The Far-upstream Enhancer of the Carbamoyl-phosphate
Synthetase I Gene Is Responsible for the Tissue Specificity and
Hormone Inducibility of Its Expression*

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The role of the proximal promoter and the far-upstream enhancer in the hepatocyte-specific and hormo-
nal regulation of the carbamoyl-phosphate synthetase I (CPS) gene was investigated in transient transfection
assays using primary rat hepatocytes, hepatoma cells,
and fibroblasts. These experiments revealed that the
activity of the promoter is comparable in all cells tested
and is, therefore, not responsible for tissue-specific
expression. The 5'-untranslated region of the mRNA is a
major, non-tissue specific stimulator of expression in
FTO-2B hepatoma cells, acting at the post-transcrip-
tional level. A 469-base pair DNA fragment, 6 kilobase
pairs upstream of the transcription start-site in the CPS
gene, confers strong hormone-dependent tissue specific
expression, both in combination with the CPS promoter
and a minimized viral thymidine kinase promoter. Se-
quences similar to a cyclic AMP-responsive element and
a glucocorticosteroid-responsive element were found in
the isolated enhancer. Subtitutional mutations in these
sites strongly affected hormone-induced expression.
Analysis of the interaction between the enhancer and
parts of the CPS promoter revealed that, in addition to
the TATA box, the GAG box, a motif similar to the GC box
near the TATA motif, is instrumental in conferring the enhancer activity.

Carbamoyl-phosphate synthetase (CPS)1 is the first enzyme
of the ornithine cycle. CPS expression can be detected from
the 15th embryonic day onward in the liver of the rat. Expression
is initially found in a few hepatocytes only, but toward the end of
the fetal period all hepatocytes have been recruited to ex-
press CPS (1–3). After birth, the expression gradually becomes
confined to the hepatocytes surrounding the portal veins (3, 4).
The only other cells producing appreciable levels of CPS mRNA
and protein are the enterocytes of the small intestine (3, 5).
After birth, CPS enzyme and mRNA levels change in parallel
under all experimental conditions (5, 8, 10, 11), suggesting that
hormonal regulation, tissue specificity, and zonal restriction of
expression of CPS is regulated at the level of transcription.
Accordingly, it was shown that glucocorticosteroids and cyclic
AMP enhance transcription of the CPS gene in adult rat hepa-
tocytes (6–9, 12).
The CPS gene is a single-copy, 110 gene that contains 38
exons and is surrounded by matrix attachment regions (13–16).
The mRNA is 5546 nucleotides in length (excluding the poly(A)
tract) and consists of a 140-nucleotide untranslated region, an open
reading frame of 4500 nt, and a 3'-UTR of 906 nt. Functional
analysis of the 5'-part of the gene showed that the minimal,
fully active promoter is located within the 161 nt upstream
of the transcription initiation site (15). DNase I footprint analysis
of this region revealed three protected sites (sites I–III (17, 18),
but the actual identity of the factors occupying these sites is not
known (19). In between the TATA motif at position –21 and
protected site I, a so called "GAG" element was identified (19)
that resembles the element recognized by the TFIIIA-like Cys67
His2 zinc finger class of transcription factors, including Sp1,
but is not a target of the Sp1 protein itself.
Two MspI restriction sites upstream of the transcription start
site were found to be differentially methylated (15). The site at
–6.3 kbp becomes demethylated shortly after birth in
liver and intestine. The other site, at –4.0 kbp relative to the
transcription start site is fully methylated in liver and partly
demethylated in postnatal small intestine. A 4-kbp fragment
containing the –6.3-kbp MspI site responds to cyclic AMP
and dexamethasone in transient expression assays, giving a 10-fold
rise in reporter gene expression in FTO-2B hepatoma cells (15).
The regulatory regions of the CPS gene were analyzed with
respect to their role in tissue-specific expression and hormone
sensitivity. A 469-bp far-upstream enhancer fragment was
found to be responsible for hormone-dependent tissue-specific
expression of the CPS gene. It was also investigated whether
the proximal promoter and the 5'-UTR of the mRNA con-
tributed to tissue-specific expression. Exploration of the inter-
action of the enhancer fragment with parts of the CPS promoter
revealed an important role for the GAG element in the trans-
duction of the hormonally induced activation signal from the
enhancer.

EXPERIMENTAL PROCEDURES

Cell Culture—FTO-2B rat hepatoma cells (20), Rat-1 fibroblasts (21,
22), Chinese hamster ovary cells (CHO-K1), and Caco-2 cells (23) were
cultured in Dulbecco's modified Eagle's medium/F-12 (Life Technolo-
gies, Inc.), supplemented with 10% fetal calf serum (FCS; Life Technolo-
gies, Inc.) and MH1C1 hepatoma cells were cultured in Ham's F-10
medium (Life Technologies, Inc.), supplemented with 17.5% FCS. The
FCS was selected for its inability to induce CPS expression in cultured
hepatocytes. All cells were cultured in a 5% CO2/air atmosphere at
37 °C. FTO-2B and MH1C1 cells express CPS, but Rat-1, CHO-K1, and Caco-2 cells do not (see "Results"). Cell lines were tested monthly for the presence of mycoplasmas.

