The Enhancer of the Human Transferrin Gene Is Organized in Two Structural and Functional Domains*

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We previously identified a 300-base pair long enhancer, located 3.6 kilobases upstream of the cap site of the human transferrin gene. A 5' deletion up to position 86 of the enhancer resulted in complete loss of the enhancer activity. Here we show by competition footprint analysis, gel retardation assays, and transient expression studies in hepatoma and HeLa cells that the enhancer is composed of two distinct structural and functional domains, A (nucleotides 1–86) and B (nucleotides 87–291). Each domain is a proto-enhancer of a different type.

Domain A is a proto-enhancer that, when multimerized, is able by itself to stimulate transcription from the heterologous SV40 promoter, both in Hep3B and HeLa cells. It contains the octanucleotide TGTTTGCT sequence and is the binding site of two liver-specific nuclear factors and of a different HeLa nuclear factor. Domain B contains four binding sites interacting with several liver nuclear proteins. In order to bind, any of these proteins requires the presence of all the others. This domain is able to block the activity of a downstream negative element, but it has no enhancer activity by itself. In the presence of the transferrin promoter, full enhancer activity requires the association of the two domains A and B.

The molecular basis of the human transferrin (Tf) gene expression was first analyzed in liver (Brunel et al., 1988; Schaeffer et al., 1989) and more recently in Sertoli cells of rat testis (Guillou et al., 1991). We previously showed that, in liver, the regulatory elements are distributed in four functionally different regions: a minimal cell type-specific promoter, a distal promoter, a negative-acting region, and an upstream-located enhancer. The Tf enhancer, situated between -3.6 and -3.3 kb relative to the transcriptional start site of the gene, fulfills the criteria established for enhancers. It stimulates transcription of an homologous and heterologous promoter in an orientation- and position-independent manner. Transient expression studies revealed that the Tf enhancer is not tissue-specific since it is able to stimulate transcription from the SV40 promoter both in hepatoma and in epithelial carcinoma HeLa cells. Thus it appears that the liver-specific expression of the Tf gene is only mediated by the nuclear protein transferrin-liver factor 1 and the CCAAT/enhancer binding protein-related factor, binding to adjacent sites of the proximal Tf promoter (Schaeffer et al., 1989; Ochoa et al., 1989).

Recent studies of cellular enhancers have started to reveal the complexity of their cis-acting elements; some of them were shown to present a modular organization resembling that of the viral SV40 enhancer (Herbst et al., 1989; Godbout et al., 1988; Grayson et al., 1988; Kruse et al., 1988; Knepel et al., 1990). An extensive analysis of the SV40 enhancer defined distinct levels of organization of an enhancer (Herr and Clarke, 1986; Fromental et al., 1988; Ondek et al., 1988). The basic structural unit was called an enhanson and was defined as the binding site for a trans-acting factor. The combination of two or more copies of the same enhanson or of different enhansons generates an enhancer module (Schafer et al., 1988; Dyman, 1989), also called a proto-enhancer. In the SV40 enhancer, each enhanson displays a characteristic cell typespecific activity (Kanno et al., 1989).

The aim of this work was to determine the structural and functional organization of the Tf enhancer. The interactions of nuclear factors with different regions of the enhancer were analyzed by DNase I footprint and competition experiments and by gel retardation assays. They defined two distinct structural domains: the domain A composed of one enhanson and the domain B composed of four enhansons.

To establish the function of each domain, single and multiple copies of each domain were cloned upstream of the Tf and the SV40 promoter in expression vectors that were introduced into Hep3B and HeLa cells.

The data demonstrate the modular organization of the Tf enhancer and clarify the role of each domain in transcriptional stimulation. They stress the importance of the octanucleotide TGTTTGCT sequence, which is also present in several other liver-expressed genes. This sequence was detected in domain A, which is the binding site of distinct proteins in different cell types.

MATERIALS AND METHODS

Materials—T4 DNA ligase, DNA polymerase I (Klenow fragment), T4 polynucleotide kinase, and restriction endonucleases were purchased from New England Biolabs; alkaline phosphatase and F-12 and Dulbecco's modified Eagle's media from Boehringer Mannheim; poly(dI-dC) from Pharmacia LKB Biotechnology Inc.; DNase I from Worthington; [32P]-labeled dNTPs from Amersham Corp.; and acetyl coenzyme A from Sigma.

