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Liver-enriched HNF-3α and ubiquitous factors interact with the human transferrin gene enhancer

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The human transferrin gene enhancer is organized in two domains. Domain A contains a single enhanson designated Ia. Domain B contains four enhansons named Ib, II, III and IV. We demonstrate here that the liver-enriched transcription factor HNF-3α interacts with enhanson Ia and that enhansons Ib and IV are binding sites for members of the NF1 family. In addition, enhansons II and III seem to be respectively the targets for the AP4 protein and for EIII, a factor not yet completely identified Analysis of mutated enhancer regions establishes that each enhanson is required for full enhancer activity and that the proteins binding to enhansons II, III and IV may interact within a multiprotein complex. This enhancer region presents no activity in the Sertoli cells of testis, where transferrin is also synthesized. We demonstrate that in Sertoli cells, the members of the HNF-3 family are not expressed; this fact may account for the inactivity of the enhancer in these cells

Transferrin; Enhancer; Liver, Hepatoma cell, Sertoli cell, Transcription factor

1. INTRODUCTION

Transferrin (Tf) is an iron-binding glycoprotein essentially synthesized in hepatocytes, and in lower amounts in other cell types like Sertoli cells of testis and different cellular populations of the central nervous system [1–3]. Human transferrin gene expression in hepatoma cells is modulated by a combination of multiple positive and negative *cis*-acting elements [4,5]. These elements are distributed in four functionally different regions, and one of these regions, situated between −3.6 and −3.3 kb upstream of the transcription start site of the gene, fulfills the criteria established for enhancers [5]. This enhancer is composed of five enhansons, interacting with at least four nuclear proteins and organized in two domains A and B (Fig. 1) which act in synergism [6]. It is active in transient expression experiments performed in Hep3B cells, but no enhancer activity can be associated to this sequence in assays performed in primary cultures of Sertoli cells [7].

Full understanding of the mechanisms leading to the differential activity of the enhancer in several cell types needs the identification and the characterization of the properties of the transcription factors involved.

In this paper, we identified three of the factors required for full enhancer activity in hepatoma cells and we showed that one of them is a liver-enriched protein. Furthermore, we have clarified the relative contribution of each of the factors to the overall activity of the enhancer as well as some aspects of its structural organization.

Finally, as a first step to understand the molecular basis of the inactivity of the enhancer in Sertoli cells, we investigated the presence of the liver-enriched factor in these cells.

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Abbreviations hTf, human transferrin; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assays; HNF-3, hepatic nuclear factor 3; EBP, enhancer binding protein; kb, kilobase pairs.

2. MATERIALS AND METHODS

2.1. *Materials*

T4 DNA ligase, DNA polymerase I (Klenow fragment), T4 polynucleotide kinase and restriction endonucleases were purchased from New England BioLabs, Inc.; DNase I from Worthington; alkaline phosphatase and Ham's F-12-modified Eagle's medium from Boehringer Mannheim; Poly(dIdC) and Taq polymerase from Pharmacia LKB Biotechnology Inc.; ^{14}C-labeled chloramphenicol, ^{35}S-labeled methionine and ^{32}P-labeled dNTPs from Amersham Corp.; acetyl coenzyme A from Sigma

2.2 *Site-directed mutagenesis*

The wild-type enhancer, obtained from the (−3,600,+39)Tf-CAT plasmid [5] by *Eco*RI and *Hin*dIII digestion and filled in, was first cloned into the *Sma*I site of the pUC19 polylinker. This construction was named (0.3)pUC19. Mutations of the hTf enhancer were introduced by the polymerase chain reaction (PCR) using the (0,3)pUC19 as a template. We used mutated oligonucleotides as internal primers, universal (U.P.) and reverse (R.P.) primers with *Nde*I ends (U.P (*Nde*I); R.P.(*Nde*I)) as external primers (for sequences see Table I). The mutated fragments were obtained after digestion of the final PCR products by *Nde*I. Altered enhancers obtained by PCR mutagenesis are named eMIb, eMII, eMIII and eMIV

2.3 *Plasmid constructions*

Four altered enhancers were cloned into the *Nde*I site of

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(−620,+39)Tf-CAT plasmid [5] to obtain (eMIb)pTf-CAT, (eMII)pTf-CAT, (eMIII)pTf-CAT, (eMIV)pTf-CAT, respectively.

