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Genetics

Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements

(cAMP/glucocorticoid/hepatocyte nuclear factors 3 and 4/signal transduction)

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ABSTRACT **Tyrosine aminotransferase (TAT; L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) gene activity is stimulated by glucocorticoids and glucagon and is repressed by insulin. Expression and responsiveness to the different signal transduction pathways are restricted to the liver, in which the gene is activated shortly after birth. Here we provide a model for the basis of this tissue specificity of the hormonal control. In the two enhancers mediating hormone induction of TAT gene activity we find the hormone response elements in combination with binding sites for constitutive liver-enriched transcription factors: proteins of the hepatocyte nuclear factor 3 family bind in the vicinity of the glucocorticoid response element located 2.5 kb upstream of the transcription start site, while hepatocyte nuclear factor 4 interacts with an essential element in the cAMP-responsive enhancer at −3.6 kb. By juxtaposing the liver-specific element and the target sequence of the signal transduction pathway the regulatory properties of either enhancer can be reconstituted. Thus, the interdependence of the respective enhancer motifs restricts the hormonal activation of the TAT gene to the liver. The coincidence of the onset of TAT gene expression around birth with the perinatal changes in the concentrations of glucocorticoids, glucagon, and insulin suggests cooperation of signal transduction pathways and cell type-specific transcription factors in the developmental activation of the TAT gene.**

Gene activity is regulated in response to temporal, spatial, and environmental cues. In the last 10 years many sequence motifs have been discovered which are involved in determining tissue-specific and developmentally regulated gene expression. These cis-regulatory elements interact with trans-acting factors which influence the rate of transcription and which often exhibit correspondingly restricted expression patterns (1, 2). Similarly, changes in gene expression in response to external signals such as growth factors and hormones are mediated by altering the function of specific transcription factors. Two major types of signal transduction pathways have been elucidated: (*i*) signaling molecules such as steroid hormones influence gene activity by inducing DNA binding of their intracellular receptors (3, 4); (*ii*) binding of many growth factors or peptide hormones to specific cell-surface receptors elicits a cascade of events mediated by second messengers, which affect the activity of protein kinases and phosphatases, as well as Ca^{2+} levels. These changes finally result in the modification of specific transcription factors, thereby changing their activities (5).

While the components of the signal transduction pathways are mostly of ubiquitous nature, hormonal stimulation of a particular gene is often restricted to certain cell types. We have chosen the tyrosine aminotransferase (TAT) gene as a model system to study the basis of this cell type-specificity of hormone induction and its role in developmental gene activation. The TAT gene is transcriptionally responsive to members of both classes of signaling pathways. It is induced by glucocorticoids and glucagon, acting via its intracellular mediator cAMP (6–8), and is repressed by insulin (9). The hormone-regulated expression is restricted to the parenchymal cells of the liver, in which the gene is activated shortly after birth (10, 11). We show here that in the two enhancers at −2.5 and −3.6 kb (relative to the transcription start site) which respond to glucocorticoids and glucagon, respectively (12, 13), the hormone response elements synergize with binding sites for the liver-enriched transcription factors hepatocyte nuclear factor 3 (HNF3; refs. 14 and 15) and HNF4 (16). Therefore, the interdependence of the hormone response elements and cell type-specific enhancer motifs seems to be the basis of the strictly liver-specific hormonal inducibility of TAT gene expression and may provide the switch for the developmentally timed activation of the gene.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. In plasmid 3.0TK the *Sal* I–*Kpn* I fragment from pTATCAT (12) was cloned into the polylinker of pBLCAT2 (17), generating a construct containing TAT 5′ flanking sequences from −2950 to −351 bp in front of the herpes simplex virus thymidine kinase (TK) promoter. Constructs −2.5HSTK and 2×GRETK are described elsewhere (18). The constructs containing one or two copies of the TAT glucocorticoid response element (GRE) in front of the TAT promoter have been described previously as GRE-351TAT and 2GRE-351TAT (19). In all other constructs depicted in Fig. 4*A* the following oligonucleotides were cloned in the sense orientation into the *Bam*HI site of GRE-351TAT: HNF3(TAT-2.5), 5′-GATCTACGCAGGACTTGTTTGTTCTAGTCG-3′; HNF3(TAT-11), 5′-GATCCGACGTTTCTCAATATTTGCTCTGGCAGATA-3′; −11mut, 5′-GATCCGACGTTTATACATATTTTATCTGGCAGATA-3′; HNF4, 5′-GATCTGCTGCTCTTTGATCTG-3′; HNF4mut, 5′-GATCTGCTGCGAGGGGATCTG-3′ (the mutated base pairs are underlined). The cAMP response element (CRE)-HNF4 and CRE-HNF4mut constructs (Fig. 4*B*) are described as plasmids BI/BIII and BI/BIII(M12) in ref. 13. The CRE-HNF3 heterodimers were constructed correspondingly, inserting the HNF3 oligonucleotides (see above) instead of the HNF4 (BIII) motif. All constructs containing oligonucleotides were verified by plasmid sequencing (20).

