Transcriptional Regulation of the *Xenopus laevis* Bβ Fibrinogen Subunit Gene by Glucocorticoids and Hepatocyte Nuclear Factor 1: Analysis by Transfection into Primary Liver Cells†

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ABSTRACT: The blood-clotting protein fibrinogen is composed of three subunits, designated Aα, Bβ, and γ, which are encoded by a family of related genes. As part of the acute-phase response, expression of the fibrinogen genes is coordinately regulated in the liver by glucocorticoids. To understand the factors underlying this hormonal response, we have examined control of transcription from fibrinogen gene fragments transfected into hepatocytes from the frog *Xenopus laevis*. This analysis is the first in any species to define transcriptional regulatory elements for the fibrinogen genes by transfection into primary liver cells, rather than liver-derived cell lines. A transfection vector was constructed containing the *Xenopus* Bβ gene transcription start site and 1293 bp of the 5′ flanking sequence linked to the firefly luciferase gene. When this construct was transfected into primary liver parenchymal cells, luciferase expression was induced approximately 14-fold by glucocorticoids, an increase similar to the transcriptional stimulation of the endogenous Bβ subunit gene. DNA fragments with as little as 284 bases of upstream sequence retained full hormone responsiveness. This region contains a sequence resembling the canonical glucocorticoid response element (GRE) at bases −148 to −162. Deletions or specific point mutations eliminating this putative GRE led to complete loss of glucocorticoid inducibility. Physical association of the steroid hormone receptor with this functional GRE was demonstrated with a truncated form of the rat glucocorticoid receptor containing the DNA-binding domain. A second possible GRE at positions −526 to −540 was not hormone-responsive, in either the presence or the absence of the more proximal GRE. The regulatory region also has a sequence similar to the binding site for a liver-specific transcription factor, hepatocyte nuclear factor 1 (HNF-1), at positions −120 to −132. Specific point mutations in the HNF-1-binding site, in a construct containing a wild-type GRE, reduced promoter activity by a factor of 10, while stimulation by glucocorticoids was retained. Binding studies confirmed specific interaction between this site and the transcription factor HNF-1α from mouse. Thus, we have identified a GRE sufficient to account for full glucocorticoid inducibility and an HNF-1 site close to the promoter that are major determinants of transcriptional control of the *Xenopus* fibrinogen Bβ subunit gene in cells from normal liver tissue.

Coordinate expression of multiple genes is essential throughout the life of multicellular organisms for the establishment and maintenance of differentiated tissues and for cellular responsiveness to external signals, such as hormones. Much of the control of gene expression occurs by direct regulation of transcription, through proteins binding to DNA near the 5′ end of genes and either stimulating or repressing the transcriptional machinery. For analyzing the mechanisms of simultaneous activation of several genes in a complex network, an attractive model is provided by the genes coding for the subunits of fibrinogen. Fibrinogen, the precursor of the blood-clotting protein fibrin, is synthesized and assembled in the liver from three nonidentical subunits, Aα, Bβ, and γ. Since the messenger RNAs for these subunits are transcribed from separate genes (Kant et al., 1985), efficient control of fibrinogen production requires coordinated expression of three independent genes.

Synthesis of fibrinogen, as well as several other plasma proteins produced by the liver, increases dramatically as the body responds to many types of physiological trauma, such as infections, inflammation, injury, burns, and neoplastic growth (Kushner, 1988). This increase is part of a broad range of metabolic and systemic changes, referred to as the acute-phase response, that collectively function to maintain homeostatic balance (Munck et al., 1984). Stimuli that induce fibrinogen production act at the mRNA and gene levels, coordinately elevating the three fibrinogen subunit mRNAs (Crabtree & Kant, 1982) by stimulating transcription of the separate genes (Moshage et al., 1988).

Fibrinogen gene expression is controlled primarily by two major physiological regulators: the adrenal steroids, glucocorticoids (Munck et al., 1984); and the cytokine interleukin 6 (IL-6),[1] produced by monocytes and other cell types

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[1] Abbreviations: DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GRE, glucocorticoid response element; HNF-1, hepatocyte nuclear factor 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-6, interleukin 6; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M sodium citrate; SEM, standard error of the mean; bp, base pair(s); kb, kilobase pair(s).

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(Baumann et al., 1984). The actions of these hormonal effectors have been analyzed in isolated hepatocytes and hepatoma cells. With regard to IL-6, Fuller and co-workers showed in rat primary hepatocytes and a rat liver cell line that monocyte-derived factors caused a coordinated 10-fold increase both in mRNA levels (Fuller et al., 1985) and in rates of transcription of the three subunit genes (Otto et al., 1987). Full stimulation of fibrinogen gene expression in rat liver, both *in vivo* and in response to IL-6 in cultured cells, requires glucocorticoids (Crabtree & Kant, 1982; Otto et al., 1987). However, when these steroids are administered alone, variable results have been obtained. One group reported a 4-fold elevation of fibrinogen mRNA levels in isolated rat hepatocytes in response to the synthetic glucocorticoid dexamethasone (Princen et al., 1984), while other investigators detected increased fibrinogen secretion but no change in fibrinogen subunit mRNA levels either in primary rat hepatocytes or in a rat hepatoma cell line (Otto et al., 1987). The converse was found in human HepG2 cells at least for one of the subunits; Bβ mRNA increased dramatically following dexamethasone treatment but secreted fibrinogen increased only slightly (Huber et al., 1990). Thus, although glucocorticoids are important in regulating fibrinogen production during an acute-phase response, it has been difficult to elucidate the molecular mechanisms underlying this response in mammalian cells.

To understand the role of glucocorticoids in controlling fibrinogen gene expression, we have developed a liver cell culture system derived from *Xenopus* frogs in which dexamethasone causes a substantial stimulation of fibrinogen synthesis. We have demonstrated previously that the large increase in secretion of fibrinogen by *Xenopus* hepatocytes correlates with a 10–30-fold elevation of the mRNAs coding for each of the fibrinogen subunits (Bhattacharya & Holland, 1991). The rate of transcription of each of the three subunit genes increases in a highly concerted manner, with a 10–20-fold stimulation within 1–2 h after glucocorticoid treatment. Thus, in frog hepatocytes, the correlation is very good between induction of gene transcription rates, elevation of subunit mRNA levels, and increased secretion of fibrinogen in response to hormone.

We have taken advantage of this reliable *Xenopus* liver cell system to characterize the DNA sequence elements that mediate transcriptional regulation of the fibrinogen genes. For this analysis, we optimized conditions for introducing cloned gene fragments into primary *Xenopus* hepatocytes by transfection. The use of cells from normal liver tissue reproduces as closely as possible the natural cellular environment, thus eliminating concerns about the aberrant patterns of transcriptional regulation in transformed cell lines. Using this gene transfection system, we have identified DNA sequences involved in the glucocorticoid-dependent and glucocorticoid-independent transcription of the Bβ fibrinogen subunit gene. The functional significance of these elements was confirmed by site-directed mutagenesis, and binding of well-defined transcription factors to these sites was demonstrated by electrophoretic mobility-shift assays.

MATERIALS AND METHODS

Genomic DNA Cloning. The *Xenopus laevis* genomic DNA library from Dr. Igor Dawid was prepared from red blood cell DNA of a homozygous diploid *Xenopus* frog (Reinschmidt et al., 1985) in the phage vector λEMBL4 (Firschauf et al., 1983). Following our general procedures for screening and subcloning (Bhattacharya et al., 1991), phage were adsorbed to the host strain LE392 (Kaiser & Murray, 1985), plated, and transferred to filters. For the primary screening, filters were prehybridized at 37 °C for 6 h in 50% formamide, 3 × SSC, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 1% SDS, 10 mM HEPES–NaOH, pH 7.5, 1 mM EDTA, 50 μg/mL yeast RNA, and 10% dextran sulfate. Hybridization was carried out for 18 h in a solution of the same composition containing (2–4) × 10^6 cpm/mL of the 425-bp *Eco*RI–*Sty*I fragment from the 5′ end of the Bβ fibrinogen cDNA (Bhattacharya et al., 1991), labeled by random priming (Holland et al., 1991). In at least three subsequent screenings, filters were hybridized at 60 °C in 0.2 × SSC, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 1% SDS, 50 mM sodium phosphate, pH 6.5, and 40 μg/mL yeast RNA. Filters were washed twice for 5 min at room temperature in 2 × SSC/0.1% SDS and twice for 1 h at 60 °C in 0.2 × SSC/1% SDS. Fragments of the purified genomic clone λBβ425:1 were transferred to the plasmid vector pBluescript SK– (Strategene, La Jolla, CA). DNA was sequenced by the dideoxy-termination method (Holland et al., 1991). The entire sequence of the 1.4-kb *Hin*dIII genomic fragment containing the transcription start site was determined unambiguously on both strands.