DNA Transfection—Exponentially growing cells were transfected by electroporation (24). The capacity of CPS gene sequences to direct expression of a reporter gene was analyzed by cloning into the vector plT1 or plBluc. The plT1 vector, based on pBluescript SK+ (Stratagene), contains the firefly luciferase gene (25) as a reporter in conjunction with SV40 small t-antigen intron and polyadenylation signal optimized for expression (26). The vector plBluc (27) contains the firefly luciferase reporter gene driven by the first 81 base pairs of the viral thymidine kinase promoter (28) and is referred to as "minimized TK promoter." To confirm equimolar amounts, 3 μg of pRSVcat (RSV LTR (29)) were transfected equimolar amounts, 3 μg of vector pRSVcat (RSV LTR (29)). After each transfection the cell suspension was divided into equal parts, one being grown in culture medium and the other(s) in culture medium supplemented with 100 μm dexamethasone or with 100 μm dexamethasone, 1 μM dbutyryl cyclic AMP (Bt2cAMP; Boehringer) and 0.1 μM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) (7). 44 hours after transfection on cells were harvested, lysed in 100 μl KHPO4/K3HPO4, pH 7.6, 0.1% Triton X-100, and tested for CAT activity (30). Luciferase activity, 31, and protein concentration (bicinchoninic acid reagents, Pierce).

Determination of Transfection Efficiency—FTO-2B hepatoma cells and Rat-1 fibroblasts were transiently transfected as described (24) with 31 μg of luciferase construct and 25 μg of pRSV-n-LacZ, encoding β-galactosidase carrying a nuclear translocation signal. 44 hours after transfection, luciferase activity and protein concentration were determined in one well. Cells in the other well were stained for galactosidase activity (32). The specific luciferase activity driven by the promoter of interest (well 1), divided by the transfection efficiency (well 2), is a measure for the activity per transfected cell (24).

Isolation and Transfection of Primary Rat Hepatocytes—Adult Wistar rats were obtained from the Brookman Institute, B.V., Someren, The Netherlands. Hepatocytes were freshly prepared from 17-day-old fetuses as described (33), except that DNase was omitted to prevent degradation of plasmid DNA during transfection. Cells were transfected as described, and plated in rat tail collagen-coated dishes containing Dulbecco's modified Eagle's medium/F-12 and 10% FCS. Non-adhering cells were removed after 5 h. The cultures were incubated in Dulbecco's modified Eagle's medium/F-12 and 2.5% FCS in the absence or presence of added hormones, and harvested after 20 h. ExoIII Deletions—ExoIII-deletion analysis of the 4-kbp fragment that contains the upstream enhancer (15) (Fig. 5) was performed as described by Brown and Ferguson (34). Under protocols and Applications Guide. Constructs B, C, D, and E are derived from construct A, in which the 4-kbp enhancer fragment is cloned in positive direction upstream of the luciferase reporter gene are shown to the right. The TATA box (black box), GAG box, and Rat-1 fibroblasts were transiently transfected as described (24) with oligonucleotides GGGAAGGAAAGCTT GAGGCTG, CATGAAGGCTTGTGTC- CAATCTGC and GTGACTAAGCTTAAATCAAAATCTC, respectively (all complementary to the sense strand; HindII sites are underlined). The T7 primer of pBluescript was used as complementary primer. All PCR products were sequenced. The putative CRE and GRE element in the 469-bp enhancer fragment (see Fig. 5, construct J) was subsequently introduced into pBluescript SK+ . Primer TTATTTA- GAATCATTAGGAGGATTTA was used to disrupt the CRE and CATACAGAGAATTGTCATGTCAAGCATC to disrupt the putative GRE sites. The resulting constructs were substituted (see Fig. 6). The T3 and T7 primers were used to fill in both sides flanking the mutagenesis primer. The PCR products were sequenced in both directions.

