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Cloning of a μ-class glutathione *S*-transferase gene and identification of the glucocorticoid regulatory domains in its 5′ flanking sequence

(secondary glucocorticoid response)

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ABSTRACT **The expression of a μ-class glutathione *S*-transferase gene (*hGSTYBX*) isolated from hamster smooth muscle tumor cells (DDT_1 MF-2) is transcriptionally up-regulated by glucocorticoids, and this hormonal regulation is dependent upon protein synthesis. To study the mechanism of regulation, we have cloned and sequenced *hGSTYBX* genomic DNA including its 5′ flanking region. The *hGSTYBX* gene contains nine exons dispersed over a 6.3-kilobase region. When linked to a chloramphenicol acetyltransferase (CAT) reporter gene, the 5′ flanking region was able to direct transcription of the reporter gene. With 5′ deletion studies, we have localized the major glucocorticoid-inducible regulatory element between nucleotides −353 and −239. Within this region no classic glucocorticoid response element (TGTTCT) was identified, but four potential helix–loop–helix binding domains are embedded in two 16-base-pair repeats. Another glucocorticoid regulatory domain has been localized between nucleotides −239 and −136. Cycloheximide blocks glucocorticoid-induced transcription of both the −353CAT and −239CAT reporter genes (nucleotides −447 to −12 and nucleotides −239 to −12 of *hGSTYBX*, respectively, ligated to a CAT reporter gene); therefore, our observations support previous results suggesting that *hGSTYBX* induction by glucocorticoids is a secondary response.**

Tumor induction in the male reproductive tract of the Syrian hamster is a highly reproducible event requiring the dual administration of androgens and estrogens. To study this event, we have placed tumor cells into tissue culture and previously reported that their growth was up-regulated by androgens and down-regulated by glucocorticoids (1). Focusing on the glucocorticoid-controlled cell cycle arrest events, we learned that a μ-class glutathione *S*-transferase (GST; EC 2.5.1.18) was up-regulated by glucocorticoids in a manner dependent upon protein synthesis (2). GSTs are a group of related enzymes that catalyze conjugation of glutathione to electrophilic metabolites of both endogenous and xenobiotic compounds including carcinogens.

Regulation of expression of GST isoforms is currently under study in many laboratories, and specific regulation has been observed in both a drug- and tissue-specific manner in response to phenobarbital, phorbol esters, and agents that bind the dioxin receptor (3–5). Hormonal regulation of GST gene expression has been observed in rat prostate (6) and the Syrian hamster tumor cell line DDT_1 MF-2 (2). A unifying feature of these observations is that the growth status of the cell appears to be involved in the regulatory pathway. A hypothesis has been developed (7) that suggests that this regulatory mechanism may be involved in androgen/estrogen carcinogenesis in Syrian hamsters where decreased GST levels during unscheduled cell growth may unduly expose the cell to carcinogenic insult. To further develop this hypothesis, we have cloned the μ-class GST gene.¶ We now report on its characterization and define both its basal level promoter activity and regulation by glucocorticoids.

MATERIALS AND METHODS

Screening of the Genomic DNA Library. A λEMBL3 hamster genomic library was screened with an [α-^{32}P]CTP-labeled *Bam*HI–*Eco*RI fragment containing 264 nucleotides of *hGSTYBX* cDNA 3′ untranslated sequence (2). Positive plaques were screened through three successive cycles. Appropriate DNA restriction fragments from resulting bacteriophage were subcloned into Bluescript vectors (Stratagene) for further characterization. Sequencing was performed on both DNA strands as described (2).

RNA Isolation and Blotting. RNA isolation and Northern blots were performed as described (2). For slot blots, total RNA (30 μg) was denatured in 2 M formaldehyde/10 mM Mops, pH 7.0, at 65°C for 15 min and then transferred to nitrocellulose filters using a Hybri-Slot cassette (BRL). The hybridization and washing conditions were as described (2).

