Volume 298, number 2,3, 245–248 FEBS 10755 February 1992

Cloning and characterisation of a gene encoding the 11.5 kDa zinc-binding protein (parathymosin-α)

Hans-Ingo Trompeter and Hans-Dieter Söling

Abteilung Klinische Biochemie, Zentrum Innere Medizin, Universität Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, Germany

A cDNA clone encoding the 11.5 kDa zinc-binding protein (ZnBP) was used to screen a genomic and a subgenomic rat liver library to isolate the corresponding genomic DNA. Positive clones were restriction-mapped and sequenced to give the primary structure of the ZnBP gene. A putative promotor region was detected.

Zinc-binding protein, 11.5 kDa; Parathymosin-α; Genomic DNA; Subgenomic library

1. INTRODUCTION

The 11.5 kDa zinc-binding protein (ZnBP) was first described by Brand and Söling [1,2] as being capable of inactivating phosphofructokinase-1 (PFK-1; EC 2.7.1.11) in a Zn^{2+}-dependent but reversible manner. Affinity chromatography revealed its ability to also bind to other glycolytic and gluconeogenetic enzymes in the presence of Zn^{2+} [3]. ZnBP is found in the cytoplasm of liver, brain, adrenal gland, smooth muscle, kidney, lung, spleen and testis, whereas it is only weakly detectable in skeletal muscle and adipose tissues [4]. Cloning and sequencing of its cDNA [5] revealed the identity of ZnBP with rat parathymosin-α [6,7]. One interesting aspect of the primary structure of ZnBP is that it contains in its C-terminal region a sequence (PKRQKT) resembling the prothymosin-α nuclear targeting signal [8].

We have now cloned the genomic DNA encoding the ZnBP transcript in order to get more information about regulation of expression of ZnBP and to possibly detect isoforms.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes were purchased from Gibco/Eggenstein (Germany), T4-ligase, Hybond-N filters, [α-^{32}P]dCTP and [α-^{35}S]-dATP were from Amersham-Buchler/Braunschweig (Germany). Calf intestine alkaline phosphatase (CIP) was obtained from Boehringer/Mannheim (Germany); proteinase K, DNase I and RNase A from Sigma/München (Germany). M13mp18/19RF-DNA was from Pharmacia/Freiburg (Germany), gt10-DNA, the Gigapack II Plus packaging extracts and the lambda DASH rat genomic library came from Stratagene/Heidelberg (Germany). The EMBL-3 rat liver genomic library was from Clontech-Renner/Dannstadt (Germany). GeneScreenPlus membranes came from NEN-Dupont/Bad Homburg (Germany), autoradiography was performed using Kodak XAR-5 films. Male Wistar rats came from Winkelbach/Dernbach (Germany).

Abbreviations: bp, base pairs; CIP, calf intestine alkaline phosphatase; H9I, insert-DNA of cDNA clone H9; kb, kilobase pairs; ZnBP, 11.5 kDa zinc-binding protein.

Correspondence address: H.-I. Trompeter, Abteilung Klinische Biochemie, Zentrum Innere Medizin, Universität Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, Germany.

2.2. *Methods*

Lambda-DNA was prepared from plate lysates, digested with the appropriate restriction enzymes and analysed on 0.4%–0.8% agarose gels. *Eco*RI insert DNA from cDNA clone H9 (H9I) [5] was eluted out of the gel and labelled with ^{32}P using the random priming method [9]. Hybridization to DNA bound to Hybond-N- or GeneScreenPlus-filters was performed as in [10].

Preparation of genomic DNA from rat liver. A fresh rat liver was washed in fresh, ice-cold sucrose buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.05 mM spermidine, 15 mM HEPES, 2 mM EDTA, pH 8.1, and 0.5 mM EGTA) and homogenized in 60 ml of this buffer. The homogenate was centrifuged at 1000 × g. The pellet containing the nuclei was washed twice with the above sucrose buffer and resuspended in 60 ml of TNE (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, pH 8.1). 1.5 ml of 20% (w/v) SDS and 1.5 ml of proteinase K (2 mg/ml in TNE) were added and the mixture was incubated overnight at 37°C with gentle shaking. The viscous solution was carefully extracted with phenol/chloroform/isoamylalcohol (25:25:1, v/v/v) and precipitated with ethanol. DNA was resuspended in 50 ml of 0.1 × SSC (15 mM NaCl, 1.5 mM Na-citrate) and dialysed against 0.1 × SSC. After treatment with RNase A (100 μl of 10 mg RNase A/ml 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 h, 37°C), the DNA was reincubated with 250 μl of proteinase K solution (1 h, 37°C), three times extracted with phenol/chloroform/isoamylalcohol, precipitated with ethanol and resuspended in 50 ml of TE-buffer. After dialysis against TE for three hours the DNA was analyzed on 0.2% agarose gels.

