Nucleic Acids Research, Vol. 19, No. 23 6383–6389

Liver specific transcription factors of the HNF3-, C/EBP- and LFB1-families interact with the A-activator binding site

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Received September 24, 1991; Revised and Accepted November 1, 1991

ABSTRACT

The A-activator binding site (AABS), present in the *Xenopus* A2 vitellogenin gene and several mammalian liver specifically expressed genes, interacts with different liver specific transcription factors including LFB1- and C/EBP-isobinders. We have now isolated some additional proteins interacting with AABS and show that they are HNF3-isobinders. The interactions between AABS and members of the HNF3 family are confirmed by binding studies using bacterially made HNF3-alpha protein. Thus a short DNA module of 24 bp is able to bind proteins of three different families of liver specific transcription factors. Competition experiments in the cell free *in vitro* transcription show that AABS dependent transcriptional activation is mediated by transcription factors belonging to at least two different families, the C/EBP- and the HNF3-isobinders. Being able to mediate the action of several distinct transactivators, AABS may thus be a prototype for a novel kind of tissue specific promoter modules with unique regulatory capacities.

INTRODUCTION

In higher eucaryotes tissue specific gene expression is mainly controlled by tissue specific transcription factors interacting with *cis*-acting DNA elements. While this general scheme has been found valid for many different cell types, it has become clear that in most cases tissue specificity is not conferred by one factor alone but rather by the combination of several distinct factors. Especially in the case of liver specific gene expression a number of regulatory elements and *trans*-acting factors have been identified recently (1).

According to their binding specificities liver specific transcription factors can be grouped into four families of 'isobinders', i.e. proteins that recognize the same or related DNA sequences:

– The LFB1 family including LFB1 (ref. 2; also called HNF1, ref. 3 or HNF1α, ref. 4) and LFB3 (ref. 5; also called vHNF1, ref. 6 or HNF1β, ref. 4), two factors that have structural similarities to the POU proteins.

– The C/EBP family including C/EBP (7), DBP (8), LAP (ref. 9; also called IL-6-DBP, ref. 10 or AGP/EBP,ref. 11), NF-IL6 (12) and others, recognizing a number of related sequences with a basic DNA binding domain.

– The HNF3 family including HNF3-alpha, -beta and -gamma, proteins that show structural similarities to the gene product of the homeotic gene *fork head* of *Drosophila* (13).

– HNF4, a member of the steroid hormone receptor superfamily (14).

Studying the regulatory elements involved in the liver specific, hormone dependent and developmentally regulated expression of the A2 vitellogenin gene of *Xenopus laevis* (15) we previously identified an estrogen responsive unit (16) and a short promoter module of 24 bp, the A-activator binding site (AABS), that mediates liver specific activity (17).

Using transfection and *in vitro* transcription experiments we showed that AABS, present in a *Xenopus* gene, mediates liver specific transcriptional activation in mammalian cells, suggesting that it interacts with transcription factors that have been highly conserved during evolution (17). Initial experiments revealed that AABS interacts with proteins belonging to two different families of transcription factors, the LFB1- and the C/EBP-isobinders (18).

In this report we describe the purification of additional factors interacting with AABS and show that they belong to a separate family of transcription factors, i.e. the HNF3-isobinders. Using a cell free *in vitro* transcription system we demonstrate that the liver specific transcriptional activity mediated by AABS is conferred by proteins of at least two distinct families of liver specific transcription factors.

MATERIALS AND METHODS

Oligonucleotides

Synthetic oligonucleotides containing the following sequences were used as probes and competitors in the gel retardation assay and in the cell free *in vitro* transcription: AABS (A-activator binding site): *Xenopus laevis* vitellogenin A2 promoter −121/−97 (18); HNF3BS (HNF3 binding site): Mouse transthyretin promoter −111/−85 (19); C/EBPBS (C/EBP binding site): Oligonucleotide used for Southwestern detection

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of C/EBP (7); HP1 (LFB1 binding site): *Xenopus laevis* albumin (68 kD) promoter −67/−50 (20); BABS (B-activator binding site): *Xenopus laevis* vitellogenin B1 promoter −56/−42 (21); ERE: EREwt (27mer; ref. 22); element 1: *Xenopus laevis* vitellogenin A2 promoter −121/−109 (18); element 2: *Xenopus laevis* vitellogenin A2 promoter −108/−97 (18). All oligonucleotides were synthesised as complementary strands to give double-stranded oligonucleotides with Hind III- and BamH I-overhangs upon annealing and purified over NACS-columns (BRL).

Preparation of nuclear extracts and gel retardation assays

Nuclear extracts from rat liver and spleen were prepared as described (21, 23).

