Control of Apolipoprotein AI Gene Expression through Synergistic Interactions between Hepatocyte Nuclear Factors 3 and 4*

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Apolipoprotein AI (apoAI) gene expression in liver depends on synergistic interactions between transcription factors bound to three distinct sites (A, B, and C) within a hepatocyte-specific enhancer in the 5′-flanking region of the gene. In this study, we showed that a segment spanning sites A and B retains substantial levels of enhancer activity in hepatoblastoma HepG2 cells and that sites A and B are occupied by the liver-enriched hepatocyte nuclear factors (HNFs) 4 and 3, respectively, in these cells. In non-hepatic CV-1 cells, HNF-4 and HNF-3β activated this minimal enhancer synergistically. This synergy was dependent upon simultaneous binding of these factors to their cognate sites, but it was not due to cooperativity in DNA binding. Separation of these sites by varying helical turns of DNA did not affect simultaneous binding of HNF-3β and HNF-4 nor did it influence their functional synergy. The synergy was, however, dependent upon the cell type used for functional analysis. In addition, this synergy was further potentiated by estrogen treatment of cells cotransfected with the estrogen receptor. These data indicate that a cell type-restricted intermediary factor jointly recruited by HNF-4 and HNF-3 participates in activation of the apoAI enhancer in liver cells and suggest that the activity of this factor is regulated by estrogen.

An emerging hallmark of transcriptional regulation in eukaryotes is the assembly of multiprotein complexes at the enhancer and promoter regions of target genes (1). These complexes are formed and stabilized through multiple protein-DNA and protein-protein interactions. Tissue specificity of many liver-specific genes, the expression of which is restricted to the liver, is imparted by combinatorial interactions between liver-enriched and ubiquitous transcription factors (2, 3). The synergy factors bound to three distinct sites (A, B, and C) are also provided (11). In the current report, we first define the minimum requirements for efficient enhancer function and then experimentally address several possible explanations for how the transcriptional synergy may arise. Our results suggest that the synergy may result from an additional factor, possibly a coactivator, acting in concert with the liver-enriched transcription factors HNF-3 and HNF-4.

MATERIALS AND METHODS

Synthetic Oligonucleotides—Oligonucleotides spanning the −124 to −192 (oligoA) and −178 to −148 (oligoB) regions of the apoAI promoter and the −110 to −85 transthyretin proximal promoter region have been described previously (5, 11). Complementary single-stranded oligonucleotides spanning the −220 to −135 region of apoAI (AB) with nucleotide substitutions (AX, AXB, and XB) and half helical (A-0.5-B) and full helical turns (A-1.0-B) were purified by polyacrylamide gel electrophoresis and annealed (12).

Plasmid Constructions—The wild-type and mutated versions of the −220 to −135 apoAI gene region were subcloned into the restriction site of the luciferase (LUC) basal promoter vector (−41LUC; Ref. 11). The LUC construct was created by subcloning the −220 to −192 site A oligonucleotide obtained from the plasmid 2X[A].CAT construct (13). The B.LUC (11) and various HNF-3β deletion constructs were described previously (11).

Cell Culture, Transfections, Nuclear Extracts, and Electrophoretic Mobility Shift Assay (EMSA)—All methods were as described previously (11). The affinity-purified rabbit polyclonal HNF-3β antibody was raised against amino acids 7–86 of the rat protein (14). The HNF-4 antibody was obtained from Dr. Frances Sladek (15). ARP-1 antibody has been described (16).

Bacterial Expression of HNF-3 and HNF-4—HNF-3 (17) and HNF-4 (18) cDNAs were subcloned into the bacterial expression vector pET-6His11d. After transformation of Escherichia coli BL21(DE3)pLYS5 (Novagen) and induction, histidine-tagged proteins were purified over Ni2+-affinity resin as described (19).