Construction of Plasmids. To insert the 5′ flanking sequence of a hamster μ-class GST gene (*hGSTYBX*) into the chloramphenicol acetyltransferase (CAT) reporter gene, the oligonucleotide primer 5-TGGTGCTGGTCTAGAGCGGGCCGACCTG was synthesized, which is complementary to the sequence from nucleotide −27 to +1 of *hGSTYBX*, where two nucleotides were substituted (underlined) to create a *Xba* I site. After PCR amplification and digestion with *Xba* I, the 5′ flanking sequences were ligated into pUMSCAT-P_L vector (a gift from Dennis Watson, Frederick, MD). A series of deletion mutants were generated by using unique restriction sites or by PCR amplification, using oligonucleotides designed to generate ends containing appropriate restriction sites. The mutants were also cloned into pUMSCAT-P_L. Each construction was confirmed by sequencing.

Transient Transfections. DDT_1 MF-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Transfections were performed by Lipofectin (BRL) as recommended by the manufacturer. Briefly, the cells were washed twice with Opti-MEM I reduced-serum medium, and 3 ml of the same reduced-serum medium was added to the cells. For CAT assays, usually 2–5 μg of plasmid DNA per 6-cm plate was cotransfected with 2 μg of a

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Abbreviations: GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase; TA, triamcinolone acetonide; HLH, helix–loop–helix; GRE, glucocorticoid response element.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. X61033).

β-galactosidase vector. After transfection, the cells were continuously incubated in reduced-serum medium for 16 hr. The cells were then changed to Dulbecco's modified Eagle's medium with 5% fetal bovine serum. Triamcinolone acetonide (TA; 0.1 μM) or 100% ethanol (1 μl/ml) was added for glucocorticoid-treated or control groups, respectively, and cells were harvested after an additional 72 hr. All transfections were repeated at least three times with similar results.

CAT and β-Galactosidase Assays. CAT assays were performed by the method of Gorman *et al.* (8) by incubating 50–100 μl (adjusted based on the β-galactosidase assay) of cell lysate protein with 0.1 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol in the presence of 4 mM acetyl coenzyme A (Sigma) for 2 hr at 37°C. For the β-galactosidase assay, a 20- to 30-μl sample of the same cell lysate prepared for analysis of CAT activity was analyzed according to the procedure described by Nielsen *et al.* (9).

RESULTS

Isolation and Sequencing of the *hGSTYBX* Gene. Since μ-class GSTs are a multigene family in hamster, we used the 3′ untranslated region of *hGSTYBX* cDNA, a region that demonstrates the least homology to other GSTs, as a probe to screen the hamster genomic library. After screening 500,000 clones, two phage clones were identified as overlapping fragments consisting of the entire GST gene including 4400 base pairs (bp) of the 5′ flanking region and 4000 bp of the 3′ flanking region. The restriction map indicates that the gene contained multiple restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst* I (Fig. 1). No appropriate restriction site was available for subcloning the whole gene; therefore, a series of overlapping fragments, 3EB4, 3H6, 3B2, 2E1, 2P2 and 2E2, were subcloned into Bluescript vectors (see Fig. 1). Sequential deletion mutants were prepared, and the nucleotide sequence was determined in both directions. Fig. 2 presents the 6296-bp sequence of the gene including the first 447 bp of the 5′ flanking domain and 380 bp of the 3′ flanking region. The *hGSTYBX* gene is composed of nine exons. The sizes of exons 1–9 are 53, 76, 65, 83, 101, 96, 111, 112, and 233 nucleotides; they encode 12, 25, 22, 27, 34, 32, 37, and 29 amino acids, respectively. All eight introns of the *hGSTYBX* gene are flanked by GT and AG dinucleotides. The exon sequence of the *hGSTYBX* gene precisely matches its cDNA sequence (2).

A comparison of the structure of *hGSTYBX* with rat YB2 GST indicated that both the organization and sequence have high interspecies homology except for two interesting differences. One is that there is an additional splice site arising in the 3′ untranslated region of *hGSTYBX*, such that 89 nucleotides are spliced out to become intron 8. Another difference is that there is an extra sequence of 759 bp observed in intron 7 close to exon 7 (underlined in Fig. 2). This sequence appears to be a repetitive element with homology to the period gene of *Drosophila* (10). By Southern blotting, we confirmed its presence in liver DNA, which suggests that it is not a recurrent consequence of a neoplastic event or a mutation due to *in vitro* culture conditions.

Structural Characterization of the 5′ Flanking Sequence. In analyzing the sequenced 5′ flanking region of the *hGSTYBX* gene, we discovered a typical eukaryotic promoter structure with two CAAT boxes and a presumed TATA box sequence, TCATAAA, 27 bp upstream of the transcription initiation site previously mapped by primer extension (2). Just upstream of the CAAT boxes at positions −126 to −120 is a potential *ras* response element AGACTCT (11).