Deletion and Site-Specific Mutagenesis. Plasmids containing sequential 5′ deletions of the upstream region of the Bβ fibrinogen gene were constructed by the general strategy of purification of appropriate restriction fragments, digestion with mung bean nuclease to create blunt ends, digestion with a second restriction endonuclease to generate a specific site at one end, and ligation of an adaptor to the remaining blunt end of the fragment (Sambrook et al., 1989). The fragments were then ligated (Sambrook et al., 1989) into the multiple cloning site of the luciferase reporter construct pLucLink 2.0 provided by Dr. Richard Maurer (d'Emden et al., 1992).

For producing internal deletions, the fragment −632/+40 of the Bβ fibrinogen gene was inserted, using an adaptor oligonucleotide, into plasmid constructs containing the Bβ gene promoter and upstream sequence up to position −108 or −136. The resulting plasmid was linearized at the junction between the −632/+40 fragment and the −108 or −136 nucleotide, and subjected to unidirectional deletion toward bp −632 with exonuclease III. It was then digested with S1 nuclease to create blunt ends and recircularized by ligation, using protocols from Promega (Madison, WI). The specific nucleotides introduced at the internal junction by the adaptor sequence are shown in the legend to Figure 8.

Mutations in the proximal GRE and HNF-1 sites were produced by the Altered Sites oligonucleotide-directed *in vitro* mutagenesis procedure (Promega). The −284/+40 Bβ fibrinogen gene fragment was cloned into the pAlter-1 vector and single-stranded DNA prepared according to the manufacturer's protocol. Mutagenesis of the GRE-binding site was accomplished using the oligonucleotide 5′-ACCTACACTGTAGGAGATATCGTGGAAGCAAACAGAGTACAA-3′. The HNF-1 sequence was mutated using the oligonucleotide 5′-CAAACAGAGTACAAATGCTCTCGAGCACTGCTAGACAGGAGG-3′. The sequence of each mutant was confirmed by DNA sequencing.

Plasmids were transformed into DH5α-competent cells (Bethesda Research Laboratories, Gaithersburg, MD), and DNA was isolated by a modified alkaline lysis procedure (Holland et al., 1991). All plasmids used for transfections were further purified on an ethidium bromide/cesium chloride gradient (Sambrook et al., 1989).

Liver Cell Culture and Transfection of Primary Hepatocytes. Female *Xenopus laevis* (100–150 g) from Nasco

(Fort Atkinson, WI) were treated by immersion in 4×10^{-6} M estradiol-17β 7 or 8 days prior to cell preparation as described elsewhere (Roberts & Holland, 1993). Estrogen treatment increases liver parenchymal cell number about 5-fold by 14 days after treatment (Spolski et al., 1985), and was used to obtain cells engaged in cell division, which increases transfection efficiency as described in the next section. We have shown previously that fibrinogen synthesis is stimulated by glucocorticoids in a similar manner in male or female frogs, either untreated or previously-treated with estrogens (Bhattacharya & Holland, 1991; Bhattacharya, 1990).

Liver parenchymal cells were purified by *in situ* perfusion of the liver, collagenase digestion, and density gradient centrifugation as previously described (Bhattacharya & Holland, 1991) except that final washes of the cells after the Metrizamide gradient were in magnesium-free Barth's solution (88 mM NaCl, 1 mM K_2SO_4, and 10 mM HEPES–NaOH, pH 7.4). Contamination of the cultures with red blood cells was typically 1–2% of the number of hepatocytes, determined by counting in a hemacytometer. For each experiment liver parenchymal cells from two animals were pooled. Freshly-isolated cells were kept on ice until used for electroporation as outlined below.

To introduce DNA into *Xenopus* primary liver cells by electroporation, the following parameters were optimized: (1) duration of estrogen treatment of frogs before cell isolation, to maximize the number of cells undergoing cell division; (2) number of hepatocytes and amount of DNA used in transfections; (3) pulse voltage, time constant, and pulse shape; and (4) duration of culture period before cell lysates were prepared. Each of these parameters influences the level of expression of transfected genes (Andreason & Evans, 1988; Chu et al., 1987; Shigekawa & Dower, 1988). The optimized protocol is as follows: (1) Hepatocytes were isolated from frogs that had been treated with estrogen 7 or 8 days before cell purification. In general, recovery of transfectants is better when actively-growing cells rather than slowly-growing or quiescent cells are used. Since estrogen treatment of *Xenopus* stimulates liver cell division (Spolski et al., 1985), we reasoned that isolation of primary liver cells during the proliferative phase would result in improved transfection efficiencies. We showed that luciferase expression was higher in cells from frogs exposed to estrogen 8 days rather than 14 days before cell isolation. (2) A total of 2×10^6 primary hepatocytes in 500 μL of Barth's solution were mixed with 25 μg of the test construct and 25 μg of the control plasmid pCMVβgal (MacGregor & Caskey, 1989). Luciferase expression increased with transfection of DNA amounts up to 100 μg. (3) Electroporation was carried out with the geneZAPPER 450/2500 electroporator (IBI, New Haven, CT) set to deliver an exponentially decaying pulse with a peak voltage of 475 V/cm and a time constant of approximately 30 ms. These conditions were obtained at a setting of 190 V and a capacitance of 1400 μF, discharging into 4-mm foil-lined cuvettes (IBI). (4) The cells were immediately resuspended in fully-defined culture medium (Bhattacharya & Holland, 1991), divided equally to conditions of minus or plus 10^{-7} M dexamethasone and 10^{-9} M triiodothyronine, plated onto a 60-mm culture dish (Primaria, Falcon, Lincoln Park, NJ) at 1×10^6 cells/4 mL, and maintained for 40–48 h at 19 °C, 5% CO_2, and 85% humidity. Triiodothyronine by itself does not induce fibrinogen synthesis, but ensures maximal glucocorticoid-responsiveness when given with dexamethasone (Bhattacharya & Holland, 1991).

Determination of Reporter Gene Activity. After the 40–48-h incubation period, cells were washed twice on the culture plate with 2 mL of Barth's solution. To lyse the cells, 0.5 mL of 1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT was added. After 10 min at room temperature, the cell extract was scraped off the plates, the lysate cleared by centrifugation at 16000*g* for 5 min, and the supernatant stored at –80 °C. For luciferase assays, 100 μL of cell extract was added to 360 μL of 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate, pH 7.8, 1 mM DTT, and 2 mM ATP (Brasier et al., 1989). To start the reactions, 100 μL of 1 mM D-luciferin K (Analytical Luminescence Laboratory, San Diego, CA) was injected automatically and integrated light output measured for 10 or 20 s in a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

β-Galactosidase assays were performed by measuring the conversion of 4-methylumbelliferyl β-D-galactoside (Sigma, St. Louis, MO), a nonfluorescent galactoside analog, to the highly fluorescent molecule methylumbelliferone (MacGregor et al., 1991). A 120-μL aliquot of cell extract was added to 1.08 mL of 100 mM sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM $MgSO_4$. Next, 300 μL of 3 mM 4-methylumbelliferyl β-D-galactoside was added, and the reactions were incubated at 37 °C for 1–2 h. The reactions were stopped by adding 750 μL of 0.3 M glycine/15 mM EDTA, pH 11.2, and the product was measured using a fluorescence spectrophotometer (Model F-3010, Hitachi, San Jose, CA) with excitation at 363 nm and emission read at 448 nm. For each cell lysate, corrected luciferase activity was calculated as (luciferase activity in sample – background luciferase activity in mock-transfected cells)/(β-galactosidase activity in sample – background β-galactosidase activity in mock-transfected cells).

