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THE NEUROENDOCRINE PROTEIN 7B2 CONTAINS UNUSUALLY POTENT TRANSCRIPTIONAL ACTIVATING SEQUENCES

Bhabatosh Chaudhuri*, Richard P. H. Huijbregts+, Jeremy J. Coen and Peter Fürst

Department of Core Drug Discovery Technologies (CDDT), K-681.4.46, Ciba-Geigy AG, Basel, Switzerland

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The expression of the 7B2 protein, secreted from a variety of neural and endocrine tissues, increases dramatically in specific neuroendocrine tumors. We have recently shown that human 7B2 can act as a molecular chaperone in the deaggregation of proteins *in vitro*. In order to identify polypeptides which might bind 7B2 *in vivo*, the yeast two-hybrid system was employed. Surprisingly, mere covalent linkage of 7B2 to the DNA-binding domains of two yeast transcription activators, Ace1 and Gal4, activates transcription from the *ACE1* and *GAL4* operon. 7B2's ability to activate nuclear transcription surpasses that of Ace1 and compares favourably with the strong activation domain of the tumor suppressor protein, p53. Our results suggest that 7B2 must possess an activating sequence, a domain which defines all transcriptional activator proteins. Like the acidic activation domains of some transriptional activators, 7B2 also binds the yeast TATA-box binding protein, an essential polypeptide in the basic transcription machinery. Deletion analysis of the gene encoding 7B2 reveals two independent transcriptional activating sequences in the 185 amino acid protein. It is therefore conceivable that 7B2 not only has a functional role in the secretory pathway but also in the nucleus. Moreover, these findings raise an intriguing question regarding the activation domains of 7B2 and their possible link to 7B2's oncogenic potential.

The 20 kDa 7B2 protein was first isolated from the human and porcine pituitary glands [1]. Subsequent studies have revealed that 7B2 is widely distributed in the central nervous system (CNS) [2]. Although the pituitaries contain the highest concentration of the protein [3], 7B2 is also found in the hypothalamus, thyroid and the adrenal medulla of a variety of species. It has been implicated as a neurotransmitter or a neuromodulator [4].

Since 7B2 is released from the many organelles of the CNS, it was suspected early on that the protein is secreted [4]. Indeed, from birth the human plasma contains variable amounts of 7B2. Very high concentrations of 7B2 are also present within peptide hormone- and neuropeptide-containing secretory granules, which indicates a role for 7B2 in the secretory process and in prohormone maturation [5-6]. Recent results suggest that 7B2 prevents premature release of active hormones by transiently associating with the precursor of a prohormone convertase, PC2 [7].

Intriguingly, neuronal and endocrine tumors contain levels of 7B2 which are significantly different from normal cells [8-9]. In fact, it has been suggested that 7B2 could be a possible tumor marker for the neuroendocrine small cell lung carcinomas and

* To whom correspondence should be addressed. Fax: (41) (61) 696 63 23.

+Present Address: Center for Biomembranes & Lipid Enzymology, University of Utrecht, The Netherlands.

ABBREVIATIONS: 7B2, the neuroendocrine 7B2 protein; aa, amino acid; Ace1N, DNA-binding domain of yeast transcriptional activator Ace1; Ace1C, transcription-activation domain of Ace1; p53AD, transcription-activation domain of the tumor suppressor protein p53; CNS, central nervous system; Gal4N, DNA-binding domain of Gal4; *GAPDH*, gene encoding glyceraldehde-3-phosphate dehydrogenase; NLS, nuclear localization signal; p53, 53 kDa tumor suppressor protein; PCR, polymerase chain reaction; TBP, TATA-box binding protein; wt, wild type; yRPA1, yeast replication factor A1; yTBP, yeast TBP.

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medullary carcinoma of the thyroid because of the abnormally high levels of the protein present in these tumors [10-13]. These studies strongly hint at a link between 7B2 and cancer.

The cDNA sequences, for the human [14] and the *Xenopus laevis* [15] 7B2 proteins, encode classical N-terminal signal sequences which normally target proteins to the secretory pathway [16]. Comparison of the 7B2 sequence from different species (i.e. human, toad, pig, mouse and rat) shows that the protein has been very well conserved during evolution [14-15, 17-19]. The 7B2 proteins, obtained from *X. laevis* and mammals, have an overall identity of ~85% at the amino acid level. This high degree of conservation, during an evolutionary time period of 350 million years from toad to man, implies that 7B2 must be biologically important. However, its true function in the cell has yet to be fully clarified.

