragment of the M1 promoter region from the human aldolase A gene. Among the eight plasmids tested, only one was shown to induce the expression of the *HIS3* gene in the two control strains, therefore suggesting that its encoded Gal4 fusion protein recognizes the CYC1 promoter region and not the –75 GPB promoter. This clone was therefore not studied any further.

The sequences of the remaining seven plasmids were compared with the GenBank database. Encoded sequences fused to Gal4 correspond to: (i) SP100 (27) and the myeloid viral ecotropic integration factor (28), two factors apparently capable of binding DNA although their target sequences were poorly characterized; (ii) the Zn15 factor (29), which recognizes the consensus sequence 5′-GACAG-3′ (a motif found within the GPB –75 region used for the one hybrid experiment); (iii) the 70 kDa subunit of the Ku autoantigen (30); (iv) three proteins [delta Max (EMBL accession no. AA045933), USF2 (31) and Mi (32)] belonging to the family of the basic helix–loop–helix (bHLH) DNA binding factors that recognize the CANNTG consensus motif (E-box). This correlates well with the presence within the GPB promoter of an E-box at position –70 which is close to the GATA box (T**CAGCTG***ATAG*G).

The –75 GPB region and the HS3 FP1 region of the human β-globin gene are recognized by similar factors

The multiplicity of the proteins isolated during the one-hybrid experiment as being capable of recognizing the –75 GPB region when they are expressed in yeast cells made it necessary to set up additional screening to assess the functional relevance of the above results.

As previously reported (9), mobility shift assays performed with nuclear extracts prepared from erythroid cells (K562) and a 5′-end-labeled wild-type GPB probe showed three major complexes (complexes 1–3, Fig. 1A). Complex 3 corresponded to the binding of hGATA1 to the –75 GPB region in erythroid cells. Mobility shift assay performed with non-erythroid nuclear extracts (HeLa) showed the same complexes 1 and 2 and a thin lower complex migrating at the same level as complex 3 in erythroid cells. Previous mutagenesis experiments indicated that complexes 2 and/or 1, but not complex 3, are involved in GPB repression (9). Therefore, complex 3 in non-erythroid cells was not investigated. Since the –75 GPB region includes an E-box sequence (CAGCTG) close to the WGATAR sequence and since three different bHLH factors were detected by the one-hybrid technique, we first tried to determine whether one of the observed complexes could be ascribed to the binding of one E-box-recognizing factor. Accordingly, competition assays were performed using an E-box oligonucleotide taken from the well-characterized adenovirus MLP (20). As shown in Figure 1A, addition of a 250-fold excess of cold E-box oligonucleotide clearly abolished complex 1 formation with erythroid as well as non-erythroid nuclear extracts in mobility shift assays. These results therefore suggest that complex 1 results from the binding of a ubiquitous protein recognizing the E-box (USF factor; see below) that juxtaposes the –75 WGATAR GPB region. Thus, we hypothesize that complex 2 represented the ubiquitous repressor factor that binds to the –75 WGATAR GPB sequence.

We examined several regulatory regions of erythroid-specific genes for the presence of a GATA box showing a high homology with the WGATAR –75 GPB sequence (TGATAGG). One was identified within the FP1 small regulatory region HS3, a component of the human β-globin LCR (19). We thus performed mobility shift assays with a 5′-end-labeled probe corresponding to this HS3 region. Specificity of the formation of the complexes was shown by testing the probe alone and self-competition. Two high molecular weight complexes migrating as complexes 2 and 3 were present in erythroid (K562) and non-erythroid (HeLa) extracts, albeit with intensities different to those observed with the GPB probe (Fig. 1B). More importantly, the EMSA profile obtained with the HS3 probe was efficiently competed by a 250-fold excess of unlabeled GPB oligonucleotide, suggesting that the same proteins bound GPB and HS3 oligonucleotides (Fig. 1B). These results indicated that the HS3 FP1 oligonucleotide could be used as a second screen to detect the protein(s) involved in complex 2 formation.

