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Liver-Specific Gene Expression: A-Activator-Binding Site, a Promoter Module Present in Vitellogenin and Acute-Phase Genes

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The A2 vitellogenin gene of *Xenopus laevis*, which is expressed liver specifically, contains an A-activator-binding site (AABS) that mediates high in vitro transcriptional activity in rat liver nuclear extracts. Footprint experiments with DNase I and gel retardation assays revealed the binding of several proteins to AABS. Using binding sites of known DNA-binding proteins as competitors in the gel retardation assay, we found that the transcription factor C/EBP and/or one of its "iso-binders" as well as LFB1/HNF1 bound AABS. These interactions were confirmed by in vitro transcription experiments using various oligonucleotides as competitors. However, saturating amounts of C/EBP- and LFB1/HNF1-binding sites as competitors only partially blocked AABS-mediated transcriptional activity. This finding implies that at least a third distinct transcription factor interacts with AABS. In vitro transcription experiments revealed that AABS was present not only in the closely related *Xenopus* A1 vitellogenin gene but also in acute-phase genes as a liver-specific regulatory element known to confer the interleukin-6 response. Both AABS and the interleukin-6 response element are promoter modules interacting with at least three distinct transcription factors, including C/EBP and LFB1/HNF1.

Control of tissue-specific gene transcription in higher eucaryotes is partially mediated by *cis*-acting DNA elements that are recognized by tissue-specific transcription factors. This general scheme has been identified in several cell types, including hepatocytes (reviewed in references 13, 24, 26, and 37), erythrocytes (18, 48), muscle cells (17, 33), and B cells (6, 39, 46). It is clear that there is not just one *cis*-acting element present in a given cell type for mediating cell-specific gene expression but rather an array of several distinct regulatory units (16). This complex pattern might allow the coordinate expression of sets of genes during development and differentiation as well as upon external stimulation.

In the case of liver-specific gene expression, several distinct promoter and enhancer elements that mediate tissue-specific gene expression have been identified (4, 8–11, 14, 20–23, 25, 30, 35, 40, 43, 54, 56). So far the best-characterized element is HP1 (30, 44, 47), which is also known as the LFB1/HNF1-binding site (10, 22). This element is present in genes of several species from *Xenopus laevis* to humans, including the genes coding for albumin (23, 30, 36), aldolase B (49), α_1-antitrypsin (22), α-fetoprotein (30), β-fibrinogen (10), transthyretin (9), and pyruvate kinase (50). It is recognized by the transcription factor LFB1/HNF1 (10, 22, 34), which has recently been cloned (2, 19). Another well-known transcription factor, the CCAAT/enchancer-binding protein (C/EBP), is found in fully differentiated hepatic cells but also in fat and lung tissue (3, 55). This factor recognizes a broad spectrum of sequences, including the so-called CCATT boxes and the enhancer core sequences (27, 31). Genes expressed in the liver that contain a C/EBP-binding site are the genes coding for albumin (23, 36), α_1-antitrypsin (8), carbamylphosphate synthetase I (25), and transthyretin (8). Recent cloning of DNA-binding proteins revealed three distinct transcription factors, DBP (38), NF-IL6 (1), and LAP (12), that bind at the same or very similar sequence and constitute a family of "iso-binders."

The *Xenopus* vitellogenin genes, coding for the yolk precursor proteins, are transcriptionally activated by estrogen exclusively in the liver (51, 52). This liver-specific expression is independent of the estrogen-responsive element, since constructs containing this element are active in nonhepatic cell lines provided they contain estrogen receptors (15). In addition to the estrogen-responsive element, we have identified in the *Xenopus* A2 vitellogenin gene a promoter element that confers cell-specific expression in transfection experiments (28). Using nuclear extracts from rat liver, we showed that this element, which we now refer to as the A-activator-binding site (AABS), mediates high-level in vitro transcription. However, in HeLa cell extracts AABS was not active, implying that AABS interacts with liver-specific transcription factors (14). As AABS-containing templates are efficiently transcribed in in vitro transcription extracts of rat liver nuclei, we wondered which transcription factors in nuclear extracts recognize AABS. We also postulated that AABS is present in genes expressed in rat hepatocytes. However, since vitellogenin genes are not present in mammals, the mammalian genes containing AABS were not obvious. In this study, we succeeded in characterizing AABS as a promoter module interacting with at least three distinct transcription factors of rat liver nuclei. The same module is found in mammalian acute-phase genes expressed in the liver.

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MATERIALS AND METHODS

Preparation of rat liver nuclear extract. All manipulations were performed in the cold; solutions, tubes, and centrifuges were chilled to 0 to 4°C. Crude rat liver nuclear extract was prepared from male Sprague-Dawley rats as described by Gorski et al. (20), with the modifications recently described (29).

Gel retardation assay. Oligonucleotides synthesized on a Gene Assembler (Pharmacia) were end labeled by polynucleotide kinase. The C/EBP-binding site used for gel retardation was obtained by annealing the two complementary strands 5'-AGCTTCAATTGGGCAATCAGGA-3' and 5'-GATCTCCTGATTGCCCAATTGA-3'. The AABS oligonucleotide was the sequence −121 to −97 or the sequence −121 to −87 of the A2 vitellogenin 5'-flanking region with *Hin*dIII and *Bam*HI overhangs. The conditions for binding and electrophoretic separation were as described previously (29).