RNA Isolation and Quantification—5 x 106 FTO-2B hepatoma cells were transiently transfected with 30 μg of construct UTR 50, UTR 115, or UTR 138 as described and divided into equal parts. After 48 h the luciferase activity and protein content were determined in the cells of one well, while the cells of the other well were lysed in guanidinium isothiocyanate and loaded onto CsCl cushions for total RNA isolation (36). In addition, the RNA samples were treated with 3 units of RNase-free DNase I (RQ1, Promega) for 1 h at 37 °C in the presence of 56 units of RNasin (Promega), extracted with phenol and chloroform, and precipitated two times in 2 M LiCl. The concentration of the RNA was determined at 260 nm and the integrity by formaldehyde agarose gels. The concentration of luciferase-SV40 mRNA hybrids was determined by reverse transcriptase-PCR (avian myeloblastosis virus-reverse transcriptase, Promega), using the micromRNA construct as internal standard (37). The micromRNA construct contains identical SV40 sequences, but, in addition, a 129-bp unrelated fragment. The primers (SV40+ , CGTTG- GGTGACATTATGG; and SV40-, TACTAAACAGCATGACTCA) recognize sites flanking the intron, leading to amplification of a 263-bp fragment from the mRNA, a 328-bp fragment from the unspliced RNA or from the plasmid DNA, and a 465-bp fragment from the mimic construct. In each reaction 1 μg of total RNA was used. The products of the RT-PCR reaction were separated on 3% Metaphor agarose (FMC).

General Methods—Plasmid DNA isolation, PCR, subcloning, restriction analysis, and Western blot analysis were performed as described.

![FIG. 1. Functional analysis of the CPS promoter.](http://www.jbc.org/)

Transfection assays of CPS promoter deletion constructs in FTO-2B hepatoma cells and Rat-1 fibroblasts. Regions of the CPS promoter that were cloned upstream of the luciferase reporter gene are shown to the left. The TATA box (black box), GAG box, and sites III, II, I of the CPS promoter (19) are indicated. The bottom-most structure, shown in gray, indicates the minimized TK promoter. The TATA box (rectangle) and the GC box of the TK promoter are given. The bars indicate the normalized specific luciferase activity (luciferase activity/CAT activity) relative to the activity obtained from the construct harboring the 161-bp CPS promoter, which was arbitrarily set at 1. The black bars indicate activities of cells grown in the absence of added hormones, while the hatched bars show activities in the presence of dexamethasone, Bt2cAMP, and IBMX ("Experimental Procedures"). Error bars indicate the S.E. of at least three independent transfections.

Deletions in the 5'-UTR of the mRNA were made by creating HindII sites in the 5'-UTR at 50, 89, and 115 nt downstream of the transcription start site of construct 2 (see Fig. 1, construct 2) with oligonucleotides GGGAAGGAAAGCTT GAGGCTG, CATGAAGGCTTGTGTC- CAATCTGC and GTGACTAAGCTTAAATCAAAATCTC, respectively (all complementary to the sense strand; HindII sites are underlined). The T7 primer of pBluescript was used as complementary primer. All PCR products were sequenced. The putative CRE and GRE element in the 469-bp enhancer fragment (see Fig. 5, construct J) was subsequently introduced into pBluescript SK+. Primer TTATTTA- GAATCATTAGGAGGATTTA was used to disrupt the CRE and CATACAGAGAATTGTCATGTCAAGCATC to disrupt the putative GRE sites. The resulting constructs were substituted (see Fig. 6). The T3 and T7 primers were used to fill in both sides flanking the mutagenesis primer. The PCR products were sequenced in both directions.

RNA Isolation and Quantification—5 x 106 FTO-2B hepatoma cells were transiently transfected with 30 μg of construct UTR 50, UTR 115, or UTR 138 as described and divided into equal parts. After 48 h the luciferase activity and protein content were determined in the cells of one well, while the cells of the other well were lysed in guanidinium isothiocyanate and loaded onto CsCl cushions for total RNA isolation (36). In addition, the RNA samples were treated with 3 units of RNase-free DNase I (RQ1, Promega) for 1 h at 37 °C in the presence of 56 units of RNasin (Promega), extracted with phenol and chloroform, and precipitated two times in 2 M LiCl. The concentration of the RNA was determined at 260 nm and the integrity by formaldehyde agarose gels. The concentration of luciferase-SV40 mRNA hybrids was determined by reverse transcriptase-PCR (avian myeloblastosis virus-reverse transcriptase, Promega), using the micromRNA construct as internal standard (37). The micromRNA construct contains identical SV40 sequences, but, in addition, a 129-bp unrelated fragment. The primers (SV40+ , CGTTG- GGTGACATTATGG; and SV40-, TACTAAACAGCATGACTCA) recognize sites flanking the intron, leading to amplification of a 263-bp fragment from the mRNA, a 328-bp fragment from the unspliced RNA or from the plasmid DNA, and a 465-bp fragment from the mimic construct. In each reaction 1 μg of total RNA was used. The products of the RT-PCR reaction were separated on 3% Metaphor agarose (FMC).