2.4. *Preparation of nuclear extracts*

Liver nuclear extracts were prepared according to the procedure of Gorsky et al. [8] with minor modifications [4].

HeLa cells and Hep3B cells nuclear extracts were prepared according to the procedure of Brunel et al. [4].

2.5. *In vitro transcription and translation*

T7 RNA polymerase was used to synthesize HNF-3α RNA from a *Bam*HI-linearized cDNA template (pGem-1). In vitro translation was performed using nuclease-treated rabbit reticulocyte lysate according to the manufacturer's protocol (Promega), in a final volume of 25 μl.

The crude reticulocyte lysate containing translated proteins was used directly in either gel mobility shift assays or in DNase I footprinting experiments.

2.6. *Electrophoretic mobility shift assays (EMSA)*

The standard assay as well as the competition experiments were performed as previously described [9] using 6 μg of nuclear extracts or 2 μl of in-vitro translated HNF-3α.

HNF-3(α, β and γ) rabbit antisera and NF1 rabbit antiserum were used to analyze purified proteins or crude nuclear extracts. 1 μl of serum, diluted 10-fold of phosphate-buffered saline (for HNF-3 antisera) or undiluted (for anti-NF1 serum), was incubated overnight at 4°C with 10 μl of protein preparations. Then, the assays were performed under standard conditions. The same experiments were carried out with serum from a non-immunized rabbit.

2.7. *DNase I footprints*

These experiments were performed as described before [4]. The probe was the mutated enhancers obtained from the different constructions by digestion with *Eco*RI and *Hin*dIII and labeled at the *Eco*RI site. Assays were performed with 30 μg of crude liver nuclear extracts.

2.8. *Cell cultures and chloramphenicol acetyltransferase assays*

Hep3B cell cultures, transfections and chloramphenicol acetyltransferase assays were performed as previously described [5].

2.9. *Northern blot assays*

Total cellular RNA were prepared from Hep3B or rat Sertoli cells using the Bioprobe System RNAzol B, according to the manufacturer's procedure. PolyA(+)RNA obtained by chromatography of total RNA through an oligo(dT) affinity column [10] were electrophoretically separated on a 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane (Hybond-C). The blot was hybridized with a DNA probe corresponding to the DNA binding domain of HNF-3α. The 356-bp probe was obtained by PCR, using the HNF-3α cDNA as template and the following oligonucleotides as primers CATCACCATGGCCATCCAGC; CAGCTACCATGGATCATTTGCGGTTTTCTGGGACCC and was labeled using the Megaprime DNA labeling system (Pharmacia).

3. RESULTS

3.1. *HNF-3α interacts with the Tf enhancer domain A*

Domain A, which has been described as the active part of the enhancer, contains a single enhanson Ia, which is a binding site for two liver proteins. These proteins, named EBP-45 and EBP-40, have been purified from rat liver nuclear extracts by affinity chromatography [11]. However, EMSA performed with oligonucleotide I1 (corresponding to the motif Ia, see Table I and Fig. 1) show that EBP-40 is largely represented in Hep3B nuclear extracts whereas EBP-45 is only poorly detected (Fig. 2a). This suggests that the activity observed in transient expression experiments is essentially due to EBP-40.

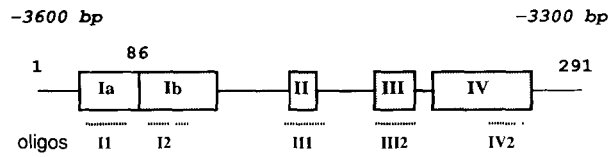

Fig. 1. Diagram of the hTf enhancer. Boxes represent the five motifs as indicated inside. Domain A is represented by a white box. Domain B by grey boxes. −3,600 bp and −3,300 bp are the limits of the enhancer relative to the cap site of the hTf gene. 1, 86 and 291 designate nucleotide numbers of the enhancer. Oligonucleotides used in this paper are depicted as broken lines under their corresponding motifs.