DNase I footprinting. Recombinant C/EBP protein or rat liver nuclear proteins were incubated in 30 μl of 50 mM KCl–0.5 mM EDTA–0.8 mM dithiothreitol–8% glycerol–15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9) for 15 min at 0°C with end-labeled DNA probes (1×10^4 to 3×10^4 cpm). The mixtures were digested with DNase I (5 to 100 μg/ml) for 2 min at 37°C in the presence of 4 mM $MgCl_2$ to obtain partial digestion. The DNA was extracted with phenol and separated on a sequencing gel.

In vitro transcription analysis. In vitro transcription reactions were performed as previously described (14, 47), using nuclear extract from rat liver. The transcription templates were generated by insertion of *Hin*dIII-*Bam*HI fragments into PL-TG (47). Oligonucleotides synthesized on a Pharmacia Gene Assembler were annealed and cloned without further purification. All plasmid constructs were verified by sequencing of the relevant portion (5). Depending on the extract preparation used, AABS conferred 15- to 50-fold stimulation of transcriptional activity.

RESULTS

AABS binds several distinct proteins, including C/EBP and LFB1/HNF1. By transfection experiments, we have previously identified in the 5'-flanking region of the A2 vitellogenin gene a regulatory sequence that mediates cell-specific expression of a reporter gene (14). In vitro transcription experiments using rat liver extracts established that this regulatory unit, composed of elements 1, 2, and 3, interacts with transcription factors. The main activity is mediated by elements 1 and 2, extending from −121 to −97 (14), and we refer to this sequence as AABS (Fig. 1). As an initial approach to identify the nature of the transcription factors recognizing AABS, we analyzed the binding of rat liver nuclear proteins to this regulatory unit. In a first series of experiments, we performed a DNase I footprint analysis of the 5'-flanking region of the A2 vitellogenin gene from positions −287 to −87 in the presence of increasing amounts of rat liver nuclear proteins. Bound proteins were identified on the end-labeled DNA by partial DNase I digestion. The DNA fragments separated on a sequencing gel are shown in Fig. 2. The extent of the footprint as well as the appearance of DNase I-hypersensitive sites changed upon addition of increasing concentrations of protein: in the upper strand, the area from −112 to −106 was clearly protected at low protein input, with induced hypersensitive sites at −116 to −113 and

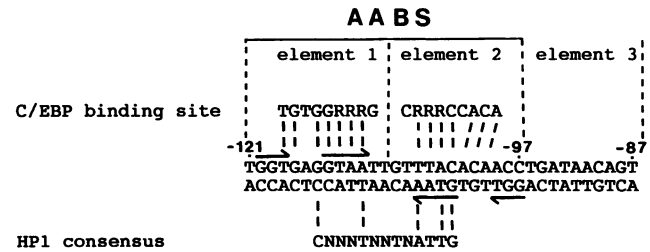


FIG. 1. Sequence of AABS and similarity to binding sites of known transcription factors. The sequence of the activator that we have previously identified in the A2 vitellogenin gene (14) is aligned with the consensus sequence of the C/EBP-binding site (27) and the HP1 consensus sequence (47). The HP1 consensus sequence is based on point mutation analysis (44) and includes some unpublished data. Nucleotides that cannot be changed without loss of function are indicated; R refers to an A or T. Arrows mark the palindrome, and positions relative to the cap site are given. The division in elements 1, 2, and 3 is as previously described (14). The sequence of the A2 vitellogenin gene is taken from reference 53.

−105 and −104 (lanes 4 and 5), whereas at high protein input the protected region extended from −124 up to the linker region at −87, with only the hypersensitive sites at −105 and −104 persisting (lane 7). A comparable situation was found for the lower strand: low protein input yielded a protected area from −116 to −110, with a prominent hypersensitive site at −108. This hypersensitive site was partially lost at high protein concentrations, and two other sites appeared at −99 and −87. In addition, the protected area was expanded from −122 to −89 at these higher protein concentrations. These changes in footprint pattern depending on the amount of nuclear extract added suggest that multiple proteins interact with AABS. The detection of footprints and hypersensitive sites outside of AABS is not surprising, since crude rat liver nuclear extracts were used and we have previously found that sequences upstream of AABS are known to influence the activity of AABS in transfection assays (14).

Complementary to the footprint experiments, we performed gel retardation assays with an oligonucleotide extending from −121 to −97 of the A2 vitellogenin promoter, designated the AABS oligonucleotide. At least five distinct complexes of different mobilities were detected (Fig. 3, lane 1). Four of these (labeled I to IV) could readily be competed for by an excess of unlabeled AABS oligonucleotide (lane 2), whereas they were not affected by the unrelated oligonucleotide B1wt (lane 6), containing the B-activator-binding site (29). The fastest-migrating complex could not be competed for by the homologous probe and thus is considered to represent an interaction with a very abundant protein whose properties we have not further investigated. We refer to the proteins forming complexes I, II, III, and IV as A-activator proteins (AAP) I, II, III, and IV, respectively.

Sequence comparison of AABS with known regulatory elements (Fig. 1) revealed some similarity to the enhancer core sequence (27) and to HP1 (44, 47), which bind the transcription factors C/EBP (27) and LFB1/HNF1 (10, 22, 34), respectively. To analyze whether these similarities are of any relevance, we used oligonucleotides containing the binding sites for these factors as competitors in the gel retardation assay. In a first series of experiments, we investigated the possibility that the transcription factor C/EBP interacts with AABS. As the C/EBP-binding site we chose the sequence used to detect the recombinant C/EBP transcription factor in a Southwestern (DNA-protein) assay (31).