General Methods—Plasmid DNA isolation, PCR, subcloning, restriction analysis, and Western blot analysis were performed as described.
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RESULTS

Three regions in the CPS gene appeared to be involved in the regulation of expression, viz. the 161-bp proximal promoter, the 138-bp 5'-UTR of the mRNA, and the 4-kbp far-upstream enhancer fragment (15). These regions were systematically investigated for their contribution to tissue specificity and response to physiologically relevant hormones.

The Proximal Promoter of the CPS Gene—Two approaches were used to investigate whether the 299-bp CPS promoter-containing fragment (from -161 to +138 relative to the transcription start site (15)) confers tissue-specific expression. As a first approach, 5' sequences of the CPS promoter were deleted, and the resulting derivatives were tested in FTO-2B hepatoma cells and Rat-1 fibroblasts (Fig. 1). Elements upstream of the GAG box did not substantially alter reporter gene expression in either FTO-2B or Rat-1 cells. Deletion of the GAG box, however, decreased activity to 30–40%. In both cell lines the CPS promoter appeared to be sensitive to added hormones, possibly due to enhanced expression of general transcription factors. The minimal fully active CPS promoter was 50 times more active in FTO-2B cells than the minimized TK promoter.

Since the activity of the CPS promoter was similar in hepatoma and fibroblast cells (Fig. 1), this sequence probably does not confer tissue specificity. To establish this conclusion more firmly, luciferase activity per transfected cell was determined. This approach (Fig. 2) clearly demonstrated that the activity of the CPS promoter was quantitatively comparable in FTO-2B and Rat-1 cells and, hence, does not confer tissue specificity. The minimized TK promoter, containing only the proximal GC box and a TATA box, was less active in the hepatoma cell lines than in the fibroblasts. The GC box, target of the Sp1 protein and the main determinant of the strength of this promoter (28, 39) may therefore be hardly functional in hepatoma cell lines, in accordance with the relatively low concentrations of Sp1 in hepatocytes (40).

The 5'-Untranslated Region—A promoter fragment lacking the 5'-untranslated region of the mRNA, showed a dramatically lower expression in transfection studies (15). Several pro-

TABLE I

|                  | Lack of tissue specificity of the 5'-UTR |
|------------------|----------------------------------------|
|                  | Comparison of the stimulation of reporter gene expression by the 5'-UTR in FTO-2B and MH1C1 cells. The activities were obtained with the a promoter fragment containing the 138-bp 5'-UTR (wt 5'-UTR, Fig. 1, construct 1) and the promoter lacking the UTR from +50 to +138 (U 50 5'-UTR, Fig. 3, construct U 50). The values are the mean ± S.E. of the normalized specific luciferase activity of three independent transfections. |
|                  | wt 5'-UTR                               | U 50 5'-UTR                           |
| FTO-2B           | 1.00 ± 0.03                             | 0.08 ± 0.01                           |
| MH1C1            | 1.00 ± 0.09                             | 0.44 ± 0.02                           |
| Rat-1            | 1.00 ± 0.03                             | 0.38 ± 0.05                           |

(32). Oligonucleotides were obtained from Eurogentec, Seraing, Belgium. Double-stranded DNA was sequenced using the dideoxy procedure (38).

Fig. 2. Lack of tissue specificity of the CPS promoter. Comparison of the activity of the CPS promoter (left panel) and the minimized TK promoter (right panel) in FTO-2B hepatoma cells and Rat-1 fibroblasts. Transfection efficiency was determined by cotransfecting the expression vector pRSV-n-LacZ ("Experimental Procedures"). Bars indicate normalized luciferase activity (relative light units/mg of protein × fraction of blue cells × 10^10). Error bars indicate the S.E. of at least four independent transfections. Black bars represent the activities of FTO-2B cells, gray bars the activities of Rat-1 cells. *, p (two-tail) = 0.39, **, p (two-tail) = 0.03, using the Student's t test for independent samples.