Plasmid Constructions—The Tf-CAT vectors containing the insert extending from -4000 to +39 and the 5' deletions ending at -3600, -3500, -3200, and -620, as well as the vectors 0.7Tf-CAT, previously called 0.7 (-620, +39) Tf-CAT, and 0.7Tf-CAT2 were con-
This fragment was sufficient by itself to block the negative action of the -1000 to -620 region. It is therefore tempting to suggest the presence of two functional domains, A and B, respectively located upstream and downstream of position 86.

Footprint Analysis of the Whole and Truncated Tf Enhancer—To identify the nuclear factors interacting with the whole (1-291) and truncated (87-291) enhancer regions, we performed a DNase I footprinting analysis, using rat liver nuclear extracts. Fig. 2, A and B, shows the protection pattern of each strand of the whole enhancer. The three protected regions numbered I, III, and IV have been described previously (Schaeffer et al., 1989). A careful analysis of the footprint pattern using several nuclear extract preparations indicated the presence of the short slightly protected region numbered II. As expected, regions II, III, and IV were protected in the truncated enhancer. Interestingly, motif I was still protected in the truncated enhancer between positions 87 and 121 (Fig. 2C), compared with positions 88 and 121 in the whole enhancer; moreover, the DNase I hypersensitive site located at position 99 in the whole enhancer disappeared. These results suggest that at least some of the proteins are able to interact with region I of the whole enhancer, one binding to region Ia (58-64) and the other binding to region Ib (87-121). Fig. 3 presents the DNA sequence of the enhancer, as well as the localization of the four protected regions as determined in Fig. 1. Nuclear proteins present in HeLa cell extracts generated an identical footprinting pattern.

**Footprint Analysis of the Whole and Truncated Tf Enhancer**—To further explore the interactions of nuclear proteins with the putative domains A (1-86) and B (87-291), footprinting experiments were performed in the presence of different competitor oligonucleotides. The synthetic oligonucleotides I1 (58-86), I2 (81-107), and I3 (100-128) correspond to overlapping sequences of region I (Fig. 4). As shown in Fig. 5a, when unlabelled oligonucleotide I1 was used as a competitor in 50- and 100-fold excess, the protection of motif Ia disappeared as expected, as well as the hypersensitive site located at position 64; the footprint patterns of regions Ib, II, III, and IV were unchanged. The exact delimitation of the 3' border of the protection of motif Ia is difficult to assess by the competition footprinting experiments; however, the results shown in Fig. 5a indicated that the presence of the hypersensitive site at position 64 on the lower strand correlates with the binding of a nuclear protein to this motif. Moreover, methylation interference data performed with oligonucleotide I1 showed that the nucleotides interfering with the interaction of the protein are located at position 64 on the upper strand and at positions 67 and 69-72 on the lower strand (Fig. 3 and results not shown). This confirms the importance of the hypersensitive site at position 64 and the correlation with the binding of a nuclear factor to the region around nucleotides 64-72. As shown in Fig. 5b, addition of increasing amounts of unlabelled oligonucleotide I2 (nucleotides 81-107) inhibited not only the protection of motif Ib, but also the formation of footprints III and IV, whereas motif Ic was not affected; the hypersensitive site located at position 99 disappeared, whereas the site located in motif Ia at position 64 was still present. The protection of motif II was in general only slightly visible; therefore, it is difficult to detect a clear modification of its pattern. Finally, oligonucleotide I3 (nucleotides 100-128) was unable to compete for the binding of any nuclear factor; the footprinting pattern obtained with a 200 molar excess of unlabelled oligonucleotide I3 was identical with the pattern observed without any competitor (Fig. 5c, lanes 0).

