Nucleic Acids Research, 1994, Vol. 22, No. 18 3685–3688

Expression cloning in K^+ transport defective yeast and distribution of HBP1, a new putative HMG transcriptional regulator

Florian Lesage, Jean-Philippe Hugnot, Ez-Zoubir Amri[1], Paul Grimaldi[1], Jacques Barhanin and Michel Lazdunski*

Institut de Pharmacologie Moléculaire et Cellulaire, CNRS UPR411, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne and [1]Centre de Biochimie, CNRS UMR134, Université de Nice Sophia Antipolis, Faculté des Sciences, Parc Valrose, 06108 Nice Cedex, France

Received June 27, 1994; Revised and Accepted August 10, 1994 GenBank accession no. U09551

ABSTRACT

The rat HBP1 cDNA was cloned by its capacity to suppress the potassium transport-defective phenotype of mutant *Saccharomyces cerevisiae* cells. HBP1 cDNA encodes a 513 amino acids protein which, unexpectedly, does not share any homology with K^+ transporters or K^+ channels. However, a search in protein databases reveals that HBP1 contains a putative DNA-binding domain called HMG-box. Northern blot analysis shows that HBP1 is expressed in a variety of tissues and that in adipocyte and myogenic cell lines, its expression is directly related to differentiation. Taken together, the results suggest that the rat HBP1 is a new member of the HMG class of transcriptional regulators involved in cell differentiation pathways.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* takes up K^+ by using high affinity (*TRK1*) and low affinity (*TRK2*) transporters as well as other unidentified transporter(s) (1). Cells bearing null mutation(s) in the *TRK1* gene (trk^- cells) are unable to grow on low-[K^+] medium. Recently, two plant K^+ channel cDNAs from *Arabidopsis thaliana* were cloned by complementation of these mutant yeast strains (2,3). The two cDNAs corresponding to the two K^+ channels called AKT1 and KAT1 restored the growth of trk^- cells on low-[K^+] medium. We have attempted to use this complementation system to isolate new mammalian K^+ channel genes. This report describes the cloning and the characterization of a rat cDNA which, similarly to AKT1 and KAT1, is able to suppress the trk^- phenotype of mutant cells. This cDNA did not encode a transmembrane protein as would be expected for a K^+ channel. Instead, it encodes a protein which bears a 70 amino acid domain similar to the basic DNA-binding domain (the HMG-box) of HMG-box transcription factors (for review see 4,5). This protein was called HBP1 for **H**MG-**B**ox containing **P**rotein **1**. HBP1 expression was studied in rat tissues and in adipocytes and myogenic cell lines.

MATERIALS AND METHODS

Preparation of the cDNA library

An oligo d(T) primed cDNA derived from poly(A)$^+$ RNA isolated from adult rat brain was constructed in the yeast/*Escherichia coli* shuttle vector pFL61 (6). Double-stranded cDNAs were synthetized and ligated to DNA adaptators (Invitrogen) with CACA cohesive ends compatible with the GTGT ends of *Bst*XI digested vector. The cDNAs were passed through a Sephacryl S-1000 column (Pharmacia) and fractions corresponding to cDNAs longer than 1.5 kb were pooled and ligated to *Bst*XI digested pFL61. After transformation of SURE *E.coli* (Stratagene) by electroporation, 8.10^5 clones were obtained. The clones were pooled, an aliquot was frozen at −80°C, and the remainder was used to prepare DNA.

Cloning and sequencing of pHBP1

The *Ura*$^-$*Trk1*$^-$ yeast strain (3) was grown at 30°C in YPB medium supplemented with 50 mM KCl. The library was introduced into trk^- cells by spheroblast transformation (7). The Ura$^+$ transformants were grown on a solid arginine-phosphate synthetic medium (minimal medium) containing 0.3 mM KCl. After 10 days at 30°C, a positive clone containing the pHBP1 plasmid was isolated. Subsequent transformations of mutant yeast strain by plasmids were performed by electroporation (8).