In Vitro Translation of Mouse HNF-1α. A plasmid clone containing a 2174-bp *Eco*RI–*Eco*RV fragment with the entire mouse HNF-1α coding sequence in pBluescript KS+ was provided by Dr. Gerald Crabtree (Kuo et al., 1990). The clone was digested with *Eco*RV, and the linearized plasmid DNA was transcribed *in vitro* in 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 mM each of ATP, CTP, GTP, and UTP, 20 μM DNA template, and T7 RNA polymerase (Promega) for 1 h at 37 °C. The mixture was treated with 100 ng/mL DNase I (Worthington, Freehold, NJ) for 15 min at 37 °C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with 2.5 volumes of ethanol. The purified *in vitro*-transcribed RNA was translated *in vitro* at 30 °C for 90 min in reactions containing 4 μg of RNA, 35 μL of nuclease-treated rabbit reticulocyte lysate, 40 units of RNasin, and 1 μL of 1 mM complete amino acid mixture in a total volume of 50 μL, using reagents and protocols from Promega. Parallel reactions contained 40 μCi of [^{35}S]methionine and 1 μL of 1 mM amino acid mixture minus methionine. Aliquots of the labeled reactions were analyzed on a 10% sodium dodecyl sulfate–polyacrylamide gel (Laemmli, 1970) followed by autoradiography (Bhattacharya et al., 1991) to confirm translation of the full-length HNF-1α protein.

Description of Oligonucleotides. Oligonucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems, Foster City, CA). Complementary DNA strands at 50 μg/mL per strand were annealed in 10 mM Tris-HCl (pH 7.5)/150 mM NaCl by heating at 65 °C for 15 min and cooling to room temperature over a period of 30 min. The sequences of the double-stranded oligonucleotides used in gel-shift assays

Table I [a]

fragment	nucleotide sequence	description
pal GRE	5′-CTACGCAGAACATGATGTTCTAGTCTT-3′	palindromic glucocorticoid response element (Zilliacus et al., 1991) containing two 6 bp half-sites separated by three noncritical nucleotides, surrounded by random sequence
Xen GRE	5′-ACTGTAGGATCAATCTGTTCAGCAAAC-3′	*Xenopus* Bβ fibrinogen glucocorticoid response element at −148 to −162
mut GRE	5′-ACTGTAGGAGATATCGTGGAAGCAAAC-3′	*Xenopus* Bβ fibrinogen proximal glucocorticoid response element with mutations in both half-sites
rat HNF	5′-ACCAAACTGTCAAATATTAACTAAAGGGAG-3′	rat Bβ fibrinogen HNF-1-binding site, consisting of two partially palindromic 6 bp half-sites separated by a single noncritical nucleotide (Courtois et al., 1987; Fowlkes et al., 1984)
Xen HNF	5′-AGAGTACAAATTATCTATTAACCTGCTAGA-3′	*Xenopus* Bβ fibrinogen HNF-1-binding site at −120 to −132
mut HNF	5′-AGAGTACAAATGCTCTCGAGCACTGCTAGA-3′	*Xenopus* Bβ fibrinogen HNF-1-binding site with mutations in both half-sites.

[a] Underlined nucleotides comprise known or putative binding sites. Boldface letters indicate nucleotides that differ from the respective wild-type sequences.

are given in Table I (top strand shown), with underlined nucleotides comprising the known or putative binding site. In the mutant fragments, the boldface letters indicate nucleotides that differ from the respective wild-type sequences.

DNA–Protein-Binding Reactions and Electrophoretic Mobility-Shift Assays. The rat glucocorticoid receptor DNA-binding domain, provided by Dr. Keith Yamamoto, consists of amino acids 440–525 of the rat glucocorticoid receptor expressed in *Escherichia coli* (Luisi et al., 1991). Binding reactions with 100 ng of this protein were carried out in a final volume of 10 μL containing 20 mM Tris-HCl, pH 7.8, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 100 ng of poly(dI-dC)·poly(dI-dC) (Pharmacia, Piscataway, NJ), and 0.2 ng of double-stranded oligonucleotide probe, end-labeled as described (Holland et al., 1991). The binding conditions for *in vitro*-translated mouse NHF-1α were 10 mM Tris-HCl, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT, 1 μg of poly(dI-dC)·poly(dI-dC), 2 μL of *in vitro*-translated mouse HNF-1α protein, and 0.2 ng of radioactively-labeled oligonucleotide in a final volume of 10 μL (Mendel et al., 1991). For both the glucocorticoid receptor DNA-binding domain and mouse HNF-1α, unlabeled double-stranded competitor oligonucleotides were added to the binding reaction 10 min before addition of radioactive oligonucleotide. After addition of radioactive oligonucleotide the reactions were incubated for a further 30 min at room temperature.

The electrophoretic mobility-shift assays (Fried & Crothers, 1981; Revzin, 1989) were performed by adding 1 μL of 250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol to the binding reactions and loading 6 μL on 5% polyacrylamide [75:1 acrylamide:bis(acrylamide) ratio] nondenaturing gels to separate DNA–protein complexes from free DNA. Gels were run in 0.25 × TBE (Sambrook et al., 1989) at 4 °C at a constant voltage of 225 V, dried without fixing at 80 °C for 30 min, and exposed to Kodak (Rochester, NY) XAR5 film at −80 °C.

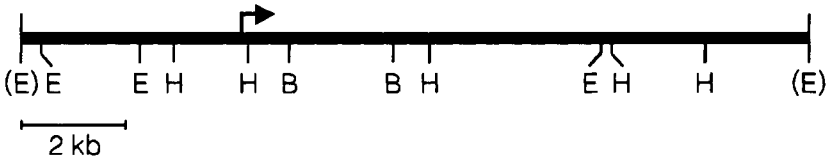
RESULTS

Isolation of Genomic DNA Clones for the Bβ Subunit of Xenopus Fibrinogen and Identification of the Transcription Initiation Site. Genomic DNA clones containing the gene for the Bβ subunit of fibrinogen were isolated by screening a *Xenopus laevis* genomic DNA library with a fragment from the 5′ end of the Bβ fibrinogen cDNA (Bhattacharya et al., 1991). Figure 1A shows the restriction enzyme cleavage map of one of these clones, designated λBβ425:1, which comprises approximately 15 kb and includes the entire coding region for the Bβ gene. The extreme 5′ end of the Bβ cDNA is contained within a 1.4-kb *Hin*dIII fragment of the genomic DNA. To locate the transcription initiation site precisely, this 1.4-kb *Hin*dIII fragment was subcloned into the plasmid vector pBluescript SK−, and the nucleotide sequence of the genomic DNA was determined. Because our cDNA clone does not include the first 20 bases of Bβ mRNA (Bhattacharya et al., 1991), it was necessary to ascertain the complete sequence at the 5′ end of the Bβ mRNA by primer extension sequencing (data not shown). Comparison of the complete mRNA and cDNA sequences to the 1.4-kb genomic DNA subclone showed that the start site of transcription is 128 bp from the 3′ end of the 1.4-kb *Hin*dIII genomic fragment and that the first exon of the gene is 116 bp long. These findings were independently confirmed by RNase protection mapping (data not shown). The genomic clone λBβ425:1 therefore contains approximately 4.3 kb of 5′ flanking sequence (Figure 1A).

DNA Sequence of the Region Upstream of the Bβ Fibrinogen Subunit Gene. The nucleotide sequence of the 1.4-kb *Hin*dIII genomic fragment containing the transcription initiation site was determined on both strands by manual dideoxy-termination sequencing (Figure 1B). It includes 1293 bp upstream of the transcription start site, the entire 116 bp first exon of the gene, and 12 bp of the first intron. Analysis of this region reveals a TATA box at position −31. This sequence, TATTTAA, is the same as the TATA box sequences of the human interferon α_1 (Ragg & Weismann, 1983), *Photinus pyralis* (firefly) luciferase (de Wet et al., 1987), and *Drosophila melanogaster* alcohol dehydrogenase adult promoters (Benyajati et al., 1983). Two sequences similar to the consensus glucocorticoid response element (GRE) are at positions −148 to −162 and positions −526 to −540. Two sequences similar to the binding site for the liver-specific transcription factor HNF-1 are present at positions −120 to −132 and positions −919 to −931.