Fig. 3. Functional analysis of the 5'-UTR. A, deletion analysis of the stimulation of reporter gene expression by the 138-nt 5'-UTR. Constructs harboring parts of the 5'-UTR were transiently transfected in FTO-2B hepatoma cells. The bars indicate the normalized specific luciferase activity ("Experimental Procedures") relative to the activity obtained from the construct containing the full-length 5'-UTR and 100 bp of the CPS promoter (UTR 138 = construct 2, Fig. 1), which is set to 1. Other constructs harbor the same upstream promoter sequences, but lack 5'-UTR regions +115 to +138 (U 115), +89 to +138 (U 89), +50 to +138 (U 50), and +7 to +138 (U 7) relative to the transcription start site. The small ORF (position 97–108) is present in constructs U 115 and U 138, and absent in the other constructs. Black bars indicate the activity of FTO-2B cells incubated in the absence of added hormones, hatched bars in the presence of dexamethasone, Bt2cAMP, and IBMX ("Experimental Procedures"). Error bars indicate the S.E. of at least three independent transfections. B, activity of the 5'-UTR of the CPS mRNA in the context of the minimized TK promoter (−81 to +52). The 5'-UTR (+3 to +138, black box) was cloned into the BglII site (position +52) of the TK promoter (gray) in both directions (arrows). The activity of the TK promoter was set to 1. Black bars indicate the activity in the absence of added hormones, hatched bars in the presence of dexamethasone, Bt2cAMP, and IBMX. Error bars indicate the S.E. of at least three independent transfections.
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**Fig. 4.** Luciferase mRNA levels produced by full-length and truncated 5'-UTR constructs in FTO-2B cells. Constructs U 138, U 115, and U 50 (Fig. 3) were transiently expressed in FTO-2B hepatoma cells and assayed by determination of luciferase-SV40 hybrid mRNA concentration (“Experimental Procedures”). Lanes 1, 4, 7, 13, and 19, U 138; lanes 2, 5, 8, 14, and 20, U 115; lanes 3, 6, 9, 15, and 24, U 50; lanes 11, 12, 17, and 18, rat liver total RNA; lanes 10 and 16, 10 fg of mimic DNA. Lanes 1, 2, and 3, 200 fg of mimic DNA; lanes 4, 5, and 6, 50 fg of mimic DNA; lanes 7, 8, and 9, 10 fg of mimic DNA. Lanes 16–21, without reverse transcriptase (RT).

**Fig. 5.** Delineation of the far-upstream enhancer. Transient-transfection assays of constructs containing sequences derived from an exonuclease III library of the 4-kbp enhancer fragment, denoned upstream of the CPS promoter-containing fragment (construct 1 in Fig. 1). The open box at position A indicates the original 4-kbp enhancer fragment (15). The “RI” indicates a landmark EcoRI site in the fragment, which is positioned 6 kbp upstream (~6 kbp) of the transcription start site in the CPS gene. On the left, size and position of the test sequences which is positioned 6 kbp upstream (469 bp) of the transcription start site in the CPS gene was shown to be transcriptionally active in primary hepatocytes (115). The putative CRE and GRE are shown. The arrow indicates the position of the most 5' nt of fragment E of Fig. 5. Sites with similarity to binding sites of liver-enriched factors HNF3 (TGACGTCA, 148–155) and the three GRE half-sites (TGTTCT, 382–394) are double underlined. On top of these sites the substitutional mutations to inactivate the putative CRE and GRE are shown. Sites with similarity to binding sites of liver-enriched factors HNF3 (CattAjgTCAAT), HNF4 (GGGCCANNNa/ga/gGTCCA), and HNF5 (TwgTTTCGcA) are given in italics.

| Fragment | Relative luciferase activity |
|----------|-----------------------------|
| RI (-6 kbp) | 0 | 10 | 20 | 4 |
| A | ND | ND | ND | ND |
| B | ND | ND | ND | ND |
| C | ND | ND | ND | ND |
| D | ND | ND | ND | ND |
| E | ND | ND | ND | ND |
| F | ND | ND | ND | ND |
| G | ND | ND | ND | ND |
| H | ND | ND | ND | ND |
| I | ND | ND | ND | ND |

**Fig. 6.** Nucleotide sequence of fragment I (Fig. 5), containing the 469-bp enhancer fragment (fragment J). The landmark EcoRI site depicted in Fig. 5 and the differentially methylated MspI/HpaI site (CCCG) at −6.3 kbp (Fig. 9) are underlined. The imperfect CRE (TGACGTCA, 148–155) and the three GRE half-sites (TGTTCT, 382–401) are double underlined. On top of these sites the substitutional mutations to inactivate the putative CRE and GRE are shown. Sites with similarity to binding sites of liver-enriched factors HNF3 (CattAjgTCAAT), HNF4 (GGGCCANNNa/ga/gGTCCA), and HNF5 (TwgTTTCGcA) are given in italics.
activity in the presence of dexamethasone, Bt2cAMP, and IBMX. The activities indicate the normalized specific luciferase activity relative to the activity obtained from the minimized TK promoter and are corrected for the effect of hormonal stimulation on the TK promoter without enhancer. Bars indicate the S.E. of four independent transfections.

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Fig. 7. Tissue specificity of the far-upstream enhancer. Transient-transfection assays of the construct containing the 469-bp enhancer fragment coupled to the minimized TK promoter in FTO-2B hepatoma cells and Rat-1 fibroblasts. Black bars represent the activity of cells incubated in the absence of added hormones, gray bars the activity in the presence of dexamethasone, and hatched bars the activity in the presence of dexamethasone, Bt2cAMP, and IBMX. The activities indicate the normalized specific luciferase activity relative to the activity obtained from the minimized TK promoter and are corrected for the effect of hormonal stimulation on the TK promoter without enhancer. Bars indicate the S.E. of four independent transfections.

