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Identification of human GC-box-binding zinc finger protein, a new Krüppel-like zinc finger protein, by the yeast one-hybrid screening with a GC-rich target sequence

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Abstract A new human zinc finger DNA-binding protein was identified by using a yeast one-hybrid selection system. Two versions of the cDNA, encoding the same protein, were detected that differ for a 584 bp extension at the 5′ region. Sequence analysis showed that the longer clone is a full length version containing part of the 5′ untranslated region. The smaller version was fused in frame with the yeast GAL4 activation domain whereas the 5′ region of the longer clone displayed a stop codon interrupting the fusion with the GAL4 domain. Nevertheless, this clone activated the yeast HIS3 reporter gene with the same efficiency as the smaller version. Sequence comparison of the derived protein with the database showed that it belongs to a family of zinc finger DNA-binding proteins which regulate the expression of genes involved in cell proliferation. Expression of the protein in an in vitro system, DNA-binding studies and genetic experiments identify this factor as a new zinc finger DNA-binding protein which binds GC-rich sequences and contains a domain probably functioning as a transcriptional activator. The new human protein identified in this study was therefore named GC-box-binding zinc finger protein).

Key words: Yeast one-hybrid screening; GC-box; Human zinc finger DNA-binding protein; Transcription activation

1. Introduction

The yeast one-hybrid system is an in vivo assay aimed to identify cDNA sequences for proteins which can bind a target DNA and activate expression of a reporter gene in yeast cells [1]. This method is of peculiar advantage especially when DNA-binding factors are present in a minute amount in the cell, making standard purification techniques cumbersome and time consuming. We wanted to apply this approach to search for DNA-binding proteins able to interact with the 3′ end of the D-loop of human mitochondrial DNA (mtDNA). This region contains conserved sequences, known as termination-associated sequences (TAS), which might constitute *cis* elements for termination of mtDNA synthesis [2–7]. In vivo and in vitro experiments previously suggested the presence of proteins that bind such sequences and might be involved in the regulation of the mtDNA copy number [8,9].

In this study, we report the cloning of a human cDNA encoding a new member of the Krüppel zinc finger protein family [10], by using a non-traditional yeast one-hybrid screening approach. Because the protein is able to bind GC-rich sequences, we have named it GC-box-binding zinc finger protein (GZP1). The observation that the protein is able to activate the expression of the HIS3 reporter gene even in the absence of the GAL4 domain points toward a transcription activation function of GZP1. Furthermore, the finding that GZP1 is most likely a nuclear factor interacting with a mitochondrial target sequence in the one-hybrid screening is of importance for further experiments with this system.

2. Materials and methods

2.1. Yeast one-hybrid screening and cDNA characterization

To construct the target DNA for selecting DNA-binding domains encoded in the human libraries, a 369 bp fragment (nucleotides (nt) 16 049–16 417) was PCR-amplified from human fibroblast total DNA with primers H-TAS-For (nt 16 049–16 070) and H-TAS-Rev (nt 16 417–16 396). mtDNA positions are according to Anderson et al. [11]. The product was subcloned into the pMOSBlue vector (Amersham), recovered as an *Eco*RI-*Xba*I fragment of 420 bp and inserted into reporter plasmid pHISi-1 (Clontech) in front of the HIS3 gene, generating the TAS-HIS3 reporter cassette. The recombinant plasmid was linearized with *Xho*I and introduced into the genome of the yeast strain YM4271. For all transformation procedures, lithium acetate-polyethylene glycol treatment was followed [12]. The basal activity of the HIS3 gene was sufficient to identify colonies that have integrated the construct into the genome. They were further checked for growth on medium lacking histidine (His^- medium) in the presence of 10 mM 3-aminotriazol (3-AT). Cells whose growth was inhibited under these conditions were chosen as the host for the library screening. Two Matchmaker cDNA libraries (Clontech) from human testis and fetal liver were used with their cDNA sequences cloned into the vector pACT2 that encodes the GAL4 activation domain.

