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Glucocorticoid Stimulates Primate but Inhibits Rodent α-Fetoprotein Gene Promoter

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Glucocorticoids inhibit rodent α-fetoprotein (AFP) gene activity but stimulate expression of the human homologue. Like human, activity of the AFP promoter from other primates was stimulated by the synthetic glucocorticoid dexamethasone (Dex) in various cell lines. A glucocorticoid responsive element (GRE) is located within 180 bp upstream of the transcription initiation site of all AFP genes examined. Comparative analysis of the GRE in the two different groups of promoters revealed a common 3′ hexamer, 5′-TGTCCT-3′, but the 5′ hexamers were different. This difference converts the rodent GRE to a DR-1 motif. DR-1 is a binding site for members of the nuclear receptor superfamily including the orphan receptor hepatocyte nuclear factor-4 (HNF-4). The presence of DR-1 in the rodent but not human may underlie the opposite actions of Dex on the AFP promoter. We tested this hypothesis using a transient transfection assay. In hepatoma cells that expressed GR and HNF-4, reporter-activity was inhibited by Dex. The same construct in nonhepatoma cells was strongly induced by over expression of HNF-4 and the induced activity was inhibited by Dex. The findings show that Dex induction of human AFP is mediated by a GRE. But Dex repression of the rodent promoter requires a DR-1 motif that interacts with GR and HNF-4. © 2001 Academic Press

***Key Words:* α-fetoprotein; glucocorticoid responsive elements; direct repeat-1; glucocorticoid receptor; and hepatocyte nuclear factor-4.**

Abbreviations used: AFP, α-fetoprotein; GRE, glucocorticoid responsive elements; DR-1, direct repeat-1; GR, glucocorticoid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; PBS, phosphate buffered saline; Dex, dexamethasone; PCR, polymerase chain reaction; HNF, hepatocyte nuclear factor; Luc, luciferase; GSTP, glutathione *S* transferase P; and MMTV, mouse mammary tumor virus.

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α-fetoprotein (AFP) is an oncofetal serum protein that is abundantly expressed in yolk sac and liver during embryonic development (1–3). Previous studies showed that glucocorticoids decreased *in vivo* transcription of the AFP gene in neonatal rodents (4–10). Similarly, this effect of glucocorticoids on AFP have also been observed *in vitro,* using Morris hepatomas grown intramuscularly (11) and in cultured McA-RH 7777 rat hepatoma cells (12, 13). The inhibitory action of glucocorticoids on AFP expression in rodent is believed to play a major role in the down-regulation or "shut-off" of this gene in the developing liver (reviewed by Chen *et al.*) (14). Studies of the rat AFP promoter showed glucocorticoid inhibition was mediated by a DNA fragment spanning −172 to −150 of the gene (10). DNase I footprinting confirmed the binding of the glucocorticoid receptor (GR) to a motif, −168 to −150 within this fragment (10). A homologous region with similar features spans nucleotides −169 to −151 of the mouse AFP gene (15).

In contrast to the rodent, previous reports including our own show glucocorticoids increase AFP expression in several human hepatoma cell lines; HuH-7, Hep 3B, huH-4, KIM-1/c-4 and PLC/PRF/5, and human hepatoblastoma cell lines; HuH-6, huH-1, and Hep G2 (16–18). This increase arises from glucocorticoid stimulation of gene transcription (19), a response that requires the AFP gene fragment −175 to −161. Within this DNA fragment, there is a motif that resembles the consensus glucocorticoid responsive element, GRE. Site directed mutation of this motif decreases response to glucocorticoids (19).

Hepatocyte nuclear factor-4 (HNF-4) is an orphan member of the nuclear receptor superfamily (20) comprised of transcription factors whose activities are triggered in response to the binding of steroid hormones, thyroid hormone, vitamins A or D, and other lypophilic ligands. Members of this family bind to motifs comprised of hexameric (5′-AGKTCA-3′) repeats separated

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by variable number of nucleotides (21–23). HNF-4 is predominantly expressed in liver (20) but it is also found in the kidney, stomach, and intestine (24, 25). HNF-4 is crucial for liver-specific transcription of AFP (26 and Nakabayashi *et al.*, unpublished data) and a number of other genes expressed during liver differentiation and development (27–31). In this report, we have analyzed the opposite effects of glucocorticoids on expression of the AFP gene in rodents and primates. Our findings suggest that a subtle difference between the GREs from the two species enables HNF-4 to act along with GR in the rodent motif and thus give rise to the differential actions of glucocorticoids on AFP.

MATERIALS AND METHODS

Cell cultures and transient transfection analysis of plasmid DNA. The human hepatoma cell-lines, HuH-7 (32) and human yolk sac tumor cell line TG-1 (33) were cultured in ISE-RPMI medium (34) supplemented with 1% fetal calf serum (FCS), 100 units/ml of penicillin and 100 μg/ml of streptomycin (GIBCO). The rat Morris hepatoma cell-line McA-RH 7777 (35) and monkey kidney derived cell line, COS-7 (36) were grown in ISE-RPMI medium supplemented with 10 and 5% FCS, respectively. Cells were transiently transfected with plasmid DNA using the calcium-phosphate DNA precipitation method (37) with minor modifications. Twenty-four hours prior to transfection, 1×10^6 cells were mixed with 2 ml of medium and plated onto a 35-mm diameter dish (Falcon 6-well multi plate). At the time of transfection, the cells were incubated in 2 ml of Dulbecco's modified Eagle medium supplemented with 10% FCS containing 0.5 μg of reporter, 0.25 μg effector, and 0.25 μg internal control pRL (Renilla luciferase)-TK (Promega) plasmid DNA in 25 μl of 0.25 M $CaCl_2$ and mixed with 25 μl of 2× BBS (50 mM BES [*N*,*N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid], 280 mM NaCl, 1.5 mM Na_2HPO_4 (pH 7.0). Four hours after transfection, the precipitate was removed by rinsing the cells with PBS, and 4 ml of serum-free fresh medium with or without Dex was added. After a further 48-h incubation, cells were harvested and luciferase (Luc) assay was performed using a Dual-Luc Reporter Assay System (Promega) according to the manufacturer's instructions. Renilla Luc activity was used to normalize transfection efficiencies among experiments.

Preparation of whole cell extract from transfected COS-7 cells. COS-7 cells were transfected with expression vectors including GR, 6RGR (gift from Dr. K. R. Yamanoto, University of California, CA, 38) and retinoid X receptors (RXR) α, RXRα/6R (39) followed by treatment with 3×10^{-6} M Dex or with 1×10^{-6} M all-*trans* retinoic acid for 48 h prior to whole cell extract. COS-7 cells were also transfected with HNF-4 expression vector, pLEN4S (gift from Dr. F. M. Sladek, University of California, CA, 20) and all cell extracts were prepared according to the method of Ladias *et al.* (40).

Electrophoretic gel-mobility shift assays. Gel-mobility shift assays were performed according to methods described previously (34). In brief, double-stranded oligonucleotides corresponding to the elements of interest were synthesized (Fig. 4A) with either a 5′-GATCT-3′ or 5′-AGCTT-3′ sequence at 5′-end and annealed together. These oligonucleotides were radiolabeled by filling-in the 3′-recessed ends with [α-^{32}P]dATP and klenow fragment. For competition assays, an excess amount of unlabeled DNA was added to the nuclear extract at room temperature and incubated for 20 min prior to the addition of labeled probe. The oligonucleotide sequences employed as specific competitors for HNF-4 is derived from element B of human ApoCIII DNA −66 to −87 (5′-GCGCTGGGCAAAGG-TCACCTGC-3′) (20, 23), the GR binding site (5′-TTGGTTACA-AACTG-TTCT-3′) is homologous to the mouse mammary tumor virus, MMTV DNA −186 to −169 (41). In supershift experiments, HuH-7 nuclear extract (34) was preincubated with a goat polyclonal HNF-4 antibody (Santa Cruz Biotechnology Inc.) or goat preimmune IgG.