Construction of a subgenomic library. Genomic DNA from rat liver was digested with *Bam*HI, *Eco*RI and *Hin*dIII and the resulting fragments were alkali-blotted onto GeneScreenPlus membranes and hybridized to H9I.

A region between 5 and 7 kb of *Eco*RI-fragments was eluted out of an agarose gel, dephosphorylated with CIP, and ligated to 1.1 μg of *Eco*RI-cut gt10-DNA in a molar ratio insert-vector of 2:1. The ligation

reaction was in-vitro-packaged using the Gigapack II Plus extracts and the resulting library was titered on *E. coli* C600hflA.

Library screening and clone sequencing. The gt10-phages were plated onto *E. coli* C600hflA, the EMBL-3 library on *E. coli* K803 and the lambda DASH library on *E. coli* P2PLK17. Bacteriophage plaques from the libraries were transferred onto Hybond-N membranes according to [11] and hybridized to insert DNA of clone H9. Positive plaques were picked and single positive plaques were obtained by two rescreening cycles using the same probe. Phage DNA of genomic clones was restriction-mapped with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I, *Sst*I and *Xho*I.

Fragments chosen for sequencing were eluted out of a gel and subcloned into M13mp18/19, insert-orientations being examined according to [11]. The fragments were sequenced using the Sequenase and Taquenase kits according to the manufacturers instructions using parallel dGTP and dITP runs respectively.

3. RESULTS

Earlier studies had led to the isolation of gt11-cDNA clone H9, which carries a 936 bp *Eco*RI insert (H9I) representing the complete translated region of the ZnBP [5], 115 bp of 5'-nontranslated region and the complete 3'-nontranslated region including a poly(A) tail. It displays a *Bam*HI restriction site at base 384 only 11 amino acid residues upstream the C-terminus of the encoded protein. H9I was used as a highly specific probe in Southern analyses of rat genomic DNA. One *Eco*RI fragment (about 5.5–6 kb), one major *Bam*HI fragment (about 1 kb) and a weakly hybridizing *Bam*HI fragment (about 2.9 kb) were detected. These results indicate, that H9I is transcribed only from a single-copy gene.

To isolate the genomic DNA corresponding to H9I, 2×10^6 clones of a rat liver genomic EMBL-3 library were screened using H9I as a probe but no positive clones could be isolated. To ensure isolation of the gene encoding the H9I transcript, a subgenomic library of 5–7 kb *Eco*RI fragments in lambda gt10 was constructed and a lambda DASH rat liver genomic library was screened additionally. 5×10^5 gt10 clones and 10^6 lambda DASH clones were screened using ^{32}P-labelled H9I as a probe. Three subgenomic gt10- and two genomic lambda DASH-clones (H9D1 and H9D2) were isolated during two rescreening cycles and subsequently analysed by Southern blotting and restriction mapping. All subgenomic gt10 clones carried a 5.7 kb *Eco*RI insert hybridizing with H9I. *Sal*I-inserts of lambda-DASH clones were about 18 kb long and cross-hybridized with H9I and the 5.7 kb *Eco*RI subgenomic fragment as well. Subsequent analyses of the lambda DASH clones with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I, *Sst*I and *Xho*I yielded a restriction map of the genomic region encoding the H9I-transcript (Fig. 1). A 5.7 kb *Eco*RI fragment hybridizing to the subgenomic 5.7 kb fragment was found as well as 1.0 kb and 2.9 kb *Bam*HI fragments. These results are in line with the above Southern analyses of genomic DNA. The 5.7 kb *Eco*RI fragment found in the subgenomic gt10 clones displayed the same *Bam*HI pattern as the 5.7 kb *Eco*RI fragment

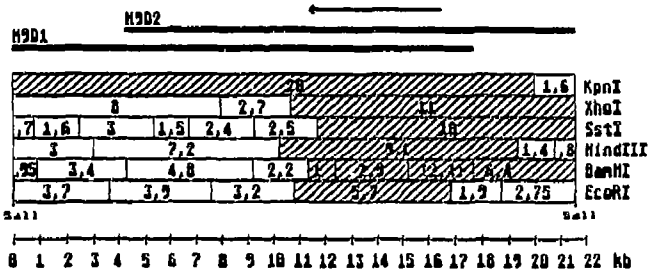

Fig. 1. Restriction map of the genomic clones. The region of DNA spanned by *Sal*I inserts of clones H9D1 and H9D2 is shown. The black bars show the position of the two clones, the arrow marks the direction of transcription. Fragments hybridizing with H9I are hatched, the 2.4 kb *Bam*HI-*Sal*I border fragment of H9D1 is marked by the interrupted vertical line in the *Bam*HI row. The position of the 1.9 kb and 2.75 kb *Eco*RI fragments is arbitrary. The lengths of fragments are given in kb.

of clones H9D1 and H9D2 and was thereby shown to represent the same DNA. The 2.9 kb *Bam*HI fragment hybridized to the 5'-*Bam*HI fragment of H9I, the 1.0 kb fragment to the 3'-one, indicating the direction of transcription.