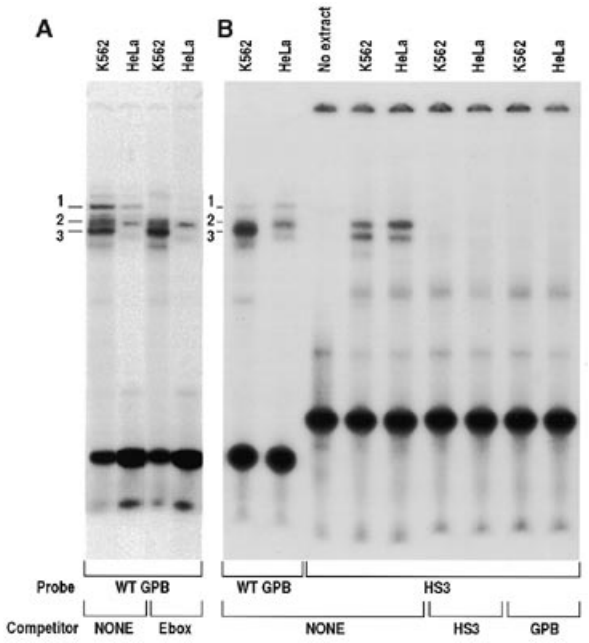

Figure 1. Protein–DNA complexes detected by EMSA. (**A**) EMSA performed with 5′-end-labeled wild-type –75 GPB oligonucleotide (WT GPB) and nuclear extracts prepared from erythroid K562 cells and non-erythroid HeLa cells. Positions of the three retarded complexes, 1–3, are indicated. A 250-fold excess of unlabeled E-box-containing oligonucleotide competed efficiently for complex 1 formation. (**B**) EMSA performed using 5′-end-labeled wild-type –75 GPB and HS3 FP1 oligonucleotides with nuclear extracts from K562 and HeLa cells as indicated. Specificity of complex formation was shown by testing the probe alone and self-competition. The unlabeled wild-type GPB oligonucleotide competed with complex 2 and 3 formation.

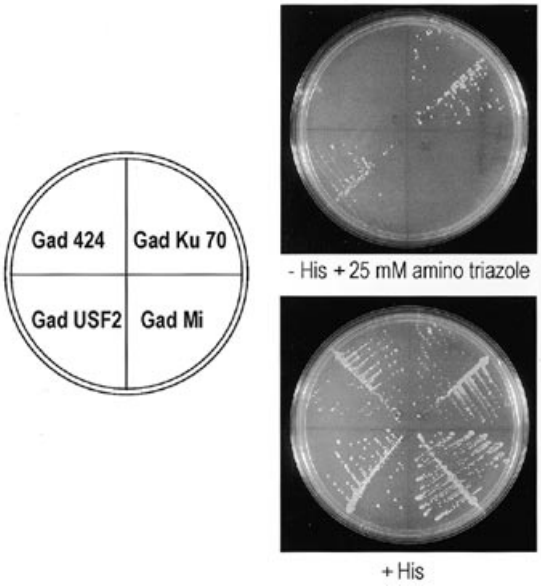

Figure 2. AT resistance of YM954 cells transformed by pH39-HS3. YM954 cells were co-transformed either by plasmids expressing the Gad Ku70, the Gad USF2 or the Gad Mi fusion proteins or by the parental vector pGad424. Transformants were streaked on a histidine-containing medium or on a medium lacking histidine but containing 25 mM AT. Only Gad Ku70 and Gad USF2 were leucine/histidine prototroph transformants capable of growing in the presence of 25 mM AT.