We have recently illustrated that 7B2 can play the distinctive role of a molecular chaperone in the *in vitro* formation of correctly-folded structures from inactive molecules [20]. Hence, it is likely that 7B2 fulfils a similar chaperone-like function [21] by acting on specific proteins in the cellular environment. We envisaged that the yeast two-hybrid system [22] would identify proteins which normally associate with 7B2 in specific cell lines. Quite unexpectedly, 7B2 reveals itself as a protein which can activate transcription when fused to the DNA-binding domain of a transcription factor. In this communication, we show that the relatively small human protein 7B2 is composed of not one but two independent transcriptional activation domains. The possibility of 7B2 acting as a true transcriptional activator creates an interesting scenario. It means that the product of a single gene could probably have diverse functions in different cell compartments [23]. We feel that our findings could engender the perception that 7B2-mediated tumor formation may be a result of unrestricted transcriptional activation.

MATERIALS AND METHODS

Construction of the plasmid pRH1, where wild type (wt) and mutant genes are subcloned for expression of proteins containing Ace1N fusions: The plasmid pRH1 has been designed to express proteins as a fusion with Ace1N (i.e. the DNA-binding domain of the yeast transcriptional activator, Ace1) [24]. pRH1 is a derivative of pTM9 [24], a yeast integrating vector containing a 544 bp *EcoRV-BglII* fragment which includes the entire promoter of the *ACE1* gene and *ACE1N*. The 3' end of the *ACE1N* sequence contains two extra nucleotides 'GC' after the last complete codon 'ATT', just prior to the *BglII* site in *ACE1*. Immediately downstream of this fragment, is a 300 bp *EcoRI-KpnI* transcription-terminator fragment of the yeast *SUC2* gene [25], followed by the *Saccharomyces cerevisiae TRP1* sequence which is a yeast selectable marker [24].

Construction of plasmids that express Ace1N-7B2 variants: The human *7B2* gene (encoding amino acids 1-192; Fig. 1) [14] was isolated by the polymerase chain reaction (PCR), using the human 7B2 cDNA [14] as template (partially represented in Fig. 1). The 5'-end primer contained a *BglII* site and the 3'-end primer an *EcoRI* site. The 579 bp *BglII-EcoRI* fragment, encoding the 192 amino acid (aa) polypeptide and a stop codon 'TAA' (Fig. 1), was subcloned in pRH1 to yield the plasmid pRH6-1. 7 aa from the 7B2 signal peptide was added to provide a spacer between the Ace1N domain [24] and 185 aa mature 7B2 [20]. Contiguous to the 5' end *BglII* site is the nucleotide 'G' which allows the coding sequence of 7B2 to be in-frame with Ace1N.

The mutated sequences, obtained by deletion of specific DNA sequences were generated by removing specific regions from the gene encoding 7B2 (Fig. 1; the deletions are described in Fig. 5). The deletions were performed using PCR, with pRH6-1 as a template and two specific primers. The 5' and 3' end primers always contained the *BglII* and *EcoRI* sites, respectively. The *BglII-EcoRI* fragments, which included an extra nucleotide 'G' before the start of the coding sequences and a stop codon 'TAA' at the 3' end, were subcloned in pRH1. The resulting plasmids were named pRH6-2, pRH6-3, pRH6-4, etc. and encoded the fragments 6-2, 6-3, 6-4, etc., respectively (all the mutant 7B2 peptides are listed in Fig. 5).

Construction of plasmids that express Ace1N-yRPA1, Ace1N-yTBP, Ace1N-p53 and Ace1N-p53AD: The coding sequences of the yeast RPA1 (yRPA1) [26] and the yeast TATA-box binding protein (yTBP) [27] were amplified by PCR, using genomic DNA from the *S. cerevisiae* strain S288C as template. The coding sequence of the human tumor suppressor protein p53 and the N-terminal 92 aa activation domain of p53 (p53AD) [28-29] were amplified by PCR, using the cDNA sequence of human p53 as template [30]. The *p53* and *p53AD* genes were subcloned as *BglII-EcoRI* fragments in pRH1. The *yTBP* gene was subcloned as a *BamHI-EcoRI* fragment in pRH1. However, *yRPA1* was isolated as a *BglII-HindIII* fragment by PCR and was subcloned,

with a 377 bp *HindIII-KpnI PHO5* transcription terminator fragment [25], in pRH1. The plasmids, bearing the *ACE1N* fusions, were named pJC4 (*yRPA1*), pJC3 (*yTBP*), pJC6 (*p53AD*) and pJC6-1 (*p53*).

Construction of pRH13 which is used for the expression of *GAL4N(1-47)-7B2(1-192)*: The coding sequence of 7B2 (amino acids 1-192, Fig. 1) was amplified by PCR, using pRH6-1 as a template. The 5'-end primer contained a *BamHI* site and the 3'-end primer a *SalI* site. The *BamHI-SalI* fragment of 7B2 (Fig. 1) was subcloned in pMA424 [31] to yield the plasmid pRH13. The 5'-end *BamHI* site is contiguous to the DNA encoding the 147 aa DNA-binding domain of Gal4 (i.e. Gal4N) [31].