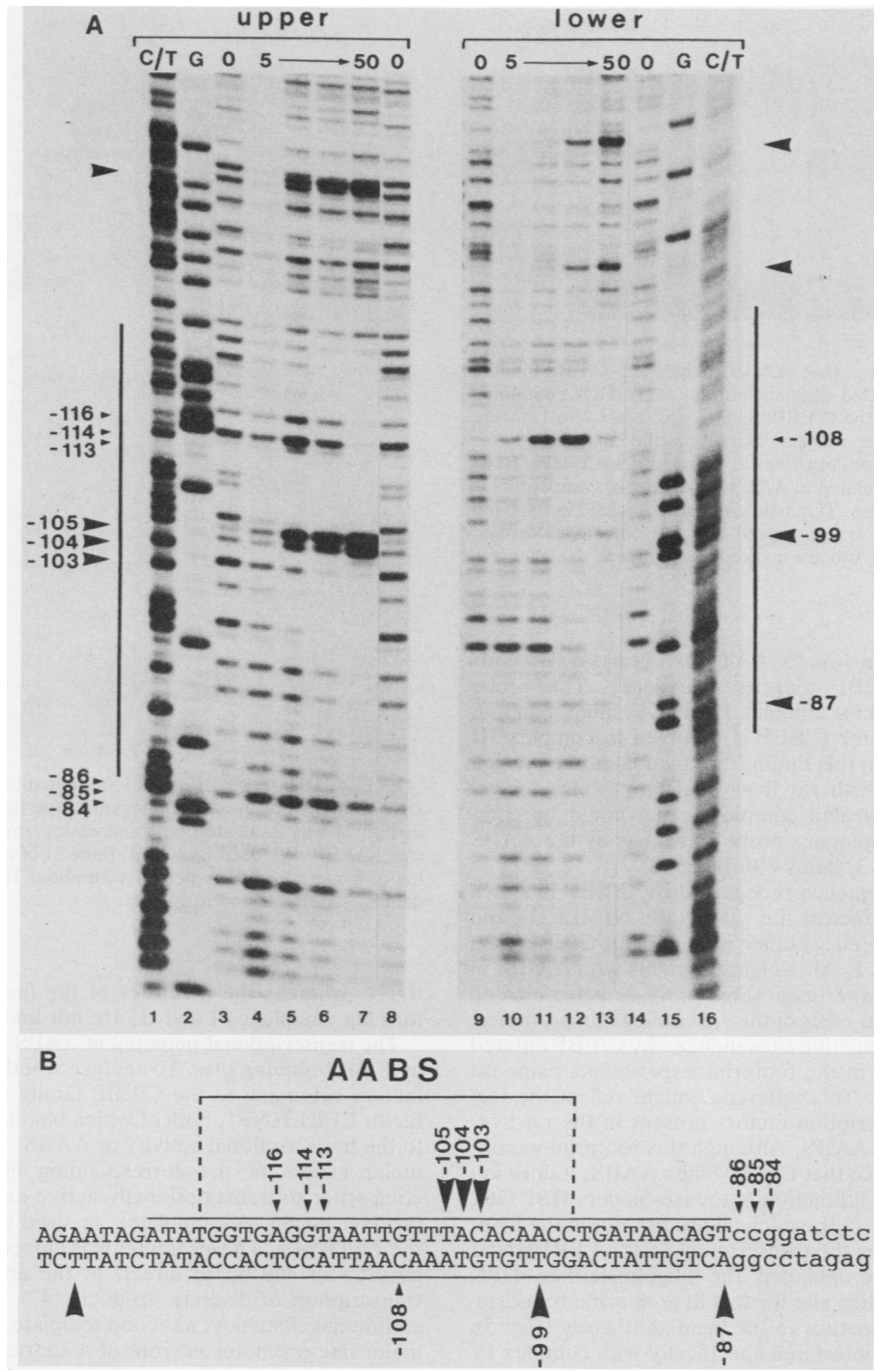


FIG. 2. (A) Analysis of AABS by DNase I footprinting using rat liver nuclear proteins. A DNA fragment containing the A2 vitellogenin gene sequence from −278 to −87 linked to the TATA box of the albumin gene (47) was isolated as either a 5′ *Hin*dIII-*Xho*I fragment or a 5′ *Hin*dIII-*Eco*RI fragment. The upper DNA strand was labeled by fill-in of the *Xho*I site with [α-^{32}P]dCTP; the lower strand was labeled by kinase reaction at the *Eco*RI site, using [^{32}P]ATP. As indicated above the lanes, 0, 5, 12, 25, or 50 μl of rat liver nuclear extract was used for binding prior to DNase I digestion (lanes 3 to 8 and 9 to 14). A C+T or G Maxam-Gilbert sequencing reaction of the same fragments was used as a reference. The protected area at the highest protein input is indicated by bars in both panels. Small arrowheads mark DNase I-hypersensitive sites transiently observed at low protein input; large arrowheads mark hypersensitive sites present at high protein input. Numbers refer to the positions upstream of the cap site of the A2 vitellogenin gene. The linker sequence is in lowercase in panel B.

