Retinoic Acid Mediates Down-regulation of the α-Fetoprotein Gene through Decreased Expression of Hepatocyte Nuclear Factors*

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α-Fetoprotein (AFP), a protein highly induced during fetal liver development, is down-regulated by retinoids in the human hepatoma cell line Hep3B, in contrast to up-regulation observed in other cell types. Previously, we have documented that such up-regulation involves direct effects through cis-retinoid X receptor-binding sites in the AFP enhancer. In this report, we show a distinctive effect of all-trans-retinoic acid (RA) in Hep3B cells. RA caused a marked decrease in AFP transcripts. Deletion analysis of the upstream regulatory region of the AFP gene revealed that cis-acting sites required for down-regulation resided near the promoter. Gel mobility shift assays for factors binding to key elements in the AFP promoter region demonstrated that hepatocyte nuclear factor (HNF) 1 binding was diminished in nuclear extracts from RA-treated cells. In addition, HNF4, which is not known to bind to the AFP promoter but does regulate HNF1, was also diminished. The levels of HNF1 and HNF4 mRNA were also decreased following RA treatment. AFP promoter-chloramphenicol acetyltransferase transient transfection assays demonstrated that the level of HNF1 had a direct impact on basal transcription as well as RA-mediated down-regulation of the AFP gene, and that co-transfection of HNF1 and HNF4, but not transfection of either factor alone, reversed the RA-mediated inhibition. Taken together these data point to an interaction among the RA, HNF1, and HNF4 signals, which is reflected in decreased expression of AFP.

Retinoic acid (RA), a vitamin A metabolite and a potent regulator of development and differentiation, acts through transcriptional regulation of gene expression. Modulation of gene expression by RA results primarily in the induction of genes but also, in some cases, repression. Genes induced by RA include RARβ2 (1–3), RARα2 (4), RARγ2 (5), rat growth hormone (6), and cellular retinol-binding protein type II (7), as well as the α-fetoprotein (AFP) gene (8, 9). RA can also repress certain gene transcripts. In F9 cells, the 2.3-kb RXRγ gene is completely repressed by RA (10). The rat stromelysin gene, encoding an enzyme involved in extracellular matrix remodeling, is also down-regulated by RA, possibly through the AP-1 signaling pathway (11).

Activation of the AFP gene by RA has been reported in Mca-RH8994 and F9 cell lines (8, 9). These RA effects are superimposed on a complex transcriptional regulatory system, since high-level tissue-specific activation of the AFP gene from mouse, rat, and human cell lines requires three enhancer elements in addition to a minimal promoter (12–14). Several transcription factors have been reported to be involved in AFP gene regulation, including fetoprotein transcription factor (15), promoter coupling factor (16), Nkx2.8 (17), HNF1 (hepatocyte nuclear factor 1) (18, 19), C/EBP (CCAAT/enhancer-binding protein) (18), NF1 (nuclear factor 1) (18), and RXR and RAR receptors (8, 9).

HNF1 and its regulator, hepatocyte nuclear factor 4 (HNF4), appear to play a key role in the liver-specific regulation of many genes expressed during liver differentiation and development (for review, see Ref. 20). HNF1 is a homeodomain protein required for the expression of AFP and many other liver-specific genes such as fibrinogen α and β, albumin, and aldolase B (Ref. 21 and references therein). HNF1α can associate with the highly related HNF1β (vHNF1) homeodomain protein to form homo- or heterodimers (21). HNF1 dimers bind at two sites in the rat AFP promoter (−131 to −116 bp; −66 to −51 bp) (13, 18, 19). Basal level expression of AFP is strongly dependent on HNF1, since selective mutation of both HNF1-binding sites abolished expression of AFP promoter-CAT constructs, with or without enhancers (22).