After subcloning into pBluescriptII SK$^-$ (Stratagene), the 2.7 kb HBP1 cDNA was sequenced in both strands by dideoxy sequencing using an automatic sequencer (Applied Byosystems model 373A).

Growth tests and potassium influx assays

Transfected cells were grown to early stationary phase in liquid minimal medium supplemented with 50 mM KCl. Yeasts were then harvested and washed twice with distilled water by centrifugation. For the growth assay, cells were resuspended in water at 3.10^5 cells per ml. A 10 µl aliquot was dropped on solid medium and plates were placed for 2 days at 30°C before they were photographed. For Rb^+ influx experiments, cells were resuspended in a 20 mM HEPES buffer at pH 7.4 containing

*To whom correspondence should be addressed

100 mM $MgCl_2$ and 2% glucose and were incubated 3 h at 28°C. Cells were centrifuged and resuspended in the same medium at 10^9 cells per ml. A 50 µl aliquot of the suspension was mixed with 150 µl of medium containing 100 µM KCl and $^{86}Rb^+$ (5 kBq/ml). After 15 min, the uptake was stopped by addition of a solution of 10 ml of ice-cold $CaSO_4$ and assayed for radioactivity.

Cell culture

Ob1771 preadipose (9) and C2C12 myogenic (10) cells were plated at a density of 2×10^3 per cm^2 and grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 200 units/ml of penicillin, and 50 µg/ml of streptomycin. Media were changed every other day. Confluence was reached within 5 days. Differentiation of Ob1771 cells was obtained by chronic addition, after confluence, of 17 nM insulin and 2 nM T3. Differentiation of C2C12 cells was induced by shifting the cells at confluence to a medium containing 4% fetal calf serum.

RNA isolation and analysis

Total RNAs were extracted (11) and resolved by electrophoresis through a 1 M formaldehyde, 1.1% agarose gel and blotted on to Hybond-N membranes (Amersham Corp.) in 20×SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). Blots were probed with a random primer-labeled 1230 bp *Eco*RI fragment of pBluescript-HBP1 in 50% formamide, 5×SSPE (0.9 M sodium chloride, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA), 0.1% SDS, 5×Denhardt's solution, 20 mM potassium phosphate (pH 6.5) and 250 µg/ml denatured salmon sperm DNA at 55°C for 18 h and washed to a final stringency of 0.1×SSC, 0.1 SDS at 55°C.

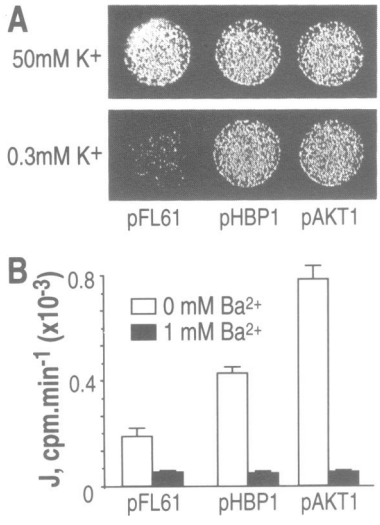

Figure 1. Heterologous expression of the rat HBP1 cDNA in K^+ transport deficient *S.cerevisiae* cells. (**A**) Growth test of trk^- cells transfected with pFl61, pHBP1 or pAKT1. Cells were plated on to minimal medium supplemented with 50 mM or 300 µM KCl and grown 2 days at 30°C. (**B**) $^{86}Rb^+$ influx (J) of pFL61, pHBP1 and pAKT1 transformed cells. Medium contained 100 µM K^+ and $^{86}Rb^+$. After 15 min, uptake was determined by radioactivity counting of washed cells. Rb^+ influx is expressed as the mean ± SEM of five independent experiments.

RESULTS

Isolation of HBP1 cDNA

In order to isolate new mammalian K^+ channels, an adult rat brain library was constructed in a yeast/bacteria shuttle vector. The library was transfected into mutant yeast trk^- and revertant cells trk^+ were selected on solid K^+-limiting medium (300 µM K^+). From 8.10^5 transformants, a single clone was obtained which allowed the growth of trk^- cells on 300 µM K^+ medium. The cloned plasmid pHBP1 was recovered by transfer from yeast to bacteria, amplified and reintroduced into trk^- cells. All transformants containing pHBP1 were able to grow on 300 µM K^+ thus demonstrating that complementation is due to the transfected cDNA.