Construction of plasmids used for the expression of *ACE1C-7B2* and *ACE1C-p53AD*: The plasmid expressing Ace1C-7B2 is described elsewhere [20], Ace1C being the transcription activation domain of Ace1 [24]. The *BglII-EcoRI* 276 bp fragment of p53AD was used to construct Ace1C-p53AD. The Ace1C fusion proteins were constitutively expressed under the control of the yeast *GAPDH* promoter from the 2μ-plasmid pDP34 [32]. To enable targeting of fusion proteins to the nucleus, the SV40 large T antigen nuclear localization signal (NLS) [32] was used.

Yeast strains and transformations: The *S. cerevisiae* strain TFY2 (*Matα his ura3-52 trp1-285 ace1 LEU2::YipCL* CUP1^r) [24,32] was used for genomic integration of pRH1-derived plasmids. TFY2 bears the *CUP1-lacZ* fusion at the *LEU2* locus [24, 32]. After integrative transformation, *TRP1* prototrophs were selected. The TFY2 strain harboring the *ACE1N-yTBP* fusion was transformed with plasmids derived from pDP34 which harbor expression cassettes for *ACE1C-7B2*, *ACE1C-p53AD*, *7B2* and *p53AD* (all gene fusions contain *NLS* at the 5'-end) [32].
For transformation of the pMA424 derivative [31], which encodes a hybrid gene consisting of *GAL4N(1-147)* and *7B2(1-192)* (Fig. 1), the yeast strain YM335::RY171 (*Mata gal4-536 URA3::RY171 ade2-101 lys2-801 his3-200 met* [31] was used as a host. A *GAL1-lacZ* fusion is integrated at the *URA3* locus of YM335::RY171. The plasmid pMA424 [31] is a 2μ-plasmid and bears the *HIS3* gene as yeast selection marker.
All transformations were performed via electroporation [33]. For verification of correct integration and gene replacement events, PCR was performed with primers flanking the insertion sites. The amplified fragments were analyzed by agarose gel electrophoresis.

Recombinant DNA techniques: Manipulation of DNA was carried out by using standard procedures [33]. All PCR-s were performed using Vent DNA polymerase (BioLabs). *E. coli* HB101 was used for the construction and propagation of plasmids. Double-stranded DNA was sequenced by J. Bietenhader (with Applied Bio-Systems DNA sequencer 370A) .

ß-galactosidase assay and induction of metallothionein expression These assays were performed exactly in the way described elsewhere [24, 32-33].

RESULTS AND DISCUSSION

Transcriptional activators are proteins which usually bind to a specific DNA element in the promoter of a gene and elevate basal levels of transcription [34]. The proteins are generally modular in nature, with a DNA-binding domain and a region which is required for activation of gene transcription. Neither of the two separate domains activates transcription on their own, even when co-expressed in the same cell. The domain, which binds to specific DNA sequences, contains unique structural motifs that permit the protein and DNA surfaces to interact [35]. The activation domain is usually characterized by a high content of acidic, glutamine or proline residues [36].

The two-hybrid system for protein-protein interactions [22] relies on reconstituting a functional transcriptional activator from its two separable domains [34]. In order to search for possible interactions between human 7B2 and other proteins, a fusion between the complete 7B2 gene (encoding 7 aa from the signal peptide + 185 aa of the mature 7B2 protein; Fig. 1) and a gene segment encoding a heterologous DNA-binding domain was constructed. The DNA-binding domain Ace1N belongs to the yeast transcriptional activator Ace1 [24]. The Ace1 protein induces expression of the metallothionein gene *CUP1*. Induction occurs after the Ace1N polypeptide binds to the *CUP1* promoter, in the presence of monovalent silver or divalent copper. Copper is ordinarily very toxic for yeast cells. The toxicity of copper is abrogated when metallothionein, a strong chelator of