For gel retardation analysis complementary oligonucleotides were annealed and labeled by filling in the Hind III- and BamH I-overhangs using the Klenow-fragment of DNA-polymerase I (E.C.2.7.7.7.; Boehringer) and α-^{32}P-dCTP (Amersham). Proteins were incubated at room temperature with poly (dI-dC) and competitor oligonucleotides in a buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 4% Ficoll 400. After 15 minutes radioactively labeled oligonucleotides were added and the reaction was incubated for another 15 minutes at room temperature before it was analysed by 4% native PAGE in 0.25×TBE (12.5 mM Tris base, 12.5 mM boric acid, 0.625 mM EDTA, pH 8.3). The gel was run for 1.5 hours, fixed, dried and exposed to X-ray film.

In vitro transcription

In vitro transcription reactions were performed as described previously (17,20,21), using cell free extracts derived from rat liver or spleen nuclei. Transcription templates were generated by insertion of AABS or HNF3BS oligonucleotides into the Hind III and Bgl II sites of PL-TG (20), thus linking them to a TATA box and a G-free cassette of 400 bp. Quantitative analysis was performed with a laser densitometer.

For the complementation of a rat spleen *in vitro* transcription system, 5 ml heparine Sepharose fractions were concentrated as follows: 500 µl of 4M $(NH_4)_2SO_4$ and 300 mg/ml of solid $(NH_4)_2SO_4$ were added to the fractions and dissolved slowly by stirring at 4°C for 15 minutes. After another 30 minutes at 4°C the proteins were precipitated by centrifugation at 35000 rpm and 4°C for 25 minutes in a type 60 TI rotor (Beckman). The precipitate was dissolved in 500 µl of nuclear extract dialysis buffer (25 mM HEPES, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.6) and dialyzed two times for 90 minutes against 500 ml of nuclear extract dialysis buffer.

Purification of proteins interacting with AABS

For the purification of HNF3-isobinders interacting with AABS, transcriptionally active rat liver nuclear extracts were adjusted to 200 mM KCl, 0.1% NP 40 and 20 mM Na_2MO_4 and loaded on a heparine Sepharose column (Pharmacia) equilibrated with buffer H (25 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% NP 40, 20 mM Na_2MO_4) containing 200 mM KCl. The column was washed with buffer H containing 200 mM KCl and eluted in several steps with buffer H containing 230−1200 mM KCl. The fraction eluting at 300 mM KCl was adjusted to 200 mM KCl and 12.5 mM $MgCl_2$ and loaded on a 7 ml AABS-affinity column prepared as described by Kadonaga and Tjian (24) by coupling oligomerized AABS-oligonucleotides to CNBr activated Sepharose CL-4B (Pharmacia) and equilibrated with buffer K (25 mM HEPES pH 7.6, 12.5 mM $MgCl_2$, 20% glycerol, 1 mM DTT, 0.1% NP 40) containing 200 mM KCl. The column was washed with buffer K containing 200 mM KCl and eluted with buffer K containing 600 mM KCl. The fractions containing the AABS binding activity were diluted to 200 mM KCl with buffer K and loaded on a 1 ml AABS-Sepharose-affinity column via an analogously prepared BABS-Sepharose precolumn. This column carries an unrelated oligonucleotide and was included in the procedure to eliminate proteins that were retarded on the first AABS-affinity column without interacting sequence specifically with AABS. After loading and washing with buffer K containing 200 mM KCl the two columns were eluted separately with buffer K containing 600 mK KCl.

For SDS-PAGE the fractions of the last affinity column were concentrated as described by Wessel and Flügge (25). Renaturation of proteins from SDS-polyacrylamide gels was performed as described by Wang and coworkers (26), the binding buffer for the renaturation of the proteins being the same as for the gel retardation assays.

Preparation of HNF3-alpha-GST fusion protein

To produce HNF3-alpha protein as a fusion protein with GST (glutathion-S-transferase), the HNF3-alpha cDNA (kindly provided by W.S.Chen and J.E. Darnell Jr.) was inserted into the BamH I site of pGEX-2T (Pharmacia). Isolation and purification of GST-HNF3-alpha fusion proteins from *E.coli JM 101* transformed with the recombinant plasmid were performed according to the manufacturers instructions.

RESULTS

AABS interacts with three different families of transcription factors

By binding and *in vitro* transcription analysis we showed previously that the promoter module AABS present in several genes which are expressed specifically in the liver interacts with proteins belonging to the LFB1- and C/EBP-families of transcription factors. Nevertheless these interactions did neither account for the total liver specific transcriptional activation mediated by AABS in an *in vitro* transcription system, nor for all the complexes seen in a gel retardation assay with rat liver nuclear proteins and AABS. Thus we concluded that other rat liver proteins we called AAP I and AAP II interact with AABS (18).

Meanwhile two novel families of liver specific transcription factors had been defined by the cloning of HNF3-alpha, -beta and -gamma (13) and HNF4 (14), and although sequence comparison of AABS (see Fig. 7) with the consensus binding sites for these factors did not reveal any obvious sequence homologies we wondered whether AAP I or AAP II might belong to one of these families. To address this question we used oligonucleotides that contained the HNF3 binding site (HNF3BS, ref. 19) or the HNF4 binding site (HNF4BS, ref. 19) as competitors in binding and *in vitro* transcription studies (see below).