Construction of Luc reporter constructs. The plasmid, pGL3-basic (Promega) is the base vector for inserting the human, gorilla, chimpanzee, orangutan, mouse, or rat AFP promoter to yield; phAF[−178/+45]-Luc, pgAF[−244/+45]-Luc, pcAF[−244/+45]-Luc, poAF[−244/+45]-Luc, pmAF[−178/+45]-Luc, prAF[−180/+25]-Luc. The numbers in the name of each construct denotes the DNA fragment obtained by PCR amplification and inserted into the *Sma*I and *Hind*III site of the base vector. The PCR products were obtained using specific oligonucleotides and template of genomic DNA from; human (*Homo sapiens*, Japanese), gorilla (*Gorilla gorilla*), chimpanzee (*Pan troglodytes*), orangutan (*Pongo pygmaeus*), mouse (*Mus musculus*, strain 129), and rat (*Rattus norvegicus*, strain Wistar), respectively. The deletional constructs; phAF[−172/+45]-Luc, phAF[−169/+45]-Luc, phAF[−162/+45]-Luc, phAF[−132/+45]-Luc, phAF[−125/+45]-Luc, phAF[−98/+45]-Luc, and phAF[−57/+45]-Luc were derived from phAF[−178/+45]-Luc using PCR and the corresponding primers. The plasmids pmAF[−170/+45]-Luc, pmAF[−131/+45]-Luc, pmAF[−99/+45]-Luc, pmAF[−60/+45]-Luc, prAF[−132/+45]-Luc, and prAF[−62/+45]-Luc were from pmAF[−178/+45]-Luc and prAF[−180/+45]-Luc, respectively. The plasmids ph/mAF[−178/+45]-Luc and pm/hAF[−178/+45]-Luc were constructed from phAF[−178/+45]-Luc and pmAF[−178/+45]-Luc using primers corresponding to the human AFP promoter −178 to −160 (5′-gatcCAAAGAGCTCTGTGTCCTT-3′) and the mouse AFP promoter −178 to −158 (5′-gaTCTGAAGTGGTCTTTGTCCTT-3′), respectively.

Construction of GST-promoter driven Luc plasmids with various AFP oligonucleotides. For functional assay of individual elements, the double-stranded oligonucleotides were inserted into *Sma*I site of pGL3-GST-P. The pGL3-GST-P was driven by 72-bp of rat glutathione *S* transferase P (GST-P) gene promoter which contains TATA- and GC-box (42) as shown in Fig. 4B. Various lengths and positions of the mouse (or rat) and human AFP 5′-flanking (Fig. 4A) were inserted at the *Sma*I site of pGL3-GST-P. These constructs were transfected separately into non-hepatic cell line COS-7.

RESULTS

Opposite Effect of Dex on Rodents and Primates AFP Promoter in Liver Cells

To demonstrate the opposite actions of Dex on the AFP promoter from rodents (rat or mouse) and primates (human, gorilla, chimpanzee or orangutan), activity of reporter constructs containing −244 to +45 or less of the respective DNA fused to Luc were transfected into 3 separate cell lines; HuH-7, TG-1, and McA-RH 7777. Luc-activity in HuH-7 cells containing the human AFP reporter, phAF[−178/+45]-Luc was stimulated 9-fold by 10^{-4} M Dex in a dose dependent fashion (Fig. 1A-a). Dex increased Luc-activity 35-fold in cells co-transfected with the reporter and 0.125 μg of a plasmid, 6RGR that expressed the glucocorticoid receptor (Fig. 1A-a).

In contrast, Dex inhibited Luc-activity to 65% of control in the same cells containing the mouse AFP reporter, pmAF[−178/+45]-Luc. A further reduction to 30% was observed following Dex treatment of cells containing 6RGR (Fig. 1A-b). Although the magnitude