Phenotype of pHBP1 transformed mutant yeasts

Figure 1A shows that pHBP1 transformed cells (pHBP1 cells) grow faster than pFL61 cells (negative control) on 300 µM KCl. Moreover, growth of pHBP1 cells was indistinguishable from that of pAKT1 cells (K^+ channel cDNA-containing pFL61, positive control) thus indicating that HBP1 completely reverted the mutant phenotype of trk^- cells. $^{86}Rb^+$ flux experiments indicated that the K^+ transport ability of pHBP1 cells was lower than that of pAKT1 cells (Fig. 1B) or wild-type yests (data not shown). However, Rb^+ influx into pHBP1 cells was significantly higher (twofold) than Rb^+ influx into control pFL61 cells (Fig. 1B). This influx into HBP1 cells was inhibited by Ba^{2+}, which is well-known for its K^+ channel blocking activity (1 mM; Fig. 1B). Taken together, these results clearly indicated that HBP1 complements the mutants cells *via* a partial restoration of a K^+ selective transport.

HBP1 sequence analysis

The 2666 nucleotides HBP1 cDNA contains only one reading frame encoding a protein of 513 amino acids (GenBank accession number UO9551). The hydropathicity profile of HBP1 protein does not predict any membrane-spanning domain and computer analysis does not show any sequence similarity between HBP1 and plant K^+ channels able to complement the trk^- cells or

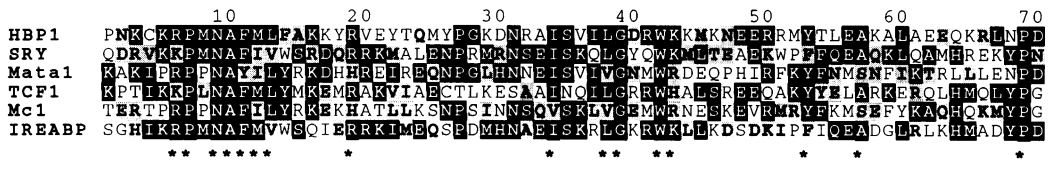

Figure 2. HBP1 homologies with HMG-boxes. The displayed region of HBP1 (nt 1287–1497; aa 429–499) was identified as a putative HMG-box by comparison with the transcription factors SRY, Mata1, TCF1, Mc1 and IRE ABP. Identical or conservatively changed residues conserved in the six sequences are indicated by stars.

between HBP1 and any known K^+ channels, K^+ channel regulator, K^+ transporters or ionic channel with another selectivity. However, homology search in GenBank, EMBL and Swiss-Prot databases reveals a significant homology between the C-terminal part of HBP1 and the DNA-binding domain of proteins belonging to the HMG-box containing transciption factor family. Figure 2 shows the alignment of the HBP1 C-terminal sequence (aa 469 to aa 499) with the HMG-boxes of the mammalian testis-determining factor SRY (12), the two fungal mating type proteins Mc of *Saccharomyces pombe* (13) and Mata1 of *Neurospora crassa* (14), the T-cell specific factor TCF 1 (15) and the mouse insulin responsive element binding protein IRE ABP (16). Residue identity and residue homology between HBP1 and these proteins varied from 23 to 31% and from 38 to 44%, respectively. All residues (identical or conservatively changed) conserved along the aligned transcription factors are present in HBP1 at the same positions except at residue 29, which is in the HBP1 sequence (Fig. 2).