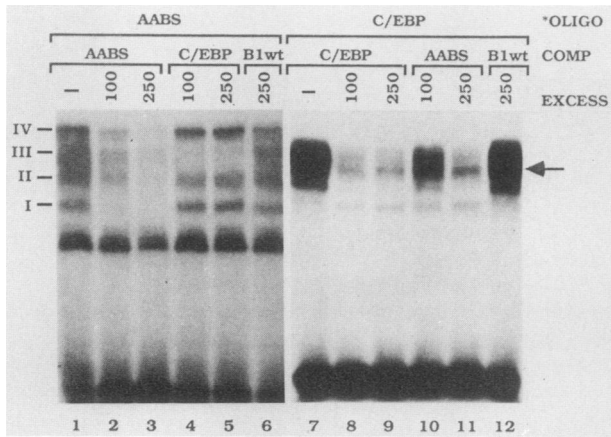


FIG. 3. Demonstration that AABS includes a C/EBP-binding site. Radioactively labeled oligonucleotides (*OLIGO) containing AABS (lanes 1 to 6) or the C/EBP-binding site (lanes 7 to 12) were incubated with rat liver nuclear extract in the absence (–) or presence of specific oligonucleotides as competitors (COMP). B1wt contains a sequence unrelated to AABS (29). Input of competitors is given as fold molar excess. The main complexes formed in the band shift assay with AABS are numbered I to IV; complexes formed with the C/EBP-binding site are marked by an arrow.

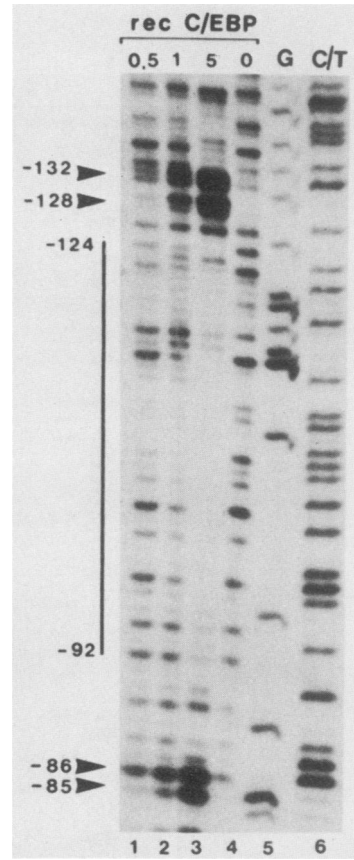


FIG. 4. DNase I footprint of recombinant C/EBP on AABS. The DNA fragment of the A2 vitellogenin gene (upper strand labeled as in Fig. 2) was incubated with increasing amounts of recombinant (rec) C/EBP. We used up to 5 μl (lane 3) of a solution containing 1 mg of bacterial proteins per ml with about 1% C/EBP. For further details, see the legend to Fig. 2.

This sequence contains the CCAAT box but has no obvious similarity to the C/EBP consensus sequence. This probe specifically competed for complex III (Fig. 3, lanes 4 and 5), implying that the factor C/EBP is involved in complex III formation. To confirm this finding, we incubated the labeled C/EBP-binding site with rat liver nuclear proteins. A gel retardation assay revealed complexes that could be competed for by the homologous probe as well as by the AABS oligonucleotide (Fig. 3, lanes 7 through 12).

Since the DNA sequence recognized by C/EBP is also a binding site for other factors, i.e., DBP (38), NF-IL6 (1), and LAP (12), we wondered whether recombinant C/EBP (32), kindly provided by S. L. McKnight, interacts with AABS. In a DNase I footprint experiment (Fig. 4), we detected binding extending from −92 to −124 of the A2 vitellogenin promoter. The DNase I-hypersensitive sites induced by C/EBP differed from the sites found in the footprint experiments using rat liver extract (Fig. 2). This difference might reflect the fact that additional transcription factors present in the rat liver extract interact with AABS. Although this footprint experiment is direct evidence that C/EBP binds AABS, it does not exclude the possible interaction of the iso-binders DBP (38), NF-IL6 (1), and LAP (12), which all are present in the liver.

To analyze whether the transcription factor LFB1/HNF1 binds to AABS, we included the oligonucleotide HP1, known to be the binding site for this liver-specific transcription factor, as a competitor in the band shift assay (Fig. 5). This oligonucleotide interfered specifically with complex IV formation (compare lane 4 with lane 1). In a reverse experiment, we used the HP1 oligonucleotide as the radioactive probe in the binding assay to reveal complex formation with LFB1/HNF1 (lane 5). This complex could be competed for by the AABS oligonucleotide as efficiently as by the homologous probe HP1 (lanes 6 and 8) but was resistant to the addition of a heterologous oligonucleotide (lane 7). Both of these experiments suggest that AABS binds LFB1/HNF1. We conclude that complexes III and IV most likely contain the transcription factors C/EBP and LFB1/HNF1, respectively, whereas the identities of the factors AAP I and II, forming complexes I and II, are not known.

The transcriptional potential of AABS is partially inhibited by C/EBP-binding sites. To analyze whether the transcription factors belonging to the C/EBP family or the transcription factor LFB1/HNF1, both of which bind to AABS, contribute to the transcriptional activity of AABS, we added a 600-fold molar excess of the corresponding binding sites as the competitor to transcriptionally active extracts derived from rat liver nuclei. As templates we used the AABS linked to the TATA box. This promoter was cloned in front of a G-free cassette of 400 bp to direct, in the absence of GTP, the transcription of discrete products (47). To quantitate transcriptional efficiency, a second template with the adenovirus major late promoter in front of a shorter G-free cassette of 200 bp was included in each reaction mixture. HP1, the regulatory element binding LFB1/HNF1, could not compete for the in vitro transcription mediated by a template containing AABS (Fig. 6; compare lanes 5 and 6). In contrast, the same oligonucleotide reduced the activity of a template containing HP1 at least 20-fold, i.e., to the basal level (lanes 1 and 2) observed with the polylinker construct (lanes 13 to 16). The specificity of competition by HP1 is shown by the finding that the template containing the C/EBP-binding site (lanes 17 and 18) as well as the internal control driven by the