Since both libraries yielded clones representing the same region of genomic DNA, the 1.0 kb and 2.9 kb *Bam*HI fragments, a 2.4 kb *Bam*HI-*Sal*I fragment (represented as a 6.4 kb *Bam*HI-*Sal*I border fragment in H9D2) and the 5.7 kb *Eco*RI fragment (to verify the borders between the short fragments) of H9D1 were subcloned into M13mp18 and M13mp19 and sequenced. The complete sequence of 6301 bp is shown in Fig. 2.

4. DISCUSSION

Since intensive screening of an EMBL-3 rat genomic library yielded no positive clones, a subgenomic library based on genomic Southern analyses was constructed in order to overcome possible cloning restrictions resulting from e.g. unstatistical distribution of *Sau*IIIA sites on the genomic DNA of ZnBP, thus leading to a low representation of this gene in a genomic library. The subgenomic library as well as an additionally screened lambda-DASH rat genomic library yielded several clones. Restriction analyses and cross-hybridizations between both clone families showed them to represent the same DNA and were in line with the Southern analyses of genomic DNA.

The genomic fragments hybridizing with H9I spanned a region of 6301 bp and contained the whole transcribed region represented by H9I with identical corresponding sequences (see Fig. 2). The translated part (printed bold in Fig. 2) is interrupted by one large (2589 bp) and three small (191 bp, 150 bp and 167 bp) introns. No introns were detected in the regions corresponding to the nontranslated parts of H9I.