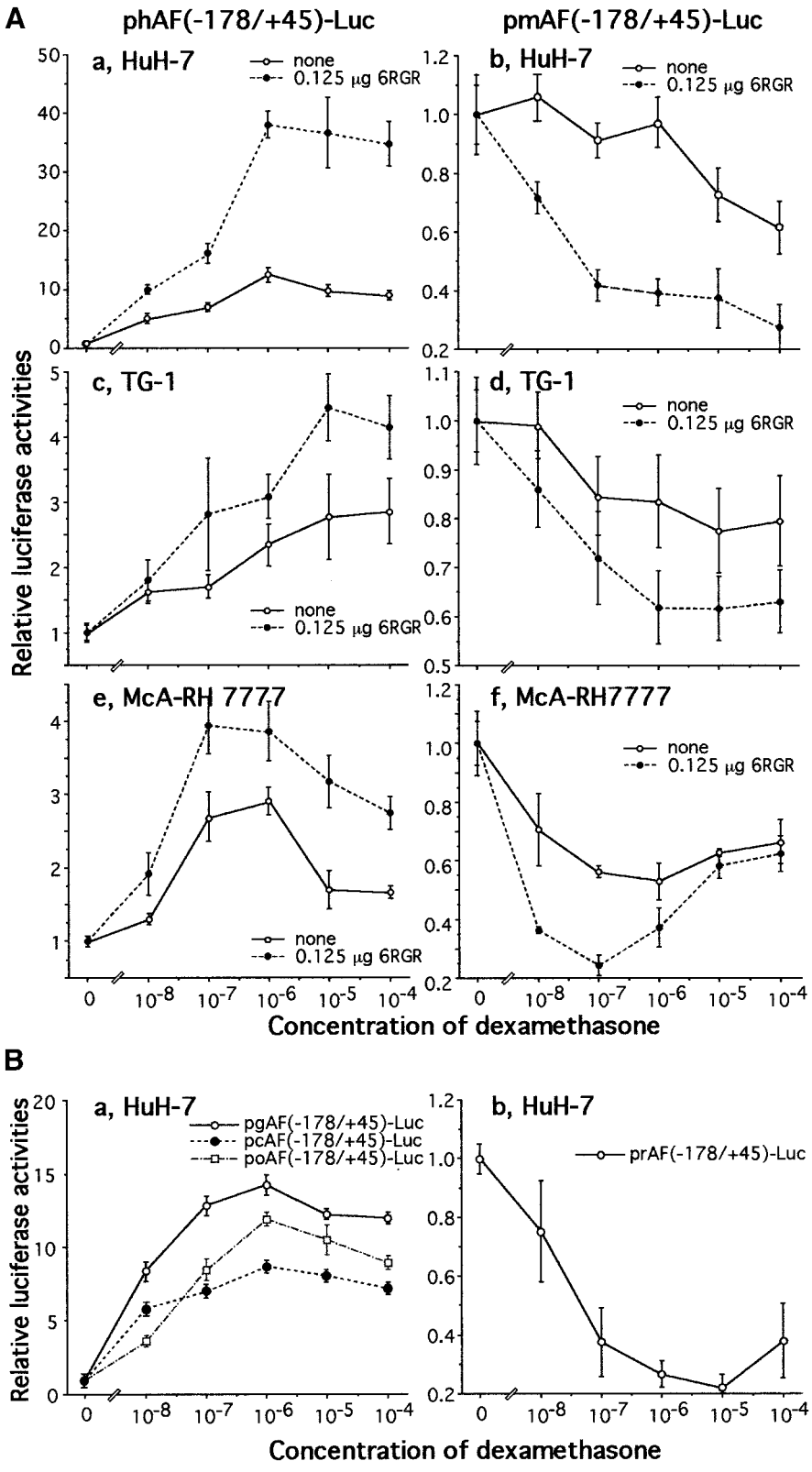

FIG. 1. Effect of Dex on the primate and rodents AFP promoter activity. (A) The plasmids phAF(−178/+45)-Luc and pmAF(−178/+45)-Luc carrying the human and mouse AFP promoter from −178 to +45 bp regions were transfected into HuH-7 (a, b), TG-1 (c, d) and McA RH-7777 cells (e, f). The cells were cotransfected with a reporter plus or minus 6RGR and then incubated with different concentrations of Dex for 2 days before assaying for Luc-activity. (B) The plasmids pcAF(−244/+45)-Luc, pgAF(−244/+45)-Luc, poAF(−244/+45)-Luc, and prAF(−180/+45)-Luc carried AFP promoter from the chimpanzee, gorilla, orangutan, and rat, respectively. These reporter constructs were cotransfected into HuH-7 cells with or without 6RGR and then exposed to different concentrations of Dex for 2 days. Each data point shows the means ± SD of $n \geq 4$.

of change was different, Dex stimulated or inhibited activity of human and mouse AFP promoter, respectively regardless of the origin of the liver cell line (Figs. 1A-c and 1A-d).

The possibility that the uniform responses of the two promoters may arise from their insertion into human cells lead us to measure activity of phAF[−178/+45]-Luc and pmAF[−178/+45]-Luc in the rat hepatoma cells, McA-RH 7777 (Figs. 1A-e and 1A-f). Whereas, Dex increased activity of the human promoter to a maximum of 2.5- and 4-fold in the absence or presence of 6RGR, respectively, that of the mouse decreased to 58 and 25%, respectively.

Effect of Glucocorticoid on AFP Promoter from Other Primates and Rodent

Next we analyzed the effect of Dex on the AFP promoter from other primates: chimpanzee, gorilla, orangutan, and an additional rodent, rat. Plasmids: pgAF[−244/+45]-Luc, pcAF[−244/+45]-Luc, poAF[−244/+45]-Luc containing the AFP promoter from chimpanzee, gorilla, and orangutan fused to Luc were cotransfected separately along with 6RGR into HuH-7 cells. In transfected HuH-7 cells, Dex stimulated Luc-activity in a dose-dependent manner (Fig. 1B-a). The stimulatory effect of Dex on the activity of these plasmids was evident regardless of the cell line tested (data not shown). However, Luc-activity in cells containing prAF[−180/+25]-Luc, the rat AFP promoter was suppressed by Dex (Fig. 1B-b). As expected, the activity of the rodent AFP promoter was inhibited by Dex in all cell lines tested. Together the results in this section and that above show the opposite effects of Dex stimulate but inhibit AFP promoter activity in primates and rodents, respectively. These effects are independent of whether the liver cell line was derived from human or rat.

Functional GRE of AFP Promoter

We previously showed that the DNA fragment between −175 and −161 of the human AFP gene contained a conserved GRE motif (19). The corresponding element in rodents spanned −168 to −150 in rat (10) and −169 to −151 in mouse, region IV (15) as noted in published DNase I protection analysis (Fig. 2A). To define the precise location of the GRE in the human AFP promoter, we constructed serial deletions of the promoter fused to Luc and transfected them into HuH-7 cells. Dex increased Luc-activity in HuH-7 cells containing human AFP promoter from −178 to +45, phAF[−178/+45]-Luc 32-fold (Fig. 2A). Further deletion of 5 and 10 bp yielded the −172 and −169 constructs, respectively. The activity of both constructs was stimulated 17-fold by Dex (Fig. 2A). However, removal of 17 bp to −162 reduced the inducibility to 7-fold in HuH-7. Further deletion to −162 and beyond to −132, −125, −98, and −57 was associated with only minimal reductions in the positive effects of Dex. These results showed that the major stimulatory actions of Dex required the presence of the hexamer, 5′-TGTCCT-3′ (Figs. 2A and 3B). In contrast, the rodent AFP promoter, mouse −178 (pmAF[−178/+45]-Luc), −170 (pmAF[−170/+45]-Luc) and rat −180 (prAF[−180/+45]-Luc) showed reductions to 25–50% of Luc-activity following treatment with Dex (Fig. 2B). Plasmids containing −131 in mouse and −132 in rat or 3′ to this point were not inhibited but rather stimulated by Dex. These findings help us locate the position of the positive GRE in human and the negative GRE in the mouse AFP genes.

Human and Rodents GRE Increase GST-P Promoter Activity in COS-7 Cells

Results of the above deletional studies suggest that the human AFP GRE is contained in a fragment spanning −178 to −132 of the gene (Fig. 3A). Within this fragment there is a 15-bp motif, −175 to −161 that resembles the consensus GRE, 5′-AGRACAnnnTGTYCT-3′. A comparison of this region amongst the primate sequences shows a single transverse mutation from a C in human to G in non-human primates (Fig. 3A). In contrast, the rodent sequences are more divergent with three transverse and three transitional mutations. These changes result in the lost of the 5′-hexameric repeat and makes the rodent motif look less like the consensus GRE.

Deletional analysis was used to test for Dex responsiveness of the hexameric motifs found in each of the AFP promoters. Thus a single copy of the human AFP fragments spanning −178/−158, −172/−144 and −162/−144 was inserted upstream of a heterologous promoter GST-P (Fig. 3B) to yield constructs called site A, B and C, respectively (Fig. 3A). Construct A contains an intact 5′ and 3′, plasmid B has only an intact 3′ and reporter C is lacking both 5′ plus 3′ hexameric motifs. The activity of these constructs was assessed in both HuH-7 and GR-deficient nonhepatoma cell line, COS-7 cotransfected with 6RGR (Figs. 4A and 4B, respectively). Results showed that activity of both site A and B were stimulated 16- to 17- and 8- to 9-fold, respectively, by Dex (Fig. 4, top panels) in the two cell lines. The activity of site A was comparable to that of the MMTV/GRE inserted into the same vector (data not shown). As predicted, site C was not responsive to Dex in either cell line. This finding suggests that the actions of Dex require an intact 3′ hexamer.

Tissue-Specific Repression of Rodent GRE by Glucocorticoid

Next we tested mouse AFP constructs −178/−146 and −170/−146 fused to the GST-P promoter to yield