Library plasmids were prepared by alkaline lysis [13] and transformed into the aploid yeast strain Y187 (MAT α) generating about 2×10^6 independent transformed yeast colonies for both libraries. Aliquots of transformed cells were mated for 16 h at 28°C with the aploid yeast strain YM4271 (MAT a) containing the TAS-HIS3 reporter cassette. The resulting diploids were plated on selective medium lacking histidine and leucine but containing 10 mM 3-AT and incubated at 28°C. After 3–5 days, positive colonies were observed from screenings with both libraries. They were tested on minimal plates with increasing concentrations of 3-AT ranging from 5 to 60 mM.

Plasmids from putative positive clones were isolated by standard techniques [13] and transferred into RR1 and DH5α *Escherichia coli* strains. For back transformation, the putative positive plasmids were individually re-introduced into yeast cells containing the TAS-HIS3 reporter cassette and tested for growth on selective medium. The

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nucleotide sequence was determined on both strands by the biochemical method of Sanger and by automated sequencing and used to query the GenBank database. Sequence comparisons were made by using Clustal software [14].

2.2. In vitro translation of GZP1 cDNA

A plasmid construct bearing the short form of GZP1 suitable for in vitro translation was obtained as follows. The corresponding cDNA insert in the pACT2 plasmid was PCR-amplified (Expand High Fidelity PCR System Boehringer) with a forward primer containing the *Sal*I site and the sequence of the initiation codon in the Kozak consensus [15] and with a reverse primer encompassing the *Xho*I site of the pACT2 polylinker. The product was then cloned into the same restriction sites of pCITE plasmid (Novagen). The correct nucleotide sequence of the construct was verified. The protein was synthesized using 1 μg of recombinant plasmid and the TNT Coupled Reticulocyte kit (Promega) according to the manufacturer's protocol, in the presence or in the absence of [^{35}S]methionine.

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was performed in a 20 μl volume containing 20 mM Tris-HCl, pH 7.9, 10 mM glycerol, 6 mM $MgCl_2$, 1 mM EDTA, 100 μM $ZnSO_4$, 0.4 ng labelled probe and 6 μl of in vitro translated protein. The gERE probe [16] was prepared by annealing the complementary oligonucleotides 5′-ATGGGGCGGGGTGGGGGG-3′ and 5′-ATCCCCCCACCCCGCCCC-3′. Probes TAS1 and TAS2 were double-stranded oligonucleotides designed on the human mtDNA sequence [11], from nt 16 143 to nt 16 196 and from nt 16 301 to nt 16 360, respectively. Probes were then end-labelled with [α-^{32}P]dATP, using Klenow enzyme. For competition experiments, varying amounts of different double-stranded oligonucleotides were included in the reaction. After incubation at room temperature for 30 min, samples were analyzed on a non-denaturing 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer.

2.4. Transcription activation activity test

A plasmid construct bearing the short form of GZP1 for an activation test was obtained as follows. The insert was PCR-amplified (Expand High Fidelity PCR System Boehringer) from the corresponding recombinant pACT2 plasmid, using a forward primer containing the *Xba*I site and the sequence of the initiation codon in the Kozak consensus [15] and a reverse primer encompassing the *Bgl*II site of the pACT2 polylinker. The *Xba*I-*Bgl*II fragment was inserted into *Xba*I-*Bam*HI sites of the YEp351-ADH vector [17] and the correct nucleotide sequence of the construct was verified. Plasmids were used to transform yeast cells containing the TAS-HIS3 reporter cassette. Transformants were tested for their growth ability on His$^-$ plates with increasing amounts of 3-AT as specified in Table 1.

3. Results

3.1. Isolation of human DNA-binding factors by using a yeast one-hybrid selection system

In order to identify and clone the cDNA for DNA-binding proteins interacting with the TAS region of human mtDNA, we used a modification of the classic one-hybrid screening [1], which makes use of the mating procedure for yeast transformation. This protocol, originally developed for the two-hybrid system [18], employs two yeast strains of a different mating type (YM4271 (MAT a) and Y187 (MAT α)) which are transformed with the reporter plasmid and with the entire cDNA expression library, respectively. After mating, the screening of an entire cDNA library can be performed much faster with respect to the traditional procedure as it is limited only by the number of plates that can be handled. Moreover, yeast cells can directly express the fusion protein without delay which avoids losses of positive clones as sometimes observed in the traditional transformation procedure.

