Sertoli Cell-specific Expression of the Human Transferrin Gene

COMPARISON WITH THE LIVER-SPECIFIC EXPRESSION

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We present a comparative study of the cis- and trans-acting elements governing the expression of the human transferrin (Tf) gene in two tissues, liver and testis, where Tf is expressed at various levels. We have previously identified the elements of the promoter, negative, and enhancer regions involved in the liver-specific expression of the gene. By transfection experiments of primary cultured rat Sertoli cells compared with hepatoma cells, DNase I footprinting, and gel retardation studies, we have analyzed 3.6 kilobase pairs of the Tf regulatory region.

The far upstream enhancer functional in Hep3B cells is inactive in Sertoli cells; in the two cell types, different nuclear factors appear to bind to a DNA domain crucial for enhancer activity. Similar negative- and positive-acting elements are present in the distal promoter in both tissues. However, different combinations of proximal promoter elements control tissue-specific expression. Liver-specific transcription is governed by the interaction of the TF-LF1 protein and a C/EBP-related factor with the -125 to -45 region. In Sertoli cells, a -34 to -18 TATA box-binding factor is sufficient to initiate basal-level transcription. Efficient expression is achieved by the association of two factors binding either to the (-82, -1) or to the (-153, -52) region. The addition of a third adjacent element decreases the promoter activity, suggesting that the balance of three factors binding to the proximal sites regulates testis-specific expression.

Transferrin (Tf) functions as the essential iron transport protein in serum and as a growth factor for a variety of cells (Kahn et al., 1987). It is synthesized at a high level in liver (Morgan, 1969) and at a lower level in other organs such as brain (Aldred et al., 1987), testis (Skinner and Griswold, 1980), mammary glands (Chen and Bissell, 1987), and fetal muscle (Levin et al., 1984). The Tf gene exhibits a diverse pattern of tissue-specific expression and developmental regulation. It represents an excellent model of a gene transcribed in more than one tissue, each of which regulates the gene by different factors such as iron, vitamins, and various hormones.

The molecular mechanisms governing the cell type-specific gene control in various tissues are still poorly understood. A limited number of studies have been performed, concerning the involvement of different cis- and possibly trans-acting factors in the regulation of gene expression in different cell types (Garabedian et al., 1986; Dietrich et al., 1987; Hammer et al., 1987; Higuchi et al., 1988; Sastry et al., 1988; Ogami et al., 1990; Yan et al., 1990).

In a first step toward the elucidation of the mechanisms generating a diverse pattern of tissue specificity and diverse levels of expression in several tissues, we have focused our studies on Tf gene expression in liver. We have determined previously the organization of the 33.5-kb-long human Tf gene (Schaeffer et al., 1987) and analyzed the regulatory function of sequences extending over 4 kb of the 5'-flanking region (Brunel et al., 1988; Schaeffer et al., 1989). By transient expression experiments, we have demonstrated that the interaction of two proximal promoter elements with the nuclear proteins TF-LF1 and a C/EBP-related factor is responsible and sufficient for liver-specific transcription. Moreover in liver, Tf gene expression is modulated by a combination of multiple negative- and positive-acting elements. When cloned upstream of the SV40 promoter, a far upstream enhancer is more active in hepatoma cells than in the epithelial carcinoma HeLa cell line where the Tf gene is not expressed.

In this report we have designed experiments to elucidate the mechanisms controlling the transcriptional regulation of the Tf gene in testis, where transferrin is synthesized at a lower level than in liver. In testis, Sertoli cells form the semiferous tubules and create the blood-testis barrier (De Kretser and Kerr, 1988). They play a major role in the maintenance and control of spermatogenesis by providing the proper microenvironment for the developing germinal cells (Bardin et al., 1988). Sertoli cells synthesize and secrete testicular transferrin (Skinner and Griswold, 1980), one of the essential components for the maturation of germinal cells. It was shown that testicular and serum Tf have the same amino acid composition, the same mRNA size, and it is likely that they are the product of the same gene (Skinner et al., 1984).

Here we report the mapping of 3.6 kb of the Tf 5' regulatory region and present the identification of the cis- and trans-acting elements involved in Sertoli cell-specific expression. These data, compared with the results of liver-specific expression, show how a different interplay between specific DNA sequences and DNA-binding proteins governs the modulation of the Tf gene in two different tissues.