KU70 and USF2 are the only two proteins recognizing both HS3 and GPB –75 regions when expressed in yeast

We took advantage of the fact that the same protein(s) could bind the –75 GPB region and the HS3 regulatory region to further analyze and discriminate between the seven different proteins isolated in the one-hybrid screen (see above). A repeat of three oriented copies of D13 and HS3 were inserted within the CYC1–HIS3 region, 200 bp upstream from the TATA box, on the yeast replicative vector pH39-670. The two resulting plasmids, pH39-D13 and pH39-HS3, are ARS-centromere-based plasmids carrying the TRP1 marker. Plasmids for the seven classes of protein identified in the first screen were transformed into the recipient yeast strain YM954 together with either the pH39-670 parental vector, the pH39-D13 plasmid or the pH39-HS3 plasmid. Transformants were selected as cells growing in the absence of both leucine and tryptophan. For each combination, four independent selected colonies were replicated and tested for their capacities to grow in the absence of histidine and in the presence of 25 mM AT. The results of this assay showed that the plasmids expressing the 70 kDa subunit of Ku and that expressing USF2 are the only ones capable of inducing the expression of both CYC1–HIS3 fusion genes which are present on the pH39-D13 and pH39-HS3 plasmids (Fig. 2). Among the 27 clones isolated by the one-hybrid screen, two different Ku70-encoding plasmids were isolated. Both encode the C-terminal parts of Ku70 (from residue 280 to 609 and from residue 363 to 609) (30) that contain a DNA binding domain (536–609) similar to a helix–turn–helix (HLH) motif and an adjacent basic domain (33–35).

In the case of USF2, two different plasmids were isolated. It appeared that one of the USF2-encoding plasmids corresponded to the USF2a cDNA (31). During the screening, we found that the clones expressing USF2 grew in the absence of histidine and in the presence of 25 mM AT to a lesser extent when co-transfected with the pH39-HS3 plasmid (partial complementation) than when co-transfected with pH39-D13. Indeed, the HS3 oligonucleotide also contained an E-box and these results might account for the different E-boxes sequences (CANNTG) present on the D13 (CAG**C**TG) and HS3 (CAG**A**TG, lower strand) oligonucleotides. It is noteworthy that the consequence of a 1 nt difference between the D13 and HS3 E-boxes is even more drastic *in vitro*, since complex 1, detected with the GPB probe in EMSA assays, was not detected with the HS3 oligonucleotide (Fig. 1B). This might be due to the different sensitivities of the two techniques used.

As the USF2 factor was previously described to bind the HS2 LCR of the human β-globin genes cluster (36), supershift assays were performed using the –75 wild-type GPB oligonucleotide and anti-USF antibodies. USF2 may effectively correspond to the bHLH protein that binds the GPB –70 E-box, since complex 1 formation was inhibited when either anti-USF1 or anti-USF2 polyclonal antibodies were added (see below).

Altogether the results suggest that Ku70 could be part of the complex 2 recognizing the core WGATAR of the –75 GPB and the HS3 FP1 regions.

Ku70–Ku80 heterodimer binding to the –75 GPB region

We used specific antibodies to directly address the possibility that Ku70 may be one of the ubiquitous factors recognizing the –75 GPB region in mammalian cells. Since this protein is known to bind to DNA as heterodimeric complexes (37), EMSA experiments were performed with antibodies specific for both the known dimerization partners and the –75 wild-type GPB oligonucleotide.

When anti-Ku antibodies were added to the EMSA mixture, we observed numerous non-specific complexes using erythroid and non-erythroid nuclear extract, which made the gels difficult to interpret (data not shown). To circumvent this problem, we used partially purified HeLa nuclear extracts. Heparin–agarose chromatography was performed (18) and we found that the fractions eluted at 200 mM KCl (called H 0.2) contained the factor(s) responsible for complex 2 but not for complexes 1 and 3 formation (Fig. 3A). When the H 0.2 fraction was used in EMSA, addition of the antibody directed against the Ku70–Ku80 heterodimer resulted in the disappearance of complex 2 (Fig. 3B). The addition of an antibody specific for the Ku80 subunit resulted in a partial inhibition of the bandshift. The result obtained with anti-Ku70 monoclonal antibody was not informative, since this antibody gave a high non-specific background (not shown). As a control, complex 2 formation was not affected by the presence of an irrelevant antibody (monoclonal antibody directed against the red cell Xg^a membrane protein). Taken together, the results of both the one-hybrid experiments and gel mobility shift assays strongly indicate that complex 2 formation corresponds to the binding of the Ku70–Ku80 heterodimer with the –75 region of the GPB promoter.