Localization of Cis-Acting Regulatory Elements Controlling Basal and Glucocorticoid-Inducible Expression. To characterize the promoter activity and its regulation by glucocorticoid, we ligated the 5′ flanking region of the *hGSTYBX* gene to a CAT reporter gene. The plasmid construct containing 435 bp of the 5′ flanking regions (−447CAT; nucleotides −447 to −12) was transfected into DDT$_1$ MF-2 cells. The results of basal and glucocorticoid-inducible CAT activity indicated that this region contains the basal promoter activity and the regulatory element(s) that respond to glucocorticoid induction (Fig. 3). To more precisely localize these regulatory elements, a series of deletion mutants of the 5′ flanking region of *hGSTYBX* gene were prepared. These constructs were transfected into DDT$_1$ MF-2 cells, and the results are presented in Fig. 3. Deletion of 5′ flanking sequence from nucleotide −447 to nucleotide −353 (−353CAT) had no apparent effect on basal or inducible CAT activity (compare −447CAT with −353CAT). However, deletion to nucleotide −239 (−239CAT) significantly reduced both basal CAT activity as well as glucocorticoid-inducible CAT activity although there is still a 2-fold inducibility observed with −239CAT. Further deletion of 5′ flanking sequence to nucleotide −136 (−136CAT) essentially abolished basal level expression, and no glucocorticoid-inducible CAT activity

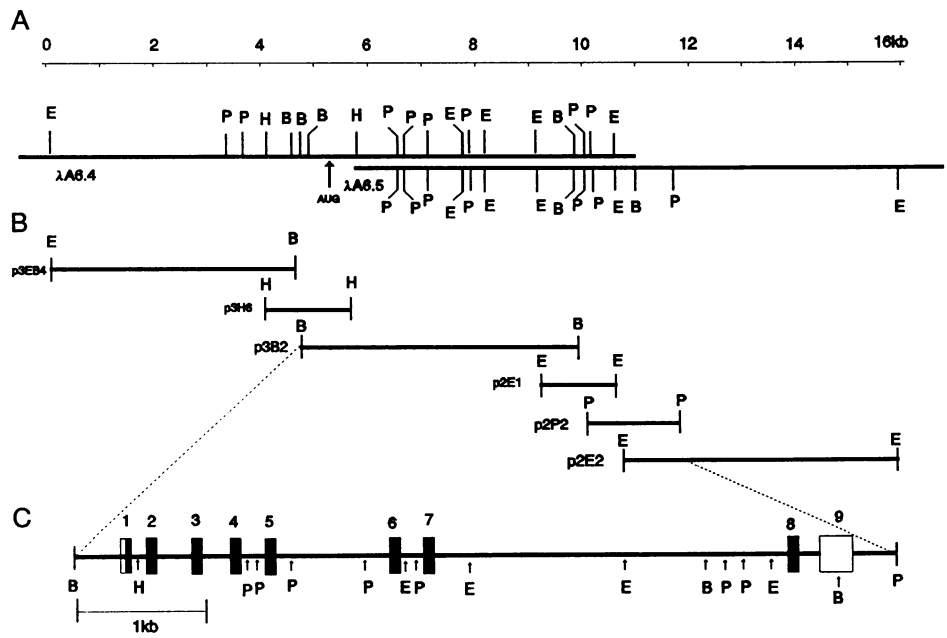

FIG. 1. Organization of the *hGSTYBX* gene and its flanking regions. (*A*) Restriction map of two positive clones, λA6.4 and λA6.5. The arrow (AUG) indicates the site of the first codon. (*B*) Subclones of the *hGSTYBX* gene, p2EB4, p3H2, p3B2, p2E1, p2P2, and p2E2, are in Bluescript plasmid vectors. (*C*) Detailed organization of the *hGSTYBX* gene. The open and filled boxes represent the noncoding and coding exons, respectively. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I.