Figure 2A shows comparisons of the putative GREs in the *Xenopus* Bβ fibrinogen gene upstream region and previously-characterized GREs from the mouse mammary tumor virus LTR and the rat tyrosine aminotransferase gene to the consensus GRE (Beato, 1989). The GRE at positions −148 to −162 of the *Xenopus* Bβ gene matches the consensus at 9 of the 12 positions. Both half-sites of the element have the G and C nucleotides shown to be critical for receptor binding in the tyrosine aminotransferase gene (Tsai et al., 1988). The distal GRE at −526 to −540 also matches the consensus at 9 of the 12 positions and has the important G and C nucleotides in the left half-site, but it has only one of the two critical nucleotides in the right half-site.

A



B

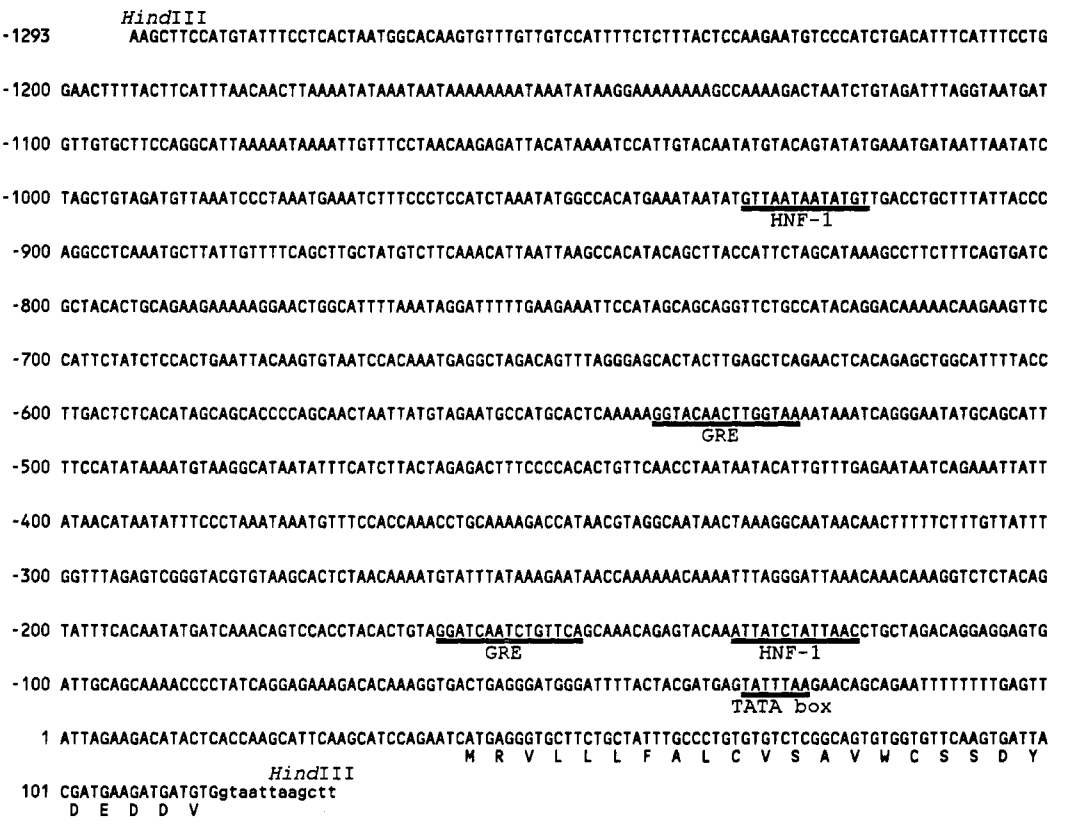


FIGURE 1: Characterization of the gene for the Bβ subunit of *Xenopus* fibrinogen. (A) Restriction map of the genomic DNA clone λBβ425:1. This approximately 15-kb *Xenopus laevis* genomic DNA fragment encompasses the entire Bβ subunit gene. The bent arrow indicates the transcription initiation site, which lies within a 1.4-kb *Hin*dIII fragment. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. (B) Nucleotide sequence of the 1.4-kb *Hin*dIII genomic DNA fragment containing the start site for transcription. This fragment includes the transcription initiation site (+1), upstream base pairs to position −1293, and the RNA coding region to position +128. Underlining marks a TATA box at position −31, two sequences similar to the consensus glucocorticoid response element (GRE) at positions −162 and −540, and two sequences similar to the binding site for the liver-specific transcription factor HNF-1 at positions −132 and −931, with these numbers indicating the 5′-most nucleotide in each site.

In Figure 2B, the putative HNF-1-binding sites in the *Xenopus* Bβ fibrinogen gene upstream region and the HNF-1-binding sites from the rat and human Bβ subunit genes are compared to the consensus HNF-1-binding site (Mendel & Crabtree, 1991). The HNF-1 site at −120 to −132 matches the consensus at 9 of the 12 positions, including a perfect right half-site. The HNF-1 site at −919 to −931 matches the consensus at 8 of the 12 positions, with a perfect left half-site. The sequences flanking the HNF-1 sites also share some similarity, as indicated in the figure.

Identification of an Upstream Element Important for Hormone-Stimulated Expression of the Bβ Fibrinogen Subunit Gene. To demonstrate whether the putative regulatory sites identified above actually play a role in transcriptional control, portions of the Bβ fibrinogen gene promoter and upstream sequence were fused to the luciferase reporter gene in the vector pLucLink 2.0 (d'Emden et al., 1992). These constructs were transfected into purified *Xenopus* liver cells, and luciferase activity was assayed after 40–48 h in culture with or without 10^{-7} M dexamethasone. Such fusion genes containing 2.2 kb of the Bβ gene 5′ flanking sequences supported a hormonal stimulation of transcription of about 14-fold (Figure 3). This level of induction is in approximately the same range as the 10-fold increase in transcription of the natural Bβ fibrinogen gene after 2 h of exposure to dexamethasone, as determined previously in transcription run-on assays (Roberts & Holland, 1993). To determine the location of cis-acting regulatory elements within this 2.2-kb fragment, we generated a set of mutants lacking progressively more of the 5′ flanking sequence. The results showed that full hormone inducibility was maintained when as little as 284 bp of upstream sequence was present (Figure 3). Further deletion to the −162

A

MMTV LTR	(-185)	GTTACAAACTGTTCT (-171)
MMTV LTR	(-129)	GGTATCAAATGTTCT (-115)
Rat TAT	(-2509)	TGTACAGGATGTTCT (-2495)
Rat TAT	(-2426)	GGACTTGTTTGTTCT (-2440)
Xenopus Bβ	(-540)	GGTACAACTTGGTAA (-526)
Xenopus Bβ	(-162)	GGATCAATCTGTTCA (-148)
Consensus GRE		5'-GGTACANNNTGTTCT-3'

B

Rat Bβ	(-104)	ACCAAACTGTCAAATATTAACTAAAGGGAG (-75)
Human Bβ	(-97)	AACCAAAAATTAAATATTAACTAAGGAAAG (-68)
Xenopus Bβ	(-140)	GAGTACAAATTATCTATTAACCTGCTAGAC (-111)
Xenopus Bβ	(-931)	AATAATATGTTAATAATATGTTGACCTGCT (-902)
Consensus HNF-1 Site		5'-GTTAATNATTAAC-3'