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 G1  W   T   P   A   F   A   Y   S   P10 R   T   P   D   R   V   S   E
GGA TGG ACT CCA GCA TTT GCT TAC AGC CCC CGG ACC CCT GAC CGG GTC TCA GAA
1                                                                   54
 A   D20 I   Q   R   L   L   H   G   V   M   E30 Q   L   G   I   A   R
GCA GAT ATC CAG AGG CTG CTT CAT GGT GTT ATG GAG CAA TTG GGC ATT GCC AGG
                                                                   108
 P   R   V   E40 Y   P   A   H   Q   A   M   N   L   V 50 G   P   Q   S
CCC CGA GTG GAA TAT CCA GCT CAC CAG GCC ATG AAT CTT GTG GGC CCC CAG AGC
                                                                   162
 I   E   G   G   A   H60 E   G   L   Q   H   L   G   P   F   G70 N   I
ATT GAA GGT GGA GCT CAT GAA GGA CTT CAG CAT TTG GGT CCT TTT GGC AAC ATC
                                                                   216
 P   N   I   V   A   E   L   T80 G   D   N   I   P   K   D   F   S   E90
CCC AAC ATC GTG GCA GAG TTG ACT GGA GAC AAC ATT CCT AAG GAC TTT AGT GAG
                                                                   270
 D   Q   G   Y   P   D   P   P   N   P100 C   P   V   G   K   T   D   D
GAT CAG GGG TAC CCA GAC CCT CCA AAT CCC TGT CCT GTT GGA AAA ACA GAT GAT
                                                                   324
 G   C110 L   E   N   T   P   D   T   A   E   F120 S   R   E   F   Q   L
GGA TGT CTA GAA AAC ACC CCT GAC ACT GCA GAG TTC AGT CGA GAG TTC CAG TTG
                                                                   378
 H   Q   H   L130 F   D   P   E   H   D   Y   P   G   L140 G   K   W   N
CAC CAG CAT CTC TTT GAT CCG GAA CAT GAC TAT CCA GGC TTG GGC AAG TGG AAC
                                                                   432
 K   K   L   L   Y   E150 K   M   K   G   G   E   R   R   K   R160 R   S
AAG AAA CTC CTT TAC GAG AAG ATG AAG GGA GGA GAG AGA CGA AAG CGG AGG AGT
                                                                   486
 V   N   P   Y   L   Q   G   Q170 R   L   D   N   V   V   A   K   K   S180
GTC AAT CCA TAT CTA CAA GGA CAG AGA CTG GAT AAT GTT GTT GCA AAG AAG TCT
                                                                   540
 V   P   H   F   S   D   E   D   K   D190 P   E   *
GTC CCC CAT TTT TCA GAT GAG GAT AAG GAT CCA GAG TAA
                                                   579
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Fig. 1. The DNA and protein sequence of human 7B2. The N-terminal residue of the mature 185 aa 7B2 protein starts with Tyrosine (Y) residue at position 8 of this sequence. The first 7 amino acids belong to the C-terminal end of the putative 26 aa signal peptide [14].

copper, is expressed. Yeast cells therefore can survive in the presence of copper only when the *CUP1* gene transcription is activated by a functional Ace1 protein.

In order to establish that the two-hybrid screen would be a valid approach, it was initially essential to confirm that 7B2 in the fusion protein Ace1N-7B2 did not contribute to the activation of gene transcription from the *CUP1* promoter. Hence, at first, only one of the two hybrid proteins (i.e. Ace1N-7B2) was expressed in the yeast strain TFY2 (see Materials and Methods). The Ace1N domain, devoid of the Ace1 activation domain, is incapable of acting as a transcription factor [24]. Two reporter genes (*CUP1* and the *E. coli* ß-galactosidase *lacZ*) linked to the *CUP1* promoter, have been used to ascertain if at all Ace1N-7B2 activates gene transcription. A qualitative estimate is obtained via metallothionein expression which leads to copper-resistant growth of cells on plates. Expression of *lacZ* provides a more accurate quantification of transcriptional activation.

Surprisingly, we find that 7B2 can efficiently substitute for the activation domain of Ace1 (Fig. 2). In fact, the ß-galactosidase assay portrays that Ace1N-7B2 protein is more efficient in activating transcription than the intact Ace1 protein (Fig. 2-II). This activation is definitely much stronger than the large number of random *E. coli* genomic fragments (pertaining to nearly 10% of the total genome) that also function as activating regions [31,34]. Most of the latter activate gene transcription relatively poorly.