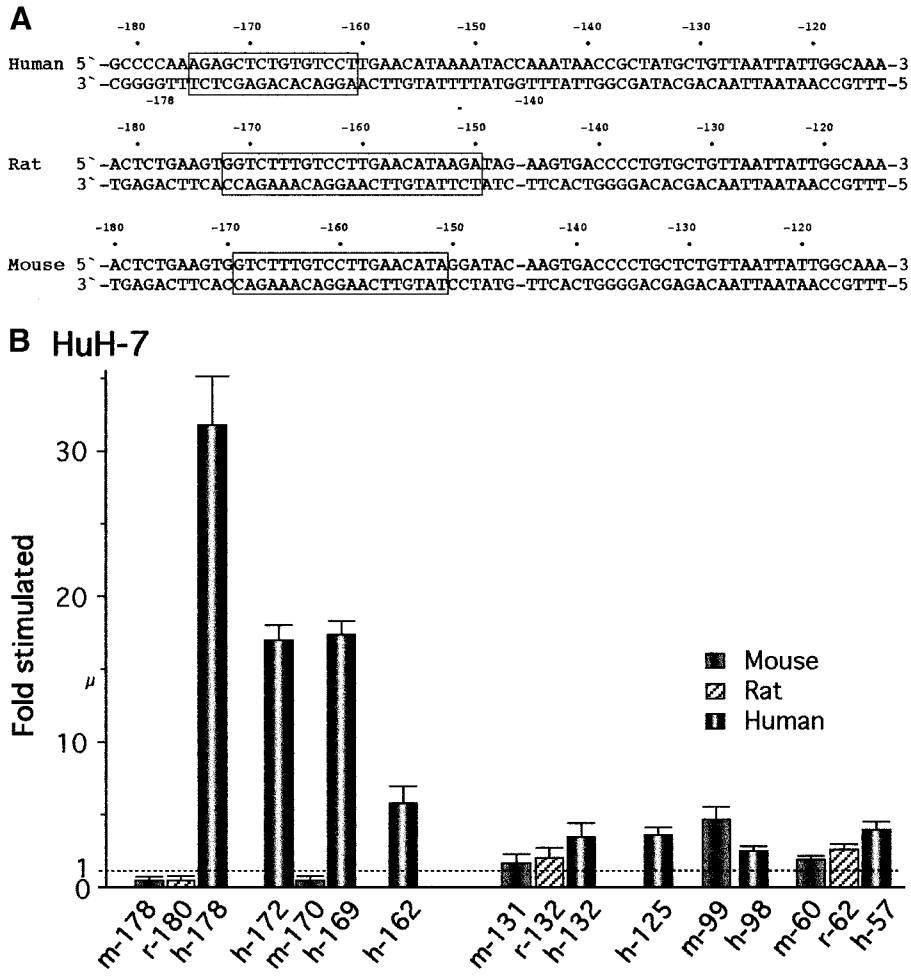

FIG. 2. Nucleotide sequence of GRE and deletional analysis to locate an element that mediates actions of Dex. (A) The nucleotide sequence of human, mouse, and rat AFP GRE. The human AFP GRE was reported previously (16) and aligned with DNase I protected sites reported in rat (9) and mouse (14). The specified motifs appear in boxes. (B) Various deletion mutants carrying the human, rat, and mouse AFP promoter were transfected into HuH-7 cells. The cells were cotransfected with the reporter of interest plus 6RGR and then incubated with 3×10^{-6} M Dex for 2 days. Each data point shows the means ± SD of $n \geq 4$.

reporters, sites D and E, respectively (Fig. 3A). Both constructs contained an intact 3′ hexamer, 5′-TGTCCT-3′ but construct E lacked the 5′ repeat. Although Dex inhibited activity of site D in the HuH-7 cells, it caused induction of site E in the same cells (Fig. 4, bottom, left panel). Furthermore, this response was dependent on dose of Dex added to the cells. In the COS-7 cells transfected with 6RGR, Dex stimulated the activity of sites D and E (Fig. 4, bottom, right panel). Together these findings show that the rodent AFP GRE is stimulated by Dex in nonhepatic cells but the same hormone inhibited the GRE in hepatic cells, thus raising the possibility that the effects of Dex is cell specific and may be limited to liver derived cells. A similar observation was reported previously using the rat AFP GRE in non-hepatic CV-1 and F9 cells (43). One potential explanation for the opposite responses of the rodent promoter in the two cell types may arise from the lack of factor(s) in non-hepatic cells that is/are present in liver cells, which enables Dex to inhibit activity of AFP.

An HNF-4 Binding Motif and DR-1 Overlaps the GRE in Rodent AFP Gene

A potential explanation for the opposite effects of Dex may arise from differences in nucleotide sequences between the rodent and primate AFP GREs. A search of the database using criteria defined by Heinemeyer *et al.* (44) of a 25-bp sequence that spans −176 to −152 in mouse and the −178 to −154 in human AFP promoter (Fig. 5A) showed high homology with the consensus and 38 other GREs yielding scores of 76.3% for mouse and 83.3% in human (Fig. 5A). However, one important feature is the presence of nucleotide differences between rodent and human at the tail end of the 5′ hexameric repeat (Fig. 5B). This difference in the sequence changes the rodent GRE to a motif that

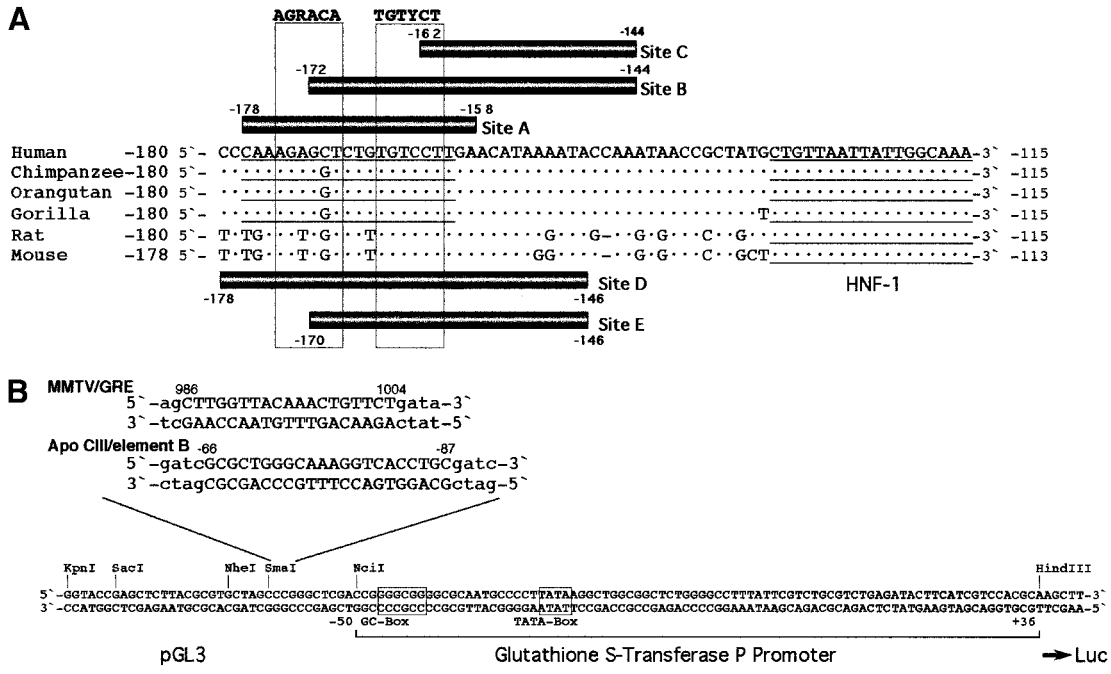

FIG. 3. Nucleotide sequences of primate and rodent AFP GRE. (A) The nucleotide sequence between −180 and −115 of human, chimpanzee, orangutan, gorilla, rat and −178 and −113 of mouse AFP promoters. The double-stranded oligonucleotides from human AFP fragments spanning −178/−158 (site A), −172/−144 (site B), and −162/−144 (site C) were inserted upstream of a heterologous promoter GST-P (42). The mouse AFP fragments spanning −178/−146 (site D) and −170/−146 (site E) were inserted upstream of GST-P. Portions of each promoter that is homologous to the consensus GRE are underlined. The 5′ hexamer "5′-AGRACA-3′ " and 3′ hexamer, 5′-TGTYCT-3′, of consensus GRE are shown above the boxes. (B) The MMTV/GRE (39), ApoCIII/element B (17), and AOX/PPRE (21, 22) motif used in this experiment are indicated.

matches best with the binding site for an orphan member of the nuclear receptor superfamily, HNF-4 (Fig. 5A, scored 84.1%). In contrast, the human and gorilla counterpart scored only 67.8% and align closely with the consensus GRE (Fig. 5A). Thus comparative sequence analysis of this region shows that −171 to −159 bp region of mouse AFP contains a motif that matches with the consensus sequence bound by HNF-4. The configuration of this element fits under the classification of a direct repeat separated by one nucleotide or so called DR-1 (Fig. 5B).