FIGURE 2: Putative regulatory elements within the *Xenopus* Bβ fibrinogen subunit gene upstream region. (A) Comparison of GREs in the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Buetti & Kühnel, 1986) and rat tyrosine aminotransferase (TAT) gene (Jantzen et al., 1987) to putative GREs in the *Xenopus* Bβ fibrinogen gene. The numbers indicate the position of each element relative to the transcription initiation site at +1. The more proximal GRE in the rat TAT gene is given in reverse orientation. Underlining denotes nucleotides identical with the consensus. The asterisks mark nucleotides within the consensus demonstrated to be most critical for receptor binding to the distal GRE in the rat TAT gene (Tsai et al., 1988). (B) Sequence comparison of HNF-1-binding sites in the promoter regions of the human (Huber et al., 1990) and rat (Fowlkes et al., 1984; Mendel & Crabtree, 1991) Bβ fibrinogen genes to putative HNF-1-binding sites in the *Xenopus* Bβ gene upstream region. The numbers indicate the position of each sequence relative to the transcription initiation site at +1. Underlining denotes identity with the consensus; a dot shows additional identity between at least three of the four sequences.

position decreased hormone responsiveness to about half. This deletion removes all sequences up to the start of the proximal GRE identified by sequence comparison. This GRE, (-162) GGATCAATCTGTTCA(-148), matches the consensus GRE, 5'-GGTACANNNTGTTCT-3', in 9 out of 12 positions, which are indicated by underlining. When this putative GRE was deleted in the construct which has its 5' end at position -136, glucocorticoid responsiveness was completely eliminated. The partial loss of hormone inducibility upon removal of sequences between -162 and -284 could be due to the removal of an independent element in that region. The simplest interpretation, however, is that glucocorticoid responsiveness is impaired by the substitution of vector sequences for the naturally-occurring nucleotides just upstream of the core GRE sequence at positions -148 to -162. Nordeen et al. have reported similar effects of specific point mutations in nucleotides immediately adjacent to the mouse mammary tumor virus GRE (Nordeen et al., 1990).

Effect of Mutations in the Glucocorticoid Response Element on Hormonal Induction. To confirm the function of the putative GRE at positions -148 to -162 both half-sites of the GRE were mutated in the fragment containing the first 284 bp of upstream sequence. The mutated sequence is shown at the bottom of Figure 4 in comparison to the putative GRE for the Bβ gene and the consensus GRE. Transfection experiments in primary liver cells were carried out with the natural and mutated constructs. The vector with the wild-type GRE, designated -284LL, had the expected glucocorticoid responsiveness of approximately 12-fold (bars on left of Figure 4), whereas hormone inducibility was completely

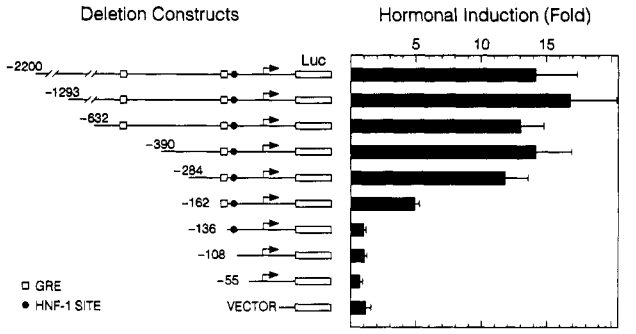


FIGURE 3: Effect of progressive deletion of the 5' flanking sequence of Bβ fibrinogen–luciferase constructs on dexamethasone-stimulated expression in transfected cells. Portions of the 5' flanking region of the *Xenopus* Bβ fibrinogen gene of progressively shorter length (from positions -2200 to -55) were inserted into plasmid vector pLucLink 2.0 upstream of the luciferase gene. All constructs contained the Bβ gene transcription initiation site (indicated by the bent arrow) and sequences to position +40. Twenty-five micrograms of each plasmid was introduced by electroporation into *Xenopus* primary liver cells, cotransfected with 25 μg of pCMVβgal to control for transfection efficiency. Transfected cells were left untreated or treated with 10^{-7} M dexamethasone and 10^{-9} M triiodothyronine. Forty to forty-eight hours after transfection, cell lysates were prepared, and each sample was assayed for luciferase and β-galactosidase activity. Luciferase activity in light units was normalized to β-galactosidase activity as described under Materials and Methods. The bar graph shows hormone-induced transcription relative to basal expression for each construct. Values represent mean fold inductions from five to eight determinations in three to four independent experiments. Error bars represent the standard error of the mean (SEM).

eliminated in the construct with the mutated GRE, designated mGRE (bars in middle of Figure 4). Even in the absence of dexamethasone, expression from the wild-type GRE was slightly elevated compared to the mutated sequence. This effect was probably due to low levels of endogenous glucocorticoids present in the liver cells.

Binding of the Recombinant Rat Glucocorticoid Receptor DNA-Binding Domain to the Glucocorticoid Response Element in the Bβ Fibrinogen Gene Upstream Region. Hormonal stimulation of fibrinogen gene expression in *Xenopus* hepatocytes is mediated by the glucocorticoid receptor since the response is blocked by the antiglucocorticoid RU486 (Bhattacharya & Holland, 1991). To confirm that the putative GRE in the Bβ fibrinogen gene upstream region physically interacts with the glucocorticoid receptor, we undertook DNA–protein-binding studies. Glucocorticoid receptor from *Xenopus* liver has been characterized (Lange & Hanke, 1988; May & Westley, 1982), but it has not been extensively purified. Our preliminary efforts to purify unactivated receptor from liver cytosol indicated that hormone-free receptor is present in relatively low abundance (unpublished data). The level of unbound receptor cannot be increased by adrenalectomy, as is done in mammals, because the adrenal and renal tissues are closely associated in amphibians (Hanke, 1978). Therefore, we used glucocorticoid receptor from rat in electrophoretic mobility-shift assays. A truncated form of the rat glucocorticoid receptor containing the DNA-binding domain of the protein was produced in *E. coli* from a cloned receptor cDNA (Luisi et al., 1991). This recombinant receptor fragment was incubated with a labeled double-stranded synthetic oligonucleotide containing the sequence of the Bβ GRE and surrounding nucleotides from -142 to -168. DNA–protein complexes were then distinguished from free DNA by their different mobility in nondenaturing polyacrylamide gel electrophoresis. Figure 5 shows bands representing the free DNA probe (F) and DNA occupied by one (I) or two (II) protein molecules (Tsai et al., 1988; Dahlman-Wright et al., 1991).

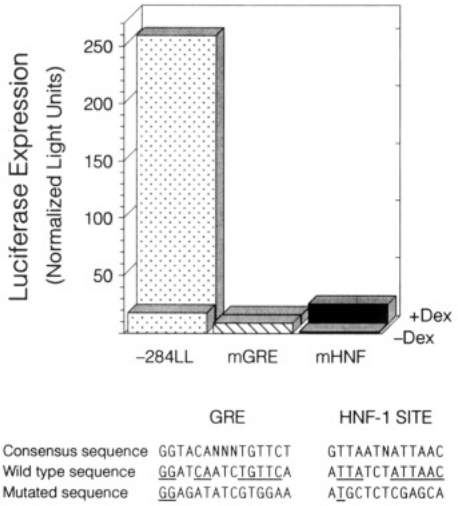


FIGURE 4: Effect of mutation of the proximal GRE and HNF-1-binding site of the Bβ fibrinogen gene on expression of Bβ fibrinogen–luciferase DNA constructs transfected into primary liver cells. The putative GRE at positions –148 to –162 in the Bβ fibrinogen–luciferase clone containing Bβ gene sequences from –284 to +40, designated –284LL, was mutated by oligonucleotide-directed mutagenesis, replacing five of six nucleotides in one half-site and three of six in the other half-site to produce plasmid construct mGRE. The putative HNF-1-binding site at positions –120 to –132 in clone –284LL was mutated by oligonucleotide-directed mutagenesis, replacing all six nucleotides in one half-site and two of six in the other half-site to produce plasmid construct mHNF. Transient transfections with the wild type and mutated constructs were carried out as before, plus or minus 10^{-7} M dexamethasone and 10^{-9} M triiodothyronine. Values represent the mean luciferase expression in light units normalized to cotransfected β-galactosidase activity from duplicate determinations in two independent experiments. The stippled bars represent the wild-type clone, –284LL; the diagonally hatched bars the mutated GRE clone, mGRE; and the solid bars the mutated HNF-1 clone, mHNF. In each set, untreated samples (–Dex) are in front of hormone-treated samples (+Dex). At the bottom, the wild-type and mutated sequences are shown in comparison to the respective consensus sequences, with underlining of the nucleotides that match the consensus.