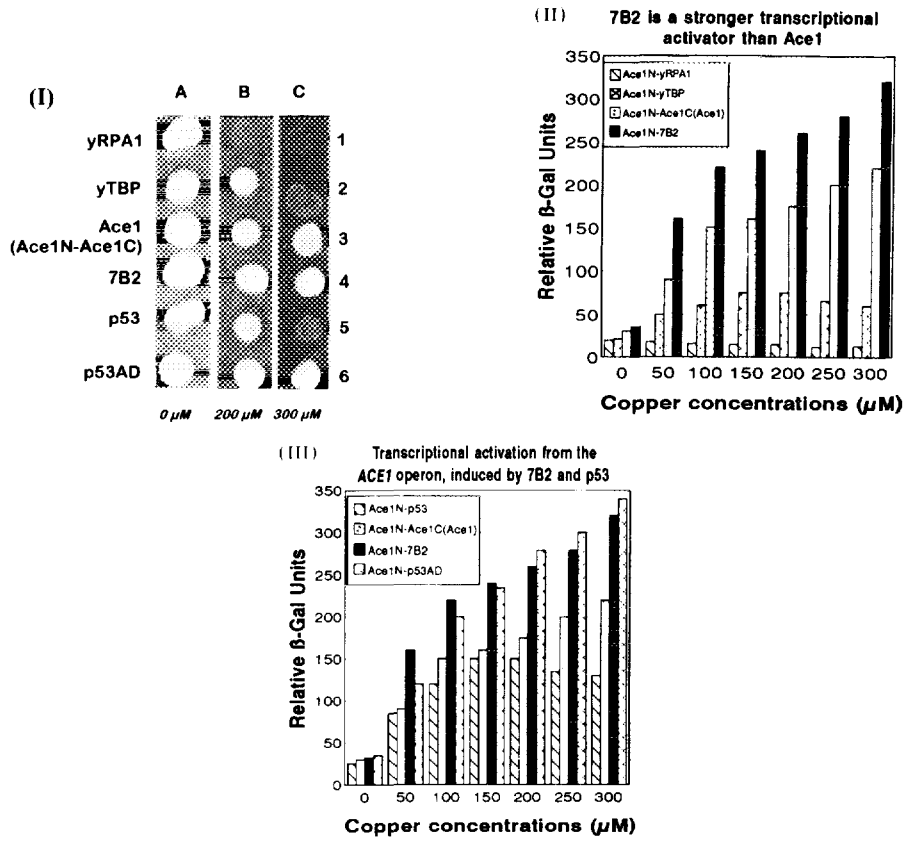

Fig. 2. Induction of metallothionein and ß-galactosidase expression by different proteins fused to Ace1N. (I) Induction of metallothionein expression by Ace1N fusion proteins [24,32]. Equal numbers of cells were spotted onto SD plates containing (A) no $CuSO_4$, (B) 200 μM $CuSO_4$ and (C) 300 μM $CuSO_4$ and were incubated for 24h at 30°C. 1, Ace1N-yRPA1; 2, Ace1N-yTBP; 3, Ace1N-Ace1C (i.e. the full length transcriptional activator, Ace1); 4, Ace1N-7B2; 5, Ace1N-p53; 6, Ace1N-p53AD. (II) Comparison of copper-dependent ß-galactosidase expression induced by Ace1N hybrids of yRPA1, yTBP, Ace1C (this fusion represents full-length Ace1) and 7B2. (III) Comparison of copper-dependent ß-galactosidase expression induced by hybrids of p53, Ace1C (i.e. full-length Ace1), 7B2, and p53AD. Activation of the *CUP1-lacZ* gene was measured at the protein level, using the ß-galactosidase assay [24,33]. Copper concentrations are that of $CuSO_4.5H_2O$. Data are expressed as means ± S.D. deviations from at least six independent transformants.

Hence, it would not be unreasonable to postulate that transcriptional activation by 7B2, although discovered fortuitously, may be a unique property of the molecule.

The human tumor suppresssor protein p53 contains a strong transcription-activating sequence at its N-terminus [28]. This has been demonstrated by expressing hybrid proteins that contain segments of p53 and the DNA-binding domain (Gal4N) of yet another yeast transriptional activator Gal4 [31,34]. Transcriptional activation was monitored via induction of a reporter gene linked to the *GAL1/GAL10* promoter which contains the Gal4-binding sites. Here, we compare the relative strengths of 7B2, p53 and the p53 activation domain in activating gene transcription from the *CUP1* promoter. The p53 activation domain (p53AD), used in this study, consists of the N-terminal 92 amino acids of wt p53 which possesses the highest level of activation amongst all p53 sequences [29].

Hybrid proteins, Ace1N-7B2, Ace1N-p53 and Ace1N-p53AD have been expressed in the strain TFY2, after integration of DNA sequences into the yeast genome. This affords expression of a single chromosomal copy of the hybrid gene. Copper dependent metallothionein and ß-galactosidase expression, induced by the hybrid proteins, is depicted in Fig.2 (I and III). The concentrations of copper, at which highest levels of expression of reporter genes are observed, also reflect the relative strengths of activation [24]. Our results demonstrate that, as a transcriptional actvator, the 192 aa 7B2 protein (Fig. 1) is much superior to the complete p53 molecule (Fig. 2-III). In fact, 7B2 is comparable to the 92 aa p53AD, a polypeptide which is as potent as the VP16 activation domain [37]. The latter is perceived to be one of the most powerful activating sequences known in the literature.

The TATA-box binding protein TBP [27] serves as a linchpin for the assembly of preinitiation complexes which are components of the basal transcription machinery. On its own TBP only permits basal levels of transcription. In association with a wide range of TBP-associated factors (TAFs), it forms multi-protein complexes that interact with proteins that regulate transcription [38]. The interaction is thought to occur via the activation domains of transcriptional activators [38]. The yRPA1 protein [26], the largest subunit of the trimeric yeast replication factor, also binds to activation domains of various transcriptional activator proteins indicating a link between the cell's replication machinery and transcriptional activation [37,39].