In previous studies gel retardation assays using rat liver nuclear proteins and radioactively labeled AABS had shown four bands corresponding to the specific complexes I-IV (18). Changing the binding buffer conditions we now succeded in increasing the resolution of our gel retardation assays, resolving the complexes III and IV into three distinct complexes each, complexes IIIa-c and IVa-c, respectively (Fig. 1*A*, lane 1). When increasing amounts of unlabeled AABS competitor oligonucleotides were included in the binding reaction (Fig. 1*A*, lanes 2−6) they

competed—with varying efficiency—for the formation of all the specific complexes. As in our previous studies, even a high molar excess of AABS did not compete completely for the formation of the complex with the highest electrophoretic mobility that was therefore considered to be due to unspecific interactions between AABS and a very abundant rat liver protein.

Increasing amounts of HNF3BS (Fig. 1*A*, lanes 7–11) competed efficiently for the complexes I,II and IVa and—less efficiently—for the complexes IVb and IVc. All these complexes may thus contain proteins that are HNF3-isobinders. By using competitor oligonucleotides containing HP1 (binding site for LFB1) and C/EBPBS (binding site for C/EBP-isobinders) as positive controls we could specify our previous results in the respect that HP1 did compete for the complexes IVa, IVb and IVc (Fig. 1*A*, lane 12) and that C/EBPBS competed more efficiently for complex IIIa than for complexes IIIb and IIIc (Fig. 1*A*, lane 13). The specificity of the observed interactions was confirmed by the finding that an unrelated oligonucleotide did not compete for the formation of any of the complexes (Fig. 1*A*, lane 14).

In an analogous experiment HNF4BS did not have any influence on the formation of complexes between AABS and rat liver nuclear proteins (data not shown), thus excluding that HNF4-isobinders interact with AABS.

The binding studies in Fig. 1*A* show that the complexes I,II and IIIa are competed for less efficiently by AABS (lanes 2–6) than by HNF3BS (lanes 7–11), indicating a higher affinity of HNF3BS for the same proteins. To verify this finding we performed a reverse binding/competiton experiment using HNF3BS instead of AABS as a radioactively labeled probe. Without any specific competitor rat liver nuclear proteins formed several complexes with HNF3BS (Fig. 1*B*, lane 1), that were competed for very efficiently by increasing amounts of HNF3BS oligonucleotides (Fig. 1*B*, lanes 2–6), whereas the same amounts of AABS oligonucleotides were not sufficient for a complete competition (Fig. 1*B*, lanes 7–11) confirming the different affinities of the two binding sites. HP1 competed only for the

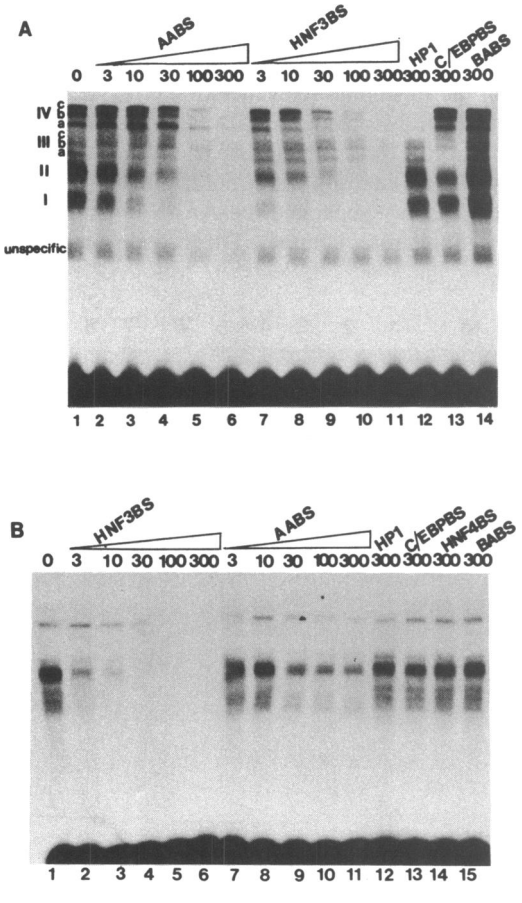

Fig. 1. AABS contains a low affinity binding site for HNF3-isobinders. For a gel retardation assay, radioactively labeled AABS (*A*) and HNF3BS (*B*) oligonucleotides were incubated with rat liver nuclear proteins and 100 ng poly (dI-dC) in the presence or absence of competitor oligonucleotides; the resulting complexes were analyzed by native PAGE. Competitor oligonucleotides were used in fold molar excess as given above the lanes; specific complexes formed with AABS (*A*) are marked at the left.