To test whether this difference between the human and rodent GREs affected function, we performed transient transfection assays using monomeric copies of human sites A, mouse site D, and ApoCIII/element B (Figs. 3A and 3B) inserted in front of GST-P. The latter two constructs contain motifs that match DR-1. These constructs were cotransfected separately into COS-7 cells along with 6RGR, pLEN4S, and RXR/6R followed by treatment with no hormone, 3×10^{-6} M Dex, or 1×10^{-6} M all trans-retinoic acid, respectively (Fig. 5C). As shown previously Dex induced both human site A and mouse site D (Figs. 4B and 4D) but it had no effect on ApoCIII/element B. Over expression of HNF-4 had no effect on human site A but on the DR-1 motif in site D and ApoCIII/element B it caused a 8- and 9-fold, respectively induction. Since ApoCIII/element B has DR-1 motif that bind HNF-4, the same sequence may also interact with RXRα (23, 40). Thus each of the reporter constructs was tested in the presence of RXR/6R. As expected, RXRα had no effect on human site A because it lacked a DR-1 motif but the factor plus its hormone induced site D and ApoCIII/element B by 3- and 6-fold, respectively, above control. These findings show that the DR-1 motif in the mouse GRE is induced strongly by HNF-4 and weakly by RXRα.

HNF-4 and Heterodimer of RXRα/PPARα Binds the Rodent Site D

Next we analyzed binding of the various receptors to site A, site D, ApoCIII/element B, and AOX/PPRE using mobility shift assay. In the first set of studies (Fig. 6A) we used a radiolabeled oligonucleotide containing the MMTV/GRE and bound it to protein(s) in whole cell extract, WCE from COS-7 cells transfected with 6RGR. Results showed the presence of a single protein/DNA complex (Fig. 6A, lane 2). The formation of this complex was inhibited by the addition of excess unlabeled site D (Fig. 6A, lanes 3–5) and human AFP site A (Fig. 6A, lanes 6–8) but not ApoCIII/element B (Fig. 6A, lanes 9–11).

Whether HNF-4 bound to the mouse site D was tested using radiolabeled oligomer of this element in

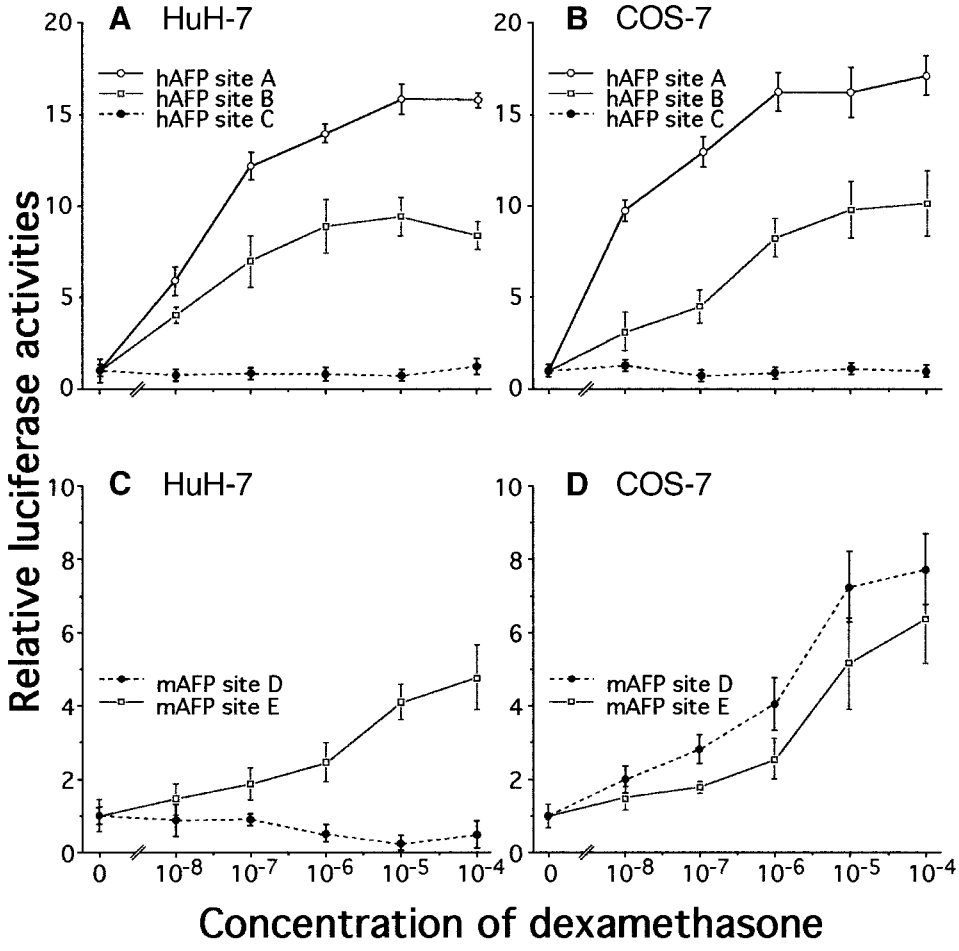

FIG. 4. Effect of Dex on Luc-activity of different oligonucleotides fused to pGL3-GST-P. Various oligonucleotides indicated in Fig. 3 inserted into the *Sma*I site of pGL3-GST-P were transfected into nonhepatic cells, COS-7 (A) and hepatic cell line HuH-7 (B). The cells were co-transfected with 6RGR followed by treatment with different concentrations of Dex for 2 days. Each data point shows the means ± SD of $n > 4$.

the presence of WCE from COS-7 cells over expressing HNF-4. We observed the formation of single band but the R_f of the dominant complex was different from that in the presence of GR (Fig. 6B, lane 2). The formation of this complex was inhibited by the addition of excess unlabeled site D (Fig. 6B, lanes 3–5) and authentic HNF-4 binding motif from the ApoCIII/element B (Fig. 6B, lanes 9–11). However, human AFP site A (Fig. 6B, lane 6–8) could displace binding to the radiolabeled probe.

Further demonstration that HNF-4 bound to mouse AFP site D arose from the use of a specific antibody against this factor in supershift studies. Results (Fig. 6C) show that whereas nonspecific goat preimmune IgG failed to supershift the protein radiolabeled mouse AFP site D complex, anti-human IgG against HNF-4 bound to the complex and retarded its mobility leading to a supershift. Together these findings showed that although the human AFP site A is very similar to mouse site D, these two motifs interact with different transcription factors. The data above shows that GR, HNF-4 and RXRα binds to mouse site D. It also raises the possibility that this interaction may interfere with the ability of GR to gain access to this motif. Thus the presence of a DR-1 motif in the rodent AFP promoters may underlie its inhibitory response to Dex.