To construct the DNA target site, a 369 bp fragment containing the TAS sequences was inserted into the reporter vector pHISi-1 in front of the yeast HIS3 gene and introduced into the genome of the YM4271 yeast strain. Two human cDNA plasmid libraries from fetal liver and testis tissue, cloned into the pACT2 vector, were transformed into the Y182 yeast strain. A total of seven independent colonies showing good growth on selective media were selected. They were tested on minimal plates with different amounts of 3-AT and good growth was observed up to a concentration of 60 mM 3-AT. Separate back transformation of the isolated recombinant pACT2 plasmids into the yeast reporter strain always resulted in the same good growth even at high levels of 3-AT. Restriction analysis of cDNA inserts from the seven clones allowed them to be grouped into two classes of five and two clones that represent two versions of the same DNA insert (Fig. 1). Five clones contained a cDNA insert of 3097 bp whereas in the other two, the insert was 584 bp shorter. Sequence analysis revealed long open reading frames for both types of cDNA inserts. The 3′ region always included the polyA tail and was found to be identical whereas the 5′ region exhibited differences. The longer insert represents a full length cDNA clone even with part of the 5′ untranslated region (5′ UTR) (EMBL accession number AJ132592). Interestingly, a stop codon was found in the 5′ UTR that interrupts the continuous reading frame from the upstream yeast GAL4 domain in the fusion protein. On the contrary, the shorter cDNA insert (EMBL accession number AJ132591) was fused in frame with the GAL4 domain.

The longer reading frame predicts a 895 amino acid long polypeptide whose main features are summarized in Fig. 2. The protein contains four C_2H_2 zinc fingers (from residue 263 to 368) that are separated by the consensus motif $S^TG^REK^RPF^Y$. This identifies the protein as a possible member of the Krüppel zinc finger family [10]. Other features of the protein are the presence of tracts of poly-glycine (residues 4–37), poly-proline (residues 86–96) and poly-histidine (residues 141–148) and of a basic domain (residues 219–236) con-

Table 1
Growth test of the YM4271 yeast strain bearing the TAS-HIS3 cassette on minimal medium with different concentrations of 3-AT

TAS-HIS3 reporter yeast strain	3-AT concentration					
	0 mM	5 mM	15 mM	30 mM	45 mM	60 mM
No plasmid	+	−	−	−	−	−
pACT2-Short GZP1	+	+	+	+	+	+
pACT2-Long GZP1	+	+	+	+	+	+
YEp351-ADH-Short GZP1	+	+	+	+	+	+

The yeast strain carries the HIS3 cassette as a genomic copy. This strain was transformed with different constructs of GZP1 cDNA cloned into the pACT2 vector with the yeast GAL4 activation domain or the yeast YEp351 vector that lacks the GAL4 activation domain but has the yeast ADH promoter. When cloned into pACT2, Short GZP1 cDNA was in frame with the yeast GAL4 activation domain, while Long GZP1 was not, as it contains a stop codon in the 5′ UTR. Growth of the colonies was evaluated after 2 days: +, good growth; −, no growth.