In addition, competition experiments were performed with synthetic oligonucleotides corresponding to motif II (oligo-
Organization of the Human Transferrin Gene Enhancer

FIG. 1. Enhancer activity of 5′ deletion mutant constructs. Tf DNA sequences are indicated by the black lines. ER and NB indicate, respectively, the enhancer region and the distal negative-acting region. The (−3600, +39) and (−3500, +39) inserts correspond to the 5′ deletion ending, respectively, at positions 1 and 86 of the enhancer, as defined in the legend to Fig. 3. Values on the right are the percentage of Tf-CAT expression in Hep3B cells and are expressed relative to the level achieved with (−4000, +39) Tf-CAT. They are the mean ± S.E. for at least three independent experiments with two different plasmid preparations. Transfection and CAT experiments were performed as described under “Materials and Methods.”

nucleotide III1, nucleotides 81–107) and to overlapping sequences of motifs III and IV (Fig. 4, upper panel). Oligonucleotides II1, II2, and IV2 at a 100-fold excess were able not only to displace the protection of their homologous sequence, but also to inhibit the protection of motifs Ib, II, III, and IV without affecting the protection of motif Ia. In each case, the resulting pattern was identical with the one observed in the competition assay performed with an excess of oligonucleotide I2 as shown in Fig. 5b. Oligonucleotides III1 (173–203) and IV1 (229–260), like oligonucleotide I3, were also unable to affect the footprinting pattern.

These results help to establish an interaction profile of the trans-acting factors with their preferential binding sites on each enhancer region (Fig. 4, lower panel).

Analysis of the trans-Acting Factors by Gel Retardation Assays—To determine the composition of the factor(s) bound to each domain, we performed gel retardation experiments. Each oligonucleotide I1, I2, II1, II2, and IV2 that was previously shown in the competition footprint assay to interact with a nuclear protein was 5′ end-labeled and used in gel mobility experiments in the presence of liver nuclear extracts (Fig. 6A, right panel). The electrophoretic profile of the shifted DNA-protein bands was clearly different with oligonucleotide I1 compared with the others. Moreover, when oligonucleotide
I1 was used as a probe, cross-competition experiments performed with 10, 50, and 100 molar excess of unlabeled oligonucleotides I1, I2, I11, and I112 showed that only the homologous oligonucleotide was able to compete for the protein binding (Fig. 6B).

Oligonucleotides I11 and I112 appear to interact with different liver protein(s) according to the different retarded bands (Fig. 6A, right panel), and the absence of cross-reactivity with all heterologous nucleotides (Fig. 6C). Interestingly, when oligonucleotide I112 was used as an unlabeled competitor, it was able to partially displace the complex formed by liver protein(s) with oligonucleotides I1 and IV2 (Fig. 6C). This suggests that oligonucleotide I112 contains sequences that may bind only the protein(s) interacting with oligonucleotides I2 and IV2.

Precisely, oligonucleotides I2 and IV2 gave rise to an almost similar retarded complex, different from all the others (Fig. 6A, right panel). Moreover, in cross-competition experiments, the complex formed with oligonucleotide I2 could be partially competed for by an excess of unlabeled oligonucleotide IV2 and vice versa (Fig. 6C). The same is true when oligonucleotide I112 was used as a competitor as indicated above, whereas no competition could be observed with oligonucleotides I1 and I11 (Fig. 6C).

Analysis of the Enhancer and Cell Type-specific Activity of Domains A and B

Transient Expression Analysis in Hep3B and HeLa Cells—As presented previously, the Tf enhancer corresponds to the EcoRI-HindIII fragment located between -3.3 and -3.3 kb relative to the cap site. The nucleotide after the HindIII site in the polylinker of (-3600, +39) Tf-CAT was defined as position 1 so that the HindIII site at -3.3 kb was position 291. Regions protected from DNase I cleavage are indicated by lines alongside each strand. The thick arrows indicate the sites of enhanced DNase cleavage. The open arrow at position 86 delineates domains a and b of region I. Roman numerals I, II, III, and IV correspond to the four protected regions.