Antagonistic Effects of HNF-4 on GR of Adjacent Repeats

To assess the transcriptional features of a motif comprised of HNF-4 and GR half-sites, we compared the activity of 4 motifs; MMTV/GRE, ApoCIII/element B, human AFP site A and mouse AFP site D fused separately to GST-P. Each one of these constructs was transfected separately into COS-7 cells because they lack endogenous HNF-4 and GR. Results showed, as expected the MMTV/GRE was induced by Dex in the presence of 6RGR and this induction was not affected by co-expression of HNF-4 (Fig. 7A). Additionally, the activity of the ApoCIII/element B was augmented in the presence of HNF-4 but not 6RGR (Fig. 7B). Like the

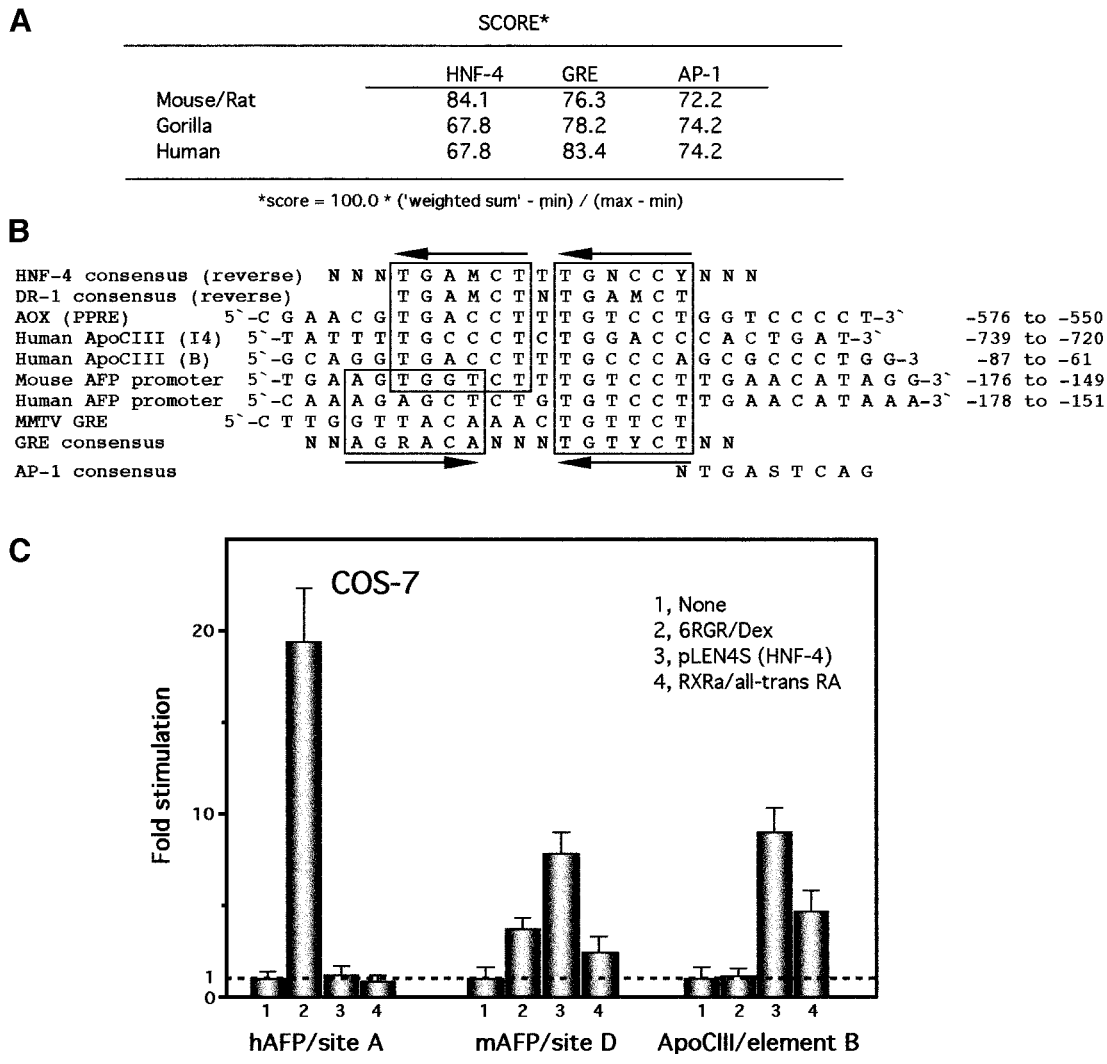

FIG. 5. Comparative analysis of GRE and DR-1 consensus sequences and mouse human AFP promoter. (A) The nucleotide sequence between −178 and −152 bp of AFP promoters was analyzed using a database search developed by Heinemeyer *et al.* (44). The homology score was calculated using the formula: score = 100.0* (weighted sum − min)/(max − min). (B) Sequence analysis of AFP promoter shows 5′-half hexamer change GRE to DR-1 motif in mouse. Sequences with homologies to the GRE and DR-1 binding motifs are boxed. (C) Transcriptional activity of GR, HNF-4, and RXRα. Various oligonucleotides inserted into the *Sma*I site of pGL3-GST-P (Fig. 3) were cotransfected into COS-7 cells along with none (lane 1), 6RGR with 3×10^{-6} M Dex (lane 2), pLEN4S (lane 3), and RXR/6R with 1×10^{-6} M all-trans retinoic acid (lane 4). Luciferase activity was analyzed using a Dual-Luciferase Reporter Assay System. Each data point shows the means ± SD of $n \geq 4$.

MMTV/GRE, activity of the human AFP site A was induced by Dex in the presence of 6RGR and this response was not affected by HNF-4 (Fig. 7C). In contrast, activity of the mouse site D was enhanced by HNF-4. However, in the presence of both 6RGR and HNF-4 the activity following treatment with Dex was inhibited to the control level (Fig. 7D). These findings provide further support for the idea that a chimeric motif comprised of both HNF-4 and GRE half-sites enable HNF-4 to bind this element and may help explain why Dex has a negative effect on this element.

To further demonstrate that the DR-1 site in the mouse AFP GRE restricted the response of this rodent motif to Dex, a chimeric reporter containing the DR-1 site from mouse was used to the replace the 5′ hexamer in the human AFP GRE (Fig. 8A). The Dex induced activity of this construct in HuH-7 cells was roughly 20% of that compared to the wild type human AFP GRE. The converse in which the 5′ hexamer of human AFP GRE was swapped with that from the mouse yielded a chimeric that was stimulated by Dex (Fig. 8). These findings confirm the potent suppressive actions of the DR-1 site and its removal unmasked the stimulatory effects of Dex on the mouse GRE.

DISCUSSION

Our ongoing interest in understanding the control of AFP expression has lead us to study the opposing effects of glucocorticoids on this gene in differing species.