The two yeast proteins, yTBP and yRPA1, have been expressed as fusions with Ace1N, to serve as appropriate controls for the study of 7B2's transcriptional activity. The levels of ß-galactosidase expressed from a genomic copy of the hybrid genes confirm that, compared to 7B2, yTBP is a poor transcriptional activator whereas yRPA1 is non-functional (Fig. 2-I and II).

To be certain that the ability of 7B2 to activate transcription was not restricted to the *ACE1* operon, we have tested 7B2's capacity to activate transcription from the *GAL1/GAL10* promoter [31]. A Gal4N-7B2 hybrid protein (as encoded in pRH13) was expressed in the yeast strain YM335::RY171 from the 2µ-plasmid pMA424 which autonomously replicates in multiple copies per cell [31]. Our results indicate that full-length 7B2 is a facile transcriptional activator in this system too (Fig. 3).

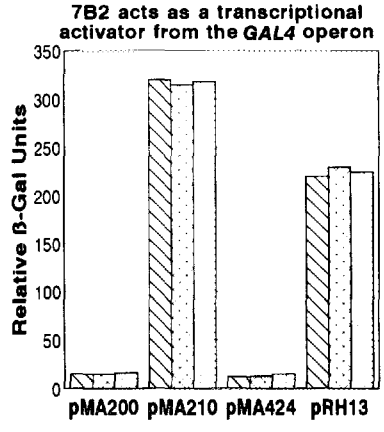

Fig.3. Comparison of transcriptional activation induced by Gal4 and 7B2. The plasmid pMA210 encodes the complete 881 aa of the transcriptional activator Gal4 [31] whereas pRH13 (see Materials and Methods) encodes the Gal4N-7B2 fusion.Three transformants were grown in minimal SD medium which contained a mixture of galactose, glycerol, ethanol (each 2%) as carbon source [40]. Induction of activation of the *GAL1-lacZ* gene was measured colorometrically [31]. The 2µ-plasmid pMA200 is the parent of pMA210 and lacks the *GAL4* coding sequence [40]. pMA424 is also a 2µ-plasmid (similar to pMA200) and contains the DNA sequence encoding 1-147 aa of Gal4 (i.e. Gal4N, the DNA-binding domain of Gal4) under the control of the yeast *ADH1* promoter [31,40].

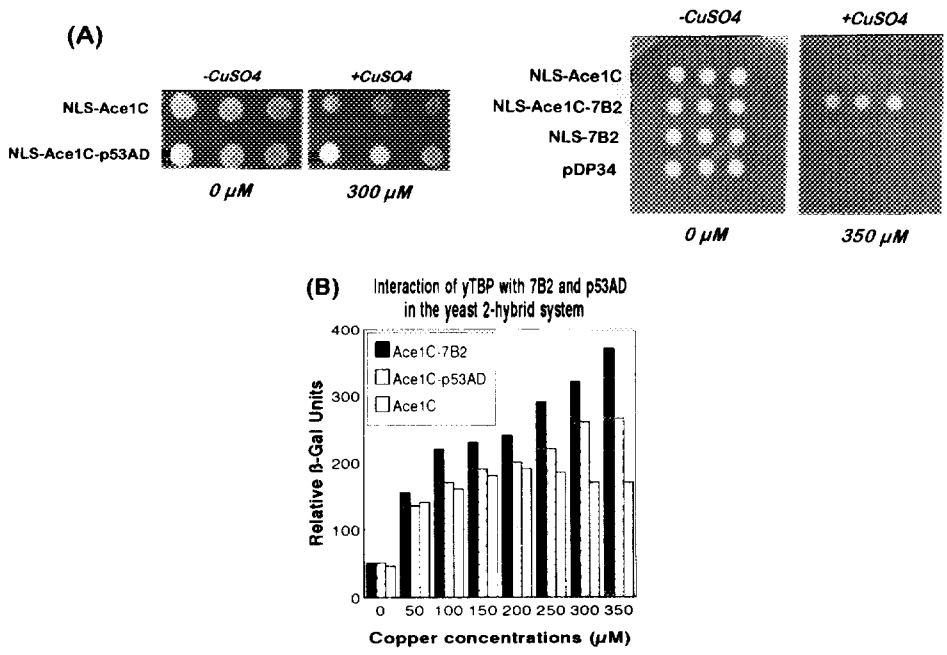

Fig. 4. Interaction of yTBP with 7B2 and p53AD. yTBP is expressed from the chromosome as an Ace1N fusion (with Ace1's own nuclear localization sequence) whereas the expression of Ace1C-7B2 and Ace1C-p53AD fusions (from the plasmid pDP34) is targeted to the nucleus with the aid of the SV40 T antigen nuclear localization sequence (NLS; see Materials and Methods). The expression of 'NLS-Ace1C' is used as a negative control for all experiments reported in this communication. 'NLS-7B2' implies that 7B2 is expressed on its own and not as a Ace1C fusion protein. (A) Induction of metallothionein expression (performed as in Fig. 2-I). Equal number of cells were spotted onto SD plates containing (I) no $CuSO_4$ and (II) 350 μM $CuSO_4$. (B) Induction of Cu^{2+}-dependent β-galactosidase expression [24,33]. Copper concentrations are that of $CuSO_4.5H_2O$. Data are expressed as in Fig. 2.