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Abbreviations: CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; GRE, glucocorticoid response element; HNF, hepatocyte nuclear factor; TAT, tyrosine aminotransferase; TK, thymidine kinase.

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Cell Culture and Transient Transfections. All experiments were performed with FTO2B hepatoma and XC fibrosarcoma ("fibroblast") rat cell lines. Cell culture, transfection experiments, and chloramphenicol acetyltransferase (CAT) assays were performed as described (13). Cells were harvested 48 hr after electroporation and hormone additions.

Gel Mobility-Shift Assays. Mobility-shift experiments were performed as described (18). Rat liver nuclear extract was prepared and partially purified by DEAE-Sepharose chromatography (21). The antisera directed against HNF3α, -β, and -γ were generously provided by V. Prezioso and J. Darnell (15). HNF4-specific antiserum was kindly provided by F. Sladek and J. Darnell (16).

RESULTS

Members of the HNF3 Family of Liver-Enriched Transcription Factors Bind to the Glucocorticoid-Inducible Enhancer of the TAT Gene. The TAT gene is induced by glucocorticoids exclusively in the liver, and this cell-type specificity of the hormone response can be reproduced in transient-transfection experiments (Fig. 1). Constructs containing TAT 5′ flanking sequences, including the glucocorticoid-inducible enhancer at −2.5 kb (12), in front of the promoter of the herpes simplex virus TK gene, are induced >50-fold in hepatoma cells by the addition of the synthetic glucocorticoid dexamethasone. Whereas two copies of the GRE from the TAT enhancer (2×GRETK) stimulate the TK promoter in both hepatoma and fibroblast cells, the complete enhancer either contained on a 250-bp fragment (−2.5HSTK) or in its natural position at −2.5 kb (3.0TK) strongly responds to hormone only in the hepatoma cell line. Thus, it appears that sequences located within the glucocorticoid-inducible enhancer restrict its activity to the liver.

We noted a sequence motif ≈60 bp downstream of the GRE with striking homology to an important element contained in the constitutive liver-specific TAT enhancer located at −11 kb (Fig. 2*D*; ref. 22), which has recently been found to bind liver-enriched transcription factors of the HNF3 family (D.N. and G.S., unpublished work). To test whether the sequence in the glucocorticoid-inducible enhancer also interacts with HNF3 proteins, we performed gel mobility-shift analyses with an oligonucleotide containing the respective sequence motif (TAT-2.5; Fig. 2). With extracts prepared from either hepatoma or fibroblast cells several prominent protein–DNA complexes were observed, only one of which was common to both extracts (Fig. 2*A*). To define the specificity of the protein–DNA complexes we performed competition experiments with different oligonucleotides, which showed that the sequence derived from the TAT enhancer at −2.5 kb contained an HNF3 binding site (Fig. 2*B*): In the presence of the HNF3 motif from the transthyretin gene (HNF3; ref. 23) or from the TAT enhancer at −11 kb (TAT-11), the hepatoma cell-specific protein–DNA complexes are not formed,