FIG. 3. Localization of the protected regions in the enhancer of the human Tf gene. The nucleotide sequence of the Tf enhancer was analyzed by gel retardation experiments in HeLa cells. These cells were transfected with a functional enhancer construct in the presence of Hep3B and HeLa cells. These cells were transfected with a series of Tf-CAT constructs, containing the domain A (oligonucleotide 11) or the 0.2-kb long domain B in single and multimerized copies linked either to the homologous Tf promoter (Fig. 4) or to the SV40 early promoter (TATA box and 21-bp repeats) (Fig. 8). The levels of CAT expression were expressed relative to the level of pSV2-CAT containing the Tf enhancer. This activation can be compared to the 3- and 5-fold stimulation of the whole 0.3-kb enhancer, respectively, in HeLa and in Hep3B cells. In contrast, multimers of the oligonucleotide I11 were hardly able to stimulate transcription from the Tf promoter. Their enhancing ability was far below that of the whole 0.7- or 0.3-kb long enhancer region, which activated transcription of the Tf promoter about 5-fold (Fig. 7).

Mobility Shift Experiments with Oligonucleotide I1 in the Presence of Rat Liver and HeLa Cell Extracts—Since the multimerized oligonucleotide I11 was able to function as an enhancer both in Hep3B and HeLa cells in the presence of the SV40 promoter, it was interesting to determine whether or not the same factor binding to this site was present in the
**Discusssion**

This report presents the structural and functional characterization of the enhancer of the human transferrin gene. The region situated between -3.6 and -3.3 kb upstream of the transcriptional start site of the Tf gene was shown in our previous paper to present the characteristics of a typical enhancer (Schaeffer et al., 1989). As shown before, this enhancer is not liver-specific since it was able to stimulate the expression of rat liver (Figs. 2, A and B, and 3) and of HeLa cells.

The first evidence of the existence of two distinct structural domains was given by the DNase I footprint analysis of the (87–291) truncated enhancer region. As shown in Fig. 2C, even in the absence of the 58–86 sequence, the remaining region I was still protected by liver nuclear extracts. This suggests that region I can be divided at position 86 into two structural domains. These assays were performed with unlabeled oligonucleotides 11, 12, 111, 112, and IV2, shown to compete for binding as described in the legend to Fig. 4, were used in the gel shift assay. A, right panel, they were incubated with 6 μg of rat liver nuclear extracts; left panel, oligonucleotide II1 was incubated with 6 μg of either rat liver or HeLa cell nuclear extracts; the two specific retarded DNA-liver protein complexes C1 and C2 are indicated. B, cross-competition experiments performed with oligonucleotide II1 as a probe. A—争相, homologous competition; X—X—X, heterologous competition with unlabeled oligonucleotides I2, I11, I12, and IV2. % indicates percent of binding of liver proteins to the probe; molar excess indicates the concentration of each unlabeled competitor. C, results of the different cross-competition assays performed with 100 molar excess of each unlabeled oligonucleotide. ++++, 100% of competition; ++, 50% of competition; −, absence of competition.
pendent experiments and are expressed relative to pSV2-CAT. Values on the right are the percentage of transient Tf-CAT expression site of the Tf gene. Positions -3600 and -3500 correspond respectively to positions 1 and of the oligonucleotide I1 described in the legend to Fig. 4. Constructs were transfected in Hep3B cells, and CAT expression in Hep3B cells. They are the mean values  and acetylated products (Ac-CM) are indicated.

Fig. 3. The limits of the inserts are indicated in base pairs, relative to the cap promoter (TATA and 21-bp repeats). The Tf-CAT constructs were transfected in Hep3B and HeLa cells, and CAT activity was measured as described under “Materials and Methods.” Values on the right are the percentage of transient Tf-CAT expression in Hep3B and HeLa cells. They are the mean ± S.E. for at least three independent experiments and are expressed relative to pSV2-CAT.

Fig. 7. Analysis of the enhancer activity of multimers of the enhancer domains linked to the Tf promoter. The Tf-CAT constructs were transfected in Hep3B cells, and the CAT expression level was measured as described under “Materials and Methods.” The limits of the inserts are indicated in base pairs, relative to the cap site of the Tf gene. Positions -3600 and -3500 correspond respectively to positions 1 and 87 of the enhancer sequence presented in Fig. 3. The black polygon in the last three constructs corresponds to the insert of the oligonucleotide I1 described in the legend to Fig. 4. Values on the right are the percentage of transient Tf-CAT expression in Hep3B cells. They are the mean ± S.E. for at least three independent experiments and are expressed relative to pSV2-CAT.