Fig. 8. Relation between expression of the endogenous CPS gene and the far-upstream enhancer activity. Panel A, transient transfection of the 469-bp far-upstream element (fragment J in Fig. 5) in combination with the CPS promoter (fragment 1 in Fig. 1) in Chinese hamster ovary cells, Rat-1 fibroblasts, and FTO-2B- and MH1C1-hepatoma cells. The bars indicate the normalized luciferase activity relative to the activity obtained from the basal CPS promoter without enhancer. The activities are corrected for the effect of added hormones on the basal CPS promoter without enhancer. Black bars indicate the activity of cells cultured without added hormones, hatched bars the activity of cells to which dexamethasone, Bt2cAMP, and IBMX were added. Error bars indicate the S.E. of at least three independent transfections. Panel B, Western blot analysis of CPS protein content in the respective cell lines in the absence (−) and the presence of dexamethasone, Bt2cAMP, and IBMX (+). In each lane 50 μg of total protein was applied.

the combination of dexamethasone and Bt2cAMP was found to be delimited by the 5′ and 3′ borders of constructs D and F, respectively (Fig. 5). This region alone (construct H in Fig. 5) was as active as either construct D or F. Further deletions confined the enhancer activity to a 469-bp fragment (construct J in Fig. 5). The differentially methylated CCGG (15) site is located within this fragment. A number of sites resembling known liver-enriched factor binding-sites (HNF3, -4, and -5) and hormone-responsive elements (CRE and GRE) were identified (Fig. 6).

Tissue Specificity of the Far-upstream Enhancer—The same enhancer fragments that caused hormonal induction of reporter gene expression in FTO-2B hepatomas, also stimulated hormone-dependent expression in Rat-1 fibroblasts (Fig. 5), but 6-fold less. This cell-specific difference in activation properties of the enhancer became more pronounced when the 469-bp enhancer fragment was tested in combination with the minimized TK promoter (Fig. 7). The expression obtained with this fragment in FTO-2B cells was 340-fold induced, compared to the TK promoter itself, while only a 12-fold induction was seen in Rat-1 cells, i.e. an almost 30-fold difference. These results show that the tissue specificity conferred by the enhancer is independent of the promoter. The strength of the 470-bp enhancer fragment was also tested in two hepatoma cell lines

Fig. 9. Enhancer analysis in enterocytes. Transient-transfection assays of constructs containing regions upstream of the transcription start site of the CPS gene in Caco-2 cells. CPS-A and TK-A represent constructs in which a 4-kbp fragment (fragment A in Fig. 5) was cloned upstream of the CPS promoter (fragment 1 in Fig. 1) or the minimized TK promoter (−81 to +52), respectively. CPS-B and TK-B represent constructs in which a 4-kbp fragment containing the differentially methylated site at position −4.0 kbp (15) was cloned upstream of the 161-bp CPS promoter or the minimized TK promoter. Bars indicate the normalized CAT activity (Panel A) or luciferase activity (Panel B). Black bars show the activities of cells grown in the absence of added hormones and hatched bars the activities of cells grown in the presence of dexamethasone, Bt2cAMP, and IBMX. The values were normalized either to the activity of construct CPS-A in the absence of added hormones (Panel A) or to the activity of construct TK-A in the absence of added hormones (Panel B). Values are the mean of two independent transfections, error bars indicating the variance.
(FTO-2B and MH1C1) and two fibroblast lines (Rat-1 and CHO-K1) and compared to the endogenous CPS expression in these cell lines. A clear relation between the strength of the enhancer (Fig. 8A) and the level of expression of CPS protein in the different cell lines (Fig. 8B) was observed. The difference in the fold stimulation of the luciferase reporter gene and the endogenous CPS gene probably results from the difference in stability of both gene products.

To verify the validity of the use of hepatoma cells as a model for assessing tissue-specific expression, the enhancer was also tested in freshly isolated hepatocytes. In these cells the CPS promoter was stimulated 4.6–5.0-fold (n = 2) by dexamethasone, Bt2cAMP, and IBMX. When combined with the 469-bp enhancer, the expression was reduced to 27 ± 3% (n = 5) of that of the promoter alone in the absence of added hormones. In the presence of hormones, however, the expression was stimulated 63–79-fold (n = 2) compared to the promoter, the hormonal induction being more than 250-fold.

**Expression in Intestinal Cells**—The enhancer element at −6.3 kbp is selectively demethylated in CPS-expressing cells. To test whether sequences surrounding the site at −4.0 kbp which is partially demethylated in the small intestine only (15), have enhancer properties in intestinal cell lines, they were tested in Caco-2 cells (Fig. 9). Only the −6.3 kbp site-containing fragment conferred enhanced expression in the presence of dexamethasone, Bt2cAMP, and IBMX, both in combination with the CPS promoter and with the minimized viral TK promoter fragment. Combined with the data from other cell lines, we conclude that only the fragment harboring the MspI site at −6.3 kbp has the capacity to enhance CPS gene expression.