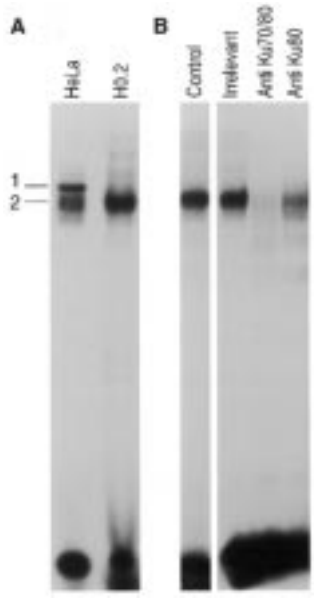

Figure 3. Effect of monoclonal antibodies to Ku protein subunits on protein–DNA complexes detected by EMSA. EMSA were performed with 5′-end-labeled wild-type –75 GPB sequence and HeLa extracts. (**A**) Retarded complexes detected with the HeLa cell nuclear extract (complexes 1 and 2) and the HeLa heparin–Sepharose purified fractions (H 0.2) (only complex 2). (**B**) Anti-Ku antibodies were added to the EMSA mixture, carried out with HeLa fraction H 0.2, as indicated at the top of the gel. A control was performed without antibody (left lane). The lane 'irrelevant' refers to the assay performed with addition of a murine monoclonal antibody which has no effect on complex 2 (antibody directed against the red cell Xg^a membrane protein). The following two lanes refer to assays performed by adding murine monoclonal antibodies specific for the heterodimer Ku70–Ku80 (clone 162) and to the Ku80 subunit (clone 111), respectively. The amount of complex 2 was severely reduced or markedly diminished in the presence of antibodies to the Ku70–Ku80 heterodimer or Ku80. Controls and samples with antibodies were run on the same gel; high protein concentration in lanes with antibodies resulted in an altered migration of free probe.

Point mutations and transient transfection experiments demonstrated that Ku is involved in the repression of GPB promoter expression

In order to evaluate the functional significance of Ku70 binding on the –75 GPB region, we first performed mutagenesis on the –75 GPB oligonucleotide looking for a sequence that allows complexes 1 and 3 but impairs complex 2 formation. As shown in Figure 4, three high molecular weight complexes were evident in the K562 cells with the mutated M9 GPB oligonucleotide (TGATAG→TGATA**A**) in EMSA. Complex 3 is present only in erythroid (K562) cells and corresponds to the binding of hGATA-1, as determined by competition assay with a 250-fold excess of cold hGATA-1 oligonucleotide. Two other complexes were present, one migrating as complex 1, and the second, indicated by an asterisk, migrating below complex 1. Addition of anti-USF1 or anti-USF2 antibodies clearly resulted in a complete disappearance of these two complexes, indicating that both of them are due to USF heterodimeric isoforms. Complex 2, corresponding to the binding of Ku70–Ku80 on the GPB wild-type oligonucleotide, is not observed with the M9 GPB oligonucleotide.

Transient transfection experiments were performed to address the functional role of Ku70 in the transcriptional regulation of GPB. All the transfections were performed in K562 erythroid cells as: (i) the –95 GPB–CAT construct was shown to be completely inactive in HeLa cells; (ii) previous mutagenesis experiments showed that the repressor is also functional in erythroid cells (9).

The GPB reporter genes consisted of the bacterial CAT gene placed under the control of either the wild-type (pBLGPB–95wt) or a mutant (pBLGPB–95M9) form of the GPB promoter. The pBLGPB–95M9 mutant contained the mutated –75 GPB sequence M9. The reporter constructs were transfected into K562 cells together with pCDNA3 plasmids expressing either Ku70 or Ku80 factor, or both. As controls, the two reporter constructs were co-transfected with the pcDNA3 parental vector.