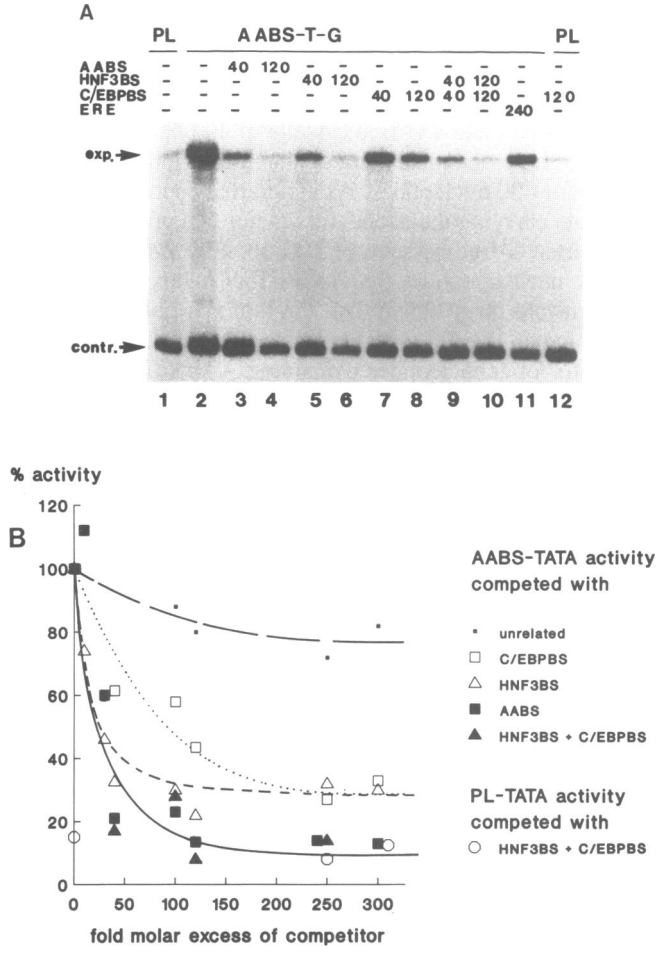

Fig. 2. AABS mediated transcriptional activity is conferred by HNF3- and C/EBP-isobinders. *A*: *In vitro* transcription analysis: A minimal polylinker-TATA box promoter (PL; lanes 1 and 12) or an AABS-TATA box promoter (AABS-T-G; lanes 2–11) was used to direct the transcription of a 400 nucleotide RNA from a G-free cassette (exp.); the 200 nucleotide control RNA was transcribed from a shorter G-free cassette under the control of the adenovirus major late promoter (contr.). Competitor oligonucleotides were added to the *in vitro* transcription reaction in fold molar excess as given above the lanes. The ERE oligonucleotide was used as an unrelated competitor. *B*: Quantitative densitometric analysis of five independent *in vitro* transcription/competition experiments using the activity of the adeno virus major late promoter for standardization. The relative activities of the AABS-TATA box promoter and the polylinker-TATA box promoter are plotted against the excess of competitor oligonucleotides included in the *in vitro* transcription reactions.

two weak complexes formed with HNF3BS (Fig. 1***B***, lane 12) which have electrophoretic mobilities comparable to complexes IVb and IVc in Fig. 1***A***. Together with the weak competition for the complexes IVb and IVc by HNF3BS seen in Fig. 1***A***, this finding suggests that the HNF3BS oligonucleotide includes a low-affinity binding site for LFB1-isobinders as it has been proposed previously by the group of Darnell (19). C/EBPBS (Fig. 1***B***, lane 13), HNF4BS (Fig. 1***B***, lane 14) and another unrelated oligonucleotide (Fig. 1***B***, lane 15) had no effect on the formation of any of the complexes.

Summarizing our binding studies we concluded that AABS is able to interact with rat liver nuclear proteins belonging to the HNF3 family of transcription factors.

HNF3-isobinders contribute to AABS mediated transcriptional activation

To test the functional significance of the observed interactions between AABS and HNF3-isobinders we performed *in vitro* transcription experiments, including HNF3BS as a competitor in the reactions. The reporter constructs used for our analysis carried either AABS or an unrelated polylinker sequence in front of a TATA box and a G-free cassette of 400 bp. In the absence of GTP, these templates direct the transcription of discrete products of 400 nucleotides. As an internal control we included a template carrying the adenovirus major late promoter in front of a shorter G-free cassette of 200 bp. Fig. 2***A*** shows that in rat liver nuclear extract the AABS-TATA box promoter was about ten-fold more active than the minimal polylinker-TATA box promoter (lane 2 in comparison to lane 1). Addition of competitor oligonucleotides containing AABS (lanes 3 and 4), HNF3BS (lanes 5, 6, 9 and 10) or C/EBPBS (lanes 7, 8, 9 and 10) reduced this stimulation, whereas the addition of unrelated oligonucleotides (lane 11) had no effect. The activity of the polylinker-TATA box promoter was not affected by the addition of oligonucleotides (lane 12 in comparison to lane 1). We conclude from this *in vitro* transcription analysis that HNF3-isobinders contribute to the liver specific transcriptional activation mediated by AABS.