Recent studies have indicated that HNF-3, a family of liver-enriched transcription factors, could recognize the motifs 5′-TGTTTGTTTCT-3′ [12] or 5′-TGTTTGTTTT-3P′ [13] closely related to the motif present in the core of the enhanson Ia. In order to test whether EBP-40 belongs to the HNF-3 protein family, we used antisera directed against HNF-3α, HNF-3β or HNF-3γ in gel mobility shift assays. Fig. 2b shows that the antiserum directed against HNF-3α is the only one that reacts with EBP-40 (which forms the slower complex in gel shift experiments) preventing the binding of the protein to the DNA. Moreover, as shown in Fig. 2c,

Table I

Sequence of oligonucleotides used in this paper

	57 86
I1	GCTCTTTGTTTGCTTTGCTTCTGTGTCAAC
I1mut-2	GCTCTT**at**TTTGCTTTGCTTCTGTGTCAAC
	81 107
I2	GTCAACTGGGCAACATTTGGAAACAACA
I2mut	TGTGTCAAC**gttta**AACATTTGGA
	140 171
III1	ACCCACCTCCTGCTGGTCAGCTTTTCCAGCTT
III1mut	ACCCACCTCCT**ttaaa**TCAGCTTTTCCAG
	188 226
	* ** *
III2	GCGCAGCTATTTCTTTAAGGGCCAACTCGTGGTTGAA
III2mut	GGGCCAAC**Ta**A**TttTTa**AAATTTGAG
	254 284
IV2	GAAAGATTTGGCTCATGCTTGGGTTGGTCTA
IV2mut	TTGGCTCATGC**Tatcg**TTGGTCTAGAA
	-193 -161
CR	CTGTGCTGGACTCCTTCCACTGGCGGGTCGTC
AP4	GATCACCAGCTGTGGAATGTGTGT
APF1	GTCAGCAGGTGACCTTTGCCCAGCGCCCTG
U P.(*Nde*I)	CTCCATATGGTTTTCCCAGTCACGACG
R.P (*Nde*I)	CTCCATATGAACAGCTATGACCATGAT

We report only the sequences of the upper strands. Numbers indicate the positions of the nucleotides in the enhancer region, excepted for the oligonucleotide CR where numbers indicate the position relative to the cap site. Mutated oligonucleotides have been realized according to the 'preferential binding sites' of the wild-type oligonucleotides (Boissier et al. [6]). The nucleotides mutated from the wild-type sequence are indicated in lower-case letters. Concerning the oligonucleotide III2, asterisks indicate the purines for which methylation interferes with binding.

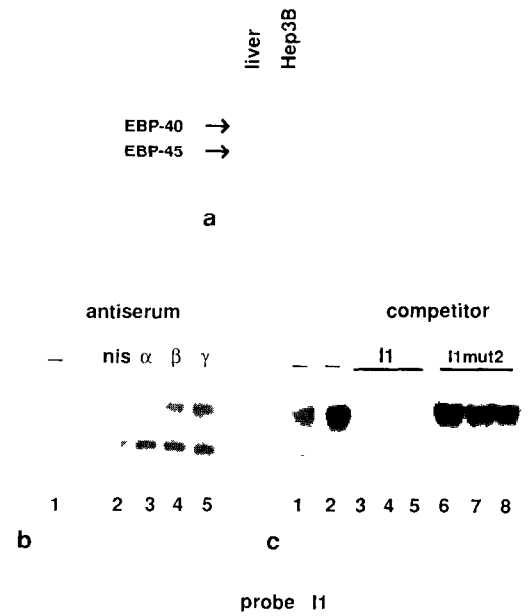

Fig. 2. Analysis of the interaction of HNF-3α with the A domain of the hTf enhancer EMSA were performed with oligonucleotide I1 as a probe. (a) Hep3B or rat liver nuclear extracts were used as indicated. (b) Assays performed in the presence of co-purified EBP-40 and EBP-45, and different sera. Lane 1, assay without serum, lane 2, assay performed with serum from nonimmunized rabbits; lane 3, assay performed with serum from rabbits immunized against HFN-3α; lane 4, assay performed with serum from rabbits immunized against HFN-3β; lane 5, assay performed with serum from rabbits immunized against HNF-3γ. (c) Lane 1, assay performed in the presence of co-purified EBP-40 and EBP-45; lane 2, assay performed with in-vitro translated HNF-3α; lanes 3–5, competition experiments were performed with a 10-, 50- or 100-fold excess of unlabeled oligonucleotide I1, in the presence of in-vitro translated HNF-3α; lanes 6–8, competition experiments were performed with a 10-, 50- or 100-fold excess of unlabeled oligonucleotide I1mut-2, in the presence of in-vitro translated HNF-3α.