MATERIALS AND METHODS

Plasmid Constructions—pUC19-CAT and the Tf-CAT vectors containing the inserts which are 5' deletions of the -3960 to +39 kb.
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fragment, as well as the inserts (–82, –1), (–52, +30), (–153, +30), and (–153, –82) were constructed as reported previously (Schaeffer et al., 1989).

The (–153, –52)TF-CAT vector was obtained by following the same procedure as to construct (–153, –41)TF-CAT, described previously (Schaeffer et al., 1989).

The (–150, –14)TF-CAT vector was obtained by Bal31 digestion of (–184, +14)Tf-CAT at the KpnI site and closing the vector on itself. To construct (–184, +14)TF-CAT, the 198-bp HindIII-HindIII blunt-ended fragment was inserted in the Smal site of pUC19-CAT. The HindIII-EcoRI fragment from –5600 to –5300 was isolated from (–20, +39)TF-CAT, filled in, and cloned in the Smal site of pUC-CAT2 (containing the SV40 early promoter), as described previously (Schaeffer et al., 1989), and in the Ndel blunt-ended site of (–620, +39)TF-CAT, to obtain, respectively, 0.3 TF-CAT and 0.3 (–620, +39)TF-CAT.

The orientation of the inserts was determined by restriction enzyme mapping. All the described inserts were in sense orientation.

Cell Culture and Transfection—Sertoli cells were isolated from 17- to 20-day-old rat testis (Wistar, Iffa Credo, France) as described by Guilhou et al. (1986). They were cultured in Dulbecco’s modified Eagle’s medium (Boehringer Mannheim) supplemented with 10% fetal calf serum (Organon). Cells were plated out at a density of 12 × 10^5 cells/cm^2 dish 24 h before transfection and refed 3 h before transfection. Plasmids were prepared by double banding in cesium phosphate precipitation method (Graham and Van Der Eb, 1973). The (-150, +1)Tf-CAT vector was obtained by following the same procedure as to construct (–153, –41)TF-CAT, described previously (Schaeffer et al., 1989). Isolated Sertoli cells were pelleted by centrifugation at 80 × g for 5 min and resuspended in 4 packed cell volumes of buffer B (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 10% glycerol) and dialyzed against the same buffer. The insoluble material was removed by centrifugation, and small aliquots of protein extract were quickly frozen and stored at –80°C. The protein concentration was determined by the method of Bradford (1976). We obtained about 5 mg of nuclear proteins from 70 rat testis.

Nuclear proteins from 70 rat testis were isolated by the method of Chomczynski and Sacchi (1987), with the described homogenization buffer containing in addition 1 mM phenylmethylsulfonyl fluoride, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 1 mM DTT, 25 mM glycerol, 1 mM pepstatin, and 40 μM bestatin) containing 136 mM NaCl. An equal volume of buffer A containing 700 mM NaCl was added dropwise with continuous gentle shaking. The extract was homogenized with seven strokes in a cell glass Dreunse homogenizer (type B pestle) and shaken gently for 15 min at 4°C. The viscous lysate was centrifuged for 30 min at 100,000 × g at 4°C and the supernatant, of supernatant with gentle shaking. The precipitated proteins were sedimented by a 40 min centrifugation at 50,000 × g at 4°C. The protein pellet was resuspended in buffer (20 mM HEPES, pH 7.9, 1 mM MgCl2, 60 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol) and dialyzed against the same buffer. The insoluble material was removed by centrifugation, and small aliquots of protein extract were quickly frozen and stored at –80°C. The protein concentration was determined by the method of Bradford (1976). We obtained about 5 mg of nuclear proteins from 70 rat testis.

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DNA-Protein Mobility Shift Assay—The standard assay (Gardner and Reverin, 1981) was performed in a final volume of 20 μl containing 1 ng of 5'32P-labeled double strand oligonucleotide, 1 μg of poly(dI-dC), 25–75 ng of sonicated salmon sperm DNA, 30 ng of sonicated Escherichia coli DNA, 10 mM MgCl2, 25 mM KCl, 0.5 mM DTT, 12.5 mM HEPES, pH 7.8, 10% glycerol, 0.05% Nonidet P-40. Then 5 μg of rat liver or rat testis nuclear proteins were added. After 15 min at 4°C, the mixture was loaded onto a 6% or 8% polyacrylamide gel in 0.25 × TBE (1 × 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.5) and electrophoresed for 90–120 min at 10 V/cm. The gel was dried and autoradiographed. For the competition experiments, various amounts of unlabelled homologous or heterologous oligonucleotide were added in the binding reaction.