**Interaction between the Enhancer and the Promoter**—In the presence of added hormones, the enhancer stimulates expression via the proximal promoter. To investigate which elements of the promoter mediate this bridging function, a series of CPS promoter deletions was tested in combination with the 1003-bp enhancer fragment (fragment H in Fig. 5) in the FTO-2B hepatoma cell line. Fig. 10 shows that both the TATA box and the GAG box were needed for an optimal transactivation by enhancer elements. When the GAG box was deleted, transactivation decreased to 50% of that of the fully active promoter. Neither sites I-III nor the 5′-UTR are involved in the interaction between the promoter and the enhancer fragment. These effects became even more pronounced when the 469-bp fragment (fragment J in Fig. 5) was used. When tested in combination with the TK promoter (Fig. 10), the hormonal stimulation decreased to 25% of that of the fully active CPS promoter. Obviously, the GC box is not able to functionally replace the GAG box.

When the distance between the enhancer and the minimized TK promoter was decreased, luciferase expression levels increased strongly (Fig. 11). Such distance effects were virtually absent in combination with the CPS promoter (Figs. 5 and 11).
Whether or not sites upstream of the TATA box of the CPS promoter are functioning as coupling elements between the enhancer and the promoter was tested. A promoter fragment in which all sites upstream of the TATA box were deleted, leaving only the TATA box and 5′-UTR (Fig. 1, construct 4) was coupled to either the 1003-bp enhancer fragment (H in Fig. 5) or the 469-bp fragment (I in Fig. 5) and transfected to FTO-2B cells (Fig. 13). The truncated promoter was more sensitive to increasing distance than the CPS promoter, but not as sensitive as the TK promoter. These results underline the role of the GAG box.

Mutational Analysis of cis-Elements in the Enhancer—Expression of the CPS gene in vivo is regulated at the transcriptional level by both glucocorticosteroids and cyclic AMP (8, 12). The activity of the upstream enhancer depends entirely on the presence of these hormones and was therefore investigated for the presence of hormone-responsive elements. The 469-bp enhancer contains sequences that qualify as a potential cAMP-responsive element (CRE, Fig. 6), the binding site for dimers of the CREB family, and a glucocorticosteroid-responsive element (GRE, Fig. 6). When 4 nt of the CRE were substituted (CREmut, Fig. 6), basal expression decreased 60% (Fig. 12A). By binding to the regulatory subunit of protein kinase A, cyclic AMP releases the active catalytic subunit (Ca) (41). Expression vectors for Ca, CREB (51, 52), and CBP (53, 54) were cotransfected with the wild-type enhancer or the CRE mutant. These additions resulted in a 2-fold increase of the expression signal of the wild-type enhancer, but the CRE mutant no longer conferred this effect (Fig. 12A). To functionally test the existence of a GRE, one nucleotide that has been shown to be essential for receptor binding (42) was substituted in each of the three putative GRE half-sites (Fig. 6). The activity of the mutated enhancer (GREmut) was decreased to 20% of that of the wild-type enhancer when tested in combination with an expression vector encoding the GR in the presence of dexamethasone (Fig. 12B). Interestingly, the fold-induction by dexamethasone of the CRE mutant was not affected.

**DISCUSSION**

The aim of this study was the functional characterization of the regulatory regions of the CPS gene with respect to their role in tissue-specific expression and hormone sensitivity. Three regions were functionally analyzed. The CPS promoter, comprising a TATA box, a GAG box, and elements I-III, associated with the binding of presently unknown proteins (19), the 138-bp 5′-untranslated region of the mRNA, and the far-upstream enhancer at ~6.3 kbp.

Our data show that, in vitro, the CPS promoter has comparable strength in hepatoma cells, which do express CPS, and in fibroblasts, which do not express CPS, demonstrating that it is not involved in tissue-specific regulation of transcription.
thermore, our findings clearly show that sites I, II, and III (−150 to −79 nt) are not essential for basal promoter activity or translocation of the hormonally induced activation signal from the enhancer. The GAG box, however, is quantitatively important because its absence reduces both the promoter activity and the effect of the enhancer on the promoter 2–3-fold. These data suggest that the protein(s) binding to the GAG box are involved in transducing the activation signal from the enhancer to the promoter as well as in the functioning of the promoter itself.