As shown in Figure 5A, co-transfection of the reporter plasmids with the control pcDNA3 vector alone indicated that the M9 GPB mutation resulted in a 2–3-fold increase in the CAT activity driven by the GPB promoter. Overexpression of the Ku70 subunit led to a 2-fold decrease in the activity of the –95 wild-type GPB promoter, whereas no effect was observed with the M9 GPB mutant. Conversely, overexpression of the Ku80 subunit had no effect on the –95 wild-type GPB promoter activity and thus this plasmid was not analyzed in co-transfections with the –95 M9 GPB promoter. Co-expression of the two subunits of Ku resulted in the same effect as co-transfection with the Ku70 subunit alone, as determined on the –95 wild-type GPB promoter.

Altogether, these results indicate that the –75 GPB region is a specific DNA target for Ku70 in mammalian cells. To provide further evidence that Ku70 recognizes the –75 GPB region, a plasmid encoding the entire Ku70 protein fused to the strong

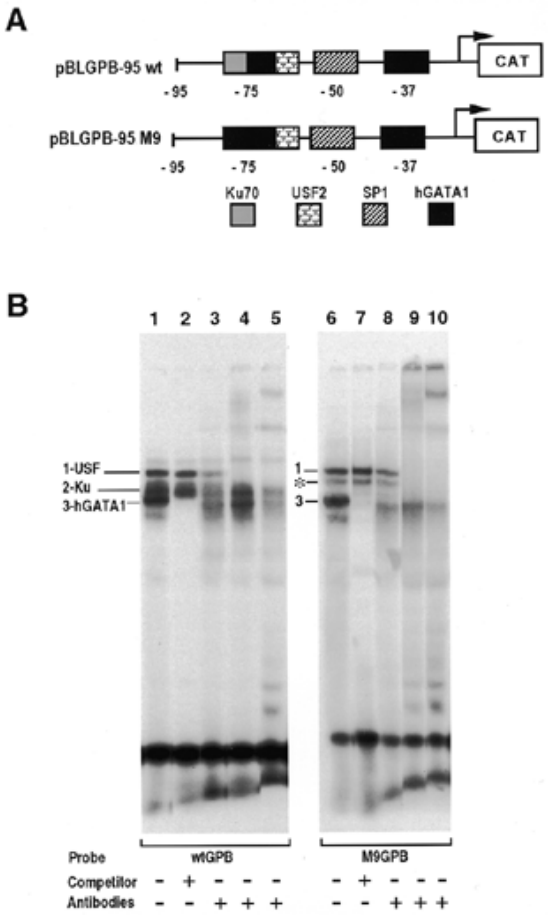

Figure 4. Reporter constructs and EMSA analysis. (**A**) Schematic representation of reporter plasmids (pBLGPB–95wt and pBLGPB–95M9) used for transient transfection of K562 erythroid cells. Symbols for the *cis*-acting sequences at positions –75, –37 and –50 of the GPB promoter and factors which bind to them are indicated by different boxes. hGATA1 and SP1 bind to boxes –37 and –50, respectively, whereas the box at position –75 of pBLGPB–95wt binds Ku, hGATA1 and USF (as shown in this report). Mutation in the –75 box on plasmid pBLGPB–95M9 abolishes Ku but not hGATA1 and USF binding. (**B**) EMSA performed using 5′-end-labeled wild-type –75 GPB and M9 GPB mutated oligonucleotides were incubated with K562 nuclear extracts. Numbers 1–3 in the left margin refer to the three complexes obtained with wild-type GPB oligonucleotide**.** The only complexes obtained with the mutated M9 GPB oligonucleotide are complex 1, complex 3 and another complex indicated by an asterisk. Using the –75 wild-type GPB (lanes 1–5) and the M9 GPB (lanes 6–10) probes with erythroid (K562) nuclear extracts, adjunction of an unlabeled hGATA-1 probe competed with complex 3 formation (lanes 2 and 7). Adjunction of immune serum directed against USF1 (lanes 4 and 9) or USF2 (lanes 5 and 10) results in the disappearance of both complex 1 and the complex marked by an asterisk (heterodimeric isoform of USF observed only with the M9 GPB probe). Preimmune serum (lanes 3 and 8) does not affect USF–DNA complex formation.