We have explored the possibility that 7B2 might physically interact *in vivo* with yTBP. *In vitro* experiments have already revealed that the p53AD binds to yTBP [37-38]. Our experiments with the yeast two-hybrid system not only verify that p53AD interacts with yTBP but also confirm that 7B2 binds yTBP (Fig. 4). It appears that, in this assay, 7B2 is superior in its capacity to bind yTBP than p53AD (Fig. 4B). Inexplicably however, the hybrid protein Ace1N-yRPA1 fails to bind both Ace1C-p53AD and Ace1C-7B2 (data not shown).

If 7B2 were to be a transcriptional activator, it could be possible that only a part of the mature 185 aa 7B2 protein (Fig. 1) activates gene transcription. Published reports indicate that a small 60 aa region of the 281 aa yeast transcriptional activator protein GCN4 can act as an activating sequence [34]. Also, two ~100 aa regions in the yeast Gal4 protein have been described to be activating regions [40]. In order to delineate the region or regions which might be involved in transcriptional activation, an extensive deletion analysis was performed on the 7B2 molecule.

Unique fragments of the 7B2 gene sequence have been expressed as Ace1N fusions from the yeast genome and are described in Fig. 5. The relative values for β-galactosidase expression are also indicated. It was quite interesting to find that a small protein like 7B2 has two distinct transcriptional activation domains (see peptide 6.10-7B2: aa 8-92, and peptide 6.16-7B2: aa 93-144 in Fig. 5). The first domain, consisting of the N-terminal 85 aa sequence from the mature 7B2 polypeptide, is highly conserved phylogenetically. About 95% similarity is observed between the human and the *Xenopus* proteins in this region [14-15]. Our results also suggest that the first three amino acids of this region may be crucial for transcriptional activation mediated