FIG. 1. CAT activity of the 5'-flanking region of the human transferrin gene in Sertoli cells compared with hepatoma cells. A, representation of the TF-CAT plasmids containing progressive 5' deletions of the TF regulatory region extending from –9600 to +29 bp. Details of construction are presented under “Materials and Methods.” The 5' end points of deletion are indicated in base pairs. The HindIII (H) and PvuII (P) restriction sites are indicated. B, transient CAT expression in Sertoli and Hep3B cells transfected with the TF-CAT plasmids described in A. The vectors pUC19-CAT without any promoter and pSV2-CAT containing the promoter and enhancer of SV40 have been used, respectively, as negative and positive controls. Activities were determined as described under “Materials and Methods” and represent the mean of at least three separate transfections. Variations between experiments in expression of TF-CAT plasmids relative to pSV2-CAT did not exceed 20%. The data concerning the Hep3B cells have been presented in our previous study (Schaeffer et al., 1989).
**RESULTS**

The Enhancer Functional in Liver Is Inactive in Sertoli Cells—We have localized previously the cis-acting DNA elements present within 4 kb of the Tf regulatory region involved in the transcriptional control of the Tf gene in liver. A series of TF-CAT vectors was introduced into human hepatoma HepG2 and Hep3B cells where the Tf gene is expressed at a high level and into the epithelial HeLa cell line where the Tf gene is not transcribed. Transient and stable expression assays revealed the presence of a positive element presenting the characteristics of an enhancer located between positions -3600 and -3300, a negative element extending from position -1000 to -620, and the promoter from -620 to the cap site (Schaeffer et al., 1989).

To examine whether the same cis-acting elements were functional or not in two tissues expressing a high and a low level of transferrin, we performed transient expression studies in primary cultures of 20-day-old rat Sertoli cells and in hepatoma Hep3B cells. TF-CAT deletion mutants extending from position -3600 to -620 (Fig. 1A) were introduced into the two cell types by the calcium phosphate coprecipitation method. The results of transient expression of CAT activity are presented in Fig. 1B. The efficiency of expression was about 40 times lower in Sertoli cells compared with Hep3B cells with the positive control vector pSV2-CAT. The 5' deletion results clearly show that the vectors with an insert extending from -3600 to -620 present a very low CAT activity in Sertoli cells. In contrast to Hep3B cells, the -3600 to -3200 deletion does not define a positive-acting region. However, the negative-acting (-1000, -620) region is present in both cell types. In Sertoli cells, the vector containing the (-620, +39) promoter region was expressed at the highest level, reaching 2% of pSV2-CAT, compared with 7% of pSV2-CAT in Hep3B cells.

We showed previously that in liver, the activity of the enhancer overrides the (-1000, -620) negative region, which functions only in absence of the enhancer. One could imagine the possibility that in testis, on the contrary, the negative element overrides the enhancer. To test this hypothesis, we measured the CAT activity of vectors containing the (-3600, -3300) enhancer region directly linked either to the transferrin promoter or to the SV40 early promoter. The results of the CAT activity of the various constructs transfected in both cell types are shown in Fig. 2. In Hep3B cells, the 0.3-kb fragment enhanced transcription both of the homologous and heterologous promoter. In Sertoli cells the 0.3-kb fragment had no effect, confirming the absence of any enhancer activity. It is interesting to note in Sertoli cells, the high activity of the SV40 early promoter containing the TATA box and the 21-bp repeats. This high activity of pUC-CAT2, reaching about 30% of pSV2-CAT, appears quite unusual and suggests the presence of a high amount of the Sp1 factor binding to the 21-bp repeats (Dynan and Tjian, 1985).

**Fig. 2.** Activity of the TF-CAT plasmids containing the 0.3-kb transferrin 5'-flanking sequence linked either to the transferrin or to the SV40 promoter. **Top panel,** the 0.3-kb fragment between -0.6 and -3.3 kb of the Tf 5'-flanking region was cloned upstream of the (-620, +39) homologous promoter and upstream of the heterologous SV40 early promoter (TATA and 21-bp repeats). Values on the right are the percentages of transient CAT expression in Sertoli and Hep3B cells. They are the mean ± S.E. for at least three independent experiments and are expressed relative to (-620, +39)TF-CAT. **Bottom panel,** one typical CAT assay in Sertoli and Hep3B cells transfected with the vectors presented in top panel. Experimental conditions are described under "Materials and Methods." Lanes 1-4 correspond to the vectors indicated in top panel.