Distinct binding occurred with the putative Bβ GRE oligonucleotide (lane 2) which was competed off by an excess of the same oligonucleotide which was not radioactively labeled (Xen GRE, lane 3) or by an oligonucleotide containing a perfectly palindromic GRE (pal GRE, lane 5). By contrast, an excess of the mutated *Xenopus* GRE (mut GRE, lane 4) or of a nonspecific oligonucleotide (rat HNF, lane 6) was unable to block the mobility shift. Since the palindromic GRE (pal GRE) appeared to compete the DNA–protein complex more efficiently than the Bβ GRE (Xen GRE), a range of lower concentrations of unlabeled pal GRE competitor oligonucleotide was used to estimate the relative difference in binding affinity between Xen GRE and pal GRE (lanes 7–9 of Figure 5). A 100-fold excess of pal GRE was shown to compete off approximately as much of the labeled Xen GRE as a 1000-fold excess of Xen GRE (Figure 5, lane 7). Therefore, the glucocorticoid receptor DNA-binding domain may bind to the Xen GRE with about 10-fold lower affinity than to the perfectly palindromic GRE.

Identification of an Upstream Element Important for Hormone-Independent Expression of the Bβ Fibrinogen Subunit Gene. To investigate the role of cis-acting sequences other than the GRE, we examined transcription in the absence of dexamethasone. Analysis of basal expression of the Bβ gene was, however, complicated by the fact that primary cells were transfected as soon as they were isolated from frogs. They therefore still contained low endogenous levels of glucocorticoids. As long as the GRE was part of the

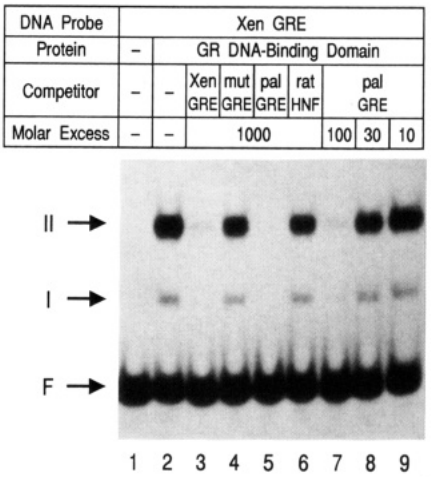


FIGURE 5: Binding of the proximal GRE of the Bβ fibrinogen subunit gene to the DNA-binding domain of the rat glucocorticoid receptor. An end-labeled 27-bp double-stranded oligonucleotide probe containing the proximal GRE of the *Xenopus* Bβ fibrinogen gene was mixed with the DNA-binding domain (amino acids 440–525) of rat glucocorticoid receptor that had been synthesized from cloned DNA in *E. coli*. Complexes were resolved by the electrophoretic mobility-shift assay. Bands representing the free DNA probe (F) and DNA occupied by one (I) or two (II) protein molecules are indicated. Double-stranded competitive oligonucleotides used were the following: Xen GRE, the proximal GRE of *Xenopus* Bβ fibrinogen; mut GRE, an oligonucleotide containing the same mutations in the proximal GRE of *Xenopus* Bβ fibrinogen as in the mutant transfection construct –284mGRE; pal GRE, an oligonucleotide containing a perfectly palindromic GRE; rat HNF, an oligonucleotide containing the HNF-1-binding site from the rat Bβ fibrinogen gene. See Materials and Methods for the complete sequences of these oligonucleotides.

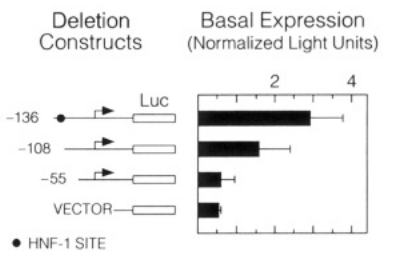


FIGURE 6: Effect of progressive deletion of the 5′ flanking sequence of Bβ fibrinogen–luciferase constructs on transcription in transfected cells. Bβ fibrinogen–luciferase constructs containing 136, 108, or 55 bases of upstream sequence were transiently transfected into primary liver cells as in Figure 3 and maintained without dexamethasone and triiodothyronine. Luciferase expression was normalized to cotransfected β-galactosidase expression as before. Values represent the mean and SEM of three determinations.

transfection construct, transcription was somewhat elevated even in the absence of added dexamethasone because of the endogenous hormone. It is likely that the core GRE sequence is the only determinant of this residual responsiveness because once the GRE sequence was removed, either by deletion in the –136 construct (Figure 3) or by specific point mutations (Figure 4), the level of transcription was no longer influenced by dexamethasone.

To identify additional DNA sequences that regulate transcription, we used deletion mutagenesis in the region between –136 and the promoter. Deletion to position –108 caused a drop to about half of the previous level, and further deletion to –55 caused another drop of about the same amount to a level of expression equivalent to that obtained from the promoterless vector (Figure 6). These results suggest that sequences between –55 and –136 are important for the function of the Bβ fibrinogen promoter. Since analysis of the nucleotide sequence of the upstream region revealed a possible binding

site for hepatocyte nuclear factor 1 from positions –120 to –132, further experiments were performed to determine the importance of this sequence in transcriptional control of the *Xenopus* Bβ gene.

Effect of Mutations in the Hepatocyte Nuclear Factor 1-Binding Site on Transcriptional Activity of Transfected DNA Constructs. To confirm the function of the putative HNF-1-binding site at positions –120 to –132, both halves of this site were mutated in a construct that contained the first 284 bases of upstream sequence. A comparison of the consensus HNF-1 site with the sequence of the putative proximal Bβ HNF-1 site and the mutated Bβ HNF-1 sequence is shown at the bottom of Figure 4. In transfection experiments, this construct, designated mHNF, showed a significant drop in expression in both untreated and hormone-treated cells to 8–10% of the levels for the wild-type clone (compare bars on right with bars on left of Figure 4). In contrast, transcription was only reduced to about 50% (Figure 6) when the HNF-1 site was eliminated by deletion from the 5′ end rather than by point mutations. The data obtained from the construct with specific nucleotide substitutions in the HNF-1 site probably more accurately reflect the importance of this sequence since more of the natural promoter/enhancer context is preserved.

Since mHNF retains the wild-type GRE at positions –148 to –162, the effect of loss of HNF activity on hormone responsiveness could be determined. The fold induction of luciferase expression in response to dexamethasone was 14-fold for the wild-type clone and 11-fold for the mutated clone. Thus, although mutation of the HNF-1-binding site significantly reduced the level of transcription of the gene, the ability to respond to glucocorticoids was not affected.

Binding of in Vitro-Translated Mouse Hepatocyte Nuclear Factor 1α to the Hepatocyte Nuclear Factor 1-Binding Site in the Bβ Fibrinogen Gene Upstream Region. To confirm that the HNF-1-like site we have identified in the Bβ gene upstream region actually binds HNF-1 protein, we used the HNF-1α isoform from mouse (Kuo et al., 1990) produced from a cDNA clone by *in vitro* transcription and translation. Figure 7 shows the results of binding of this protein to DNA in mobility-shift experiments. The HNF-1α protein formed a complex with the putative HNF-1-binding site oligonucleotide (Xen HNF, lane 3) which was identical in gel mobility to that formed with the well-characterized rat Bβ fibrinogen HNF-1-binding site (rat HNF, lane 2). Complex formation was specifically blocked by an excess of the rat HNF or Xen HNF oligonucleotides which were not radioactively labeled (lanes 4 and 5). In contrast, an excess of the mutated *Xenopus* HNF-1-binding site (mut HNF) or of a nonspecific oligonucleotide (pal GRE) was unable to block the gel shift (lanes 6 and 7).