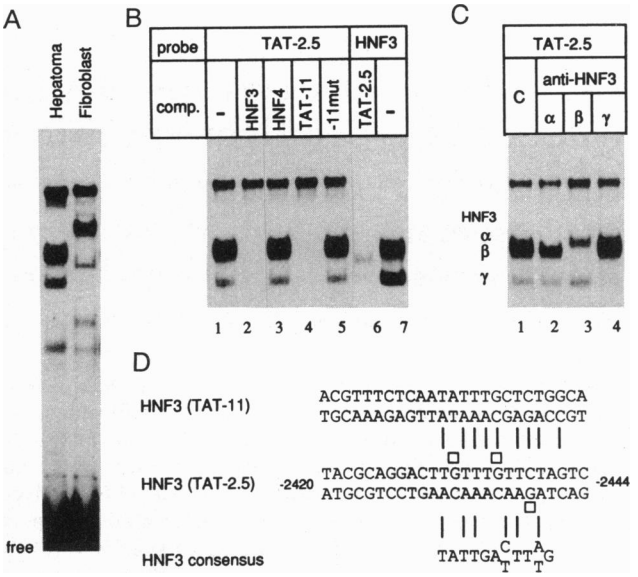
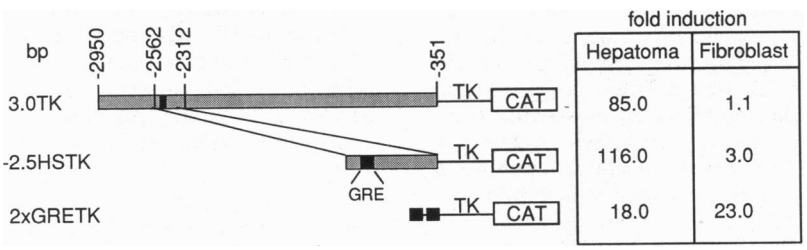


FIG. 2. HNF3α, -β, and -γ bind to the glucocorticoid-inducible enhancer at −2.5 kb. (*A*) Comparison of protein–DNA complex formation with nuclear extracts prepared from hepatoma or fibroblast cells. Position of unbound oligonucleotide probe containing the HNF3 site at −2.5 kb (TAT-2.5) is indicated (free). (*B*) Labeled oligonucleotides containing either the TAT-2.5 sequence (lanes 1–5) or an HNF3 binding site from the transthyretin gene (ref. 23; lanes 6 and 7) were incubated with hepatoma cell extracts in the absence (−) or presence of 100-fold molar excess of the indicated unlabeled oligonucleotide competitor (comp.). The HNF4 binding site is also derived from the transthyretin gene (23). (*C*) Hepatoma cell extracts were incubated with labeled HNF3 oligonucleotide TAT-2.5 in the presence of preimmune serum [control (c) lane 1] or antiserum directed against HNF3α (lane 2), -β (lane 3), or -γ (lane 4). (*D*) Comparison of the sequence derived from the TAT enhancer at −2.5 kb [HNF3 (TAT-2.5)] with the HNF3 consensus binding site (23) and the HNF3 motif from the TAT enhancer at −11 kb [HNF3 (TAT-11)]. Homologous positions are indicated by vertical lines. Squares above and below the sequence illustrate the HNF3-specific DNA contacts as determined by methylation interference analyses (data not shown).

whereas complex formation is not influenced by excess of an HNF4 binding site (Fig. 2*B*, lanes 1–4). Consistent with these results, the TAT-2.5 oligonucleotide competed for formation of the complexes obtained with the HNF3 motif from the transthyretin gene (Fig. 2*B*, lanes 6 and 7). Further, with the help of specific antisera (kindly provided by V. Prezioso and J. Darnell), we demonstrated that HNF3α, -β, and -γ were contained in the hepatoma cell-specific protein–DNA complexes obtained with the sequence from the TAT enhancer at −2.5 kb (Fig. 2*C*).