**Interactions of Testis Nuclear Proteins with the Tf (-3600, -3300) Region—**To explore further the molecular basis for the lack of the enhancer activity in Sertoli cells, we performed a DNase I footprinting analysis of the (-3600, -3300) region. We have shown previously the existence of at least three protected regions, I, II, and III, with liver nuclear extracts (Schaeffer et al., 1989). Fig. 3 shows the footprinting pattern obtained with nuclear extracts from rat testis compared with rat liver. Quite surprisingly, nuclear proteins present in testis were able to interact with the 0.3-kb DNA fragment. Even more, they appeared to protect regions I-III. However, only part of region I from position 83 to 121 appeared protected with Sertoli extracts; in the 58-83 remaining region I protected in liver, only the hypersensitive site at position 64 was observed. Moreover, the hypersensitive sites located at positions 231 and 233 between motifs II and III with liver proteins were absent with testis proteins. The same results were obtained with nuclear extracts of either 20-day-old rat testis or of isolated Sertoli cells.

A fine mapping of the cis- and trans-acting elements of the enhancer demonstrated that in liver, the enhancer is composed of two structural and functional domains A and B. The domain A corresponds to position 58-86 of the region I, and the domain B corresponds to the remaining parts of the enhancer (Boissier et al., 1991). To determine whether each domain interacts with the same Sertoli and liver nuclear proteins, we performed gel retardation experiments. We used synthetic oligonucleotides I1 and I2, corresponding, respectively, to the sequences 57-86 (domain A) and 81-107 (part of domain B). As shown on Fig. 4, with oligonucleotide I1 Sertoli extracts gave rise to a specific DNA-protein complex, whereas liver extracts gave two bands of a different electrophoretic mobility. A different excess of unlabeled oligonucleotide I1 was able to compete for the formation of the specific complexes. An excess of a heterologous unlabeled oligonucleotide DRO had no effect on the complex formation. With oligonucleotide I2, liver and Sertoli extracts gave rise to a
distinct pattern of specific retarded bands of almost similar mobility. This result suggests that the nuclear factors interacting with the domain A and at least one region of the domain B are different in the two tissues.

**Interactions of Testis Nuclear Factors with the Tf Promoter**—The interactions between the (-620, +39) Tf promoter region and DNA-binding proteins were determined with the DNase I footprinting technique (Fig. 5). Seven protected areas were detected with testis nuclear extracts; five out of them correspond to the sites protected with liver extracts, identified in our previous study (Brunel et al., 1988). There are the distal regions DRII (-614, -598) and DRII (-474, -455), the central region CR (-191, -164), the proximal regions PRII (-102, -78) and PRI (-72, -50). In Fig. 5 are shown the protection pattern of PRI, PRII, CR, and DRI. In the same figure, we can observe that two additional sites were protected with testis extracts: the distal region DRO (-440, -425) and the TATA box region (-34, -18). It is interesting to mention that the protection of the DRO site could also be detected with partially purified liver extracts, suggesting that this factor is present in liver at a lower concentration than in testis. The footprints of the central and distal sites appear very clearly and present an identical pattern with both tissue extracts (Fig. 5, C–E; Brunel et al., 1988). The footprinting pattern of the proximal region is shown on each DNA strand with both Sertoli and liver nuclear extracts (Fig. 5, A and B). These data clearly show the presence of a protein on the TATA region with Sertoli extracts and the absence of protection with liver extracts. The comparison between the two cell extracts indicates that the protection of the proximal sites PRI and PRII is less visible with testis extracts than with liver extracts. Moreover the footprinting pattern is different with the two cell extracts; the hypersen-

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**F. Brunel, I. Petroopoulos, and M. M. Zakin, unpublished results.**
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Fig. 5. Footprint analysis of the transferrin promoter region with Sertoli and liver nuclear extracts. A1, A2, A'2, a 180-bp Ddel-Ddel fragment was 3' end-labeled at position -150 (lower strand) and B1, B2, B'2 at position +31 (upper strand). C, a 210-bp HindIII-PstI fragment was 3' end-labeled at position -620 (lower strand). D, a 300-bp PstI-NarI fragment was labeled at position -10 (upper strand); E, a 210-bp HindIII-PstI fragment was 3' end-labeled at position -405 (upper strand). Lane G + A represents the sequence ladder, F corresponds to assays with DNA without nuclear extract, S to assays with rat Sertoli nuclear extracts, and L to assays with rat liver nuclear extracts, as described under "Materials and Methods." Numbers along the autoradiographs indicate the position relative to the cap site of the gene determined in liver. Horizontal arrows indicate sites of enhanced DNase I cleavage. Brackets delineate sequences protected against DNase I digestion by Sertoli cells or liver nuclear extracts. A similar analysis performed with human and rat liver nuclear extracts has been described previously by Brunel et al. (1988).