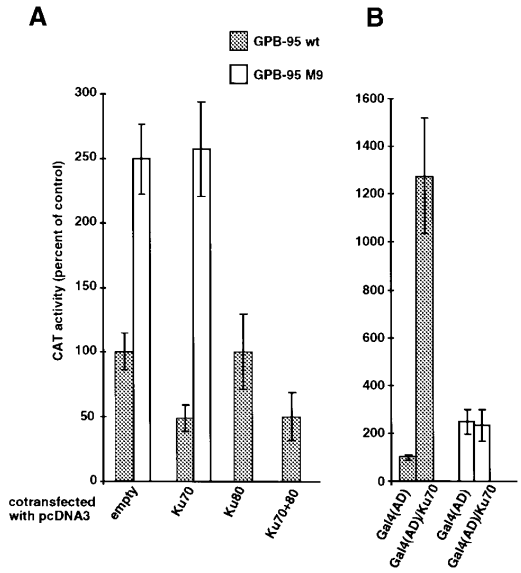

Figure 5. CAT assays in transfected K562 cells. CAT assays performed on K562 cells transfected with the reporter gene pBLGPB–95wt (dark bars) or pBLGPB–95M9 (open bars). (**A**) CAT activity obtained after co-transfection of pBLGPB-CATwt with an empty plasmid was used as reference. Basal level of the mutant GPB–95M9 reporter gene (which does not bind Ku70) results in a 2–3-fold increase in CAT activity compared with the GPB wild-type. Co-transfections with plasmids encoding Ku70 alone or Ku70+Ku80 show ~50% reduction in CAT activity of the GPB–95wt reporter gene, but have no effect on the CAT activity of the mutant GPB–95M9 reporter. Co-transfections with plasmids encoding the Ku80 subunit alone have no effect on the CAT activity of the pBLGPB-CAT–95wt reporter plasmid. (**B**) Co-transfections were performed using Ku70 cDNA fused in-frame to the Gal4 activation domain in a pcDNA3-derived expression plasmid or Gal4(AD) domain cDNA alone. A very high CAT activity was detected with the GAL4(AD)–Ku70 chimera only when the reporter plasmid contained a target sequence able to bind Ku (GPB–95wt but not GPB–95 M9). Control assays showed that GAL4(AD) alone did not induce any CAT activity.

transactivator domain of the Gal4 transcription factor protein was constructed. The encoded chimeric protein should associate the binding capacity of Ku with an activator property. Overexpression of Gal4(AD)–Ku70 resulted in a huge increase in –95 wild-type GPB transcription activity in K562 cells compared with that of the plasmid expressing Gal4(AD) alone. As expected, no effect was observed on the transcription activity of the M9 mutant (Fig. 5B).

DISCUSSION

In this report, we were able to identify, by the one-hybrid system in yeast and EMSAs, Ku70 as the ubiquitous protein which binds to the –75 GPB promoter region. We demonstrated that binding of Ku70 to the –75 region of the GPB promoter accounted in part for complex 2 formation and decreased the GPB promoter activity by 50% in co-transfection assays. Conversely, the GPB promoter mutant that did not allow the binding of Ku70 resulted in a 2–3-fold increase in GPB promoter activity, as evaluated in transfection assays.

The Ku factor is a nuclear DNA binding protein composed of two subunits, p70 and p80, with apparent molecular masses of 70 and 80 kDa, respectively. When associated with a third catalytic subunit (p350) it forms a complex which has DNA-dependent kinase activity (38–40). Ku is an abundant nuclear factor with several functional roles. Binding of Ku was shown to occur at the ends of double-stranded DNA, independently of the DNA sequence, but it binds also to nicks, gaps and DNA transitions (39). Once bound to DNA ends, Ku is able to translocate along the DNA fragments (41). These properties are important for several Ku functions like V(D)J recombination, DNA double-

strand break repair (38,39) and nucleotide excision repair (42). It was also claimed that Ku plays a role in DNA replication (43–45).