HNF4 is an orphan receptor of the nuclear receptor superfamily (23). HNF4 forms dimers and binds to the promoter sites of the transthyretin (24), phosphoenolpyruvate carboxykinase (25), and HNF1 genes (26). In the promoter of the HNF4 gene, an HNF1 transcriptional response has been demonstrated, suggesting the presence of a regulatory loop between HNF4 and HNF1 (27). Additionally, deletion of HNF4 from cell somatic hybrids eliminated expression of HNF1, demonstrating that these two genes form a network of transacting factors controlling multiple liver-specific genes (28). HNF1 also negatively regulates its own expression and that of other HNF4-dependent genes that lack HNF1-binding sites. This repression is due to direct interaction between HNF1 and HNF4 (29).

Previous studies have shown RA activation of AFP gene expression, primarily through elements that we characterized in the nearest upstream enhancer, at −2.4 kb (8). This activation is present in several AFP-expressing cell lines and has been characterized in detail in Mca-RH8994 cells (8). However, we observed an unusual paradoxical response in Hep3B cells in that AFP expression was strongly inhibited by RA. The mech-
anism of this repression is likely to involve important features of RA-mediated gene control that are obscured by the AFP-activation observed in other cell lines. The studies in this paper demonstrate a distinctive RA-induced repression of the AFP gene and further show that repression is mediated by elements of the proximal promoter that interact with HNF1, a factor which is in turn controlled by HNF4.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Cell lines Hep3B and McA-RH8994 were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in minimal essential medium (Sigma) supplemented with 10% fetal calf serum. Calcium phosphate transient transfection assays were performed essentially as described with the modification that assays were performed in six well plates rather than 10-cm plates (30). Reagents were scaled down accordingly. In brief, 1 day before transfection, cells were seeded in each well at $8.45 \times 10^4$ cells. AFP-CAT plasmid constructs (3.2 mg) were mixed with 2.5 M CaCl$_2$ and 2 $\times$ HeBS (280 mM NaCl, 50 mM Heps, and 1.5 mM Na$_2$PO$_4$ at pH 7.05) and the plasmid precipitate was applied to cells for 5 h. Cells were next treated with 10% glycerol for 2 min, washed three times with 1 ml of phosphate-buffered saline, and incubated with 2 ml of MEM with or without RA (Sigma). Cells were re-fed with MEM with or without RA each day for total of 2.5 days. After treatment, the cells were harvested, and Northern blot hybridization was performed for AFP and control rRNA or $\beta$-actin mRNA.

Plasmid pGEM was added to equalize the amount of plasmid per well. The figures show simultaneous platings for each plasmid, and each data point is the average of three separate transfections. Each experiment described was reproduced at least three times. The following plasmids for transfection have been previously described: AFP-gene expression plasmids pAFP7300, pSpe$_D$X, pNsi$_D$X, p1483$_D$BN, p3195$_D$XN, and p1334 (14); AFP-gene plasmids with promoter deletions 2191, 2178, 2155, 2134, and 2125 (31); HNF4 reporter plasmid pAPF-HIVCAT and HNF4 expression plasmid pLEN4S (23); and HNF1 expression plasmid pONHNF1 (32). Among the AFP constructs, pSpe$_D$X, which is deleted to 260, contains only half of the HNF1–2 site and the basal promoter and serves as a negative control. The RXR-specific agent LG100153 was a generous gift from Ligand Pharmaceutical Co.

Northern Blot Hybridization—Total RNA was extracted by the guanidinium isothiocyanate method (33). Twenty mg of total RNA per lane was resolved by electrophoresis on a 1.2% agarose gel containing 2.2M formaldehyde and then transferred to nylon membranes by capillary blotting. $^{32}$P-Labeled HNF1a (28), HNF4 (23), AFP (34), rRNA, and $\beta$-actin cDNA (35) restriction fragments were labeled by random priming and hybridized to membranes in 7% (w/v) SDS, 0.5 M sodium phosphate, pH 6.5, 1 mM EDTA, and 1 mg/ml bovine serum albumin at 68 °C overnight. The membranes were washed twice in 1% SDS, 50 mM NaCl, and 1 mM EDTA at 68 °C for 15 min each and autoradiographed using intensifying screens.