Fig. 6. Localization of the binding sites for Sertoli nuclear proteins in the transferrin promoter. Sequences protected from DNase I cleavage are indicated by lines alongside the strand; only the coding or upper strand sequence is shown. The lines above and below the sequence correspond, respectively, to the upper and lower strand protection. The DNase I footprint experiments were performed as described under "Materials and Methods."

The interactions of testis nuclear factors with the proximal sites were further analyzed by mobility shift experiments. As shown in Fig. 4, the synthetic oligonucleotides TATA, PRI, and PRII were 5' end-labeled and used with testis extracts compared with liver extracts. Each oligonucleotide gave specific retarded DNA-protein complexes, which disappeared with an excess of unlabeled competitor oligonucleotide and did not disappear with an excess of unlabeled heterologous DRO oligonucleotide. In the competition experiments performed with the TATA region, the addition of the homologous TATA competitor resulted in the disappearance of the two specific faster bands and in an increased amount of the slowest band (Fig. 4, TATA). We explain these results by the sequestering of the specific nuclear factors which allows the increased binding of nonspecific proteins to the TATA oligonucleotide. Interestingly, the mobility of the specific retarded bands was the same with either Sertoli or liver nuclear proteins. This result suggests that similar or closely related transacting factors may be present in the two different tissues.

The results obtained with the TATA region using liver nuclear extracts differ according to whether it is analyzed through footprinting or mobility shift experiments. Indeed, no protection was found over the TATA sequence with liver extracts in footprinting experiments using the proximal promoter region (Fig. 5, A1 and B1). On the contrary, the mobility shift assays performed with the synthetic TATA oligonucleotide gave rise to specific retarded DNA-protein complexes (Fig. 4, TATA). This difference is probably due to the fact that in the case of the retardation assays the oligonucleotide used contains only the TATA site, whereas in the footprinting experiments the analyzed fragment contains the PRI and PRII sites. It may be that the liver proteins interacting with the PRI and PRII sites prevent the binding of the liver protein specific of the TATA region. This seems not to be the case in the Sertoli cells.

Functional Analysis of the Tfr Promoter in Sertoli Cells Compared with Hepatoma Cells—The functional mapping of the (-620, +39) promoter region was performed by transient
expression studies of a series of 5' deleted Tf-CAT vectors shown in Fig. 7A. The ability of the truncated promoter regions to activate the transcription of the CAT gene in Sertoli and Hep3B cells is shown in Fig. 7B. The level of CAT activity reached by the (−620, +39)Tf-CAT vector containing the promoter region was chosen as 100% in each cell type. In Sertoli cells, the −620 to −493 deletion did not modify the CAT level, in contrast to the decrease of CAT activity detected in Hep3B cells. Except for this difference, the distal promoter region located between −620 and −125 bp presented a similar CAT expression pattern in both cell types. A strong positive element is defined by the −493 to −387 deletion, since CAT expression was reduced at least 5-fold. The further 5' deletion from position −150 to −125 resulted in an increase of the CAT level different in each cell type. The CAT level increased 7-fold in Hep3B cells and less than 2-fold in Sertoli cells, which indicates the presence of a negative-acting element. In hepatoma cells this element was previously localized within a larger area extending from −323 to −125.

The proximal promoter region located downstream position −125 is strikingly different in hepatoma and Sertoli cells. In Sertoli cells the CAT activity increased steadily with the progressive 5' deletions ending at −150, −125, and −82. The

(−82, −1)Tf-CAT vector was expressed at the same level as (−620, +39)Tf-CAT containing the whole promoter region. The further 5' deletion up to −52 bp decreased the CAT level 2-fold. In contrast, in hepatoma cells CAT expression reached its maximum level with (−125, +39)Tf-CAT and was reduced to zero with (−82, −1)Tf-CAT. A finer analysis of this (−125, +39) region in hepatoma cells was presented previously (Schaeffer et al., 1989).

The proximal promoter was further analyzed in Sertoli cells using 3' deletions of the −150 to +14 region. Fig. 8 shows the Tf-CAT constructs which were used for the CAT expression studies, along with the localization of the binding sites of the testis nuclear factors, as determined in Figs. 5 and 6. The level reached with (−150, +14)Tf-CAT was chosen as 100%. The 3' deletion removing the TATA box up to position −52 in (−153, −52)Tf-CAT resulted in a 2-fold increase in CAT expression. The further 3' deletion up to −82 bp which eliminates the TATA box and the PRI site abolished transcription; this shows that the PRII site is not sufficient by itself to promote transcription.