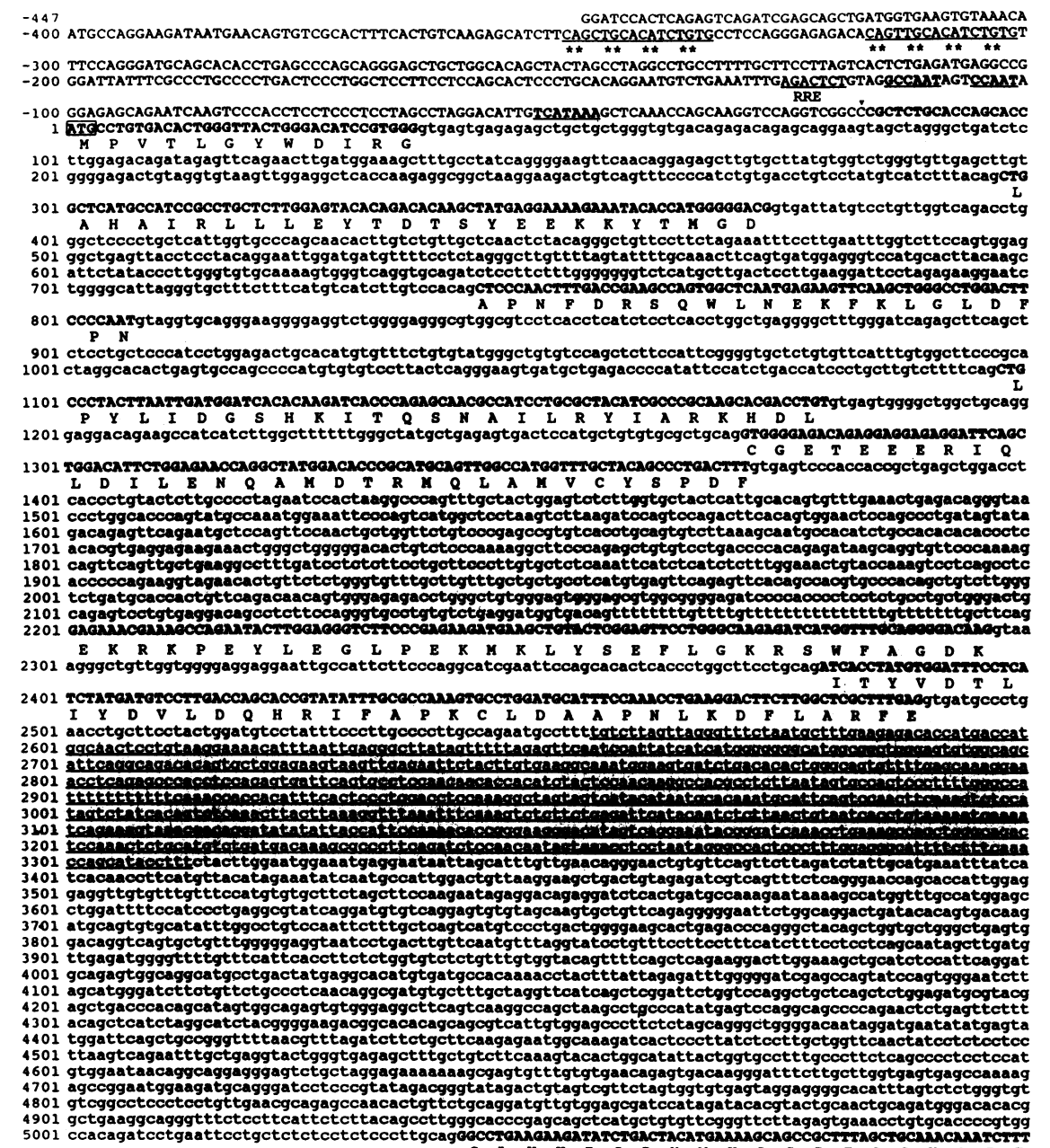

FIG. 2. Nucleotide sequence of the *hGSTYBX* gene and its flanking regions. Nucleotide 1 corresponds to the first ATG codon. The sequence of the 5′ flanking region is in uppercase letters, the sequences of exons are in boldface uppercase letters, and introns are in lowercase letters. The amino acid sequence is provided under the nucleotide sequence. Two CAAT boxes, the TATA box, and a poly(A) additional signal are underlined. The transcription initiation site (nucleotide −18) is marked by an upside-down triangle. The start codon is boxed. The potential helix–loop–helix (HLH) protein-binding sites are underlined and starred. The potential *ras* response element (RRE) is also underlined and indicated. In intron 7, the underlined 759 bp are the extra sequence with homology to the *Drosophila* period gene.

was detected. Interestingly, the −119CAT vector always demonstrated slightly higher basal level CAT activity than −136CAT, although no glucocorticoid inducibility of this vector was observed. Within this region is the putative *ras* response element located between nucleotide −126 and nucleotide −120, which may function as a repressor element affecting basal gene expression. Finally, to examine if other glucocorticoid regulatory elements might be present further upstream in the 5′ flanking region of *hGSTYBX* gene, we constructed a CAT vector containing 2.2 kilobases of 5′ flanking sequence (−2200CAT). No significant difference was found in comparing this vector to −353CAT.