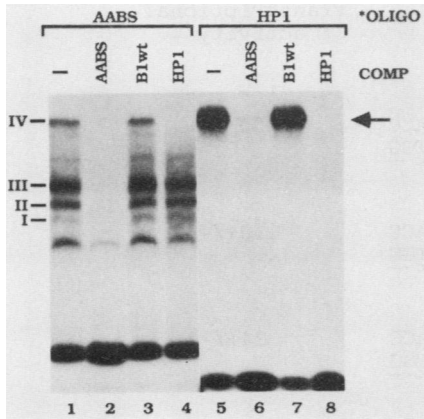


FIG. 5. Demonstration that AABS includes an HNF1/LFB1-binding site. Radioactively labeled oligonucleotides (*OLIGO) containing AABS (lanes 1 to 4) or representing HP1, the binding site for HNF1/LFB1 (lanes 5 to 8), were incubated with the specific oligonucleotides as competitors (COMP) and analyzed in a band shift assay. Complexes I, II, III, and IV formed by incubation with AABS are marked; the position of the complex formed with HP1 is indicated by an arrow. The pattern of complexes I to IV differs from the one shown in Fig. 3 because the oligonucleotide containing AABS was larger, extending from −121 to −87 rather than from −121 to −97. B1wt contains a sequence unrelated to AABS (29).

adenovirus major late promoter were not significantly affected. Inclusion of the C/EBP-binding site as a competitor resulted in significant reduction of the transcripts derived from templates containing AABS (lanes 7 and 8). From densitometry, we estimate a sevenfold decrease. This reduced level is still sixfold higher than the activity derived from the constructs containing the polylinker sequence (lanes 13 to 16). In control experiments, the C/EBP-binding site completely inactivated the activity of a template containing this C/EBP-binding site (lane 19) but did not affect transcripts derived from an HP1-containing template (lane 3). To analyze whether addition of HP1 increases the inhibitory effect of the C/EBP-binding site, we added both oligonucleotides simultaneously. Competition by the C/EBP oligonucleotides could not be increased further by addition of HP1 (lanes 7 and 8). Since substantial activity remained in the presence of both oligonucleotide competitors, i.e., sixfold higher than the activity derived from the polylinker construct, we conclude that at least one other transcription factor acts on AABS. Data for the hemopexin promoter (HpxA) construct (lanes 9 to 12) are discussed below.

AABS is composed of two equivalent elements. Sequence analysis of AABS revealed a partial palindrome with the sequence 5′-GGTNNNGTAA-3′ in the upper strand of element 1 and in the lower strand of element 2 (Fig. 1). This feature suggested that elements 1 and 2 might be identical. Therefore, we synthesized an oligonucleotide with an inverted duplication of element 1 [AABS (1/1)] or of element 2 [AABS (2/2)]. These perfect palindromes (Fig. 7) were inserted in front of a TATA box into the vector used for in vitro transcription. Both templates mediated high-level in vitro transcriptional activity, at least 10-fold higher than the construct containing a polylinker instead of the AABS derivatives. AABS (1/1) was even slightly more active than the original AABS representing the A2 vitellogenin sequence from −121 to −97, whereas AABS (2/2) was less active. The higher activity of a template containing a duplication of element 1 than of the corresponding construct with element 2 agrees with our previous observation that element 1 as a

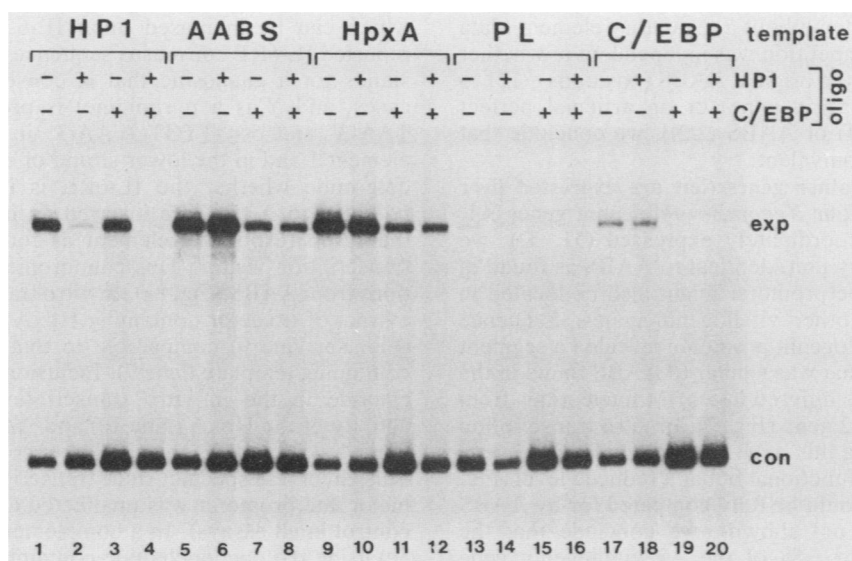


FIG. 6. Partial reduction of the transcriptional activity of AABS by addition of oligonucleotides known to bind C/EBP. Transcription vectors containing either HP1 (47), AABS (see Fig. 1), HpxA (see Fig. 8a), a polylinker (PL), or the C/EBP-binding site (see Materials and Methods) cloned in front of the *Xenopus* albumin TATA box were incubated in rat liver nuclear extract. A 300-ng sample of HP1 and/or C/EBP-binding site oligonucleotide (oligo) was used as indicated to compete for HNF1/LFB1 and/or C/EBP. The amount of competitor used was about 10-fold higher than the minimal amount required to obtain complete inhibition of an HP1-driven template (44). A further increase in competitor led to nonspecific inhibition of transcription. As an internal control, the adenovirus major late promoter was included in all reactions. The transcription vectors give transcripts of about 400 nucleotides (exp), whereas the internal control generated a 200-nucleotide RNA (con).