Effect of Internal Deletions within the Upstream Sequence on Hormonal Induction of Bβ Fibrinogen–Luciferase Constructs. Figure 3 showed that in deletions proceeding from the 5′ end, elimination of the putative distal GRE did not affect glucocorticoid responsiveness of the Bβ promoter. This element, (–540)GGTACAACTTGGTAA(– 526), matches the consensus GRE perfectly in the left half-site, as shown by the underlining. However, both the G at position –529 and the A at position –527 would be unfavorable for response element function, based on mutation analysis of the mouse mammary tumor virus GRE (Nordeen et al., 1990). Because of this suboptimal sequence, the element should perhaps be considered an isolated half-site. While half-sites are ineffective by themselves, they can contribute to the hormonal inducibility of genes in which they occur (Chalepakis et al., 1988).

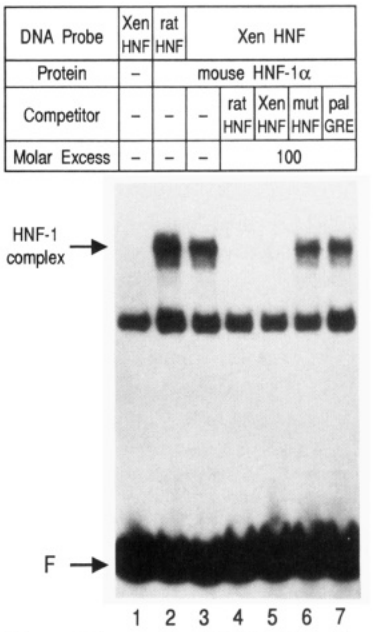


FIGURE 7: Binding of the proximal HNF-1-binding site of the Bβ fibrinogen gene to *in vitro*-translated mouse HNF-1α. End-labeled 30-bp double-stranded oligonucleotide probes containing the proximal HNF-1-binding site of the *Xenopus* Bβ fibrinogen gene or the rat Bβ gene HNF-1-binding site were used. The binding reactions were carried out with *in vitro* translation mix containing no added mRNA (lane 1) or *in vitro*-translated mouse HNF-1α protein (lanes 2–7), and the indicated molar excesses of double-stranded competitor oligonucleotide. Competitor oligonucleotides used were as follows: rat HNF, the rat Bβ fibrinogen HNF-1-binding site; Xen HNF, the proximal HNF-1-binding site of the *Xenopus* Bβ gene; mut HNF, an oligonucleotide containing the same mutations in the proximal HNF-1-binding site of the *Xenopus* Bβ gene as in the mutant transfection construct –284mHNF; pal GRE, an oligonucleotide containing a perfectly palindromic GRE. See Materials and Methods for the complete sequences of these oligonucleotides. The electrophoretic mobility shift assay was used to distinguish free DNA (F) from the DNA–HNF-1 complex. The additional complex present in all lanes is formed by a component of the rabbit reticulocyte lysate.

To determine whether the function of this or other regulatory elements is masked by more proximal sequences, two sets of internal deletion mutations were constructed. One set included the start site and sequences upstream to position –108, followed by deletion of a variable number of nucleotides upstream of that position (Figure 8A). The second set had the start site and sequences upstream to position –136, followed by a similar series of internal deletions (Figure 8B). This second set contained the putative HNF-1-binding site at positions –120 to –132.

Transfection experiments showed that none of the first set of internal deletion constructs, commencing from position –108 and proceeding upstream, retained full hormone responsiveness. The only construct of the first set that was partially hormone responsive was missing the HNF-1 site, bases –109 to –135, but still contained the proximal GRE (Figure 8A). It is likely that the decrease in hormonal activation in this construct was due to the relative change in position of the GRE rather than to loss of the HNF-1 site itself, since point mutations in the HNF-1 element did not interfere with hormone responsiveness (Figure 4). Progressing further upstream with the internal deletions, glucocorticoid inducibility was completely lost once the proximal GRE was eliminated. Thus, the GRE at positions –526 to –540 was not able to substitute for the proximal –148 to –162 GRE, even when brought into close proximity with the promoter.

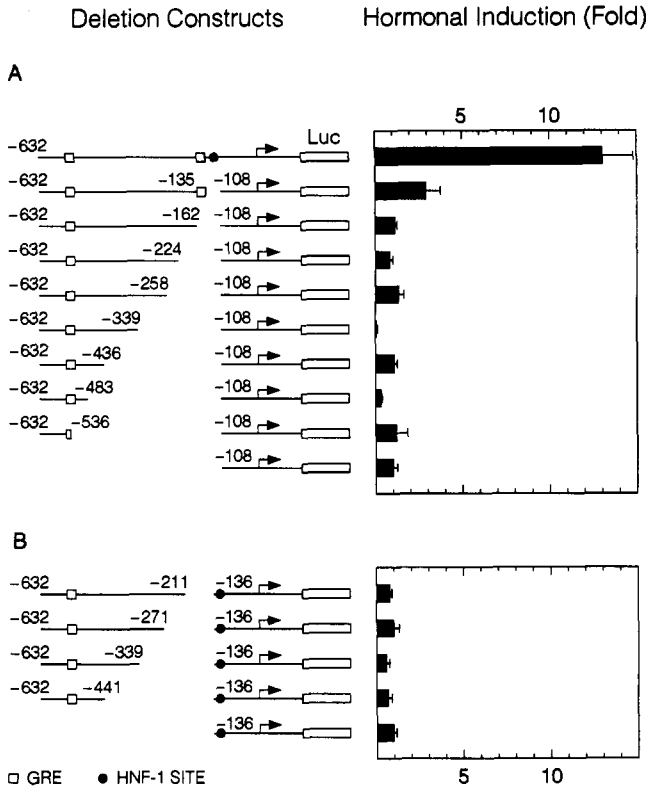


FIGURE 8: Effect of internal deletions within the upstream sequence on hormonal induction of Bβ fibrinogen–luciferase DNA constructs containing promoter sequences to −108 or −136. All Bβ fibrinogen–luciferase constructs contained the Bβ gene transcription initiation site (indicated by the bent arrow), sequences downstream to position +40, and sequences upstream either to position −108 (A) or to position −136 (B), but were missing increasing lengths of upstream internal sequence. The adaptor sequence at the internal junction was 5′-CTGCGGCCGCAAGCTTG-3′. In some cases, one or two nucleotides that were originally at the ends of the adaptor matched the reconstructed sequence of the Bβ DNA and were, therefore, included in the numbering of the upstream nucleotides. These constructs were transiently transfected into primary liver cells as before. Luciferase expression was normalized to cotransfected β-galactosidase expression as described under Materials and Methods and the fold induction over basal levels determined. Values represent the mean and SEM of three to six determinations in two to three independent experiments.

The results were similar with the second set of 3′ internal deletion constructs, which removed sequences upstream from position −136 (Figure 8B). Members of this set of constructs retained the HNF-1-binding site but were uniformly unresponsive to hormone in the absence of the proximal GRE. Therefore, the inability of the distal GRE to function in the previous experiment was not due to the absence of the HNF-1 site. In summary, it is unlikely that other functional glucocorticoid-responsive sequences besides the one at −148 to −162 are present within 632 bases upstream of the start site.

DISCUSSION

We have isolated the *Xenopus laevis* Bβ fibrinogen subunit gene and have identified two DNA sequences close to the promoter that are critical for transcriptional regulation: a glucocorticoid response element (GRE) and a binding site for the liver-specific protein hepatocyte nuclear factor 1 (HNF-1). These elements were characterized by transfection of portions of the fibrinogen gene regulatory region into highly-purified primary hepatocytes from *Xenopus*. This homologous transfection system mimics the natural context of transcriptional control of the Bβ gene, and we have shown that transcription of transfected gene constructs accurately reflects the fold stimulation of the chromosomal Bβ gene measured by run-on transcription assays. Endogenous genes are, of course, exposed to additional levels of control through distal DNA sequences and complex chromatin organization. Nonetheless, transfection methodology is a powerful approach for revealing crucial DNA–protein interactions that operate in intact cells to control gene expression. The use of freshly-isolated liver cells is important because transformed cells do not retain all normal functions and can lead to misinterpretation of the role of specific factors in regulating particular genes (Johnson, 1990).