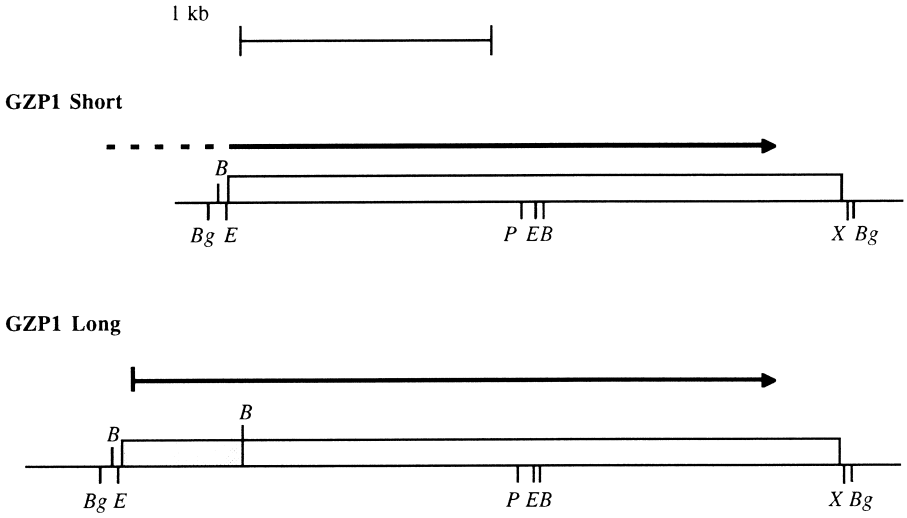

Fig. 1. Restriction map of GZP1 Short and GZP1 Long cDNA clones identified by the one-hybrid screening. The human cDNA inserts, cloned as *Eco*RI-*Xho*I fragments, are represented by blocks. A thin line indicates flanking sequences of the pACT2 vector. The gray box in the long insert labels sequences that are not found in the shorter fragment. The difference between the short and long clone is due to 584 bp starting from the identical priming site used for cDNA construction. Arrows mark the length and direction of the open reading frames. The reading frame of GZP1 Short cDNA is in frame with the upstream GAL4 activation domain (indicated by a broken line), whereas the GZP1 Long insert has a reading frame that starts from its own first ATG. Abbreviations for restrictions are the following: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; X, *Xho*I).

taining a bipartite nuclear localization signal [19]. Another nuclear localization signal is placed between residues 403–409. A BLASTP search of the protein database revealed a consistent similarity (28% identity plus 30% similarity) with the human protein ZBP-89 [20]. ZBP-89 is a zinc finger DNA-binding protein that recognizes GC-rich sequences and is involved in the control of the expression of several promoters and inhibits cell proliferation [21–25]. Therefore, it is likely that our protein was selected for its ability to recognize and bind the GC-rich tracts contained in the mtDNA target fragment [11]. On these bases, the protein was named GZP1. The alignment of the amino acid sequences of GZP1 and ZBP-89 is reported in Fig. 3. GZP1 is 134 amino acid longer than ZBP-89, this difference being mostly due to the amino-terminal extension of GZP1. This implies that ZBP-89 lacks the poly-histidine, poly-proline and poly-glycine tracts present in GZP1. On the other hand, GZP1 does not contain the acidic domain observed in ZBP-89 between residues 56 and 98. The four zinc fingers, the consensus motif and the nuclear localization signals are found in corresponding positions in both GZP1 and ZBP-89.

3.2. Structure-function studies of recombinant GZP1

In order to assess the binding capacity of GZP1, the shorter version of its cDNA was subcloned into the expression vector pCITE and subjected to in vitro transcription-translation in a rabbit reticulocyte lysate. A single product of about 90 kDa was observed after SDS-gel electrophoresis (Fig. 4A). The synthesized protein was assayed in gel-shift experiments using an oligonucleotide as a probe (gERE) which is known to be bound by zinc finger proteins that, like ZBP-89, recognize GC-rich sequences [21]. Fig. 4B shows that the protein is able to bind the probe, thereby producing a retarded band. The binding is sequence-specific as the retarded band disappears when increasing amounts of the same cold oligonucleotide are added to the reaction mixture. On the contrary, large amounts of an heterologous oligonucleotide did not compete with the gERE probe in binding to the protein (data not shown). The synthesized protein also formed a complex (Fig. 4C) with the oligonucleotide TAS1 which is comprised in the target used for the one-hybrid screen and contains a GC-box. The complex was specific as confirmed by a competition assay. No complex was formed when probe TAS2, which does not contain GC-rich sequences, was used (data not shown). This confirms that our protein was selected for its ability to recognize and bind the GC-rich tracts contained in the mtDNA target fragment [11].

The finding that the complete GZP1 gene is not fused with the yeast GAL4 activation domain but still activates the HIS3 gene efficiently in the yeast one-hybrid screening prompted us to address the question whether the short form is also able to activate the HIS3 gene on its own when separated from the GAL4 activation domain. For this purpose, the short fragment was cloned into the yeast expression vector YEp351 under the control of the yeast ADH promoter but without the GAL4 activation domain. After introduction of this construct into the yeast reporter strain bearing the TAS-HIS3 cassette, good growth was observed in 3-AT-containing His^- medium (Table 1). The growth under selective conditions was comparable to that observed for plasmids pACT2-Long GZP1 and pACT2-Short GZP1 when transformed into the same reporter strain. These results indicate that the GZP1 gene product does contain a transcription activation domain of its own. Additionally, the presence of a nuclear localization