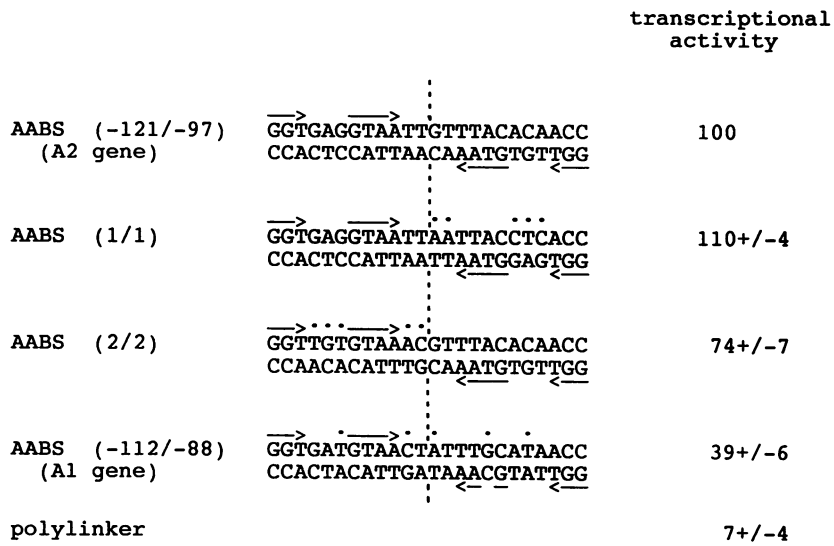


FIG. 7. Transcriptional activities of AABS variants. Oligonucleotides corresponding to a duplication of element 1 [AABS (1/1)], element 2 [AABS (2/2)], or the sequence of the A1 vitellogenin gene from −112 to −88 [AABS (−112/−88)] were inserted into the transcription vector PL-TG (47). Transcriptional activities of the various constructs (means of at least three experiments) were quantitated by densitometry from autoradiograms (see Fig. 6) and compared with the activity found for the construct containing AABS (−121/−97). The activity for the polylinker construct, PL-TG, is given for comparison. The palindrome found in these constructs is indicated by arrows. Nucleotides that differ from the AABS (−121/−97) sequence are indicated by dots.

monomer mediates higher in vitro transcription than does element 2 (14).

To investigate whether AABS (1/1) and AABS (2/2) represent equivalent *cis*-acting elements, we added the oligonucleotides AABS (−121/−97), AABS (1/1), or AABS (2/2) in large molar excess into the in vitro transcription assays with AABS-containing templates. All AABS oligonucleotides competed for transcriptional activity very efficiently, by about a factor of 20 relative to the transcription level observed with a template without a regulatory element (data not shown). As this competition was independent of whether the template contained the original AABS (positions −121 to −97 of the A2 vitellogenin gene) or an artificial perfect palindrome [AABS (1/1) or AABS (2/2)], we conclude that elements 1 and 2 are equivalent.

AABS is present in other genes that are expressed liver specifically. Since the four *Xenopus* vitellogenin genes A1, A2, B1, and B2 are coordinately expressed (51, 52), we assume that a regulatory unit identical to AABS as found in the A2 vitellogenin gene promoter might also be located in the promoters of the other vitellogenin genes. Sequence analysis of the A1 vitellogenin promoter revealed a segment between −112 to −88 that was similar to AABS found in the A2 gene. This sequence differed in 5 of 24 nucleotides from AABS present in the A2 gene (Fig. 7). In vitro transcription of a template containing this AABS homolog of the A1 gene demonstrated that it is functional but at a reduced level (Fig. 7). Since this activity could be fully competed for by AABS of the A2 gene (data not shown), we conclude that the sequence from −112 to −88 of the A1 vitellogenin gene corresponds to AABS in the A2 gene. A search for AABS in the B vitellogenin genes revealed no obvious sequence similarity to AABS. In fact, functional tests showed a distinct regulatory element, the B-activator-binding site (29), which has also been described as the A-binding site (7).