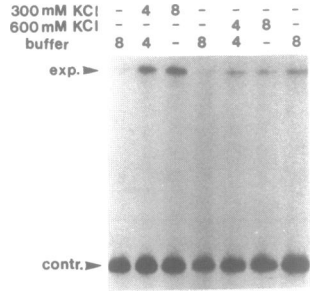

Fig. 3. Partially purified HNF3-isobinders stimulate AABS mediated transcriptional activity. Fractions of a rat liver nuclear extract eluting at 300 mM KCl and 600 mM KCl from a heparine Sepharose column were concentrated ten-fold and added to an *in vitro* transcription system derived from rat spleen nuclei. The autoradiography shows an *in vitro* transcription/complementation analysis with the two signals in each lane deriving from the transcription of a 400 bp G-free cassette driven by the AABS-TATA box promoter (exp.) and of a 200 bp G-free cassette under the control of the adeno virus major late promoter (contr.). Microliters of concentrated fractions and/or dialysis buffer added to the rat spleen nuclear extract are given above the lanes. A quantitative densitometric analysis of three independent *in vitro* transcription/complementation experiments using the adenovirus major late promoter activity for standardization shows that the addition of the 300 mM fraction stimulates the AABS dependent activity as follows: a) 4μl: 2,1-, 2,6- and 2,4-fold; b) 8μl: 2,5-, 3,6- and 2,8-fold.

A quantitative analysis of several independent competition experiments in the cell free *in vitro* transcription system (Fig. 2***B***) showed that addition of a large excess of AABS oligonucleotides reduced the transcriptional activity of the AABS-TATA box promoter to the activity of the polylinker-TATA box promoter (10–15% of the uncompeted activity). Interestingly, HNF3BS or C/EBPBS reduced the AABS mediated activity only to about 30% when added separately to the transcription reaction. Yet, when both oligonucleotides were included simultaneously (e.g. fig. 2***A***, lanes 9 and 10), the activity was reduced to 10–15%, the level reached by the AABS competiton, indicating that the C/EBP- and HNF3-isobinders are the predominant transactivators. In a reverse experiment using a template carrying HNF3BS instead of AABS in front of the TATA box and the 400bp G-free cassette, HNF3BS was also able to mediate transcriptional activation in the cell free *in vitro* transcription system derived from rat liver. This activation could be competed for specifically by AABS and HNF3BS oligonucleotides with AABS oligonucleotides competing—in agreement with our binding data—less efficiently than HNF3BS oligonucleotides (data not shown).

Thus the interactions between AABS and HNF3-isobinders we had observed in our binding studies (Fig. 1) contribute to AABS mediated transcriptional activation in a cell free *in vitro* transcription system (Fig. 2).

Isolation of HNF3-isobinders interacting with AABS

Because of the functional relevance of the interactions between AABS and HNF3-isobinders we were interested in a further characterization of these proteins we had previously named AAP I and AAP II (18). Starting with transcriptionally active rat liver nuclear extracts, we separated the AABS binding activities on a heparine Sepharose column. The proteins included in the

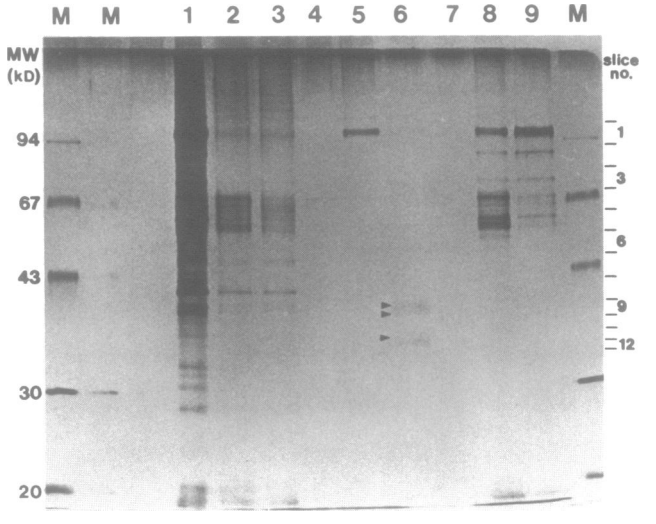

Fig. 4. Purified HNF3-isobinders have molecular weights of 40, 38 and 34 kD. SDS-polyacrylamide gel showing fractions of the pool retained on the first AABS affinity column (lane 1), the flowthrough (lanes 2 and 3), wash (lanes 4 and 5) and eluate (lane 6) of the second AABS affinity column as well as of the eluate of the BABS column (lanes 8 and 9; see *Materials and methods*). The wash fraction shown in lane 5 containes contaminating proteins that were retarded weakly by the column and thus could only be eliminated by extensive washing. The slices of lane 6 used for the renaturation experiment (Fig. 5) are marked at the right. The three bands containing the purified HNF3-isobinders are indicated with arrows and molecular weight markers (Pharmacia, Cat. no. 17-0446-01) are given.

complexes I and II (Fig. 1) eluted at 300 mM KCl and 600 mM KCl, respectively whereas LFB1-isobinders eluted at lower and C/EBP-isobinders at higher ionic strenghts, thus being separated from the HNF3-isobinders (data not shown). The 300 mM KCl- and 600 mM KCl-fractions were concentrated and added to a cell free *in vitro* transcription system derived from rat spleen nuclei which are devoid of any AABS dependent transcriptional activity. The addition of the 300 mM KCl-fraction to the rat spleen nuclear extract led to an up to three-fold stimulation of transcriptional activity, whereas the addition of 600 mM KCl-fraction had no effect (Fig. 3). Since the activity of the adeno virus major late promoter included as an internal control was not affected in these experiments, the observed stimulation was specific for the AABS-TATA box promoter. These experiments demonstrated that HNF3-isobinders which transactivate the template containing AABS *in vitro* were contained in the 300 mM KCl-fraction.