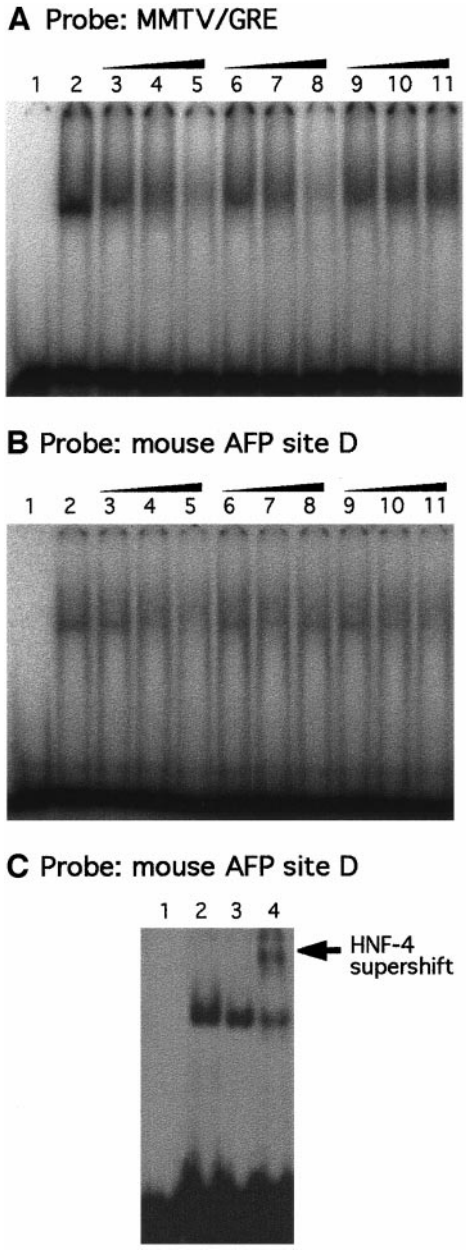

FIG. 6. Electrophoretic gel-mobility shift assay using synthetic oligomers corresponding to GRE and DR-1 of AFP promoter. (A) A 18-bp double-stranded oligonucleotide corresponding to the MMTV/GRE (Fig. 3) was labeled with [^{32}P], incubated with 40 μg of COS-7 whole cell extract transfected with 6RGR and then treated with 3×10^{-6} M Dex in the absence or presence of competitor DNA added in 50-, 100-, and 200-fold molar excess of labeled probe. Lane 1, no cell extract; lane 2, no competitor; lanes 3–5, mouse AFP site D; lanes 6–8, human AFP site A; and lanes 9–11, human ApoCIII/element B. (B) A [^{32}P]-labeled 33-bp oligonucleotide corresponding to the mouse AFP site D (Fig. 3) was incubated with 40 μg of COS-7 whole cell extract transfected with pLEN4S in the absence or presence of competitor DNA added in 50-, 100-, and 200-fold molar excess of labeled probe. Lane 1, no cell extract; lane 2, no competitor; lanes 3–5, mouse AFP site D (self-competitor); lanes 6–8, human AFP site A; and lanes 9–11, ApoCIII/element B. (C) Supershift analysis using anti-human HNF-4 IgG. Radiolabeled 33-bp site D was incubated with 20 μg of HuH-7 nuclear extract in the presence or absence of excess competitors and polyclonal HNF-4 antibody. Lane 1, no cell extract; lane 2, no IgG; lane 3, goat preimmune IgG; and lane 4, anti-human HNF-4 IgG.

We postulated that this opposite response might arise from differences in the DNA sequences of the AFP promoters. Close inspection of this DNA fragment revealed a motif, 5′-AGAGCTnnnTGTCCT-3′, comprised of two hexamers separated by 3 bp and this sequenced resembled the consensus GRE. To determine whether this motif mediated the stimulatory actions of Dex, the −178 to −158 sequence containing the preceding motif, called site A was fused to the heterologous GST-P promoter linked to Luc and cotransfect along with 6RGR into non-hepatic COS-7 cells (Figs. 3 and 4). In the transfected cells, Luc-activity was strongly stimulated in the presence of Dex. Similarly, the −172 to −144 segment called site B that contained an incomplete 5′ hexamer was also inducible by Dex. However, a further truncation that destroyed the 3′ hexamer to yield site C abrogated the response to Dex. These findings are in keeping with the idea that a GRE motif of 15-bp with partial or perfect symmetry is necessary for glucocorticoid induction of human AFP (45). Although the human AFP GRE has significant differences from the consensus motif, it retains its induction by Dex and GR. This feature may be explained by previous studies showing that each GRE half-site binds to the receptor (46). It is possible that GR recognizes an imperfect palindrome with the conserved 3′-hexamer, 5′-TGTCCT-3′.

The actions of glucocorticoids are well known to regulate gene expression in both a positive and negative fashion. Whereas, there are numerous models of the positive responses to the steroid, models of suppression by glucocorticoids are less common. The rodent AFP promoter provides a good opportunity to study this effect because Dex inhibits its activity. A proposed mechanism for the inhibitory actions of GR is through its interference with transcription factors that enhance activity of the gene (42, 47–51). In this regard, Zhang *et al.* has examined the −172 to −150 sequence in the rat AFP promoter. This DNA fragment contains a GRE that overlaps with an AP-1 binding site as shown in Fig. 6B (43). Although not proven, the overlap of the two elements offers one explanation for why Dex negatively regulates AFP. It is possible that the GRE interferes with the binding of c-Jun or c-Fos to the adjacent AP-1 site.

In support of this idea, studies of the human AFP promoter revealed that the GRE and AP-1 overlap spans −166 to −152 and this DNA segment is identical to that of rat and mouse (Fig. 5). Saegusa *et al.* showed that Dex stimulation of the human AFP promoter activity was effectively blocked by c-jun and c-fos (52). The elements mediating the actions of Dex and c-jun are adjacent to each other and their close proximity offers an opportunity to interfere with each others binding to their respective motifs. In addition to the AP-1 motif, Galarneau *et al.* reported that the rat −166 to −158 (5′-TCAAGGTCA-3′) has the potential to

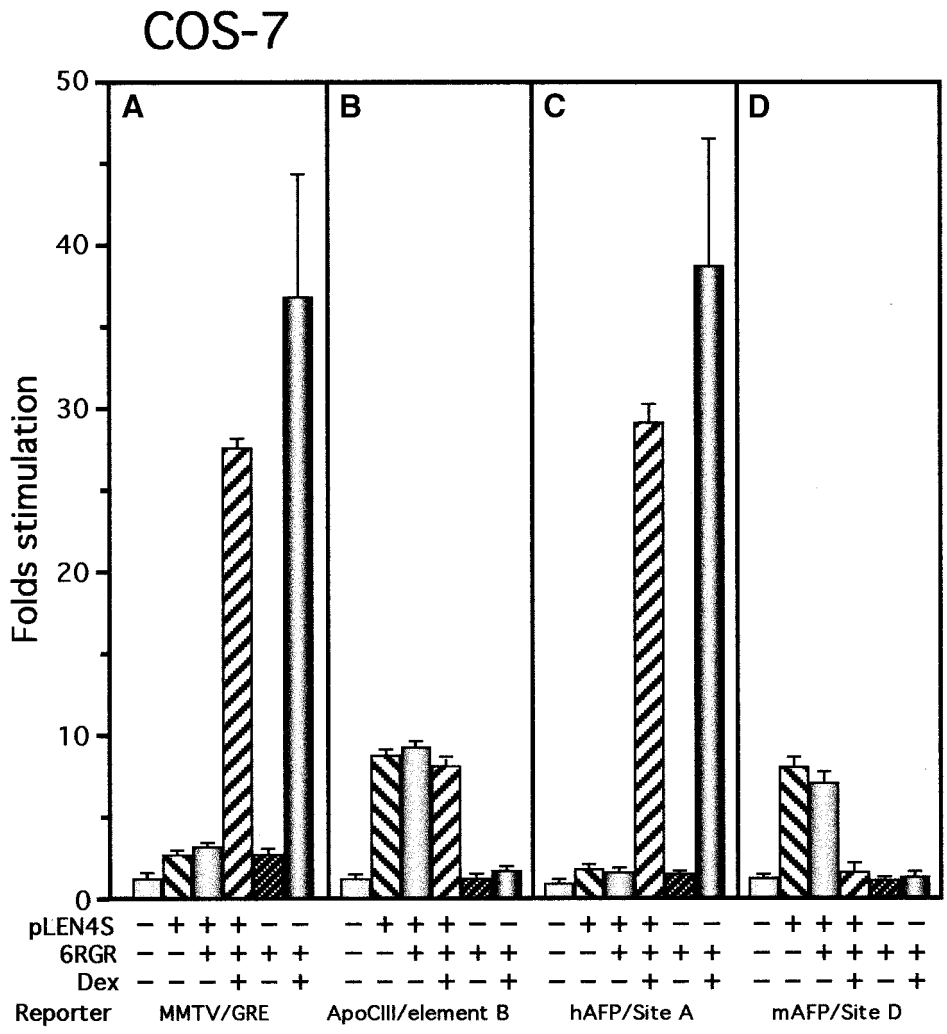

FIG. 7. Effect of HNF-4 on Dex response in various oligonucleotides inserted into the pGL3-GST-P. Various oligonucleotides inserted into the *Sma*I site of pGL3-GST-P were cotransfected with effecters (pLEN4S and/or 6RGR) into COS-7 cells and incubated with or without Dex for 2 days. Each data point shows the means ± SD of $n \geq 4$.