Fig. 8. CAT activity directed by multimers of the Tf enhancer domains linked to the SV40 early promoter. Top panel, the Tf enhancer regions were directly linked to the SV40 early promoter (TATA and 21-bp repeats). The Tf-CAT constructs were transfected in Hep3B and HeLa cells, and CAT activity was measured as described under “Materials and Methods.” Values on the right are the percentage of transient CAT expression in Hep3B and HeLa cells. They are the mean ± S.E. for at least three independent experiments and are expressed relative to pSV2-CAT. Bottom panel, one typical CAT assay in Hep3B cells transfected with the vectors indicated in the top panel. Lane 1, pSV2-CAT; lanes 2–9, constructions are presented in the top panel. Unreacted chloramphenicol (CM) and acetylated products (Ac-CM) are indicated.

performed with unlabeled synthetic oligonucleotides corresponding to overlapping sequences of the enhancer (Fig. 4). The results provided a precise mapping of the preferential sites of DNA-protein interactions (Fig. 4, lower panel). They indicated that only sequences of oligonucleotide I1 and part of oligonucleotides I2, III1, I112, and I122 correspond to the binding sites of nuclear factors. Second, they clearly showed the absence of cross-competition between oligonucleotide I1, corresponding to region Ia, and all other oligonucleotides, corresponding to regions Ib, II, III, and IV. When the competition assay was performed with oligonucleotide I1 (58–86), only the corresponding motif Ia was modified (Fig. 5a). To the contrary, competition with oligonucleotide I2 (81–107), corresponding to motif Ib (Fig. 5b) or with oligonucleotides III1, I112, and I122, modified the footprinting pattern and DNase 1-hypersensitive sites of regions Ib, II, III, and IV, without affecting the protection of motif Ia. Thus, it appears that the four protected regions can be divided into domain A, containing region Ia, and domain B, including regions Ib, II, III, and IV (Fig. 4, lower panel). Moreover, the fact that the elimination of any one of the proteins interacting with any one of regions Ib, II, III, or IV strongly modified the protection pattern of the three other regions suggests a cooperation for binding between the trans-acting factors of the B domain. It is likely that protein-protein interactions play an essential role in the stability of the final B domain DNA-nuclear protein complex.

Gel retardation studies performed with liver nuclear extracts (Fig. 6A, right panel) and cross-competition assays (Fig. 6, B and C) indicated that the proteins binding to oligonucleotide I1 are different from the proteins interacting with the oligonucleotides corresponding to domain B. Oligonucleotides III1 and I112 seem to bind distinct proteins, different from the others; oligonucleotides I2 and I122 may bind similar proteins, but different from the others. These data suggest that the four protected motifs of domain B are the binding sites of at least three different liver nuclear factors.

Liver nuclear extracts gave rise to two specific retarded DNA-protein complexes (C1 and C2) with oligonucleotide I1, whereas HeLa nuclear extracts gave a unique band of a higher electrophoretic mobility (Fig. 6A, left panel). This result strongly suggests the existence in liver and HeLa cells of different proteins able to interact with domain A.

Existence of Two Functional Domains—Transient expression studies demonstrated that the two structural domains A (1–86) and B (87–291) correspond to two functional active domains. Compared to the fully active (−3600, +39) Tf-CAT vector, the 5’-deleted (−3500, +39) Tf-CAT construct had lost all enhancer activity; in the hepatoma Hep3B cells, its level of CAT expression was identical with that reached by the (−620, +39) Tf-CAT vector containing only the Tf promoter (Fig. 1). This showed that the deleted sequence, which corresponds to position 1–86 of the enhancer, is crucial for the enhancer activity. It also indicated that the 0.2-kb long domain B had no enhancer activity by itself. However, the presence of this domain B was essential to maintain the activity of the promoter and to block the action of the (−1000, −620) negative-acting element described in our previous paper (Schaeffer et al., 1989).

The enhancer activity of each isolated domain was further tested in transient expression experiments by inserting each domain either in single copy or in multimers in front of different promoters. The resulting data clearly indicate that domain B is unable to enhance the activity of either the Tf promoter (Fig. 7) or the heterologous SV40 early promoter, either in Hep3B or in HeLa cells (Fig. 8). In contrast, multi-
mers of the oligonucleotide I1, corresponding to domain A, were able to strongly stimulate the expression of the SV40 promoter 6-fold in Hep3B cells and 8-fold in HeLa cells (Fig. 8). Thus the active liver and HeLa nuclear factors binding to domain A (Fig. 6, left panel) are able to form functional interactions with the factor Sp1 (Dynan and Tjian, 1983) binding to the SV40 promoter.