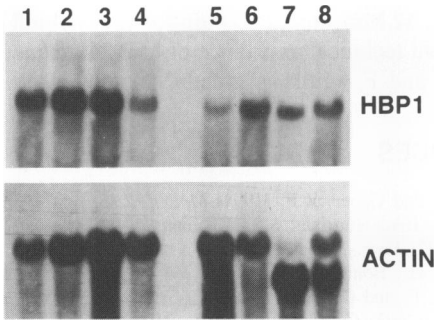

Figure 3. Tissue distribution of HBP1 mRNA. Total RNA (30 μg per lane) was isolated from adult rat tissues, blotted and hybridized as described in Materials and Methods. Lanes: 1, liver; 2, adipose tissue; 3, lung; 4, brain; 5, spleen; 6, kidney; 7, skeletal muscle; 8, heart.

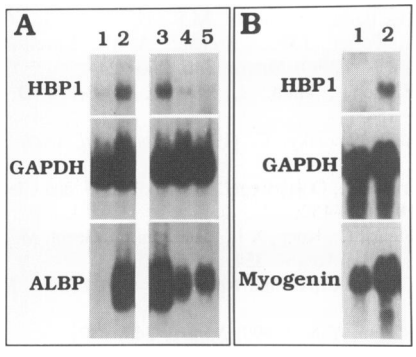

Figure 4. Expression of HBP1 mRNA during C2C12 (**A**) and Ob1771 (**B**) differentiation processes. Cells were maintained as described in Materials and Methods and RNA was isolated at the indicated days relative to confluence and analyzed by Northern-blot (20 μg per lane). (**A**) RNA from Ob1771 cells, lanes: 1, undifferentiated cells (day 1); 2, differentiated cells (day 11); 3, differentiated cells in control medium; 4, differentiated cells exposed to 60 nM okadaic acid for 24 h; 5, differentiated cells exposed to 0.5 nM TNFα for 24 h. (**B**) RNA from C2C12 cells, lanes: 1, undifferentiated cells (day 1); 2, differentiated cells (day 5).

Tissue distribution of HBP1 mRNA and activation of expression during cell differentiation

The HBP1 mRNA distribution was analyzed by Northern blot analysis in various rat tissues. A 2.7 kb transcript was detected in all examined tissues. It was abundant in white adipose tissue and lung. Lower signals were obtained in liver, kidney, skeletal muscle, heart and spleen (Fig. 3) as well as in uterus, intestine and salivary glands (data not shown). Despite the high stringency used in the hybridization experiments and the finding of only one band in all tissues examined, we cannot completely exclude that the probe could also detect closely related members (which remain to be discovered) of a putative HBP family.

The HBP1 mRNA expression was next investigated as a function of cellular differentiation of preadipose and myogenic cells, using Ob1771 (preadipocytes) (9) and C2C12 (muscle) (10) cell lines as cellular models, respectively. The differentiation was monitored by measuring the accumulation of RNAs encoding specific proteins for each tissue, i.e. adipocyte lipid binding protein (ALBP) in adipose cells (Fig. 4A) and myogenin in muscle cells (Fig. 4B). In both cell lines, HBP1 mRNA was undetectable in undifferentiated cells (lanes 1) and accumulated during the terminal differentiation process (lanes 2). Interestingly, the relationship between the adipocytic phenotype and HBP1 expression was reinforced by the observation that HBP1 mRNA expression was strongly decreased in differentiated Ob1771 cells exposed to agents known to promote adipocyte dedifferentiation (17). As illustrated in Fig. 4A, exposure of differentiated Ob1771 cells to 60 nM okadaic acid (lane 4) or to 0.5 nM tumor necrosis factor α (TNFα) (lane 5) led to a strong decrease of ALBP mRNA signal and in parallel, to a deinduction of HBP1 mRNA which became undetectable after 24 h of treatment. Conversely, a factor which is a positive effector of adipose cell differentiation, such as insulin, increased HBP1 mRNA expression. Figure 5 illustrates this regulation by insulin in differentiated Ob1771 cells maintained for 24 h in a medium containing concentrations of insulin ranging from 0 to 30 nM. This treatment resulted in a