Consequently, we used this fraction, which represented about 10% of the total rat liver nuclear proteins for a further purification using DNA affinity chromatography. The final preparation contained three proteins of apparent molecular weights of 40, 38 and 34 kD (Fig. 4, lane 6). To verify that the AABS binding activity contained in the eluate of the second AABS affinity column was due to these proteins, we cut the corresponding lane of the SDS-polyacrylamide gel into slices and eluted the proteins. After renaturation the proteins were assayed for DNA binding activity by gel retardation analysis (Fig. 5). Using AABS as a probe we found that the AABS binding activity was contained mainly in two slices corresponding to molecular weights of 38−42 kD (slice 9) and 30−34 kd (slice 12), the slices which contained the purified proteins (Fig. 5, AABS). The specificity of the observed complexes was verified by binding/competition experiments which also demonstrated that the purified proteins were no C/EBP-isobinders (data not shown). The gel retardation assay using HNF3BS instead of AABS as a radioactively labeled probe showed exactly the same pattern of complex formation (Fig. 5, HNF3BS). Thus, the proteins we had purified based on their affinity towards AABS were members of the family of HNF3-isobinders.

Bacterially synthesized HNF3-alpha binds specifically to AABS

For a further investigation of the interactions between transcription factors of the HNF3 family and AABS we inserted the cloned HNF3-alpha cDNA (kindly provided by W.S. Chen and J.E. Darnell Jr.) into the bacterial expression vector pGEX-2T (Pharmacia) to produce glutathion-S-transferase-HNF3-alpha fusion proteins in *E.coli*. The purified protein formed specific complexes with radioactively labeled AABS (Fig. 6*A*) and HNF3BS (Fig. 6*B*).

To study the affinity of HNF3-alpha towards HNF3BS and AABS, we performed binding/competition experiments using HNF3BS (Fig. 6*A* and 6*B*, lanes 2−4 each) and AABS (Fig. 6*A* and 6*B*, lanes 5−7 each) as competitor oligonucleotides. In agreement with the results we obtained using rat liver nuclear extract (Fig. 1), HNF3BS competed more efficiently for the formation of complexes than AABS.

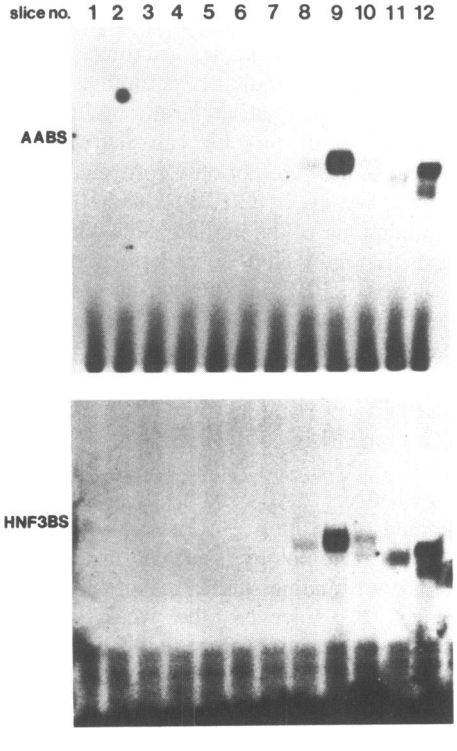

Fig. 5. Purified HNF3-isobinders form equivalent complexes with AABS and HNF3BS. After SDS-PAGE of the affinity purified HNF3-isobinders, the gel lane was cut into slices. The proteins were eluted, renatured and used as samples in gel retardation assays with AABS and HNF3BS as radioactively labeled probes. The numbers above the lanes refer to the gel slices marked in Fig. 4.