the complex formed in EMSA between the Ia sequence and the purified EBP-40 is identical to the one obtained with the in-vitro translated HNF-3α protein (tHNF-3α). Furthermore, unlabeled oligonucleotide I1 is able to compete for the formation of the DNA/tHNF-3α complex. On the contrary, its formation is not inhibited by the addition of increasing amounts of the mutated oligonucleotide I1mut-2 (see Table 1 and Fig. 2c). Indeed, this oligonucleotide fails to generate complexes with EBP-40 [11] and with tHNF-3α (not shown).

From all these data we conclude that EBP-40, the protein that interacts with the enhanson Ia, is HNF-3α.

3.2. *Identification of the proteins interacting with the Tf enhancer domain B*

3.2.1. Factors belonging to the NF1 protein family interact with motifs Ib and IV

In-vitro DNA–protein binding experiments suggested that oligonucleotides I2 and IV2 (corresponding respectively to motifs Ib and IV) are the target for the same protein [6]. To identify this protein we performed cross competition gel shift assays with a 100-fold excess of several oligonucleotides described as binding sites for known nuclear factors. One of them, the oligonucleotide CR (Table I) is able to compete for the formation of the complex obtained with liver extracts and the labeled oligonucleotides I2 or IV2 (Fig. 3a). CR corresponds to a region of the hTf promoter which interacts with transcriptional factors belonging to the NF1 protein family [4]. In order to clearly identify the factors binding on the motif Ib and IV, we used antibodies directed against members of the NF1 protein family [14] in gel retardation assays. As shown in Fig. 3b, the antiserum interacts with most of the proteins involved in the formation of the retarded complexes, preventing their binding to the probes IV2 and I2.

Finally, mutation of the TGG sequences present in motifs Ib and IV, and corresponding to a half binding site for the NF1 factors, inhibits their interactions with the liver proteins (see below). From all these results, we conclude that motifs Ib and IV, as the CR region of the hTf promoter, interact with proteins belonging to the NF1 family.

3.2.2. The factor interacting with motif II

Motif II corresponds to the sequence 5′-CTGCTGGTCAGCTT-3′ [6]. This sequence contains part of the transcription factor AP4 binding site CAGCTG [15,16], in two repeats (nucleotides corresponding to the AP4 binding site are underlined in the motif II sequence). First, EMSA have been realized with HeLa and liver nuclear extracts using an oligonucleo-

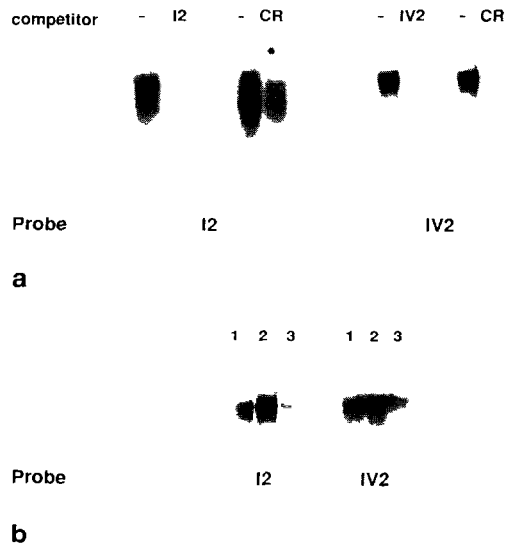

Fig. 3. Gel shift analyses of oligonucleotides I2 and IV2. In each case, assays were performed with crude liver nuclear extracts. Probes are the oligonucleotides IV2 or I2 as indicated. (a) Cross competition experiments using a 100-fold excess of homologous or CR oligonucleotide as indicated (b) Analyses using serum from rabbits immunized against NF1 Lane 1, assay without serum; lane 2, assay with the pre-immune serum, lane 3, assay performed in the presence of antiserum.