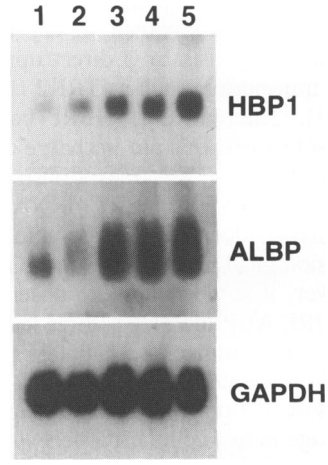

Figure 5. Effect of insulin on HBP1 mRNA expression in differentiated Ob1771 cells. Cells were differentiated for 11 days post-confluence in medium without insulin, and exposed for 24 h to medium containing increasing concentrations of insulin. Lanes: 1, no insulin; 2, 1 nM; 3, 3 nM; 4, 10 nM; 5, 30 nM insulin. RNA was analyzed as in Fig. 5.

dose-dependent accumulation of HBP1 mRNA which paralleled ALBP mRNA accumulation.

DISCUSSION

We have cloned a rat brain cDNA encoding a protein called HBP1 which is able to suppress the growth-deficiency phenotype of trk^- mutant cells. Despite the fact that HBP1 expression in mutant yeasts induces a Ba^{2+}-sensitive K^+ uptake as demonstrated by Rb^+ flux experiments, sequence analysis failed to detect any homology between HBP1 and previously cloned proteins involved in K^+ transport in plant and mammalian cell (2,3,18–21). The hydropathicity profile of HBP1 does not predict the usual membrane-spanning domains expected for ion channel and other ion transport systems. Moreover, electrophysiological analysis of *Xenopus* oocytes injected with HBP1 cRNA failed to demonstrate channel activity (data not shown). All these observations taken together supported the idea that HBP1 was not a channel but more probably an indirect regulator of the K^+ transport activity in transformed yeasts.

That conclusion led to a search of a possible mechanism of action for HBP1. Comparisons in protein databases revealed a significant sequence homology between a C-terminal sequence of HBP1 (aa 429–499) and the basic DNA-binding domains (the HMG-box) of a large family of transcription factors (for review see refs 4, 5). Among the transcription factors, the more homologs to HBP1 are the testis-determining factor SRY (12) and the related SOX proteins (for review see ref.22), the insulin responsive element binding protein IRE ABP (16), the T-cell specific factor TCF 1 (15) and the mating type proteins Mata1 (14) and Mc1 (13). The HMG proteins are widely distributed among eukaryotic cells in which they have diverse functions (for reviews see refs 4, 5). These factors regulate differentiation and mating type in fungi (Mata1, 14; and Mc1, 13) and tissue-specific gene expression in mammals (SRY, IRE ABP (23) and TCF1 (15)) *via* a specific interaction of HMG-box with DNA. The homology of HBP1 with HMG proteins strongly suggests that it belongs to this family of transcriptional regulators. The finding that HBP1 might function as transcriptional regulator in yeast provides the potential basis of future work on mechanisms of regulation of fungal genes involved in K^+ transport. HBP1 could complement trk^- yeasts by a direct upregulation of the expression of K^+ transporters such as TRK2 (1) or endogenous K^+ channels (24). Alternatively, HBP1 could act on the expression of yeast factor(s) that are in charge of controlling the activity of otherwise 'silent' K^+ transporter(s) or channel(s).

The ubiquitous distribution of HBP1 mRNA in excitable and non-excitable rat tissues does not provide much information on the possible physiological role of this HMG-like protein in mammals. However, it is worthwhile to observe that HBP1 is homolog to SRY, IRE ABP and TCF1, three factors that regulate expression of genes that initiate and maintain the differentiated phenotype (23). To elucidate whether there is a relation between HBP1 and cell differentiation, we have examined the expression of the HBP1 message in two cellular models which can undergo a spectacular differentiation *in vitro*. One is the preadipocytic cell line Ob1771 and the other one the myogenic cell line C2C12. It was found that in both models there is a large increase of HBP1 mRNA production during terminal differentiation. It was shown with adipocyte cells that HBP1 expression is directly related to the differentiation because factors that promote adipocyte differentiation as insulin provoked a potent accumulation of HBP1 mRNA and inversely, factors that promote dedifferentiation such as okaidic acid and TNFα induced a strong decrease of HBP1 expression.