Thus, an HNF3 binding site is located in the vicinity of the GRE in the glucocorticoid-inducible TAT enhancer and it



	fold induction	
	Hepatoma	Fibroblast
3.0TK	85.0	1.1
-2.5HSTK	116.0	3.0
2xGRETK	18.0	23.0

FIG. 1. The glucocorticoid-inducible enhancer of the TAT gene is active in hepatoma cells but not in fibroblasts. Reporter constructs containing different parts of the TAT gene 5′ flanking sequence cloned in front of the herpes simplex TK promoter driving the CAT gene were transiently transfected into hepatoma and fibroblast cells. TAT sequences are depicted as shaded bars with the GRE (−2509 to −2495 bp; ref. 12) indicated by a black square. The table on the right gives the values for the fold induction in response to 0.3 μM dexamethasone from a representative transfection experiment.

interacts with the three liver-enriched proteins HNF3α, -β, and -γ. The identity of the other proteins binding to this sequence is unclear. While the low-mobility shift formed with extracts from both hepatoma and fibroblast cells could not be blocked by oligonucleotides containing either an HNF3, an HNF4, or a mutated HNF3 motif (Fig. 2*B*, lanes 2–5), formation of the fibroblast-specific protein–DNA complex (Fig. 2*A*) was abolished by addition of a 40-fold molar excess of the HNF3 oligonucleotide and, moreover, showed a methylation interference pattern which was indistinguishable from that of the HNF3-containing complexes (data not shown). This may suggest that the latter protein–DNA complex contains a member of the forkhead/HNF3 gene family (refs. 14, 15, 24, and 25; K. Kästner and G.S., unpublished results).

HNF4 Binds to an Essential Motif in the cAMP-Responsive Enhancer of the TAT Gene. The TAT enhancer at −3.6 kb is composed of two essential elements, a CRE and a cell type-specific motif, which synergize to confer liver-specific and cAMP-inducible activity (13). The CRE is recognized by CREB and possibly other members of the CREB/ATF family (21). To identify proteins interacting with the liver-specific component of the enhancer, we performed gel mobility-shift experiments with an oligonucleotide containing the cell type-specific enhancer motif (BIII in ref. 13). Fig. 3*A* shows a comparison of protein–DNA complexes obtained with extracts prepared from hepatoma and fibroblast cells. Several shifted bands were formed with the hepatoma cell extract, some of which were absent in the fibroblast lane, including

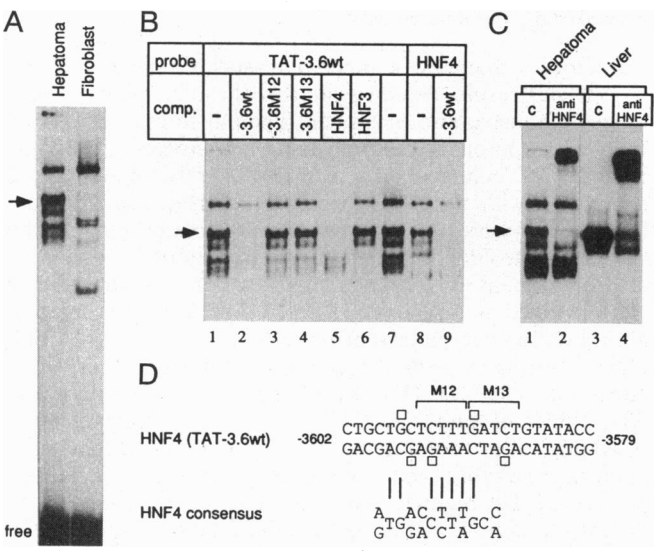


FIG. 3. The HNF4 protein binds to the cAMP-responsive enhancer at −3.6 kb. (*A*) Comparison of protein–DNA complex formation with nuclear extracts prepared from hepatoma or fibroblast cells. Position of unbound oligonucleotide probe containing the wild-type HNF4 binding site found at −3.6 kb (TAT-3.6wt) is indicated (free). Arrow indicates the HNF4-specific protein–DNA complex. (*B*) Labeled oligonucleotides containing either the TAT-3.6wt sequence (lanes 1–7) or the HNF4 binding site from the transthyretin gene (lanes 8 and 9) were incubated with hepatoma cell extracts in the absence (−) or presence of 100-fold molar excess of the indicated unlabeled oligonucleotide competitor (comp.). (*C*) Hepatoma cell extracts (lanes 1 and 2) or partially purified liver extracts (lanes 3 and 4) were incubated with labeled oligonucleotide TAT-3.6wt in the presence of 3% bovine serum albumin [control (c), lanes 1 and 3] or antiserum directed against HNF4 (lanes 2 and 4). (*D*) Comparison of the sequence derived from the TAT enhancer at −3.6 kb [HNF4 (TAT-3.6wt)], with the HNF4 consensus binding site (26). Homologous positions are indicated by vertical lines. The base pairs mutated in oligonucleotides −3.6M12 and −3.6M13 are bracketed (see ref. 13). The squares above and below the sequence illustrate the DNA contacts of the HNF4 protein derived from liver as determined by methylation interference analysis (data not shown).