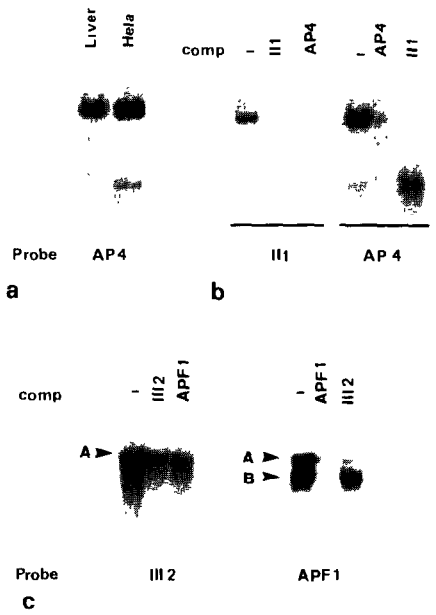

Fig. 4. Characterization of the proteins interacting with the oligonucleotides III and III2. (a) Gel shift assays were performed with liver or HeLa cells crude nuclear extracts as indicated. The oligonucleotide AP4 was used as a probe. (b) Cross-competition experiments were performed in the presence of crude liver nuclear extracts. Oligonucleotides III or AP4 were alternatively used as probes. A 100-fold excess of competitor oligonucleotide III or AP4 was used as indicated. (c) Assays were performed with crude liver nuclear extracts. Oligonucleotides III2 and APF1 were alternatively used as probes. For competitions, a 100-fold excess of unlabeled oligonucleotides III2 or APF1 was used. Arrow A indicates the band corresponding to EIII complex, arrow B indicates the band corresponding to HNF-4, COUP-TF and Arp-1 complexes.

tide containing the AP4 binding site present in the SV40 enhancer A-domain [15] (Table I). As shown in Fig. 4a, a similar complex is obtained with both extracts, indicating that the factor AP4, first purified from HeLa cells [15], is also present in liver extracts. Then, cross competition assays were performed between the oligonucleotides AP4 and III (corresponding to the hTf enhancer motif II). Data shown in Fig. 4b indicate that with liver extracts, the heterologous as well as the homologous oligonucleotide is able to compete for the formation of the oligonucleotide-protein complex. Results are the same with HeLa extracts (not shown). In addition, disruption of the GCTG sequence present in motif II, essential for AP4 binding, inhibits its interaction with the factor present in the liver nuclear extracts (see below). Taken together, these results indicate that AP4 is likely to interact with motif II of the hTf enhancer.

3.2.3. The factor interacting with motif III

EMSA have shown that a single liver nuclear factor interacts with the oligonucleotide III2 which corresponds to motif III. In order to identify this factor, we performed cross competition assays with several oligonucleotides described as binding sites for known nuclear proteins. One of them, APF1 (Table I), is able to compete for the formation of the retarded band between the liver protein and the oligonucleotide III2 (Fig. 4c). The oligonucleotide APF1 corresponds to an element of the apolipoprotein CIII promoter [17,18] which interacts with several liver nuclear factors giving rise to two retarded complexes [19]. The protein CIIIB1, forming the slower complex, was recently purified but not yet cloned [20]. The proteins involved in the formation of the faster complex were demonstrated to be HNF-4, COUP-TF and Arp-1 [21]. The retarded band we observed in EMSA with the oligonucleotide III2 has the same electrophoretic mobility as the complex due to CIIIB1. Moreover, unlabeled oligonucleotide III2 is able to compete for the formation of the CIIIB1 complex but not for the faster one (Fig. 4c). In addition, the protein interacting with motif III is, like CIIIB1, a heat stable factor (data not shown). These results indicated that the liver nuclear factor interacting with the motif III of the hTf enhancer might be CIIIB1. However, competition experiments showed that the oligonucleotide III2 was unable to compete for the binding of purified CIIIB1 to the oligonucleotide CIIIBM5, a mutated probe that binds only CIIIB1 [20] (Dr. Zannis, personal communication).