The induction of the *hGSTYBX* gene by glucocorticoid can be blocked by a low concentration of cycloheximide, suggesting that transcriptional regulation of *hGSTYBX* is a secondary response to glucocorticoid (2). To further charac-

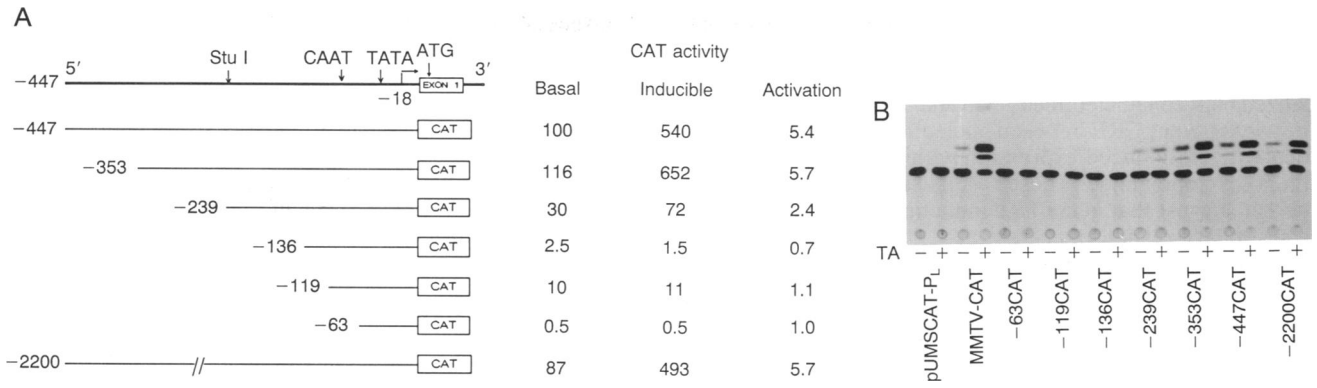

FIG. 3. Schematic illustration of the *hGSTYBX* 5′ flanking region–CAT chimeric genes and respective CAT activities of transiently expressed vectors. (*A*) Basal and glucocorticoid-inducible CAT activity of 5′ deletion mutants of *hGSTYBX* gene. The size of the flanking insert in each construct is specified by the number of base pairs included in each construct. The bent arrow represents the transcription initiation site. Each construct was transfected into DDT_1 MF-2 cells, and the basal and inducible CAT activities were monitored by TLC and quantitated by liquid scintillation spectroscopy. The CAT activities presented are the average activities from three separative transfections, which deviated from each other by <15%. (*B*) Autoradiography of a representative CAT assay. pUMSCAT-P_L and MMTV-CAT are the negative and positive controls, respectively; "+" and "−" represent the presence or absence of 0.1 μM TA.

terize this regulatory mechanism, the −353CAT and −239CAT vectors and the MMTV-CAT (positive control) vector were transiently expressed in DDT_1 MF-2 cells, which were then treated with ethanol (1 μl/ml), TA (0.1 μM), or the steroid plus cycloheximide at 1.0 μg/ml. RNA was prepared and blotted with a CAT-specific probe. The results, presented in Fig. 4, demonstrate that cycloheximide had no effect on the MMTV-CAT vector as expected since its induction is a primary response. However, glucocorticoid induction of both −353CAT and −239CAT reporter genes was abolished by cycloheximide.