This contrasts with the results obtained with the homologous Tf promoter. Two or four copies of the oligonucleotide I1 were unable to significantly enhance the activity of the Tf promoter in Hep3B cells (Fig. 7). It appears that the interactions of the cis- and trans-acting elements of the enhancer and promoter are quite stringent. Only the combination of the two domains A and B results in full enhancer activity in the presence of the Tf promoter. This emphasizes the importance of protein-protein interactions in bringing together the two enhancer domains and the Tf promoter, either directly or via some component(s) of the transcriptional machinery, as proposed by Ptashne's group (Lin et al., 1990; Carey et al., 1990).

Each Domain A and B Is a Proto-enhancer of a Different Type—A fine dissection of the SV40 enhancer revealed that it is composed of multiple motifs, binding nuclear proteins which work synergistically to generate enhancer function. Distinct types of motifs have been identified and three levels of enhancer organization have been defined (Herr and Clarke, 1986; Fromental et al., 1988; Ondek et al., 1988; Dynan, 1989). The first level is the enhanson, which corresponds to a protein binding site; the second level corresponds to combinations of identical or different enhansons to form a proto-enhancer, the enhancer minimal element. Where a proto-enhancer is not active by itself, a third level of organization exists, corresponding to the combination of at least two proto-enhancers (Fromental et al., 1988).

It is tempting to define the Tf enhancer organization following the SV40 enhancer structure. Consequently, the Tf enhancer is composed of five enhansons: Ia, Ib, II, III, and IV. Enhanson Ia corresponds to the structural and functional domain A. According to the definition proposed from Fromental et al. (1988), it is an enhanson of class C, since it exhibits enhancer activity when a single copy of the motif is oligomerized. Thus, it is also a proto-enhancer of type III. Enhansons Ib, II, III, and IV of domain B correspond to a proto-enhancer that has no activity by itself; only the association with the enhanson Ia generates enhancer activity. In conclusion, the Tf enhancer is made up of two proto-enhancers of different types.

The cooperation between the proteins interacting with B domain and the synergistic activation observed when the two proto-enhancers A and B are present may have important functional consequences. A variation in the amount of a single factor should result in a modification of the Tf enhancer activity. This emphasizes the importance of each single factor in the overall modulation of the Tf enhancer function.

The Proto-enhancer A Interacts with Cell Type-specific Proteins—The 30-bp long proto-enhancer A, corresponding to the oligonucleotide I1, contains what appears more and more like a liver-specific sequence TGTTTTGC, since the identical motif has been described in control regions of other liver-specific genes. The same octanucleotide is also found in the promoter of the α-fetoprotein and α1-antitrypsin genes (Scott and Tilghman, 1983; Ciliberto et al., 1985), in the human α-fetoprotein enhancer (Watanabe et al., 1987), and in the E site of the hepatitis B virus enhancer (Shaul and Ben-Levy, 1987). The heptanucleotide sequence TGTGTTGC is present in the mouse α-fetoprotein enhancer (Godbout et al., 1988) and in the mouse albumin enhancer (Herbst et al., 1989; Zaret et al., 1990).

The absence of liver specificity of the Tf enhancer previously observed (Schaeffer et al., 1989) could have resulted from a different cell type specificity of each proto-enhancer. However, our mobility shift data (Fig. 6A, left panel) strongly suggest that the proto-enhancer A interacts in liver and in HeLa cells with different nuclear factors. Like the octamer sequence of immunoglobulin genes (Schaffner, 1989), the sequence of the Tf proto-enhancer A appears to stimulate cell-specific transcription by binding distinct factors in different cell types. In this case, the Tf enhancer behaves in hepatoma and in HeLa cells as a tissue-specific enhancer, each proto-enhancer interacting in a particular cell type with its cognate cell-specific proteins. Purification and characterization of the different nuclear proteins are obviously necessary to confirm this attractive model.

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