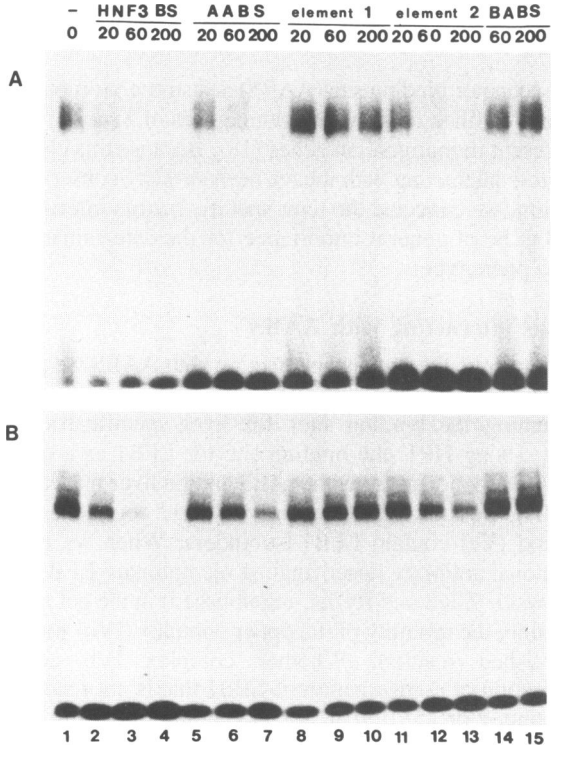

Fig. 6. Recombinant HNF3-alpha protein binds with different affinities to AABS and HNF3BS. Gel retardation assay with bacterially synthesized GST-HNF3-alpha fusion protein using AABS (*A*) and HNF3BS (*B*) as radioactively labeled probes. Competitor oligonucleotides were used in fold molar excess as given above the lanes. Elements 1 and 2 represent the 5'- and 3'-part of AABS, respectively (see Fig. 7).

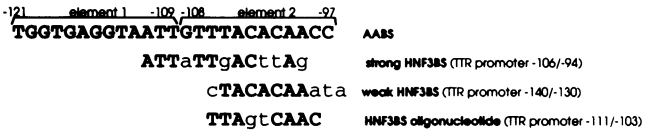

Fig. 7. The 3'-part of AABS shows sequence homologies to known HNF3 binding sites. Sequence comparison of AABS with the two HNF3 binding sites definded in the mouse TTR promoter (19) and with the 5'-part of the HNF3BS oligonucleotide. Matches are shown in bold capitals.

In addition to this quantitative analysis we were also interested in a closer determination of the binding site for HNF3-isobinders within AABS. We had previously shown that AABS consists of the two elements 1 and 2 forming an imperfect palindrome (compare Fig. 7) and that perfectly palindromic constructs of both of these elements (1/1 and 2/2) mediate liver specific transactivation (18). We therefore included oligonucleotides in the binding reactions representing only one of these elements. Element 1, representing the 5' part (−121/−109) of AABS did not compete for the formation of complexes (Fig. 6*A* and 6*B*, lanes 8−10 each), whereas element 2, corresponding to bp −108/−97 competed as efficiently as the entire AABS (Fig. 6*A* and 6*B*, lanes 11−13 in comparison to lanes 5−7 each). We therefore conclude that the binding site for HNF3-isobinders is located predominantly in the 3' part of AABS, the 12 bp sequence from −108 to −97. In both gel retardation assays, unrelated oligonucleotides had no effect (Fig. 6*A* and 6*B*, lanes 14 and 15 each).

DISCUSSION

The A-activator binding site (AABS) is a strong promoter element originally defined in the vitellogenin genes of *Xenopus laevis* but also present in mammalian genes (18). Because this element and the factors interacting with it have been strongly conserved during evolution, we expected the liver specific factors interacting with AABS to be of general importance for the determination of the hepatic phenotype.

Proteins interacting with AABS

To characterize the proteins interacting with AABS we performed binding/competition experiments with oligonucleotides representing the binding sites for liver specific transcription factors. Using HP1, the binding site for LFB1 as a competitor in a gel retardation assay with AABS and rat liver nuclear proteins (Fig. 1*A*), we showed that at least two of the complexes observed (IVb and IVc) contain LFB1-isobinders. When we included a monoclonal antibody raised against recombinant LFB1 (Eberle, Bartkowski, Zoidl and Ryffel, unpublished) in the gel retardation assay, only the mobility of the upper complex (IVc) was reduced (unpublished results). Whether complex IVb contains a modification or conformation of LFB1 that is not recognized by this antibody or a different, yet isobinding protein (e.g. LFB3, ref. 4,5,6) remains to be established.

The second family of transcription factors interacting with AABS are the C/EBP-isobinders. These proteins have been shown to bind to a number of different sequences as homo- and heterodimers (8,9,10). According to our binding/competition data, proteins of this family are contained in the complexes IIIa-c seen in a gel retardation assay with AABS and rat liver nuclear proteins (Fig. 1*A* and ref. 18). These findings are supported by observations of two other groups: Burch and coworkers have identified a regulatory element related to AABS in the chicken vitellogenin II gene and found that VBP, a factor interacting with this element, is a member of the C/EBP family (27). Cortese's group could show that a promoter module from the rat hemopexin promoter (Hpx A; ref. 28) that we found previously to be related to AABS (18) interacts with the C/EBP-isobinder IL-6DBP (10).