Evidence is accumulating that Ku could also bind to DNA on a specific target sequence. It is involved in enhancement or repression of gene transcription mediated by Pol I and Pol II (reviewed in 38,39). For instance, Ku binding to the NRE1 sequence (negative regulatory element 1) in the long terminal repeat of mouse mammary tumor virus represses glucocorticoid-induced MMTV transcription (46,47). It also binds specifically to the HSE motif involved in the regulation of heat shock protein HSP70 expression and down-regulates HSP70 gene transcription. Interestingly, Ku competes for binding at this site with another up-regulating transcription factor (48,49).

Ku binds to a specific sequence within the GPB promoter. This is based on a convergent series of arguments: (i) EMSA using wild-type and mutated oligonucleotides from the –75 GPB region determined the WGATAR sequence and the surrounding nucleotides as the anchor point of the ubiquitous protein (now identified as Ku); (ii) oligonucleotides which were able to compete with complex 2 formation all contained the internal WGATAR sequence; (iii) absence of a DNase I footprint at the extremities of the –166 to +43 GPB promoter fragment (9) suggested that binding at the ends of double-stranded DNA fragments did not occur in our *in vitro* experiments; (iv) co-transfection in K562 cells of a plasmid coding for Ku70 along with the GPB promoter fused to a CAT reporter gene showed a reduced transcriptional activity, while no effect of Ku70 was seen in co-transfection with the M9 mutant which did not allow complex 2 formation; (v) the experiments with the GAL4(AD)–Ku fusion protein showed that transactivation occurred when Ku70 bound DNA on the specific –75 region of the GPB promoter containing the WGATAR sequence.

Previous results indicated that the absence of hGATA-1 in non-erythroid cells was not sufficient to inactivate the GPB–CAT reporter gene (9). In fact, when Ku70 binding was impaired by mutation in the promoter sequence and hGATA-1 was absent (non-erythroid cells) or impaired by mutation (erythroid cells), a residual 25% of activity remained. However, when hGATA-1 binding alone was impaired (erythroid cells) or absent (non-erythroid cells), binding of the repressor to the –75 GPB promoter region led to complete repression of GPB transcription.

Experimental data presented in this report support the evidence that the Ku70 factor is the DNA binding subunit of the GPB transcription repressor. Nevertheless, co-transfection experiments performed with the Ku70 factor did not result in a complete abolition of GPB–CAT activity. We conclude that we did not obtain the full repressor activity with an exogenous Ku70 recombinant construct. One explanation for the absence of complete repression might be that Ku70 acts as a strong repressor only after some post-translational modifications, which could not be obtained with the recombinant constructs we used in co-transfections. In this case, the decreased wild-type GPB–CAT activity that we observed in co-transfection experiments might be due to the displacement of hGATA-1 activator factor from the –75 GPB promoter region.

Recently, Shelton *et al*. (50) described a phylogenetic analysis of the hypersensitive site 3 of the β-globin LCR (HS3) and reported the presence of the CSBP-2 factor (conserved sequence binding protein 2), which recognizes several sequences within the HS3 core fragment. Among these, the WGATAR sequence was previously identified as a binding site for an unknown ubiquitous protein called factor X (19). Here, we have shown through one-hybrid experiments that Ku recognizes this sequence. From a comparison of the sequences bound by CSBP2 in several species (50), it was deduced that the SHBAGAYAS (S is for G or C; H for A, T or C; B for T, C or G; and Y for C or T) sequence was the DNA recognition core motif for CSBP-2. Interestingly, this sequence diverges by only 1 nt from the GATA-containing motif around position –75 of the GPB promoter (9). This is also in agreement with the previously proposed Ku binding site (51). These findings, together with the relative migration position of retarded CSBP2 complex in EMSA (which is similar to the mobility of complex 2), strongly suggests that CSBP2, factor X and Ku (the protein forming complex 2 with the wild-type GPB probe) are one single protein entity. Moreover, Shelton *et al*. (50) indicated that CSBP2 was not likely to be a member of the zinc finger class of transcription factors. As reported in this paper, the Ku70 ubiquitous factor we cloned belongs to the bHLH class of transcription factors. No information is available on the functional role played by CSBP2 (Ku) in the HS3 context.

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