It is interesting to compare these data with the 5' deletion analysis presented in the same figure. The 5' deletion up to −82 which eliminates the PRII sequence in (−82, −1)Tf-CAT increased the CAT level 2-fold. The further deletion up to −52, which removes the PRII and PRI sites and leaves the TATA box region in (−52, +30)Tf-CAT, resulted in the same expression as the non-deleted vector.

Taken together, the 5' and 3' deletion results seem contradictory. The −52 to +30 TATA box region acts as a positive element since it is sufficient to promote transcription; however the removal of this sequence in (−153, −52)Tf-CAT resulted in an increase in CAT activity, which would suggest that the TATA box region is a negative-acting element. Moreover, the removal of the PRII site in (−82, −1)Tf-CAT increased CAT expression, suggesting that the PRII sequence is a negative-acting element; however, the expression of the (−153, −52)Tf-CAT vector containing PRII and PRI was as high as that of (−82, −1)Tf-CAT containing the PRI and the TATA box regions which both act as positive elements.

It appears that the highest CAT activity is obtained with the presence of two adjacent elements, either PRI/PRII or TATA/PRII in (−153, −52)Tf-CAT or TATA/PRI in (−82, −1)Tf-CAT. The importance of the positive action of the PRI element is clear, since its combination with the TATA box element in (−82, −1)Tf-CAT doubles the CAT activity. Moreover the combination in (−153, −52)Tf-CAT of PRI with the PRII element, which is inactive by itself, results in the highest CAT expression. Whenever a third element is added, either PRII to the TATA/PRII couple or the TATA box element to the PRII/
PRII couple, transcription is decreased. The fact that a third element diminishes the transcriptional activity compared with a promoter composed of two elements helps clarify the above contradictions.

**DISCUSSION**

This report presents the mapping of the cis- and trans-acting elements involved in the regulation of transcription of the Tf gene in Sertoli cells of rat testis. The regulatory function of sequences extending over 3.6 kb of the 5'-flanking region was analyzed in Sertoli cells and hepatoma Hep3B cells. This comparative study was designed to learn about the still poorly understood mechanisms devised by different tissues to generate diverse levels of expression of a same gene. It was reported that rat testis contains less than 2% of the amount of Tf mRNA found in liver. Upon culturing, Sertoli cells activate the Tf gene and thus contain about one-third of the amount of Tf mRNA present in liver (Lee et al., 1986).

**Analysis of the Enhancer Region in Sertoli, Hepatoma, and HeLa Cells**—Our previous studies showed that the regulatory region controlling liver-specific transcription is composed of multiple positive- and negative-acting elements, most of them interacting with DNA-binding proteins present either in human or rat liver nuclear extracts (Brunel et al., 1988; Schaeffer et al., 1989). We showed that the region located between -3.6 and -3.3 kb presents the characteristics of an enhancer, more active in hepatoma than in the epithelial carcinoma HeLa cells, which do not express the Tf gene. It was also demonstrated that the region located between -3.6 and -3.5 kb corresponding to positions 1-86 of the enhancer is crucial for the enhancer activity. (Schaeffer et al., 1989; Boissier et al., 1991).

To study the ability of the 0.3-kb region to function as an enhancer in Sertoli cells, we tested the activity of 5' deleted Tf-CAT vectors (Fig. 1) and of constructs containing the 0.3-kb region linked either to the homologous (-620, +39) Tf promoter or to the heterologous SV40 early promoter (Fig. 2). Transient expression results clearly demonstrated the absence of any enhancer activity in Sertoli cells. Our footprinting analysis revealed that nuclear proteins present in testis or cultured Sertoli cells are able to bind to regions II and III, but only to part of region I, shown to be protected by nuclear extracts of either liver (Fig. 3) or HeLa cells (Boissier et al., 1991). It is striking to consider that the 58-83 sequence of the region I, which is the binding site of a liver or HeLa nuclear factor and does not bind a Sertoli factor, corresponds to a region shown to be essential for the enhancer activity. Although this sequence is not protected, mobility shift data showed that distinct proteins present in liver and in testis extracts are able to interact with oligonucleotide I (position 57-86; Fig. 4).