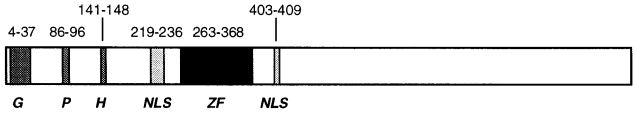

Fig. 2. Domain structure of the GZP1 protein. The protein is 895 amino acids long. Characteristic domains and their amino acid positions are shown. G: glycine-rich region; H: poly-histidine region; P: proline-rich region; ZF: four zinc finger domains of the C_2H_2 type; NLS: nuclear localization sequences.

domain is confirmed, since YEp-351 vector does not provide the expressed protein with a nuclear localization signal [17], as does the GAL4 fusion plasmid pACT2.

4. Discussion

In this paper, we report the isolation of a human cDNA for a new zinc finger DNA-binding protein, GZP1, that belongs to a protein family interacting with GC-rich sequence elements [10]. By using a yeast one-hybrid selection system, we found two classes of clones as the only positive signals differing only in their 5′ regions. The short version of GZP1 does not contain the initiating methionine and is in frame with the yeast GAL4 activation domain. On the contrary, the long version, which represents the complete protein, is not fused in frame with the GAL4 domain, as a stop codon between the two sequences, probably belonging to the 5′ UTR of GZP1, interrupts the frame. However, both versions of GZP1 mediate the same good growth of the yeast strain even at high concentrations of 3-AT. The conclusion from these findings is that in yeast, the long version is translated from the first possible ATG as the start codon. This would imply that the human protein contains a nuclear import sequence (or sequences) of its own and also a transcription activation domain that is functional in yeast. The GZP1 sequence indeed displays two canonical nuclear targeting sequences, one in the main basic domain between residues 219 and 236 and the other between residues 403 and 409. This finding is further verified by expressing the short version of the GZP1 gene in the yeast YEp vector that lacks the GAL4 activation domain. The construct is able to activate the TAS-HIS3 cassette with the same efficiency as pACT2 plasmids bearing the short and complete version of GZP1. As the shorter version of the GZP1 gene does not contain the tracts of poly-glycine, poly-proline and poly-histidine at the amino-terminus, those domains may not be essential for the transcription activation activity.

GZP1 shows a substantial sequence homology to an ubiquitous zinc finger DNA-binding protein, ZBP-89, that controls several promoter systems such as that of the rat gastrin gene [21], human ht β T-cell receptor [22], mouse BFCOL1,