Since AABS derived from a frog gene functions in mammalian cells, we speculated that some promoters of mammalian genes that are specifically expressed in the liver might contain AABS. Reviewing DNA sequences known to confer liver-specific expression or to bind factors predominantly present in liver nuclear extracts, we observed significant sequence homology in the interleukin-6 (IL-6)-responsive element (IL-6RE) present in the promoters of acute-phase genes such as the genes coding for hemopexin, haptoglobin, and C-reactive protein (42). In transfection experiments, this regulatory unit mediates hepatocyte-specific expression, which can be increased upon IL-6 stimulation (41). The reported IL-6RE consensus sequence 5′-gTgNNGYAA-3′ (g stands for a guanosine that is conserved in three of four cases, and Y is a pyrimidine) is present as 5′-GTGAGG TAA-3′ and 5′-GTTGTGTAA-3′ in the upper strand of element 1 and in the lower strand of element 2 (Fig. 8a). To determine whether the IL-6RE is identical in functional potential to AABS, we inserted an inverted duplication of the 12-bp responsive element as found in HpxA into the transcription vector. This palindrome differs at seven positions from AABS (Fig. 8a). In vitro transcription in a rat liver extract of a vector containing HpxA revealed high activity (Fig. 8b, lane 9) comparable to that found for an AABS-containing template (lane 7). Inclusion of the HpxA oligonucleotide in the in vitro transcription assay reduced the activity of the HpxA (lane 10) and AABS (lane 8) templates to the level found for the polylinker construct (lanes 5 and 6). This effect was specific, since transcription of the adenovirus major late promoter was unaffected (lane 2 and the internal control in all assays). In a complementary experiment (Fig. 8c) using the oligonucleotide containing the A2 gene AABS as a competitor, complete reduction of the activity derived from templates containing either HpxA (lanes 7 and 8) or AABS (lanes 5 and 6) was obtained. All of these in vitro transcription experiments suggest that the IL-6RE found in HpxA binds transcription factors identical to those bound by AABS.

Using the AABS oligonucleotide and HpxA probe as

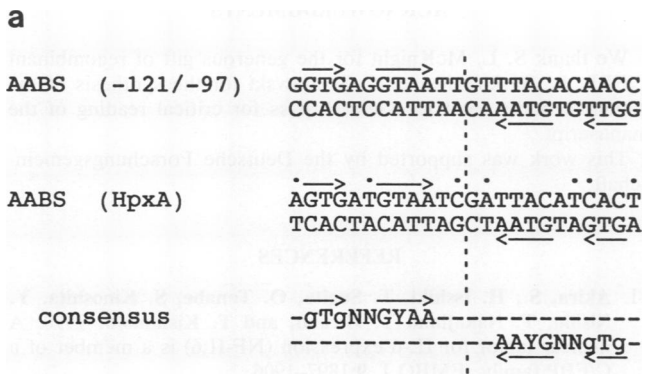
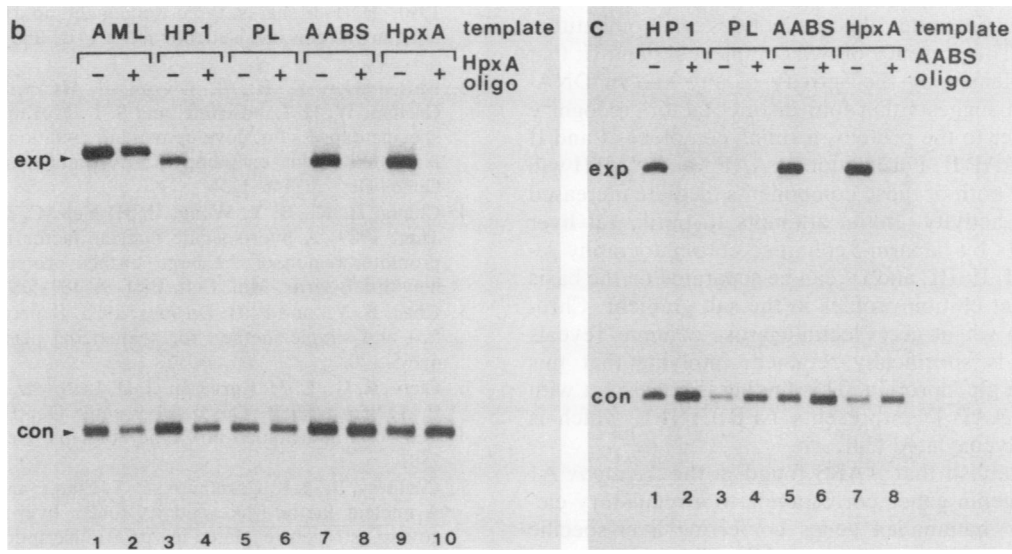


FIG. 8. Demonstration that AABS corresponds to an inverted repeat of the IL-6RE. (a) An inverted repeat of the 12-bp fragment of HpxA containing the IL-6RE (41) is aligned with AABS (−121/−97). Nucleotides that differ between AABS (HpxA) and AABS (−121/−97) are marked by dots. The consensus sequence of the IL-6RE as reported by Poli et al. (42) is given; g indicates that three of four sequences have a G at this position, Y is a pyrimidine, and N represents any of the four nucleotides. The consensus present in AABS (−121/−97) and AABS (HpxA) is marked by an arrow and forms a palindrome. (b and c) Transcription vectors containing either the adenovirus major late promoter [AML; pML(C_2AT)$_{19}$ in reference 45] or the regulatory element HP1 (47), AABS (−121/−97) (see panel a), or AABS (HpxA) (see a) replacing the polylinker sequence in PL-TG were used for in vitro transcription as for Fig. 6. The vector without a regulatory element, PL-TG, was also used (PL). Transcription was done without (−) or with (+) 100 ng of oligonucleotide (oligo) corresponding to AABS (HpxA) (b) and AABS (−121/−97) (c). For further details, see the legend to Fig. 6.



competitors, we also obtained complete competition of transcripts derived from the template containing HP1 (Fig. 8b, lanes 3 and 4; Fig. 8c, lanes 1 and 2). This result is not surprising because binding studies have shown that AABS binds LFB1/HNF1, the factor specifically recognizing HP1 (complex IV in Fig. 5).