the most prominent complex (arrow). Transient-transfection experiments with wild-type and mutated enhancer sequences identified a 10-bp motif in which clusters of five point mutations abolished enhancer activity (M12 and M13 in ref. 13). Competition experiments with oligonucleotides containing the wild-type or mutated sequences revealed that loss of enhancer function correlated with the inability to form the hepatoma cell-specific protein–DNA complexes (Fig. 3*B*, lanes 1–4).

Partially purified liver nuclear extracts gave rise to one major protein–DNA complex, which corresponded in mobility and sequence specificity to the most prominent DNA-binding activity observed with hepatoma cell extracts (Fig. 3*C*). Reciprocal oligonucleotide competition (Fig. 3*B*, lanes 5 and 9) and immunoshift experiments with HNF4-specific antiserum (a kind gift from F. Sladek and J. Darnell; Fig. 3*C*) clearly showed that this protein–DNA complex contained HNF4. Comparison of the competition experiments and the immunoshift analyses revealed that only one of the specific complexes was formed by HNF4 (arrow). Formation of the weak protein–DNA complexes of higher mobility was affected by an excess of the HNF3 motif (Fig. 3*B*, lane 6). This observation may be related to the fact that the HNF4 and HNF3 motifs from the TAT enhancers show a high degree of sequence similarity, with identity in 8 of 10 positions (TNTT-TGNTCT; compare Figs. 2*D* and 3*D*). However, only with a 400-fold molar excess of the respective oligonucleotides did we observe weak cross competition (data not shown).

A comparison of the sequence from the TAT enhancer at −3.6 kb and the published HNF4 consensus binding site (26) reveals homology in 7 of 12 positions (Fig. 3*D*). The DNA contacts determined by methylation interference analysis (open squares in Fig. 3*D*) are centered in the region of homology and lie within a mutation-sensitive domain of the enhancer as determined in transient-transfection experiments (13). The phenotypes of the neighboring clusters of point mutations also fit to the HNF4-specific protein–DNA contacts: the 5′-adjacent mutant, containing mutations at two guanines whose methylation interferes with HNF4 binding, has only ≈50% of wild-type activity; the 3′-adjacent mutant shows wild-type activity and its base-pair exchanges lie outside the region of protein–DNA contacts (M11 and M14 in ref. 13). Thus, the experiments depicted in Fig. 3 document that the essential hepatoma cell-specific motif of the cAMP-responsive TAT enhancer interacts with the transcription factor HNF4.

Binding Sites for Liver-Specific Transcription Factors Synergize with the Hormone Response Element. Having identified binding sites for the liver-enriched transcription factors HNF3 and HNF4 near the GRE and CRE, respectively, we wanted to test whether the characteristics of each enhancer could be reconstituted by combining the respective enhancer motifs. Previously, a single GRE has been shown to function proximally but not distally to the transcription start site (19). We therefore cloned the GRE and the HNF3 binding site in the promoter-distal position at −351 bp with respect to the TAT transcription start site. In addition we tested the HNF3 motif derived from the enhancer at −11 kb in an analogous construct. As a positive control, a construct containing a tandem repeat of the GRE was tested, which has been shown to confer inducibility by glucocorticoid in several cell lines (19). The constructs were transfected into rat hepatoma and fibroblast cells and their activities were measured in the presence and absence of glucocorticoid hormone (Fig. 4). The construct containing two copies of the GRE showed a strong hormone-dependent activity in hepatoma and fibroblast cells, as expected; in contrast, both GRE–HNF3 combinations were inducible only in the hepatoma cell line. As a control and to rule out any effect of spacing or sequence environment, we also tested a construct in which the HNF3