A possible promotor region was detected in a region

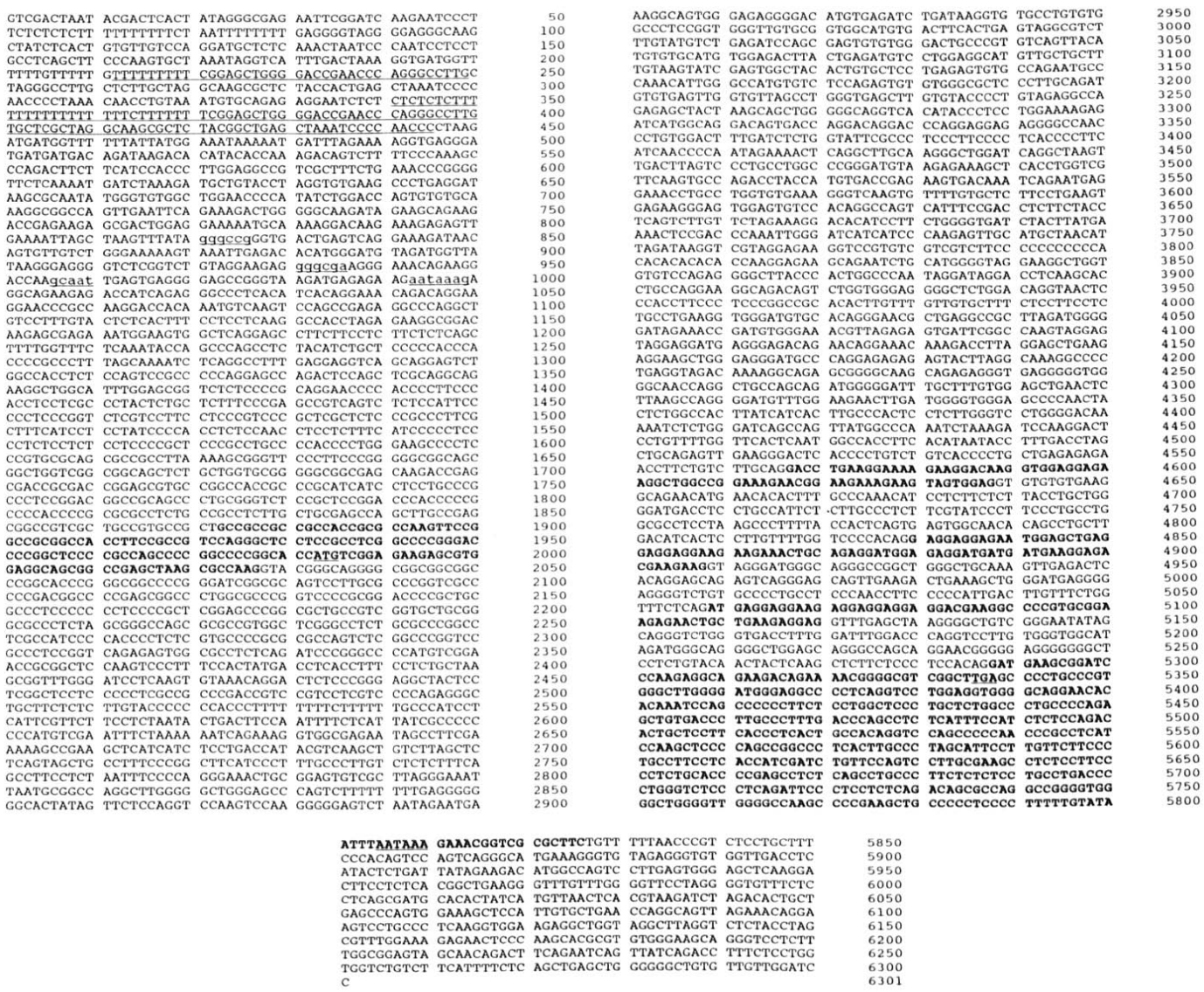

Fig. 2. Sequence of the genomic DNA encoding ZnBP. The 6301 bp region of the subcloned fragments is printed in CAPITAL letters, beginning with the *Sal*I site and ending with the *Bam*HI site. Possible promotor regions are printed in small underlined letters. DNA represented in H9I (mainly exons) is printed **bold**; start- and termination-codon as well as the polyadenylation signal are **underlined**. The underlined region at the beginning of the sequence marks the 'rat identifier'.

from base 830 to base 1000 (underlined in Fig. 2). The sequence AATAAAG, beginning at base 993 shows strong homology to the consensus sequence of the TATA-box, GCAAT (base 956) a good homology to the CAAT-box. Two possible GC-boxes are also underlined in Fig. 2. This configuration would implicate a possible transcription start around base 1020 which would give a transcript of about 1770 bases (excluding the poly(A) tail), assuming that there are no introns in the 5′-nontranslated region. This value is in good agreement with the finding of a 1800 base transcript for parathymosin-α by Clinton and colleagues [13].

No hint of a possible isoform of ZnBP synthesized by differential splicing of a premature mRNA was found in any reading frame of the gene. Only one additional donor site (AGGT; base 1284) could be detected in the 5′-nontranslated region. Therefore a differentially spliced isoform seems unlikely. If at all, a possible isoform should be transcribed from a different gene which could not be detected by H9I under stringent hybridization conditions.

A computer search in the EMBL database/Heidelberg (Germany) showed no relationships of the genomic DNA with other known sequences except for the existence of a 'rat identifier' sequence [14] upstream the promotor region.

Acknowledgements: We thank Dr. Klaus-Peter Reiners (Institute for Genetics, University of Cologne, Germany for performing the computer search in the EMBL data base/Heidelberg. This work was

supported by a grant given to H.D.S. by the Deutsche Forschungsgemeinschaft (So 43/45-2).

REFERENCES

[1] Brand, I.A. and Söling, H.D. (1986) J. Biol. Chem. 269, 5895–5900.
[2] Brand, I.A., Heinickel, A., Kratzin, H. and Söling, H.D. (1988) Eur. J. Biochem. 177, 561–568.
[3] Brand, I.A. and Heinickel, A. (1991) J. Biol. Chem. 266, 20984–20989.
[4] Brand, I.A., Heinickel, A. and Söling, H.D. (1991) Eur. J. Cell Biol. 54, 157–165.
[5] Trompeter, H.-I., Brand, I.A. and Söling, H.D. (1989) FEBS Lett. 253, 63–66.
[6] Frangou-Lazaridis, M., Clinton, M., Goodall, G.J. and Horecker, B.L. (1988) Arch. Biochem. Biophys. 263, 305–310.
[7] Panneerselvam, C., Clinton, M., Wellner, D. and Horecker, B.L. (1988) Biochem. Biophys. Res. Commun. 155, 539–545.
[8] Manrow, R.E., Sburlati, A.R., Hanover, J.A. and Berger, S.E. (1991) J. Biol. Chem. 266, 3916–3924.
[9] Feinberg, A.D. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[10] Beginnen, K. (1988) Ph. D. Thesis, University of Cologne, Germany.
[11] Benton, U.D. and Davis, R.W. (1977) Science 196, 180–182.
[12] Howarth, A.J., Gardner, R.C., Messing, J. and Shepherd, R.J. (1981) Virology 112, 678–684.
[13] Clinton, M., Frangou-Lazaridis, M., Panneerselvam, C. and Horecker, B.L. (1989) Arch. Biochem. Biophys. 269, 256–263.
[14] Sutcliffe, J.G., Milner, R.J., Bloom, F.E. and Lerner, R.A. (1982) Proc. Natl. Acad. Sci. USA 79, 4942–4946.