GREs vary greatly between different glucocorticoid-regulated genes with respect to their number, spacing, orientation, and nucleotide sequence, with some genes requiring additional factors that function through a complex glucocorticoid response unit (GRU) (Lucas & Granner, 1992). Therefore, for any glucocorticoid-regulated gene, it is necessary to establish the nature of the hormone-responsive sequences. The GRE for the frog Bβ fibrinogen gene is located at positions −148 to −162 and matches the consensus GRE at 9 out of 12 positions. The consensus GRE has been compiled from the sequences required to regulate a variety of glucocorticoid-inducible genes from several species (Beato, 1989). It consists of 12 conserved nucleotides arranged as 2 half-sites of 6 nucleotides each separated by 3 noncritical nucleotides. The half-sites are imperfect inverted palindromic repeats (see Figure 2).

Transfection experiments, site-directed mutagenesis, and electrophoretic mobility-shift assays were used to confirm the functional significance of the GRE in the *Xenopus* Bβ gene. Mutations within this sequence completely abolish glucocorticoid responsiveness of a fibrinogen–luciferase chimeric clone containing Bβ gene sequences from −284 to +40. That the site physically associates with the glucocorticoid receptor was shown in mobility-shift assays with an oligonucleotide containing the GRE and a protein consisting of the DNA-binding domain of the rat glucocorticoid receptor. The receptor binds to the frog GRE about 10-fold less efficiently than to a perfectly palindromic GRE, probably because of the unfavorable bases at −159 (T instead of A) and −148 (A instead of T). This finding is not unusual, however, because most natural hormone-response elements are suboptimal (Nordeen et al., 1990; Jantzen et al., 1987). A sequence further upstream at positions −526 to −540 contains a perfect GRE half-site, but has a poor fit to the consensus at the other putative half-site. Deletion of this GRE had no effect on hormone responsiveness of the gene. Constructs with internal deletions which eliminated the proximal GRE and brought the distal sequence closer to the promoter were noninducible. Therefore, the upstream sequence apparently has no role in mediating glucocorticoid stimulation of the Bβ gene.

In addition to the glucocorticoid-dependent site described above, a hormone-independent transcriptional regulatory element was found between the GRE and the transcription initiation site. In transfection vectors containing the −284 to +40 region of the Bβ gene, mutations between −120 and −132 reduced expression by 10–12-fold, without affecting the fold stimulation of transcription by glucocorticoids. The region contains a consensus binding site for hepatocyte nuclear factor 1 which was shown in mobility-shift assays to interact with the α isoform of HNF-1 from mouse. The mammalian protein was used for these investigations because it could be produced in a highly-purified form. The *Xenopus* homologue of HNF-1 has recently been identified in frog liver, and its cDNA has

been cloned (Bartkowski et al., 1993). When *in vitro*-expressed *Xenopus* HNF-1 becomes available, it will be of interest to confirm that it binds to the regulatory element between −120 and −132. HNF-1 plays a key role in regulating the expression of several liver-specific genes. Although it is also found in other tissues, HNF-1 is one of a small number of transcription factors implicated in establishing and maintaining the liver phenotype (Mendel & Crabtree, 1991; Bartkowski et al., 1993). Consistent with this role, HNF-1 contains structural elements distantly related to the POU domain and homeodomain, two sequence motifs present in several proteins involved in determining cell fate (Mendel & Crabtree, 1991).

Since transcription of the fibrinogen subunit gene family is highly coordinated in several species, it is instructive to compare the underlying regulatory mechanisms operating in distantly-related animals. Such analyses will show which aspects of control have been conserved, and which are quite different, revealing different means of achieving the same final outcome of concerted control of these genes. Other laboratories have developed transfection systems in continuous cell lines to investigate transcriptional regulation of human and rat fibrinogen genes, and the most detailed information is available for the Bβ subunit gene. With regard to liver-specific transcription, both the human and rat Bβ genes, like that of *Xenopus*, have an HNF-1-binding site near the start site which enhances basal transcription (Huber et al., 1990; Courtois et al., 1987; Baumann et al., 1990). The rat Aα fibrinogen subunit gene and several other liver-expressed genes also have HNF-1 sites close to their promoters (Mendel & Crabtree, 1991; Courtois et al., 1988), but it is interesting that the rat γ subunit gene does not have a recognizable site within at least 650 bp upstream (Courtois et al., 1987). Apparently the γ gene uses either a modified mechanism involving a more distal HNF-1 site or entirely different factors to signal liver-specific expression.

The glucocorticoid-responsive sequences in the Bβ genes are arranged quite differently in human, rat, and frog. We have shown here that the *Xenopus* gene has a single canonical GRE close to the promoter at positions −148 to −162, which is sufficient to account fully for the hormonal regulation of the endogenous gene in normal liver cells. In the rat Bβ gene, a fragment slightly further upstream, from position −193 to position −349, confers dexamethasone inducibility on constructs transfected into the human HepG2 cell line (Baumann et al., 1990). The specific nucleotides responsible have not been precisely located, but this stretch of DNA does not contain a sequence resembling the consensus GRE, suggesting that an unusual mechanism may be involved. The hormone stimulates transcription about 5-fold when this regulatory region drives expression of the Bβ gene promoter and about 60-fold when it is upstream of a heterologous promoter (Baumann et al., 1990). It is uncertain how accurately these transfection data represent natural regulation of the gene since variable results have been obtained for dexamethasone induction of the fibrinogen genes in rat liver primary cells (Otto et al., 1987; Princen et al., 1984). The rat Aα and γ subunit genes also contain glucocorticoid-responsive sequences within 600–800 bp of the promoter, but these elements have not been analyzed in detail (Baumann et al., 1990). In contrast to the frog and rat, the glucocorticoid regulatory region of the human Bβ subunit gene lies quite far upstream. Using transfection into HepG2 cells, Huber et al. located the hormone-responsive domain between −1500 and −2900 but did not map more closely the exact position or sequence (Huber et al., 1990). This region supports a 2-fold glucocorticoid stimulation of transcription of transfected gene constructs. In the same study, the chromosomal Bβ gene in the HepG2 cells was dramatically induced after 24 h of hormone treatment, though no change was detectable after 5 h. The discrepancy between the endogenous and transfected genes could be due to the presence of as yet unidentified glucocorticoid-responsive sequences, to greater stability of Bβ mRNA, or to biological differences between untransfected and transfected hepatoma cells.

The mammalian fibrinogen subunit genes are also regulated by interleukin 6 (IL-6). In the human Bβ gene, the IL-6-responsive element lies between −82 and −150 (Huber et al., 1990), and in the rat gene between −134 and −168 (Baumann et al., 1990). Thus, in these two species, the IL-6 domain is close to both the transcription start site and the tissue-specific HNF-1-binding site. The HNF-1 site in the human Bβ gene not only regulates the basal level of transcription but also is necessary for inducibility by IL-6 through the IL-6 response element, indicating that the two elements are functionally coupled in this case (Dalmon et al., 1993). We have no evidence that human IL-6 can regulate transcription of either endogenous or transfected fibrinogen genes in *Xenopus* liver cells, using two different preparations of this cytokine (Bhattacharya, Roberts, Woodward, and Holland, unpublished experiments). It seemed plausible that human IL-6 might function in the frog system because it induces fibrinogen synthesis in chicken hepatocytes (Amrani, 1990). On the other hand, perhaps the negative result is not surprising since other cytokines are not highly conserved between mammals and amphibians (Watkins & Cohen, 1987; Watkins et al., 1987).

In summary, we have developed a transfection system in primary liver cells that accurately reflects the hormonal control of transcription of the chromosomal Bβ fibrinogen subunit gene. In addition to the major glucocorticoid receptor and HNF-1-responsive sequences described here, experiments are in progress to define the full complement of factors utilized in normal cells for transcriptional control, not only of the Bβ gene but also of the Aα and γ subunit genes as well. Because expression of the three genes is tightly coordinated, the mechanisms of control probably have some common features. It is also likely, however, that differences have evolved over the 500 million years or more since these genes diverged from a common ancestral sequence (Doolittle, 1983; Henschen et al., 1983; Fu et al., 1992). Thus, comparison between the three genes and between species will give insight into how different combinations of transcription factors elicit similar gene responses.

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