In conclusion, we have cloned a new protein and presented evidence that this protein is related to the HMG protein family as well as results that suggest that it might play a role in the control of gene expression. Clearly, more work is necessary to confirm the status of HBP1 as a transcriptional regulator. Identification of its specific binding DNA motif could next lead to the identification of target genes and allow to define the role played by HBP1 during differentiation.

ACKNOWLEDGEMENTS

This work was supported by the Centre National de la Recherche Scientifique. We are very grateful to Pr.F.Lacroute (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France) for the gift of pFL61 vector and to Dr H.Sentenac (URA 573, CNRS, Montpellier, France) for the gift of the ura3Δtrk1Δ yeast strain and the pAKT1 plasmid. We are also grateful to Pr.C.Grignon (URA 573, CNRS, Montpellier, France) for providing laboratory facilities for some preliminary experiments. We thank Drs H.Sentenac, M.Minet and P.Daram for very helpful discussions. The excellent technical assistance of M.M.Larroque, R.Pichot, C.Roulinat and F.Aguila is greatly acknowledged.

REFERENCES

1. Ko, C.H. and Gaber, R.F. (1991) *Mol. Cell. Biol.*, **11**, 4266–4273.
2. Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W. J. and Gaber, R.F. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3736–3740.
3. Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.-M., Gaymard, F. and Grignon, C. (1992) *Science*, **256**, 663–665.
4. Ner, S.S. (1992) *Curr. Biol.*, **2**, 208–210.
5. Landsman, D. and Bustin, M. (1993) *BioEssays*, **15**, 539–546.
6. Minet, M., Dufour, M.-E. and Lacroute, F. (1992) *Plant J.*, **2**, 417–422.
7. Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) *Proc. Natl Acad. Sci. USA*, **75**, 1929–1933.
8. Becker, D.M. and Guarante, L. (1991) *Methods Enzymol.*, **194**, 182–187.
9. Doglio, A., Dani, C., Grimaldi, P. and Ailhaud, G. (1986) *Biochem. J.*, **238**, 123–129.
10. Yaffe, D. and Saxel, O. (1977) *Nature*, **270**, 725–727.
11. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156–159.
12. Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.-M., Lovell-Badge, R. and Goodfellow, P.N. (1990) *Nature*, **346**, 240–244.
13. Kelly, M., Burke, J., Smith, M., Klar, A. and Beach, D. (1988) *EMBO J.*, **7**, 1537–1547.
14. Staben, C. and Yanofsky, C. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4917–4921.
15. Van de Wetering, M., Oosterwegel, M., Dooijes, D. and Clevers, H. (1991) *EMBO J.*, **10**, 123–132.
16. Nasrin, N., Buggs, C., Kong, X.F., Carnazza, J., Goebl, M. and Alexander-Bridges, M. (1991) *Nature*, **354**, 317–320.
17. Ailhaud, G., Grimaldi, P. and Négrel, R. (1992) *Annu. Rev. Nutr.*, **12**, 207–233.
18. Jan, L.Y. and Jan, Y.N. (1990) *Nature*, **345**, 6472.
19. Pongs, O. (1992) *Physiol. Rev.*, **72**, 569–588.
20. Takumi, T., Ohkubo, H. and Nakanishi, S. (1988) *Science*, **242**, 1042–1045.
21. Attali, B., Guillemare, E., Lesage, F., Honoré, E., Romey, G., Lazdunski, M. and Barhanin, J. (1993) *Nature*, **365**, 850–852.
22. Laudet, V., Stehelin, D. and Clevers, H. (1993) *Nucleic Acids Res.*, **21**, 2493–2501.
23. Alexander-Bridges, M., Ercolani, L., Kong, X.F. and Nasrin, N. (1992) *J. Cell. Biochem.*, **48**, 129–135.
24. Gustin, M.C., Martinac, B., Saimi, Y., Culbertson, M.R. and Kung, C. (1986) *Science*, **233**, 1195–1197.