Gel Mobility Shift Assay—Double-stranded oligonucleotides to the HNF1-binding sites in the rat AFP promoter (AFP HNF1-1, −135 to −112, 5'-GTGCTTAAATTTGGCAGACT-3'; AFP HNF1-2, −70 to −47, 5'-CTGAAGTACATAGTTACAGACA-3'); the HNF1 consensus binding site (5'-GTGTAATTTAAC-3') (36); the HNF4...
consensus binding site (5'-CTTGGGAAAGTCATAGG-3') (23); the NF-1 and C/EBP-binding sites in the rat AFP promoter (AFP NF-1, -126 to -105, 5'-TGGCCAAATGTCCTA-3'; AFP C/EBP, -118 to -97, 5'-CAATTCTGCTAATACTG-3'; and the NF-1 and C/EBP consensus binding sites (NF-1, 5'-ATTGTCGCTAGGGCTCTG-3'; C/EBP, 5'-ACCTCTACCTGAAT-3') (24, 37) were end-labeled using the Klenow fragment of DNA polymerase I and [32P]dCTP. Additionally, oligonucleotides spanning the AFP promoter but with no identified site effect. To analyze the RA-mediated inhibitory effect, contrast to other hepatoma cell lines where RA had the opposite effect of RARs and RXRs. For both RA and c-RA, inhibition was noted some inhibition, although the inhibition might be a dual effect of inhibition can be mediated solely by liganded RXR. Together, these observations indicate that activation of RXRs alone cannot mediate the observed phenomena. However, RXRs mediated some inhibition, although the inhibition might be a dual effect of RARs and RXRs. For both RA and c-RA, inhibition was clear at the physiological dose of 10^-7 M.

To assess whether down-regulation of the AFP gene required ongoing protein synthesis, cycloheximide was added concurrently with RA for 48 h. Hep3B cells treated with cycloheximide (10 µg/ml) and RA at 10^-6 or 10^-5 M showed down-regulation of mRNA to the same extent as for treatment with RA alone (Fig. 1d). This result indicated that no new protein synthesis was required for RA to mediate the repression of AFP gene expression. Even with cycloheximide, we saw no detrimental effects on the cells such as necrosis or apoptosis, as determined by light microscopic evaluation of trypan blue exclusion (data not shown). Since these data demonstrated that RA (10^-5 M) showed strong inhibition without cytotoxicity, this agent and dosage were chosen for further studies.

The time course of RA-mediated mRNA down-regulation on the AFP gene was also examined. Equal numbers of cells (5 x 10^6) were grown with or without RA for up to 48 h (Fig. 2a). Without RA treatment, the level of AFP mRNA increased continuously as the cells reached confluence. RA treatment blocked the increase and by 48 h caused an absolute decrease in mRNA level. The inhibition was relatively slow; it was first noted at 16 h and became evident after 24 h of RA treatment. By 48 h, there was a 10-fold difference between RA-treated and untreated cells (Fig. 2b). Since the inhibition was not blocked by cycloheximide, the effect was most consistent with transcriptional inhibition of a relatively stable mRNA with a long half-life. It has been reported that RA (10^-5 M) can block AFP gene expression in Hep3B cells by inhibiting cell proliferation when cells are treated for long periods of time ranging up to 72 h. Cells were detached from plates using trypsin and counted by light microscopy. Each data point consists of the average of three independent determinations.

**RESULTS**

*Retinoid Effects on AFP mRNA*—Preliminary studies revealed that AFP expression in Hep3B cells was decreased by RA treatment (all-trans-RA, or RA as shown in the Fig. 1), in contrast to other hepatoma cell lines where RA had the opposite effect. To analyze the RA-mediated inhibitory effect, Hep3B cells were treated with retinoids (10^-9 to 10^-5 M) (Fig. 1a). This caused clear reduction of AFP mRNA in Hep3B cells. In contrast, 9-cis-RA (c-RA) increased AFP mRNA in McARH8994 cells. A representative study (Fig. 1b) is presented to illustrate this difference. In Hep3B cells, RA and c-RA caused comparable inhibition of AFP mRNA. Both agents are ligands for RARs, whereas only c-RA is a ligand for RXRs. To further clarify receptor specificity, we also used LG100153, an RXR-specific ligand. This agent also inhibited, but the effect was weaker than with RA and c-RA (Fig. 1, a and c). The I_50 values were calculated as 1 x 10^-7 M for c-RA and 2 x 10^-7 M for t-RA, a difference that was not significant within the accuracy of our measurements. In contrast, the I_50 for LG100153 was 3.3 x 10^-6 M. Though this agent is expected to have comparable potency to c-RA for RXR activation, the data show that a degree of inhibition can be mediated solely by liganded RXR. Together, these observations indicate that activation of RXRs alone cannot mediate the observed phenomena. However, RXRs mediated some inhibition, although the inhibition might be a dual effect of RARs and RXRs. For both RA and c-RA, inhibition was clear at the physiological dose of 10^-7 M.