The identity of AABS derived from the A2 vitellogenin gene with the element found in HpxA was supported by the competition experiments using the HP1 oligonucleotide and C/EBP-binding site as competitors in the in vitro transcription experiments (Fig. 6). AABS (−121/−97)- and HpxA-driven transcriptional activities were both insensitive to HP1 oligonucleotide competition (lanes 6 and 10), whereas transcription of both templates was partially inhibited by C/EBP oligonucleotides (lanes 7 and 11). Clearly, the HpxA element is also a regulatory unit interacting with at least three distinct transcription factors, as does AABS in the A2 vitellogenin gene.

DISCUSSION

In this study we have determined that AABS, a regulatory unit conferring cell-specific expression (14), binds at least three distinct transcription factors. Two of these proteins probably correspond to the well-known transcription factors C/EBP (27, 31) and LFB1/HNF1 (2, 19), both of which show a tissue-specific distribution.

C/EBP, originally isolated from rat liver as a viral enhancer-binding protein, was found to correspond to the CCAAT-box-binding protein (27). This factor is found predominantly in terminally differentiated cells metabolizing lipids and thus is present in hepatocytes (3). Three C/EBP-related proteins, DBP (38), NF-IL6 (1), and LAP (12), recently have been identified in hepatocytes. Since these proteins recognize similar DNA sequences, i.e., are iso-binders, it is conceivable that they all also recognize AABS. The heterogeneity of C/EBP-related proteins is reflected in the broad distribution of complex III in gel retardation assays using AABS as the ligand (Fig. 3, lane 1) as well as in the complexes formed with C/EBP-binding sites (Fig. 3, lane 7).

LFB1/HNF1 was originally identified as a liver-specific transcription factor recognizing the promoter element HP1 in several genes specifically expressed in hepatocytes (10, 19, 30). However, with use of a cloned cDNA probe, mRNA coding for LFB1/HNF1 has been found also in other tissues such as the intestine and kidney (2).

The evidence that LFB1/HNF1 binds AABS is based primarily on specific competition of complex I formation by adding the LFB1/HNF1-binding site as a competitor. Moreover, we found that an AABS oligonucleotide completely inhibited the transcriptional activity mediated by HP1, i.e., the LFB1/HNF1-binding site (Fig. 8b and c). In the reverse experiment, HP1 did not reduce the activity of AABS-mediated transcriptional activity (Fig. 6). This result may be explained by assuming that the competition of LFB1/HNF1 is overcome by the binding of another transcription factor to

AABS. Alternatively, LFB1/HNF1 may bind AABS in a nonfunctional manner. This notion is supported by our previous finding that the sequence most related to the HP1 consensus, −117 to −102 of AABS, does not support transcriptional activity (14). Furthermore, we have previously observed that binding of LFB1/HNF1 to a mutated HP1 is not strictly correlated to the transcriptional activity mediated by the mutant (44).

Besides C/EBP (and possibly its iso-binders) and LFB1/HNF1, at least a third transcription factor interacts with AABS. This conclusion is based on the fact that AABS-mediated transcriptional activity can be inhibited only partially by adding C/EBP- and LFB1/HNF1-binding-site oligonucleotides (Fig. 6). Further support is provided by the gel retardation experiments: formation of the AABS protein complexes I and II cannot be inhibited by adding C/EBP- or LFB1/HNF1-binding-site oligonucleotides as competitors (Fig. 3 and 5). The presence of two complexes, I and II, that cannot be explained by the activity of any known DNA-binding protein suggests that four distinct factors recognize AABS. We refer to the proteins forming complexes I and II as AAP I and AAP II. Purification of AAP I and AAP II will reveal whether both of these components mediate increased transcriptional activity. Initial attempts to purify rat liver nuclear extracts by heparin-Sepharose chromatography reveal that AAP I, II, III, and IV can be separated on the basis of their different elution profiles in the salt gradient. Chromatography on wheat germ lectin-agarose columns reveals that AAP IV is specifically retained, implying that this component is a glycoprotein. This finding is consistent with our data that AAP IV represents LFB1/HNF1, which is known to be glycosylated (34).

Our data establish that AABS found in the *Xenopus* A1 and A2 vitellogenin genes corresponds to a regulatory element found in mammalian genes conferring liver-specific activity in transfection experiments (42). This element was found to be distinct from previous regulatory elements. It is found in acute-phase genes (hemopexin, haptoglobin, and C-reactive protein genes) and can also mediate the response to IL-6, the main signal in the acute-phase reaction. It is therefore known as IL-6RE (41). Gel retardation assays with the IL-6RE revealed a complexity of protein-DNA interaction (42) similar to that observed in our experiments (Fig. 3 and 5). However, none of the proteins involved in complex formation with IL-6RE have been identified. Our data establish that the IL-6RE as found in HpxA mediates transcriptional activity in vitro (Fig. 8) and binds transcription factors equivalent to those bound by AABS of the A vitellogenin genes, including C/EBP and LFB1/HNF1. The conservation of such a multifunctional module throughout evolution from frog to human suggests that it plays an essential role in gene regulation.

Possibly the same or at least a related module can be found in a series of other genes that have recently been found to be induced by IL-6 (1). A cloned transcription factor, NF-IL6, recognizing these sequences was found to be related to C/EBP. We assume that it is involved in the C/EBP-binding activity that we have found to bind AABS. The data available show that this factor cannot represent AAP I or AAP II, the proteins that recognize AABS and are not yet purified.

It remains to be determined whether AABS of the vitellogenin genes not only participates in liver-specific expression but also acts as an element to confer IL-6 response.

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