recognize FTF, an orphan nuclear receptor of the Drosophila FTZ-F1 family (53). The binding site for this protein also overlaps with GRE. The preceding discussion speculates on the potential mechanism(s) underlying the inhibitory actions of Dex but the actual mechanism remains unknown. Nevertheless, the findings all point to the participation of the GRE in the inhibition. Therefore, the simplest explanation is that the opposite effect of glucocorticoids on AFP expression between rodents and primates could arise from intrinsic properties of the motif.

A comparison of the AFP GRE from rodents and primates revealed nucleotide differences at the 5′ hexamer (Fig. 3). These changes convert the hexamer at the 5′ end of the rodent GRE to a so called DR-1 sequence (Fig. 5B) and motifs of this type may bind to factors such as; HNF-4, RXR and PPAR. HNF-4 is of specific interest because it is a liver-enriched transcription factor that plays an important role in liver differentiation (54, reviewed by Hayashi *et al.*). The role for HNF-4 in AFP gene expression has not been established, although Magee *et al.* presumed that retinoic acid might down regulate rat AFP gene expression through 2- to 3-fold decreased mRNA expression of HNF-4 (26). Their findings clearly identified an HNF-4 binding site in the rat and mouse AFP promoter.

To show that HNF-4 did indeed interact with the mouse but not the human AFP promoter, we measured transcriptional activity and protein binding to both elements (Figs. 5–7). The results of co-transfection studies allowed us to assess the functional contribution of the various factors; GR, HNF-4, and RXR. Results (Fig. 5) showed that whereas HNF-4 induced the activity of the mouse site D, it had no effect on the human site A. Furthermore, RXRα had no effect on site A and only minimal effects on site D. As expected they did modulate the activities of the control ApoCIII/element B (Fig. 5).

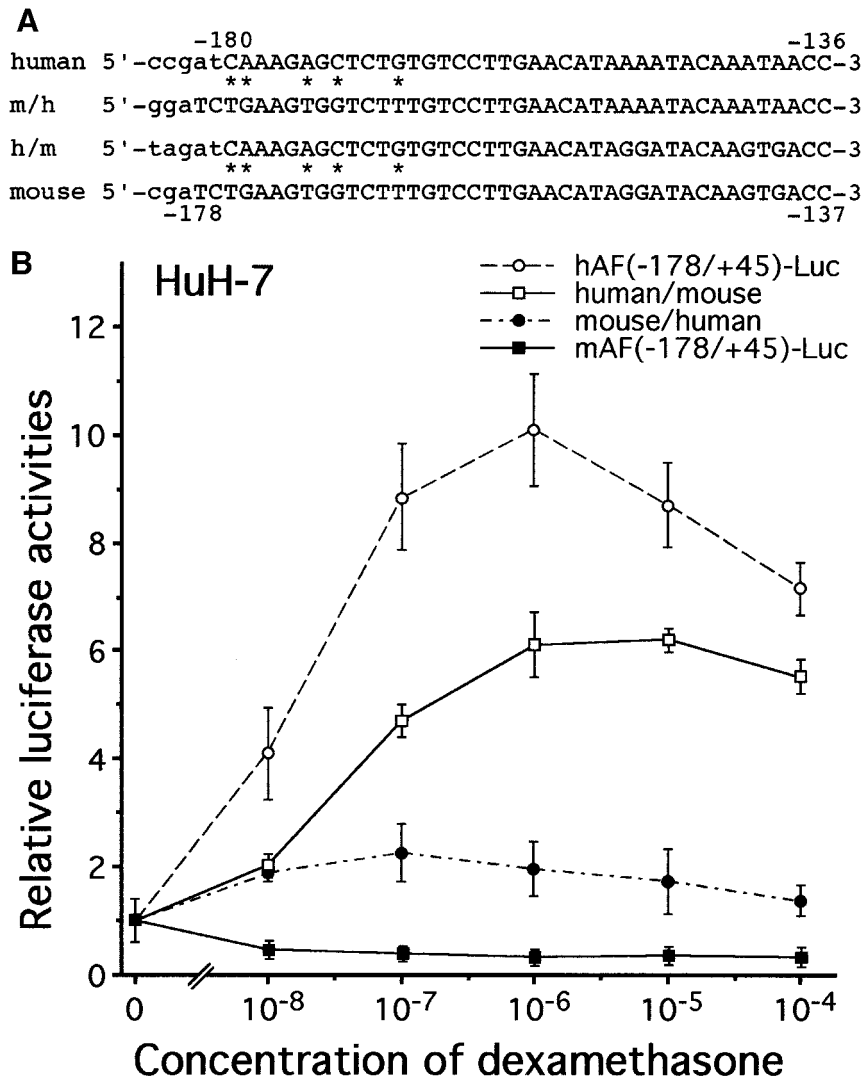

FIG. 8. Removal of the HNF-4 site unmasks Dex induction of the mouse AFP GRE. (A) The sequences at the top of the panel show two chimeric constructs comprised of the mouse HNF-4 placed in front of the 3′ hexamer from human and the converse with the human AFP GRE 5′ hexamer fused to the tail end hexamer of mouse. The mutated nucleotides are indicated asterisk. Panel B, the Dex stimulated Luc-activity of the chimeric and wild type promoter is shown in the graph below when transfected into HuH-7 cells. Each data point shows the means ± SD of $n \geq 4$.

In the gel-retardation analysis (Fig. 6), competition studies showed that authentic HNF-4 recognition motif displaced protein binding to the mouse GRE. That HNF-4 interfered with the actions of GR and Dex (Fig. 7) at mouse site D was demonstrated in COS-7 cells where the function of this site was induced by GR plus Dex (Fig. 4D). The expected induction of site D in the presence of GR and Dex was blocked by expression of HNF-4 in COS-7 cells (Fig. 7). These results clearly demonstrate that the HNF-4 binds and affects the function of the mouse AFP GRE.

Further proof that the HNF-4 site did indeed suppress the actions of the GRE was demonstrated by the creation of chimerics containing the mouse HNF-4 site fused to the 3′ hexamer of the human AFP GRE. Dex induction of this chimeric in HuH-7 cells was severely restricted (Fig. 8). Removal of the HNF-4 motif and replacing it with the 5′ hexamer from the human AFP GRE yielded a chimeric element that was induced by Dex in liver cells. These findings are consistent with the idea that HNF-4 participates in the negative regulation of the rodent GRE.

In summary, the findings in this report show that we have localized the glucocorticoid response elements in the AFP promoter to sequences that share marked homologies with the consensus GRE. The stimulatory effect of the steroid on the primate AFP promoter and its inhibitory actions on the rodent counterpart appears to be specific to liver. This effect is likely due to the presence of a HNF-4 binding motif at the 5′ end of

the rodent GRE. This HNF-4 site may interfere with the actions of GR. Thus the opposite response to glucocorticoid on expression of the AFP gene in rodents and primates appear to arise from evolutional differences between species.

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