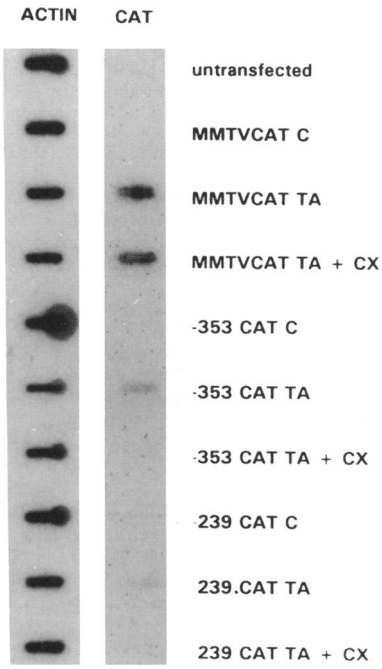

FIG. 4. Slot blots of CAT RNA. The indicated CAT vectors were transiently transfected into DDT_1 MF-2 cells as described in *Materials and Methods*. The cells were harvested 24 hr after treatment with 0.1% ethanol (C), 0.1 μM TA, or with TA plus cycloheximide at 1 μg/ml (TA + CX); total RNAs were isolated. Blots were hybridized with probe (10^6 cpm/ml) prepared by random priming from a *Hin*dIII–*Eco*RI CAT gene fragment excised from the pCAT-Promoter plasmid (Promega). The film was exposed for 3 days with an intensifying screen. The same filter was rehybridized with an actin probe (10^6 cpm/ml), and the film was exposed for 24 hr with an intensifying screen.

Tissue-Specific Expression. To determine tissue specificity of *hGSTYBX* gene expression, total RNA was isolated from the hamster epididymis, testis, diaphragm, seminal vesicles, vas deferens, brain, kidney, liver, and skeletal muscle and hybridized with an [α-^{32}P]UTP-labeled RNA probe generated from the *hGSTYBX* cDNA 3′ untranslated region (2). The results, presented in Fig. 5, indicated that the *hGSTYBX* gene is normally expressed in hamster liver, kidney, epididymis, and vas deferens but not in the testis, diaphragm, seminal vesicle, and brain.

DISCUSSION

Glucocorticoids are able to induce both primary and secondary responses in target tissue (12). A primary response is characterized by direct association of the hormone–receptor complex with a specific DNA sequence in the target gene, resulting in modification of transcription, and is independent of protein synthesis. Conversely, a secondary response requires protein synthesis for transcription of the induced RNA and a time lag between administration of the hormone and initiation of the response. We have previously described and characterized the *hGSTYBX* cDNA, expression of which is increased by glucocorticoid in a protein synthesis-dependent manner (2), suggesting that *hGSTYBX* induction may be a secondary glucocorticoid response. To further analyze this response at the molecular level, we have now cloned the entire *hGSTYBX* genomic sequence and have localized the glucocorticoid response element(s) (GRE) in its 5′ flanking

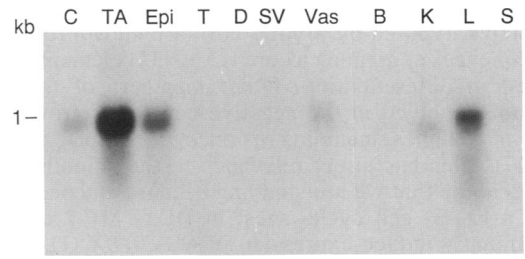

FIG. 5. Expression of *hGSTYBX* in different tissues. Total RNA (20 μg) isolated from intact nontreated hamster tissues was hybridized with 1 × 10^7 cpm of an [α-^{32}P]UTP-labeled RNA probe transcribed using T7 polymerase from *Bam*HI-linearized *hGSTYBX* DNA (3′ untranslated region). The C (control) and TA (0.1 μM) lanes are RNA isolated from DDT_1 MF-2 cells. The remaining abbreviations represent RNA isolated from tissues: Epi, epididymis; T, testes; D, diaphragm; SV, seminal vesicle; Vas, vas deferens; B, brain; K, kidney; L, liver; and S, skeletal muscle.

region. By fusing the 5′ flanking sequence into a reporter gene, we were able to demonstrate that glucocorticoid inducibility of the CAT vector is the property of the sequence localized between nucleotides −353 and −239. There appears to be a less potent GRE between nucleotides −239 and −136. Glucocorticoid-induced expression within this region is sensitive to cycloheximide. These results support our previous data that suggest that *hGSTYBX* induction by glucocorticoid is a secondary response.