**Fig. 2. Time course of RA effects on AFP mRNA and cell growth.** a, equal number of Hep3B cells (5 x 10^6) were plated and cultured in the absence (C) or presence of 10^-7 M RA for various periods of time ranging from 4 to 48 h. RNA was extracted from the cells and Northern blot hybridization was performed. b, the mRNA determinations were scanned and the measurements plotted versus time in culture. Values are relative to the mRNA level at initial plating. c, Hep3B cells (5 x 10^6) were plated and cultured in the absence (C) or presence of 10^-5 M RA for various periods of time ranging up to 72 h. Cells were detached from plates using trypsin and counted by light microscopy. Each data point consists of the average of three independent determinations.

*The Proximal AFP Promoter Mediates RA-dependent Down-regulation of the AFP Gene*
The AFP 5’-flanking region has three enhancers that have been shown to stimulate expression of the AFP promoter in an additive fashion, as well as a complex promoter (31). A region between enhancer 1 and the promoter has been reported to be involved in repression of the AFP gene and has been termed a DNA silencer region (39–41). Accordingly, RA-mediated down-regulation of AFP gene expression might involve upstream elements, the promoter, or interaction among them. Transfection analysis was employed to verify that RA effects were transcriptional and to localize the gene controls that mediate the effects.

For determination of general regions that might be critical for RA-induced down-regulation, a series of AFP-CAT plasmids derived from the parent plasmid pAFP7300 (14), p1483ΔBN, p3195ΔXN, p1334 contained single enhancer regions combined with the promoter, whereas pNsiΔX contained only the full promoter region, and pSpeΔX, a minimal promoter. b and c, cell transfections were carried out as described under “Experimental Procedures.” Transfectants were treated with or without 10^{-5} M RA for 2.5 days. Results, the average of three samples (n = 3), were plotted as total activity (counts/min (CPM) of acetylated chloramphenicol). The error bars are S.D. values.

**Fig. 3. Transfection to localize the general region of RA-repression.** a, the 5’-upstream region of the rat AFP gene is shown with enhancer and promoter regions detailed. A series of AFP gene expression plasmids, derived from the parent plasmid pAFP7300 (14), p1483ΔBN, p3195ΔXN, p1334 contained single enhancer regions combined with the promoter, whereas pNsiΔX contained only the full promoter region, and pSpeΔX, a minimal promoter. b and c, cell transfections were carried out as described under “Experimental Procedures.” Transfectants were treated with or without 10^{-5} M RA for 2.5 days. Results, the average of three samples (n = 3), were plotted as total activity (counts/min (CPM) of acetylated chloramphenicol). The error bars are S.D. values.