A promoter fragment lacking the 5′-UTR of CPS mRNA (+7 to +138) is hardly active in FTO-2B cells (Ref. 15 and Fig. 3). Such a finding can be due to an incorrect mapping of the transcription start site, or to the involvement of downstream sequences in the basal transcription complex (15). These possibilities can now be virtually ruled out because addition of up to 115 nt of the 5′-UTR downstream of the transcription start site does not restore expression levels to those obtained with the full-length 5′-UTR (Fig. 3A). When combined with the minimized TK promoter (Fig. 3B), the full-length 5′-leader of CPS mRNA inhibits, rather than stimulates expression. This result makes the presence of cis-elements in the 5′-UTR DNA, which, in combination with a promoter, are able to stimulate transcription, rather unlikely. Quantification of RNA levels (Fig. 4) shows that the 5′-UTR acts at the translational level. The 5′-UTR contains a small upstream open reading frame (uORF) of four codons (Met-Arg-Tyr-Leu) starting with the initiation codon at position 97 (position relative to the transcription start site) in a suboptimal context compared to the more downstream initiation codon of the CPS reading frame (50 and 70% similarity to the consensus sequence (43), respectively), followed by three stop codons. uORFs are thought to be able to suppress translation of downstream cistrons (44–46). Strikingly, other eukaryotic CPS genes of which the 5′-UTR sequences are known, the human CPS I gene (47), the shark CPS III gene (48), and the yeast CPA1 gene (46), also contain one or more uORFs. The uORF of the CPA1 gene is known to suppress translation in a regulated manner. Constructs U 115 and U 138 (Fig. 3) both contain the uORF, but differ markedly in luciferase activity. Deletion of the 23 nt between the uORF and the luciferase start codon affects inter-cistronic length, the sequence context at the uORF stop codon, and secondary structure, parameters which were found to be important for translational control (49).

Scanning sequences up to 12 kb upstream and 4 kb downstream of the promoter fragment, only one fragment located at 6 kb from the transcription start site could be found that had the capacity to stimulate reporter gene expression in FTO-2B cells and Rat-1 fibroblasts (15). This enhancer element has now been confined to a 469-bp fragment (Fig. 5). Transgenic mice, harboring the CPS promoter and 12-kb upstream DNA in combination with the CAT reporter gene, give rise to hepatocyte-specific expression of CAT mRNA, which co-localizes with the endogenous CPS mRNA in the liver. Mice harboring only the proximal promoter show extremely weak CAT activity, which is not tissue-specific. Combination of in vivo and in vitro results suggests that the proximal promoter and the 469-bp far-upstream enhancer are both necessary and sufficient for tissue-specific CPS expression.

Sites homologous to an imperfect CRE, with the structural characteristics of a so-called "low affinity site" (50) and a GRE were found to be essential elements in the minimal enhancer fragment (Fig. 12); in the absence of added hormones, the enhancer was inactive, while mutations of the CRE and GRE significantly decreased hormone-dependent enhancer activity.

Expression of the construct carrying the CRE mutation was still responsive to glucocorticoids (Fig. 3B), indicating that the CRE and GRE are not functionally linked. On the other hand, when sequences upstream of position 339, including the CRE (Fig. 6) are deleted (construct E of Fig. 5), the enhancer loses all activity. The deleted area contains nearly perfect consensus sequences for HNF3 and, to a lesser extent, for HNF4.

In our transfection analysis the hormonal stimulation by the CPS enhancer was found to be independent of the distance between the CPS promoter and enhancer, whereas this distance was important when combining TK promoter and enhancer (Fig. 11). This promoter-specific effect is probably highly relevant in vivo, because the CPS promoter and the enhancer are approximately 6-kbp apart. One way to explain the differences is to hypothesize that transcriptional activity is related to the probability of the enhancer to contact the promoter. The curves, proportional to (bp), represent this probability and exponent q the slope in a double logarithmic plot (Fig. 11). Mathematically, when the promoter and the enhancer are randomly distributed in space, q = −1.5 (the Gaussian limit (55)). q = 0 (CPS promoter) means that the probability is −1, i.e. that the promoter and the enhancer are connected independently of distance. q = −1 (truncated CPS promoter) approaches a random distribution and q = −3.5 (TK promoter) indicates hindrance of enhancer-promoter interaction. The main difference between the CPS and the truncated CPS promoter is the GAG box (see also Fig. 10), indicating that this motif is instrumental in conferring the hormonal activation of the enhancer to the transcriptional complex. The main difference between the minimized TK promoter and the truncated CPS promoter is the GC box. This GC box might be the actual cause of the distance dependence of the minimized TK promoter in conjunction with the CPS enhancer, possibly by preventing interaction of enhancer and promoter through steric hindrance.

In summary, our data support a model in which the tissue-specific expression of CPS is determined by the far-upstream enhancer. The activity of this enhancer is strictly dependent on the presence of glucocorticoids and cyclic AMP. The activated enhancer complex will stimulate transcription through interaction with factors bound to the GAG box and the TATA region.

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