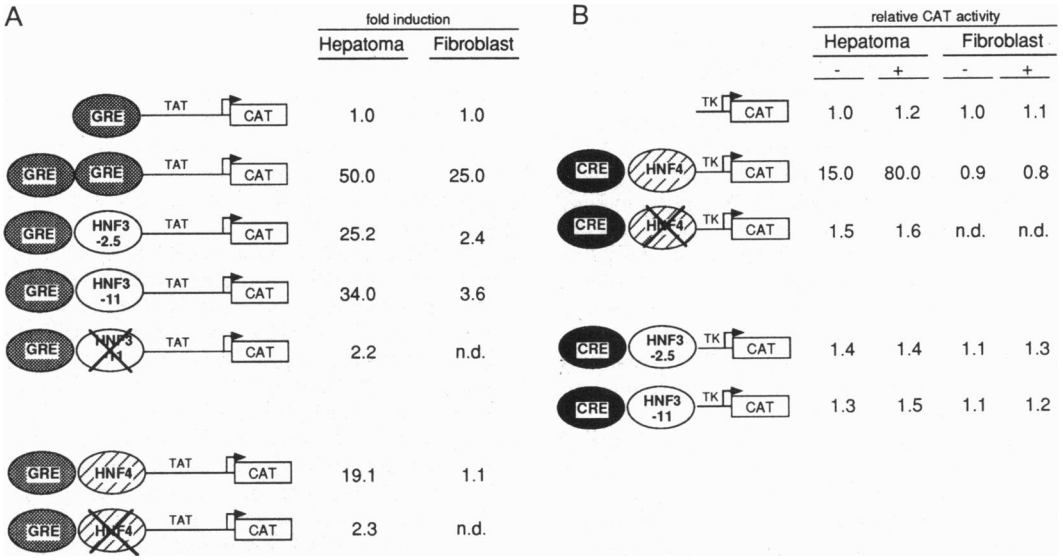


FIG. 4. Synergism between hormone response elements and binding sites for liver-specific transcription factors. Oligonucleotides with the indicated enhancer motifs (for sequences, see *Experimental Procedures*) were cloned at −351 bp of the TAT promoter (*A*) or −105 bp of the herpes simplex virus TK promoter (*B*), both driving the CAT gene. Constructs were transiently transfected into hepatoma or fibroblast cells, and the cells were split onto two plates and either mock-induced (solvent control) or induced with 0.3 μM dexamethasone (*A*) or 1 μM forskolin (*B*). The constructs are depicted with the different transcription factor binding sites represented by hatched and shaded symbols. Crossed-out symbols represent binding sites inactivated by mutation M12 (see ref. 13). The tables give the fold induction by glucocorticoid (*A*) or the relative CAT activities in the absence (−) or presence (+) of forskolin, with the activity of the TK promoter construct set to 1 (*B*). All GRE-containing constructs (*A*) show only vector-background activity in the absence of hormone. Each construct was tested at least three times in hepatoma cells and twice in fibroblasts. Results of representative experiments are depicted. n.d., Not determined.

motif was mutated. Mutation of five nucleotides which abolished protein–DNA binding *in vitro* (Fig. 2*B*, lane 5) impaired the hormone-dependent stimulation of the TAT promoter, indicating that the HNF3 motif synergizes with the GRE. Thus, the regulatory properties of the TAT enhancer at −2.5 kb can be reconstituted by combining its GRE and HNF3 binding site. Likewise, the liver-specific and cAMP-responsive activity of the TAT enhancer at −3.6 kb can be reconstituted by juxtaposing the HNF4 motif and the CRE in front of the heterologous TK promoter (Fig. 4*B*). Mutations in the HNF4 binding site which affect DNA binding (Fig. 3*B*, lane 3) reduce basal as well as cAMP-induced activities of the corresponding construct.

To see whether the synergism between the cell type-specific and the hormone response elements was specific to the combinations found in the two TAT enhancers, we constructed reporter plasmids in which the binding sites for the two liver-enriched factors were swapped. As documented in Fig. 4*A*, the HNF3 motif can be replaced by the HNF4 binding site from the TAT enhancer at −3.6 kb, and again mutation of the latter results in loss of synergism with the GRE in a promoter distal position. In contrast, neither HNF3 binding site tested in combination with the CRE was able to confer liver-specific and cAMP-responsive stimulation of the TK promoter (Fig. 4*B*). Whereas the HNF3 motifs were functional in either orientation in combination with the GRE, both HNF3 motifs (TAT-11 and TAT-2.5) when linked to the CRE were inactive in either orientation (data not shown). These results may indicate a strict requirement for a specific alignment of the CRE and an adjacent binding site not met by our constructs.