From these results, we conclude that at the moment we are not able to fully identify the protein that interacts with the motif III of the hTf enhancer. This protein is provisionally named EIII.

3.3. *Effects of domain B mutations on protein binding and on transcription activation*

In order to determine the contribution of each motif of the domain B to the enhancer activity, we performed mutagenesis experiments. The mutated oligonucleotides used for this purpose are depicted in Table I. The oligonucleotides I2 and IV2 were modified in a TGG motif corresponding to a half binding site for the NF1 factor family (oligonucleotides I2mut and IV2mut). The oligonucleotide III which seems to be a target site for the AP4 factor, was modified in the motif GCTG corresponding to part of the AP4 binding site (oligonucleotide IIImut). Concerning the oligonucleotide III2, interacting with EIII, the mutations were chosen in order to disrupt the main protein-DNA contact points determined by methylation interference assay (oligonucleotide III2mut, Table 1).

Each mutated oligonucleotide has lost the ability to bind the nuclear factor interacting with the corresponding wild type sequence, as demonstrated by gel mobility shift and DNase I footprinting competition experiments (data not shown).

3.3.1. In vitro analysis of mutated enhancers

Mutations were then introduced by PCR in the hTf enhancer to produce four modified enhancers: eMIb, eMII, eMIII and eMIV respectively mutated in the mo-

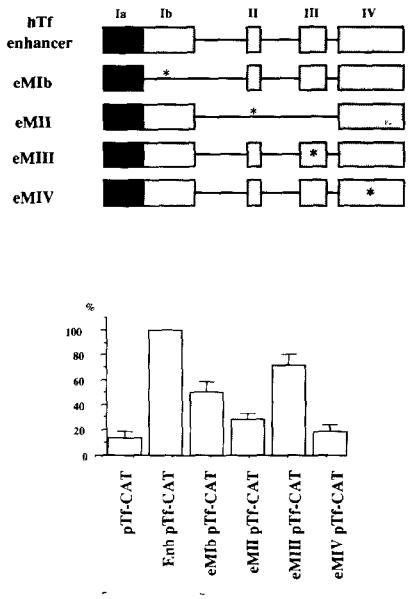

Fig. 5. Effect of region B mutations on protein binding and on transcription activation. (Upper part) Schematic representation of results obtained in footprinting experiments with the wild type and the four modified enhancers eMIb, eMII, eMIII and eMIV (as indicated on the left). Boxes representing the five motifs are indicated and summarize the presence or the absence of DNA–protein interactions. Motif Ia is represented as a dark box. Motifs of the domain B (Ib, II, III and IV) are represented as clear boxes. Stars are pointing out the mutated motifs. (Lower part) Analysis of the transcriptional activity of the mutated enhancers. Values are the percentage of CAT activity in transient expression experiments using Hep3B cells They represent the means ± S.E M. for at least three independent experiments and are expressed relative to the Enh pTf-CAT activity.

tifs Ib, II, III and IV. DNase I footprint analyses were performed using the four modified enhancers, in the presence of crude nuclear extracts. The results are summarized in Fig. 5 (upper part). As expected, eMIb affords no protection on the mutated motif Ib, indicating that NF1 is unable to interact with the Ib modified site. Mutations in the motif II of eMII prevent not only the binding of AP4 to this motif but also interfere with the binding of EIII to motif III. On the other hand, no modification of the protection over the five motifs Ia, Ib, II, III and IV is observed in both modified enhancers eMIII and eMIV. These results indicate that protein–protein interactions may exist between the factors AP4, EIII and NF1 bound respectively to motifs II, III and IV (see section 4).