A more detailed analysis of the enhancer demonstrated that it is composed of two distinct structural and functional domains A (position 1-86) and B (87-291). Each domain requires the association with the other domain to enhance transcription from the Tf promoter (Boissier et al., 1991). This synergistic effect results in a dramatic modification of the enhancer activity when one single enhancer factor is missing or different. It is therefore likely that the different Sertoli factor, interacting with the isolated sequence I1 but unable to footprint the A domain, is mostly responsible for the absence of enhancer activity.

It may appear surprising that the enhancer is totally inactive in Sertoli cells which do express a low level of transferrin, whereas it is functional in HeLa cells which do not transcribe the Tf gene. In fact, in HeLa cells the activity of the enhancer is revealed only in the presence of a functional promoter, such as the SV40 early promoter; although potentially active proteins bind to the enhancer, transcription is blocked from the Tf promoter which is inactive in these cells. In contrast, in Sertoli cells the enhancer has no potential for stimulating the activity of either the Tf or the SV40 promoter. It is the promoter which is solely responsible for transcription of the Tf gene.

**Similar Distal Promoter cis-acting Elements Exist in Sertoli and Hepatoma Cells**—The regulatory region located between -3200 and -125 bp presents a similar pattern of cis-acting elements in Sertoli and Hep3B cells, as shown by our transient expression studies (Figs. 1 and 7). A negative-acting element situated between -1000 and -620 bp is present in both cell types. Most of the cis-acting elements defined by the deletion analysis of the (-620, +39) promoter region could be correlated with the binding sites of nuclear factors, determined by DNase I footprinting data (Fig. 6). A weak positive element located between -620 and -493 bp, which correspond to the DRI binding site, is active only in Hep3B and not in Sertoli cells. A strong positive element situated between -493 and -387 bp correlates with the binding of TF-LF2 to the DRI site in Hep3B cells (Schaeffer et al., 1989) and to both DRI and DRO sites in Sertoli cells. The negative-acting element localized within the -150 to -125 region does not correspond to the binding site of a factor detectable with crude nuclear extracts. Whether the DRI, DRO, and CR cis-acting elements interact with the same or different trans-acting factors in liver and testis remains to be investigated.

**Different Combinations of Proximal Promoter cis- and trans-acting Elements Exist in Testis and Liver**—Of particular interest are the results concerning the proximal promoter region located downstream position -125. In Sertoli cells, like in liver, this proximal region is sufficient for the tissue-specific expression of the gene. However a different combination of cis-acting elements controls Tf gene transcription in the two cell types. This was clearly demonstrated by the transient expression analysis of the 5' and 3' deleted Tf-CAT vectors (Figs. 7 and 8), which correlates perfectly with the binding of trans-acting factors, as shown by the DNase I footprinting data (Figs. 6 and 7).

As shown previously (Schaeffer et al., 1989), liver-specific transcription is solely mediated by the binding of the TF-LF1 protein and a C/EBP-related factor to their respective PRI and PRII binding sites. The TATA box region is unable by itself to activate transcription of the Tf gene in hepatoma cells. This explains why the TF-LF1 CAT constructs containing the 5' deletion ending at -82 or -52 bp are totally inactive in Hep3B cells (Fig. 7).

In contrast, in Sertoli cells, the -52 to +30 TATA box region is sufficient to activate a basal level of transcription. It is interesting to note the cell-specific activity of the (-52, +30)Tf-CAT vector, which is expressed only in Sertoli cells and not in Hep3B cells. This result correlates well with the data reported by Dierich et al. (1987) who found that the first 44 bp of the 5'-flanking sequence of the chicken ovoTf gene are sufficient to promote a basal level of cell-specific expression in chicken hepatocytes and oviduct tubular gland cells.

This region contains the -34 to -18 binding site, centered on the TATA box, of a Sertoli nuclear protein that we call the TATA box-binding factor. The presence of this factor is detected both by its DNase I footprint (Fig. 5) and by the specific retarded DNA-protein complex in a competition mobility shift experiment (Fig. 4). The Sertoli-specific activity of the (-52, +30)Tf-CAT vector could suggest the existence of a cell-specific TATA box factor, able to initiate transcrip-
tion in Sertoli cells and not in hepatoma cells. However, the presence of a similar specific retarded DNA-protein complex with either factor, either in liver or in testis, may rather indicate a quantitative difference of a similar factor. In Sertoli cells, this TATA box factor appears in a larger amount than in liver.