DISCUSSION

The work presented above has documented that the liver-enriched transcription activators HNF3α, -β, and -γ and HNF4 specifically interact with two hormone-responsive enhancers of the TAT gene. While binding sites for these transcription factors have been characterized in constitutive regulatory sequences of other liver-specific genes (16, 23, 26–30), our results demonstrate that these elements in hormone-responsive enhancers can function to restrict the activity of ubiquitous signal transduction pathways to the liver. In the TAT enhancer at −3.6 kb an HNF4 binding site synergizes with the CRE to confer cAMP-inducible hepatoma cell-specific stimulation. HNF3 proteins bind in the vicinity of the GRE in the glucocorticoid-inducible enhancer at −2.5 kb, at a sequence motif which was previously characterized as a second GRE (12, 31). Since the methylation interference pattern of the HNF3 protein–DNA complexes correlates with the genomic footprint observed in hepatoma cells (31), HNF3 proteins most likely interact *in vivo*. Which of the three HNF3 proteins cooperates *in vivo*, or whether they form a redundant system, has to remain an open question until gene knockout experiments have been performed. The regulatory properties of the enhancer can be reconstituted by juxtaposing the HNF3 binding site and the GRE. *In vivo*, it is likely that liver-enriched factors of the C/EBP family also participate in determining the strong glucocorticoid response in the liver (32, 33). The recently postulated liver-enriched protein HNF5 (32, 33) is probably identical with HNF3, since the HNF3 binding site that we have identified coincides with one of the two putative HNF5 motifs described in the TAT enhancer at −2.5 kb and displays a methylation interference pattern identical with that published for HNF5. Moreover, the second postulated HNF5 binding site also interacts with HNF3 proteins (data not shown).

In some systems, including the tryptophan oxygenase gene and the mouse mammary tumor virus long terminal repeat, sequence elements for ubiquitous factors adjacent to a GRE have been documented to be important for hormone-dependent stimulation (34–38). Transfection experiments suggest that the presence of steroid hormone receptors is sufficient to allow a transcriptional response upon administration of the respective hormone (e.g., Fig. 4*A*). We have shown here that a tissue-specific response can be elicited through ubiquitous signaling pathways by coupling the target

sequence (hormone response element) to binding sites for cell type-specific transcription factors. The interdependence of two or more essential transcriptional control elements permits a finely tuned response also to small differences in factor or hormone concentrations (39). Whereas a single GRE is functional in close proximity to the transcription start site, cooperation with a second GRE or another transcription factor binding site is required for activation from a promoter-distal position (Fig. 4*A*; ref. 19). As shown in Fig. 4*B* and by the mutational analysis of the cAMP-responsive enhancer (13), a CRE alone is not capable of eliciting a cAMP response. Thus, the upstream location combined with the modular organization of the two hormone-inducible enhancers of the TAT gene determines the tissue-specificity of the response.

The results presented in this study ascribe an important role to HNF3 and HNF4 proteins in determining liver-specific and hormone-inducible TAT gene activity. Expression of HNF3α, -β, and -γ and HNF4 is activated early in liver cell differentiation and remains unchanged in the perinatal period (P. Monaghan and G.S., unpublished work). Since the TAT gene is switched on only shortly after birth, concomitant with changes in hormone concentrations, these findings also indicate a role of signal transduction pathways in the developmental activation of the gene. At birth, insulin concentrations decrease whereas glucagon/cAMP and glucocorticoid levels rise (40). The increase in intracellular cAMP levels, a response to the neonatal hypoglycemia, affects the binding of and trans-activation by the CRE binding protein CREB (21, 41, 42) thereby counteracting the inhibitory effect of insulin on CRE function (R. Ganss, F. Weih, and G.S., unpublished work). We propose that the synergism between constitutive liver-specific control sequences and targets of hormonal signal transduction pathways determines the correct developmental activation of the TAT gene in the liver.

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