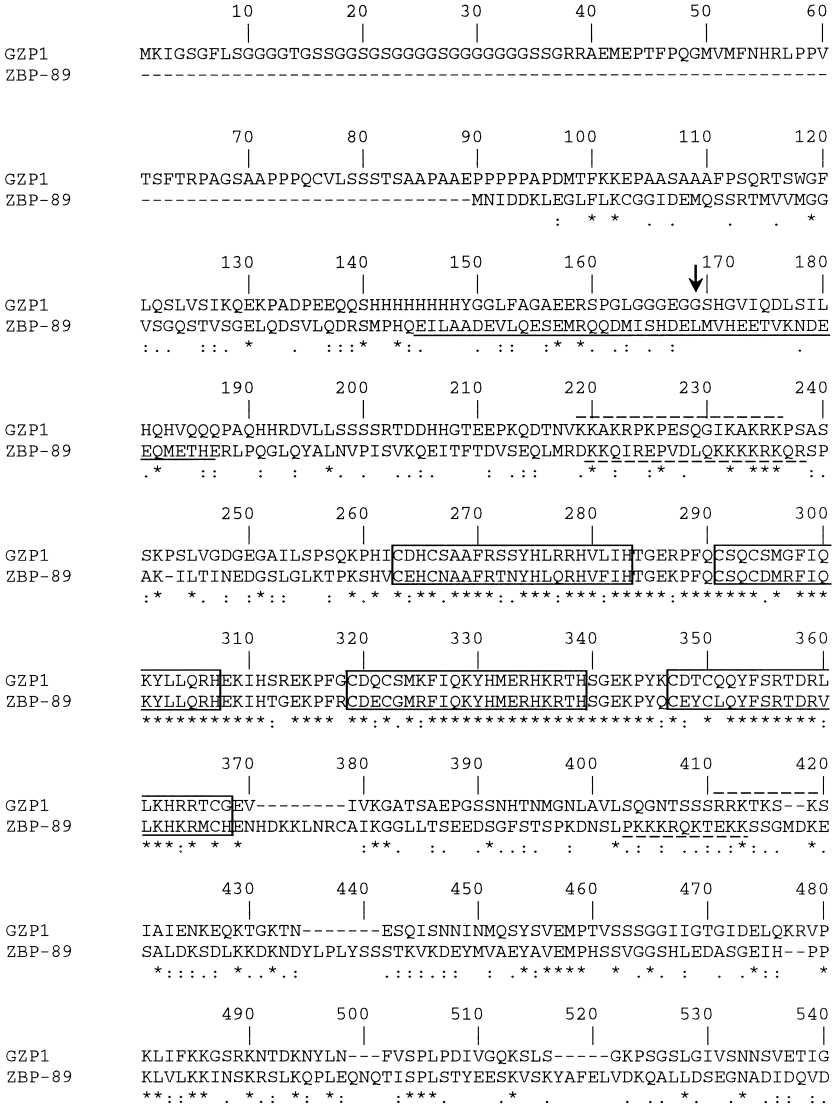

Fig. 3. Sequence alignment between GZP1 and ZBP-89. Asterisks indicate identical nucleotides, colons and dots indicate very similar and similar residues, respectively, according to Thompson et al [14]. Zinc finger domains are boxed. Acidic (solid line) and basic (broken line) motifs are shown. The arrow marks the start point of GZP1 Short.

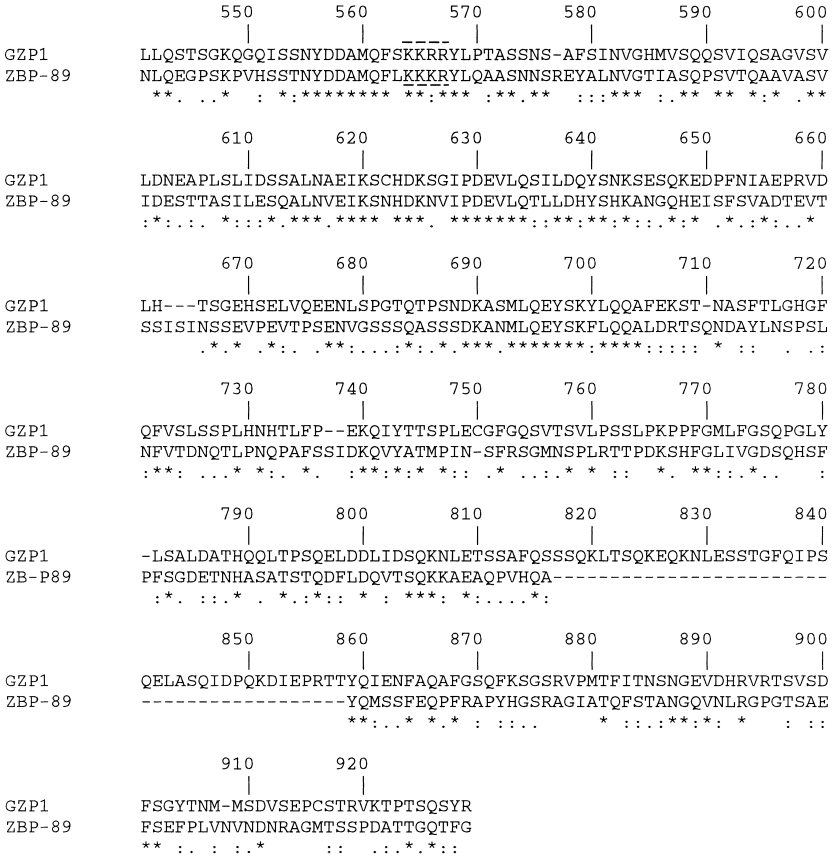

Fig. 3 (*continued*).