It is known that the binding of the general transcription factor TFIID to a 20-bp sequence including a TATA box is the first step in the assembly of an active eukaryotic transcription complex (Van Dyke et al., 1988; Lewin, 1990). The TFIID protein purified from Drosophila and yeast was shown to activate basal-level transcription (Hoey et al., 1990; Hori-koshi et al., 1990). Whether the TATA box-binding factor corresponds to the general transcription factor TFIID remains to be determined. Moreover the characteristics of the factor present in testis and in liver have to be further investigated.

Although the unique TATA box element is sufficient to support a basal level of transcription in Sertoli cells, the efficiency of transcription is increased 2-fold by the presence of the upstream adjacent PRI element, in the (−82, −1)Tf-CAT vector. Surprisingly, the PRI-TATA box combination can be efficiently substituted by the PRI-PRII couple in the (−153, −52)Tf-CAT vector (Fig. 8). It appears that optimal transcriptional activation is achieved by the combination of the PRI element with either the upstream PRI element or the downstream TATA box element. Thus the PRI-binding factor plays the central role of a switch, able to interact with either the 5' or the 3' located factor. The addition of a third adjacent element, either to the PRI-TATA box couple or to the PRI-PRII couple, reduces the efficiency of transcription. This suggests that transcriptional activation of the Tf gene in Sertoli cells is the result of a delicate balance between the three factors binding to the adjacent TATA box, PRI, and PRII sequences. Judging by the different level of expression of the gene, it is obvious that the combination of Sertoli nuclear factors has a reduced ability to activate the Tf promoter, compared with the very efficient combination of the Tf-LF1 and the C/EBP-like hepatic factors.

Our mobility shift data suggest that similar or closely related trans-acting factors interact with the proximal promoter sequences, (−34, −18) TATA box, (−72, −50) PRI, and (−102, −78) PRII in the two tissues. However, the different protection pattern observed with liver and testis nuclear extracts in DNase I footprinting assays suggests different DNA-protein interactions. In liver, a C/EBP-like factor binds to the PRII region; in testis, it was recently demonstrated that the C/EBP mRNA is present at a very low concentration (Birkenmeier et al., 1989). The question whether the nuclear factors binding to the PRII sequence and to the TATA box and PRII sites are identical or not in liver and testis awaits further characterization of these proteins.

Importance of trans-acting Factors in the Cell Type-specific Expression—Our data on liver- and testis-specific expression of the Tf gene represent a preliminary contribution to the exciting problem of generation of the tissue-specific diversity. Evidence is now increasing about the presence of various cis-elements controlling distinct cell-type expression. Results on the expression pattern of the Drosophila yolky protein gene have shown that different cis-acting elements are active exclusively in either fat bodies or ovaries (Garabedian et al., 1986). The existence of different cis-acting elements governing the tissue-specific diversity has been reported in transgenic mice studies of the α-fetoprotein gene (Hammer et al., 1987) and of the transthyretin gene (Yan et al., 1990). Recently, different cis-acting elements as well as trans-acting factors have been shown to control the hepatic and intestinal transcription of the apolipoprotein AI (Sastry et al., 1988; Higuchi et al., 1988) and the apolipoprotein CIII gene (Ogami et al., 1990); these elements present a complex pattern extending over more than 1 kb. Distinct cis- and trans-acting elements located within the proximal promoter region were reported for the transcription of the gastrin gene in pituitary cells (Godley and Brand, 1989) and in pancreatic islet cells (Wang and Brand, 1990).

Our results demonstrate that liver- and testis-specific Tf gene expression is mediated by a distinct combination of cis- and trans-acting proximal promoter elements. In addition, in both tissues similar upstream elements located between −1000 and −125 bp are involved in the modulation of the proximal promoter activity. The major difference resides in the enhancer activity of the (−3600, −3300) region. In contrast to liver factors, Sertoli factors binding to the two enhancer domains are unable to enhance transcription. The Sertoli protein interacting with the crucial sequence of the A domain most probably is responsible for the loss of enhancer function. This reveals the importance of cell-specific trans-acting factors which contribute to the specific regulation of Tf gene expression in two cell types.

Besides the general transcription factors necessary for the basal tuning of transcription, there exist other specific regulatory factors. Retinol, testosterone, and folliculo-stimulating hormone are known to stimulate Tf gene expression in rat Sertoli cells (Hugly and Griswold, 1987; Huguenik et al., 1987) and are inactive in liver. Our preliminary data are a necessary step for the understanding of the exciting mechanisms of Tf gene regulation by the specific physiological factors.

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Transcriptional Regulation of the Human Transferrin Gene