3.3.2. Transcriptional activity of the four modified enhancers

The four mutated enhancer regions (eMIb, eMII, eMIII and eMIV) have been cloned upstream of the hTf promoter in a CAT reporter plasmid to be tested in transient expression experiments in Hep3B cells. As controls, we used a vector containing the wild type enhancer (Enh pTf-CAT) and a vector containing only the hTf promoter (pTf-CAT). The four mutated enhancers present reduced activity (Fig. 5, lower part). eMIb pTf-CAT and eMII pTf-CAT show residual activities which are respectively 50% and 28% of that of the wild type. eMIII pTf-CAT has a slighter effect on the enhancer activity (residual activity of 71%). On the contrary, eMIV pTf-CAT has lost the ability to enhance the effect of the hTf promoter. It is interesting to underline that these functional results are not always in keeping with the footprinting analyses of the mutated enhancer regions (see Fig. 5).

3.4. *The members of the liver-enriched HNF-3 transcription factor family are not expressed in the Sertoli cells of testis*

The proteins we have already identified as binding to the domain B are rather ubiquitous than liver-specific. On the contrary, domain A interacts with HNF-3α, a liver-enriched transcription factor [22]. One of the questions that may be addressed concerns the possible correlation of the efficiency of the enhancer in different cell types with the presence or the absence of HNF-3. This prompted us to analyze the existence of the HNF-3 messengers in Sertoli cells where the transferrin gene is functionally transcribed but the enhancer inactive [7]. Northern blot analyses of poly(A)$^+$ RNA from Sertoli and Hep3B cells demonstrate that no messenger of any of the members of the HNF-3 protein family is detected in Sertoli samples while they appear in Hep3B samples (Fig. 6). Nevertheless, a Sertoli protein is able to interact in EMSA with an oligonucleotide containing the A domain sequence [7]; however, this protein is not recognized by antisera directed against members of the HNF-3 family of proteins (not shown).

4. DISCUSSION

4.1. *Identification of the nuclear proteins interacting with the hTf enhancer*

As a first step in the understanding of the molecular mechanisms involved in the Tf enhancer activity we identified most of the nuclear proteins interacting with this region.

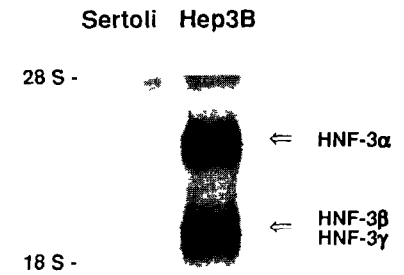

Fig. 6. Northern blot analysis of poly(A)$^+$ mRNA from Hep3B and Sertoli cells. Messengers coding for members of the HNF-3 protein family were detected with the region of the HNF-3α cDNA corresponding to the DNA binding domain of the protein. Traces of 28 S and 18 S rRNAs were present in the samples.

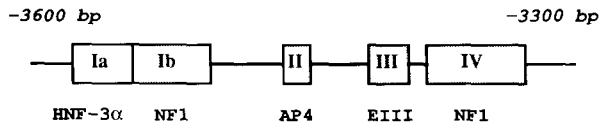

Fig. 7. Diagram of the hTf enhancer organization. Boxes represent the five motifs as indicated inside The motif Ia (domain A) is represented as a white box. The four motifs of the domain B (motifs Ib, II, III and IV) are represented as grey boxes. Factors interacting with each motif are indicated under each box. Numbers localize the position of the enhancer relative to the cap site of the human transferrin gene.

We have previously described the purification of two liver nuclear factors, EBP-40 and EBP-45, interacting with the enhanson Ia of the domain A [11]. Compared to EBP-45, EBP-40 is largely represented in Hep3B nuclear extracts (Fig. 2a); furthermore, EBP-40 presents a higher affinity than EBP-45 for the motif Ia [11]. These data indicate that EBP-40, rather than EBP-45, is involved in the hTf enhancer activity detected in hepatoma cells. We thus focused our attention to its identification. Using specific antisera we showed that EBP-40 is identical to HNF-3α (Fig. 2b). Since we purified a protein of 40 kDa and since HNF-3α is a protein of 50 kDa [22], it seems likely that we purified a proteolytic form of HNF-3α.

The use of specific antisera allows the identification of the proteins interacting with motifs Ib and IV of the hTf enhancer as belonging to the NF1 family (Fig. 3b) [14]. In addition, mutagenesis as well as gel shift experiments have shown that the protein interacting with motif II is likely to be AP4 (Fig. 4) [16]. Finally, it has not yet been possible to clearly identify EIII, the protein interacting with enhanson III.