**HNF1 and HNF4 Binding Is Reduced following RA Exposure**—Because transfection analysis localized the RA effect to a 125-bp promoter region, gel shift studies were set up to survey this region for altered transcription factor binding. Two HNF1 recognition sites were located within 131 bp of the transcription start site (13, 18, 19, 22), and HNF1 has been shown to be
an important activator of AFP gene expression (13). To accommodate the two HNF1 sites, the survey was enlarged to encompass the region to \(-213\) (Fig. 5\(a\)). We also studied HNF4 binding. Although the region contained no known binding sites for this factor, HNF4 is the primary transcriptional regulator of HNF1 and may also be down-regulated (26). Oligonucleotides representing the two AFP HNF1-binding sites were \(^{32}\)P-labeled and incubated with nuclear extracts from cells treated with or without RA (Fig. 5\(b\)). These were compared with the HNF1 consensus binding site in both direct binding and competition assays, demonstrating that all three oligonucleotides bound in a fashion specific for HNF1. The binding pattern consisted of two specific bands, marked by arrows in the figure. The upper band represented HNF1\(\alpha\) homodimers and the lower HNF1\(\alpha\):\(\beta\) heterodimers (42). Specific and nonhomologous competition demonstrated that additional bands in the patterns were non-specific. Both AFP promoter HNF1 sites were weaker than the consensus site, though the more upstream site was the stronger of the two. Notably, after RA treatment, binding of HNF1 to all three oligonucleotides was reduced by more than half compared with the controls. HNF4 binding to an HNF4 consensus binding site was similarly reduced in nuclear extracts following RA exposure (Fig. 5\(b\), right panel). For this factor, the specificity of the assay was verified by supershifting of HNF4 protein with an HNF4-specific antibody (Fig. 5\(b\)).

Other assays examined the remainder of the promoter region for additional effects (Fig. 5\(c\)). This region contained known C/EBP and NF1-binding sites. Oligonucleotides were also designed to survey regions without known binding factors, in case RA-mediated effects were predominant to cryptic binding sites that had not been described previously. In contrast to the HNF1 and HNF4 binding, NF-1 and C/EBP binding were increased by RA treatment. Observation of these effects was helpful, because it demonstrated that the RA effects were specific for HNF1 and HNF4 and did not reflect a general effect on the Hep3B nuclei or other effects that might have altered the entire nuclear extract. Gel shift with oligonucleotides A–D indicated some additional weak binding factors that had not been characterized, but none of these showed significant change with RA. Together, these data indicated that RA selectively decreased binding of both HNF1 and its regulator, HNF4, and the magnitude of the decreases was comparable with the reduced gene expression observed in the transfection assays. Moreover, the localization of the RA effect to the \(-125\)-bp region of the promoter could be explained by the effects on HNF1, even though there was an additional binding site outside this region, since earlier studies indicated that both sites make significant contributions to AFP gene expression (13, 22). Thus the shorter promoter was weaker, but still regulated through its remaining HNF1-binding site.

HNF1 and HNF4 mRNA Expression Is Reduced by RA Treat-
Northern blot analysis of mRNA levels was carried out to follow up the observation of reduced nuclear levels of HNF1α and HNF4. Fig. 6 demonstrates that both HNF1α and HNF4 mRNA expression decreased 2–3-fold following treatment of Hep3B cells with RA. These observations verify the transcription factor binding study by also indicating that the RA effect on AFP expression is mediated by transcriptional down-regulation of HNF1α and HNF4.
Down-regulation of the AFP Gene by RA

**DISCUSSION**

Down-regulation of AFP gene expression can occur by a variety of mechanisms. Previous studies have implicated AP-1 transcription factors in glucocorticoid-mediated down-regulation (43), c-Jun in 12-O-tetradecanoylphorbol-13-acetate-mediated down-regulation (44), and COUP-TF as a general negative regulator (45, 46). In this report we have demonstrated a distinctive effect in which RA-mediated down-regulation is dependent on the inhibition of transcription factors HNF1 and HNF4. This effect suggests a relationship between retinoids and the two main transcription factors that control the hepatocyte phenotype.

RA both activates and represses AFP expression in different settings. In cases where RA activates the AFP gene, the mechanism appears to involve direct activation by binding of RXR/RXRα homodimers to cis-acting sites in the 5′-flanking region of the AFP gene. We have identified an RXRE at −2406 to −2378 bp active in McA-RH8994 cells that mediates this stimulation (8). Other studies have indicated additional binding sites (9, 41, 47). Thus, RA has dual effects in controlling the expression of the AFP gene. As reported previously (46), a weak RXRE exists within the AFP promoter region at −143 to −131 bp. However, deletion of the AFP promoter to within 125 bp of the transcriptional start site did not prevent RA-mediated repression (Fig. 4), indicating that this RXRE had little effect on AFP expression with or without RA treatment. Study of the various enhancer-promoter combinations also ruled out a role for various upstream RAR- or RXR-binding sites. Thus, in contrast to stimulation, the RA-mediated repression of AFP is indirect, through regulation of HNF1α and HNF4 gene expression.