type 1 collagen gene [23], human β enolase gene [24] and ornitine decarboxylase gene [25]. The homology concerns primarily the zinc fingers and a few other regions of the two proteins. However, there are several domains that differ between the two proteins and that could suggest diverse functions in the cell. In particular ZBP-89 lacks the poly-glycine, poly-histidine and poly-proline tracts present at the N-terminus of GZP1. Conversely, GZP1 does not contain the long acidic domain present in ZBP-89 which might be responsible for protein-protein interactions [26]. An indication that GZP1 may also be an ubiquitous protein is given by our finding that the same cDNA clones were isolated from libraries from very different tissues like liver and testis. While ZBP-89 appears to act as a negative regulator of gene expression competing with the Sp1 factor for binding the 5′ half of gERE [21], it has been reported that a minimum of three proteins can recognize the GC-rich element [16] and at least one of these might exert a positive control in transcription [24]. Our data suggest that the newly identified GZP1 factor could be the one playing a positive function in transcription activation. The identification of GZP1 will allow to perform studies to address the problem of its function in human cells and the possible interactions with other proteins in regulating the expression of genes under the control of GC-rich sequence elements.

In spite of extensive library screenings with large numbers of transformed yeast cells, no additional positive clones have been identified. In particular, we did not find any positive clone encoding mtDNA-binding protein(s) able to specifically interact with the TAS region of human mtDNA. This means that either the respective cDNAs are not contained in the library or the specific proteins, though present, are not detected by the one-hybrid test, probably because, in order to bind DNA, they may require post-translational modifications that do not occur in yeast. An alternative explanation is that mtDNA-binding proteins, as all mitochondrial proteins translated in the cytoplasm and addressed to the mitochondrion, are synthesized as precursors containing a N-terminal import sequence of variable length [27]. The presence of the import domain has been shown to prevent proteins from binding the target DNA sequences [28–30], however, their DNA-binding capacity is restored upon removal of the import sequence. Since in our screening, we used full length cDNA libraries, it is very likely that mitochondrial proteins were expressed as precursors, being therefore unable to bind their target sequences. It follows that the application of the one-hybrid system for the search of mitochondrial proteins could present some limits. It is therefore advisable in those cases to use cDNA libraries with short 5′ deletions, which should allow mtDNA-binding proteins to acquire the proper folding for best contacting the DNA target.

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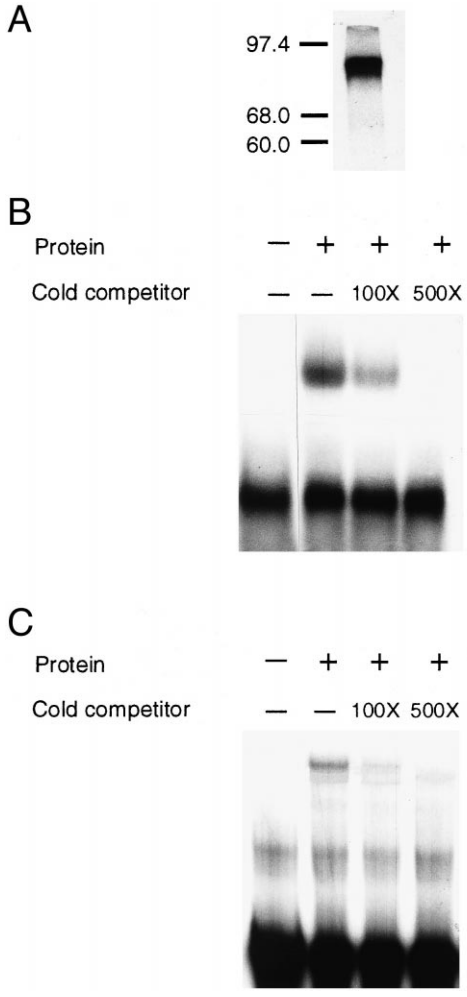

Fig. 4. DNA-binding properties of GZP1. (A) SDS-polyacrylamide gel analysis. The shorter version of GZP1 was expressed in vitro in the presence of [^{35}S]methionine, separated on a SDS-polyacrylamide gel and subjected to autoradiography. The positions of molecular mass markers (in kDa) are shown to the left. (B) and (C) EMSA. Aliquots of 6 μl from in vitro transcription-translation reaction mixtures were incubated with the α-^{32}P-labelled double-stranded gERE (B) or TAS1 (C) oligonucleotide. Where indicated, a 100-fold and 500-fold molar excess of unlabelled oligonucleotide competitor was added.

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