All these results are summarized in Fig. 7.

4.2. *Organization of the hTf enhancer*

After identification of the proteins interacting with the different motifs of the enhancer, mutagenesis experiments have clarified the relative contribution of each enhanson to its activity. The data concerning domain B, obtained in this paper from transient expression experiments (Fig. 5, lower part), as well as the results previously reported with domain A [11], indicate that each motif (or module) is necessary to obtain full enhancer activity.

Furthermore, results of the footprinting experiments performed with the mutated enhancers, illustrate some aspects of the enhancer organization.

Analyses of the footprinting patterns obtained with eM1b, modified in region 1b, indicate that this region is unable to bind NF1 (Fig. 5, upper part). As all other motifs of the enhancer are still protected, we conclude that the binding of NF1 to motif Ib occurs independently of the other factors. The same conclusion was obtained previously concerning the binding of HNF-3α (EBP-40) on motif Ia [11].

eMII, modified at the AP4 binding site, is unable to bind AP4 and surprisingly, is also unable to bind EIII (Fig. 5, upper part). On the contrary, the footprinting pattern of the eMIII enhancer, shows a protection at the mutated EIII binding site as well as at the other sites. Taken together, these results suggest that there is a direct interaction between AP4 and EIII. Indeed, the EIII protein seems to bind to enhanson III only when AP4 interacts with its own target, enhanson II. It is then tempting to suggest that EIII protects the modified motif III in the eMIII footprinting experiments, through its interaction with AP4. Consequently, in the wild-type enhancer, EIII may have two contacts, with the DNA and with the neighboring factor AP4. In eMIII, EIII would only have one contact, with AP4, sufficient to protect enhanson III against DNase I cleavage.

Similar analyses were made with eMIV; in spite of the fact that the mutation introduced in motif IV was shown to abolish NF1 binding in in-vitro experiments, all the motifs are protected in the footprinting assays (Fig. 5, upper part). These results suggest, in this case, the existence of interactions between the NF1 factor and other proteins. Since the binding of HNF-3α and NF1 to motifs Ia and Ib occurs independently of the other factors (see above), it is logical to propose that the NF1 protein, binding to motif IV, may interact with the proposed AP4-EIII complex. The hypothesis concerning a multimeric complex formation between AP4, EIII and NF1 is supported by the known properties of the AP4 factor. Indeed, Hu et al. [16] suggested that AP4 could interact selectively with a variety of different proteins.

The hTf enhancer presents, as others cellular and viral enhancers, a modular organization and an absolute requirement of each module for full enhancer activity. The hTf enhancer, associated with liver-enriched and ubiquitous proteins constitutes a particular molecular edifice specifically maintained through DNA–protein and protein–protein interactions. Such a molecular organization may have important physiological consequences: a variation in the amount of a single factor should result in a drastic modification of the enhancer activity. Indeed, the absence of efficiency of the enhancer sequence in Sertoli cells [7] could be explained in this way.

4.3. *Molecular basis of the inactivity of the enhancer in Sertoli cells*

The factors we have sofar identified as interacting with domain B of the enhancer in the hepatic system are rather ubiquitous than tissue-specific. On the contrary, domain A is the binding site for a member of the HNF-3 transcription factor family. Since this factor is a liver-enriched protein [22], we asked whether or not HNF-3 proteins are also present in Sertoli cells. It was previously shown that messengers for the HNF-3γ protein are present in whole testis RNA [23]. We demonstrate

here that no messengers of the HNF-3 family can be detected by Northern blots in Sertoli cell preparations (Fig. 6). Nevertheless, a protein is able to interact with an oligonucleotide containing the enhanson Ia in gel shift mobility; this interaction was shown to be specific by competition experiments [7]. However, this protein is not recognized by antisera directed against any of the members of the liver-enriched HNF-3 family. Moreover, footprinting experiments performed with the whole enhancer DNA region indicate that domain A is not protected or very weakly protected when using Sertoli or testis crude nuclear extracts [7]. Therefore, it is tempting to correlate the absence of the HNF-3 proteins with the non-functioning of the enhancer in Sertoli cells.

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