A variety of signaling pathways has been implicated in the down-regulation of the AFP gene, but all can be distinguished from RA-mediated repression. Similar to the effect of RA, glucocorticoid can either turn AFP gene expression on and off (reviewed in Ref. 48) through binding sites in the upstream promoter region. Postnatal repression may involve the action of a DNA silencer element in a region upstream of the minimal promoter between −1010 and −250, which also can act as a repressor for heterologous gene expression (39, 40, 49). However, deletion of the glucocorticoid-binding sites or the silencer element had no effect on the actions of RA in Hep3B cells. In fibroblasts, the AFP gene is repressed by the binding of unknown transcription factors to the promoter region between −57 and −43 bp (50), but our findings showed no increased binding of factors to this region. A variety of other transcription factors and signaling pathways such as c-Ha-Ras (51), c-Jun (AP1) (44), ATBF1 (A-T binding factor), which binds only the human upstream enhancer (52), transforming growth factor-β (53), and hepatocyte growth factor (54) have also been associated with some degree of AFP gene repression. A possible role for RA in these signaling pathways remains to be investigated.

A number of mechanisms can be envisioned for the negative regulation of the AFP gene by RA. One possibility is that RA reduces the expression of HNF4, which in turn reduces expression of HNF1α. Hence down-regulation of AFP would be due to decreased binding activity of HNF1 to the two AFP HNF1-binding sites. It is not known whether HNF4 has a direct effect on the AFP gene, because no HNF4-binding site has been identified within the AFP gene. Moreover, reduced expression of HNF4 by itself was not sufficient for RA-mediated down-regulation, since overexpression of HNF4 did not prevent down-regulation of an AFP-CAT reporter plasmid (−134). In contrast, overexpression of HNF4 and HNF1α together could completely overcome the RA-mediated down-regulation. It is not clear why HNF4 worked only when both HNF1 and HNF4 were overexpressed, but this observation does indicate a level of regulation that is not recapitulated by transfection of either factor alone. Nevertheless, the data strongly suggest that the decreased expression of HNF1α is the immediate effect of decreased AFP expression. Another case where HNF1 activity modulates AFP gene expression comes from genetic persistence of AFP expression in humans. Analysis of the AFP promoter sequence in affected individuals identified a single point mutation, a G to A transition at −119 in the AFP HNF1-binding site (55). This mutation strengthened the site, increased HNF1 binding, and also increased the expression of the AFP promoter region. A number of other genes are down-regulated when HNF1 expression is decreased in a manner strikingly similar to the AFP gene. For example, diabetes mellitus decreases HNF1 and albumin gene expression in the rat (56). Human renal cell carcinoma provides another example, where glutathione S-transferase mRNA expression is reduced concomitantly with reduction of HNF1 binding to its promoter (57).
How might RA regulate the expression of HNF1 and HNF4? The specific mechanism for RA inhibition of each gene remains to be investigated. One possibility is that RA treatment might reduce expression or activity of particular RARs or RXRs. We have found that RXRα mRNA expression is selectively reduced following RA treatment of Hep3B cells and that total transcription factor binding to DR1 and DR5 sites in RA-treated Hep3B nuclear extracts is also decreased (58). Though neither the HNF4 nor the HNF1α promoter appears to have retinoic acid receptor element (DR5) sites, the HNF4-binding site in the HNF1 promoter is also a DR1 site. The role of possible RXR or RAR binding sites in the HNF1α and HNF4 promoter regions is currently being investigated. There are several known cases where DR1 sites bind both HNF4 and RXR dimers. The hepatitis B virus enhancer I, for example, contains an RXRE-like sequence where DR1 sites bind both HNF4 and RXR dimers. The hepatitis B virus enhancer I, for example, contains an RXRE-like sequence where DR1 sites bind both HNF4 and RXR dimers. The hepatitis B virus enhancer I, for example, contains an RXRE-like sequence where DR1 sites bind both HNF4 and RXR dimers.

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