In this report we now show that AABS is interacting also with the HNF3-isobinders which include to date the three proteins HNF3-alpha, -beta and -gamma (13). To find out whether the HNF3-isobinders interacting with AABS correspond to some of the known or to novel family members, we purified them to homogeneity. The final purified preparation showed three bands in the SDS-PAGE corresponding to 40, 38 and 34 kD (Fig. 4). Although none of these molecular weights matches the molecular weights of HNF3-alpha (50 kD), HNF3-beta (47 kD) or HNF3-gamma (42 kD), the pattern 40/38/34 is remarkably similar to 50/47/42, suggesting that our purified proteins might be identical to the three HNF3's with the discrepancy between the observed apparent molecular weights being due to the purification procedure and/or to the method of molecular weight determination. This assumption is supported by binding studies showing that recombinant HNF3-alpha binds sequence specifically to AABS, yet with a lower affinity than to HNF3BS (Fig. 6). While we were using a purification procedure comparable to the one used for the initial purification of HNF3-alpha from rat liver (29), this protein can therefore be expected to be included in the proteins we isolated.

Based on the binding studies in the gel retardation assay it seems possible that proteins of different families bind to AABS in a mutually exclusive manner, as all major complexes can be assigned predominantly to one specific family of isobinders. However, the pattern is very complex and minor bands can hardly be distinguished, so that simultaneous binding of factors from different families cannot strictly be excluded; especially complexes II and IVa are possible candidates for heteromeric complexes, because they are not only competed for strongly by HNF3BS but also weakly by C/EBPBS and HP1, respectively. More detailed mapping of the binding sites within the 24 bp of AABS suggests that the HNF3-isobinders interact with element 2 (Fig. 6), whereas LFB1 binding can be assigned mainly to element 1 (data not shown). Footprint analysis using recombinant C/EBP had shown that C/EBP-isobinders interact with both of the elements (18). Based on these mapping data simultaneous binding to partially overlapping sites might be possible.

A sequence comparison of AABS with previously defined binding sites for HNF3-isobinders (Fig. 7) shows only an 8/13-homology between AABS and the strong HNF3 recognition sequence defined at position −106/−94 in the mouse TTR promoter (19). This homology is located in the central part of AABS, whereas our binding studies mapped the HNF3 binding site to element 2. However, the weak binding site for HNF3-isobinders defined at position −140/−130 in the mouse TTR promoter (19) is identical to element 2 over seven contiguous base pairs. Additionally, the same part of AABS is also homologous to the 5'-part of the HNF3BS oligonucleotide used as a representative of the strong HNF3 binding site in the mouse TTR promoter, indicating that the active HNF3 binding site within this oligonucleotide might not be located in its central part corresponding to the 'consensus' sequence but rather in the 5'-part from −111 to −102.

AABS – a liver specific promoter module with unique regulatory capabilities

Vitellogenin is the precursor protein for the main yolk proteins in oviparous vertebrates. It is produced exclusivly in the liver of adult females and although there are only four vitellogenin genes in the genome of *Xenopus laevis*, vitellogenin mRNA may constitute half of the polyadenylated mRNA in the liver of fully estrogen stimulated individuals (30). This high level of inducible and tissue specific expression requires a strong promoter containing estrogen responsive elements as well as elements that mediate a very efficient liver specific transcriptional activity. In the case of the vitellogenin A2 gene, our group has identified both of these elements, an estrogen responsive unit at position −331/−314 (16) and the A-activator binding site (AABS) at position −121/−97 (17). This promoter module AABS has been highly conserved during evolution (17,18) indicating that its compact structure with binding sites for proteins belonging to three of the four known families of liver specific transcription factors within a 24 bp sequence meets some crucial requirements for certain regulatory processes.

AABS related sequences have been identified to date in vitellogenin genes and in mammalian acute phase genes. All these genes have in common that they are dramatically induced specifically in hepatocytes upon an appropriate stimulation. We speculate that the binding of several distinct liver specific transcription factors to the same module might be important for this kind of inducibility in several respects: The capability to bind factors belonging to different families increases the concentration of available transactivating factors resulting in an increased activation of the corresponding promoter. Thereby the activation of a promoter containing an AABS-like module might become independent from variations in the concentration of a particular transcription factor due to other regulatory controls such as circadian rhythms (e.g. DBP, ref. 31). Furthermore, the compact structure of promoter modules like AABS may help to avoid the expression of the corresponding genes in inadequate tissues: It is known that all the liver specific transcription factors are not restricted to the liver but are also found in other tissues (32). However, the liver is the only tissue where all of these factors are expressed at high levels. Thus a promoter module that not only enables but rather requires the action of more than one factor, would restrict gene expression to the liver.

ACKNOWLEDGEMENTS

The authors like to thank Christiane Zoidl and Sabine Senkel for excellent technical assistance, Wilfried Kugler for the introduction to transcription factor purification and Kirsten Sendowski for the synthesis of oligonucleotides. In addition we thank W.S. Chen and J.E. Darnell, Jr. for providing the HNF3-alpha cDNA and Heike Weber for the construction of the GST-HNF3-alpha expression vector. This work was supported by the Deutsche Forschungsgemeinschaft.

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