As discussed above, primary transcriptional responses to glucocorticoids involve direct binding of the glucocorticoid–receptor complex to specific DNA sequences called GREs. The structure of GREs varies but generally contains the canonical hexamer TGTTCT. Examination of the sequence between nucleotides −353 and −136 of *hGSTYBX* failed to reveal any classical GREs, which suggests that glucocorticoid–receptor complexes are not directly interacting with this region of the gene. Therefore, induction of *hGSTYBX* seems to be a true secondary glucocorticoid response. However, we need to consider other possibilities. For example, rat α1-acid glycoprotein was also initially thought to be a glucocorticoid secondary response gene (13). However, subsequent studies identified a functional GRE in the gene's 5′ flanking domain. The authors hypothesize that a labile protein interacts with the GRE–glucocorticoid–receptor complex to regulate expression, and because the second factor presumably turns over rapidly, its concentration is affected by cycloheximide. This model of regulation, if it is substantiated, appears to be different than classical primary or secondary responses described above, although clearly transcription is dependent upon the presence of both the glucocorticoid–receptor complex and the translationally dependent renewal of additional factors. An example of a mammalian gene expressed in the liver of rats that does demonstrate secondary hormone responsiveness is α2u-globulin (14). Although footprinting studies identified protected domains, the respective nuclear proteins were determined to be constitutively expressed rather than induced by glucocorticoids (15). Therefore, although α2u-globulin falls in the secondary response class of glucocorticoid-regulated genes, the cycloheximide-sensitive step controlling gene expression remains to be identified. Neither of the GREs, structurally similar to α2u-globulin or α1-acid glycoprotein, have been identified in *hGSTYBX*, suggesting that the *hGSTYBX* is induced by glucocorticoids in a different way.

We have now analyzed the glucocorticoid-regulated domains in the 5′ flanking sequence of the *hGSTYBX* gene by transient expression assays. Two glucocorticoid-regulated regions have been identified. First, a 114-bp responsive region (nucleotides −353 to −239) was found to contain two 16-bp repeats containing four canonical, helix–loop–helix (HLH) binding domains (CANNTG) (16). HLH transactivator proteins, typified by MyoD1 (16), are known to interact with sequences containing a core CANNTG element and are involved in differentiation. Other members of the HLH family are involved in the negative control of growth of a variety of cell lines, including *ras* oncogene-transformed cell lines (17). This has direct relevance to the smooth muscle tumor cell line that we are studying because glucocorticoids induce a G_0/G_1 cell cycle arrest in DDT_1 MF-2 cells and concomitantly induce expression of *hGSTYBX* (1). Certain features of G_0/G_1-arrested DDT_1 MF-2 cells suggest they develop a more differentiated smooth muscle phenotype (18). Therefore, identification of HLH domains (emblematic of the response elements of genes controlling skeletal muscle differentiation) within the region conferring glucocorticoid responsiveness to *hGSTYBX* may indicate that glucocorticoids regulate expression via a transactivator protein with properties and functions similar to the MyoD1-like gene family. Consequently, with this data it may be possible to identify and clone the smooth muscle analogs for a hypothesized MyoD1-like protein that regulates smooth muscle cell differentiation and further may implicate transcription regulation of these genes in hormonal carcinogenesis. Second, by analyzing the results from CAT assays and slot blots, it seems that there is another potential glucocorticoid regulatory domain between nucleotide −239 and nucleotide −136. Since this region is downstream of the HLH domain and is also sensitive to cycloheximide inhibition, it is possible that the real glucocorticoid secondary response element is localized in this region. If this is the case, the potential HLH protein-binding domain probably functions as a transcriptional enhancer active in both basic and inducible transcription.

In conclusion, we have presented the characterization of a member of the GST family of genes. Until now, attention has basically been focused on the biology of this class of genes with respect to their regulation in multi-drug-resistance syndromes associated with chemotherapy. Much less is known about their role in chemoprevention, and very little is known about GST function in hormonal carcinogenesis. Our interest in the role of *hGSTYBX* in carcinogenesis developed when we determined that it was a glucocorticoid-regulated gene whose expression was dependent on protein synthesis (2) and that regulation of this gene was a function of both glucocorticoid treatment and cell cycling status (7). Because of the potential role for GST in protecting cells against quinone metabolites of estrogens and a suspected DNA repair function, the importance of understanding the mechanisms regulating expression of this class of genes is relevant to understanding their role in hormonal carcinogenesis.

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