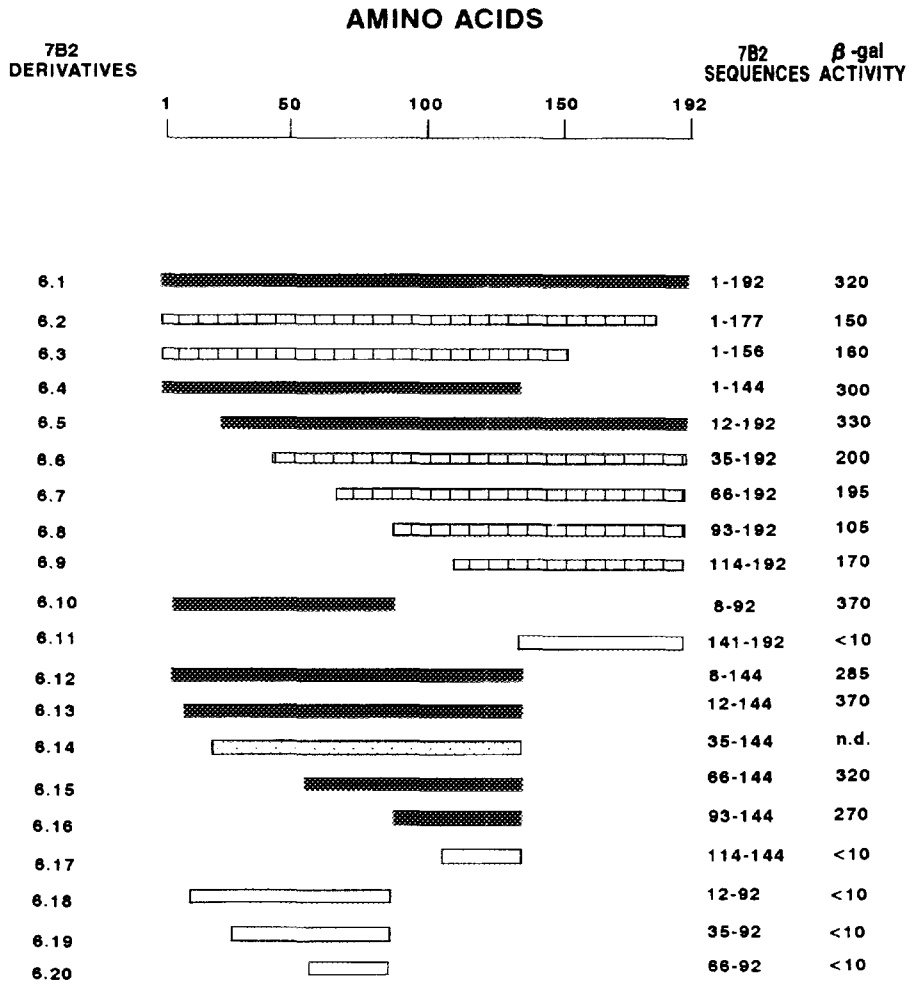

Fig. 5. Deletion analysis to define the transcriptional activation domain(s) in the human 7B2 protein. pRH1 derived plasmids, encoding complete 7B2 (Fig. 1) or deletion mutants (nineteen in all and characterized in the column '7B2 Sequences') fused to Ace1N, were integrated into the genome of the yeast strain TFY2 (see Materials and Methods). TFY2 bears a *CUP1-lacZ* fusion [24]. Six individual integrants, obtained from each of the transformations, were tested for activation of the *lacZ* gene. The described results are an average of six β-galactosidase assays measured in duplicate. The mutants that strongly activate transcription are indicated by solid bars, those that activate less efficiently by stippled bars and those that are inactive by open bars. The mutant 6.14, that could not be stably integrated, is indicated by hatched bars. Transcriptional activity was not determined for this mutant.

by this domain (compare peptide 6.18-7B2 with 6.10-7B2; Fig. 5). Moreover, it appears that the N-terminus of the second activating sequence may overlap with the C-terminal region of the first activation domain (compare peptides 6.16-7B2 and 6.15-7B2). But, it is clear that the second activating sequence can function independent of the first. Furthermore, a 52 aa polypeptide (peptide 6.11-7B2: aa 141-192; Fig. 5) at the C-terminus of 7B2, which is processed and removed during secretion from yeast and *X. laevis* [41], is completely devoid of transcriptional activity. It is also noteworthy that the *in vitro* molecular chaperone activity of 7B2 is maintained in the absence of this C-terminal domain [20].

Analysis of the 7B2 aa sequence has failed to divulge a motif which would spontaneously identify the molecule as a transcriptional activator. The two activating sequences of 7B2 are not overtly acidic (Table 1) [36]. Clear 'acidic blobs' [31,34]

Table 1. Amino acid analysis of the two transcription-activation domains present in 7B2 (Fig. 1)

7B2 Sequences; No. of aa	No. of Pro residues	No. of Gln residues	No. of Asp/ Glu residues	No. of Lys/ Arg residues
8-92; 85 aa AD	8	6	12	6
93-144; 52 aa AD	8	2	10	3

'AD' signifies activation domain and 'aa' denotes amino acid.

manifest themselves in GCN4, Gal4, p53 and VP16 activation domains. It is also apparent that the two activating regions in 7B2 do not show (Table 1) a preponderance of prolines (P) or glutamines (Q) in their primary sequences (Table 1) like the proline- or glutamine-rich transriptional activators [36].

It is well documented that similar molecular mechanisms of transcriptional regulation are shared by different eukaryotic organisms, ranging from yeast to humans [34,36]. Thus, even though our studies have been conducted in yeast, the revelation that 7B2 is a transcriptional activator portends that these observations could be biologically meaningful. In fact, a close examination of the polypeptide sequence of 7B2 divulges a telltale bipartite nuclear targeting sequence (NLS; 7B2: aa 145-159; Fig. 1) [42]. The presence of an NLS is thought to be obligatory for many transcriptional activators because of their necessity to function in the nucleus. The possibility of a secreted protein having an alternate function in the nucleus is startling. Calreticulin, a Ca^{2+}-binding protein, is one of few proteins which display similar properties. It is normally resident in the lumen of the endoplasmic reticulum but also modulates glucocorticoid receptor gene expression in the nucleus [43]. Another extracellular molecule, lactoferrin, a cationic secretory protein with iron-chelating antimicrobial activity, was recently shown to activate its own gene transcription by gaining entry into the cell nucleus [44]. Like 7B2, lactoferrin is also stored in the secretory granules but is released from neutrophils only by pathogen stimulation.

Further studies should elicit the actual role of 7B2 in the activation of gene transcription. At first, it would be important to evaluate if the protein binds DNA. It may be that the 7B2 polypeptide itself is not a DNA-binding protein. Gene transcription could be triggered if 7B2 were to form a complex with a DNA-binding protein. At this point one can only conjecture that the activating sequences of 7B2 may have a role in neuroendocrine tumorogenesis. The oncogenes *c-fos*, *c-jun*, *c-myb* and *c-myc* are transcriptional activators. Mutant oncogenes deficient in normal transforming function are also deficient in the activating function [34]. It is tempting to speculate that activating sequences of 7B2 remain subdued in a masked state under normal circumstances. Probably during the process of tumor formation, the functionality of the activating domains is manifested via the dissociation of the 7B2 protein from a dormant complex. The yeast systems, described here, could readily provide further insight into 7B2's role as a mammalian transcriptional activator.

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