RESULTS

Delineation of a Minimal apoAI Enhancer in Hepatoma HepG2 Cells—We have shown previously that interactions mediated by sites A and B are major determinants of the apoAI

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§ The abbreviations used are: apoAI, apolipoprotein AI; HNF, hepatocyte nuclear factor; LUC, luciferase; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor.
Therefore, a reporter construct AB.LUC in which LUC activity is under the control of sites A and B (Fig. 1A) was transiently transfected into human hepatoblastoma HepG2 cells. As shown in Fig. 1B, this construct displayed elevated (16-fold) transcription levels over that observed with -41.LUC. Moreover, consistent with earlier data, the activity of this construct was critically dependent upon both sites A and B since mutations that disrupted transcription factor binding at either site reduced transcription to near basal levels (Fig. 1B).

The factor(s) that binds to sites A and B in HepG2 nuclei was identified by EMSA using each of these sites as probes. The major specific complex formed on site A (Fig. 1C) was competed by an oligonucleotide (α1AT) derived from the nuclear receptor binding site in the α1 antitrypsin gene (lanes 6 and 7). This oligonucleotide has been shown to selectively interact with the liver-enriched orphan receptor HNF-4 (Ref. 20 and data not shown). Minimal competition was also observed with a syn-
thetic palindromic oligonucleotide TRE (Fig. 1C, lanes 4 and 5) that selectively competes for the ARP-1/COUP-TF subfamily of nuclear receptors (Ref. 20 and data not shown). Furthermore, although the anti-HNF-4 antibody almost quantitatively supershifted the site A complex, the anti-ARP-1/COUP-TF-II and anti-Ear2 antibodies had only marginal effects on the complex (Fig. 1C, lanes 8–11). These results suggest that under these conditions, HNF-4 is a predominant factor occupying site A, in agreement with previous reports (16).

The factor(s) that binds site B was identified by similar EMSA experiments using a high affinity variant of site B derived from the rat homolog of the apoAI enhancer as probe (Fig. 1D). Four major complexes (designated C1 to C4) were observed (Fig. 1D, lane 1), and their specificity was ascertained by oligonucleotide competition. Each of them could be competed by wild-type site B (Fig. 1D, lane 2); also, inclusion of a mutant site B oligonucleotide revealed that all complexes except C4 are specific (Fig. 1D, lane 3). Supershifting with a panel of antibodies revealed that the slowest mobility complex, C1, is composed of HNF3a, whereas the next complex, C2, contains HNF3b. No supershift was observed with an anti-HNF3γ antibody. The identity of the remaining complex (C3) is currently unknown. These results establish that minimal apoAI enhancer activity in HepG2 cells resides in sites A and B and their cognate factors HNF-4 and HNF-3, respectively.

Minimal Enhancer Activity in Non-hepatic Cells—We then asked if the minimal enhancer components as defined above would suffice to reconstitute transcriptional activity directed by the partial apoAI promoter in CV-1 cells, a cell type that neither expresses the apoAI gene nor any of the liver-restricted

FIG. 2. Reconstitution of minimal apoAI enhancer activity in a non-hepatic cell. A, the A.LUC and B.LUC constructs were assayed for LUC activity by transient cotransfection into CV-1 cells, as described in the legend of Fig. 1, with 10 ng of pCMV.HNF-3β or 250 ng of pMT2.HNF-4 vector as indicated (+). An equivalent amount of either pCMV or pMT2 parent vector lacking insert was included as a negative control (−). Relative LUC activity values represent LUC:β-galactosidase enzymatic activity ratios relative to the control. B, the AB.LUC, XB.LUC, and AX.LUC constructs were assayed for LUC activity by transient cotransfection into CV-1 cells as in A.

Fig. 3. Characterization of recombinant HNF-3β and HNF-4. A, affinity-purified HNF-4 (lane 1) and HNF-3β (lane 2) proteins (1 μg) expressed in E. coli (see "Materials and Methods") were analyzed by SDS-PAGE (12.5%). Protein was visualized by staining with Coomassie Brilliant Blue. Sizes (in thousands) of molecular weight markers (mw; lane 3) are indicated. B, EMSA reactions contain bacterially produced HNF-4 and an end-labeled site A probe. A 100-fold molar excess of unlabeled competitor oligonucleotide was included as indicated: lane 2, apoAI site A (A); and lane 3, apoAI site B (B). Other additions include lane 4, a preimmune serum; or lane 5, HNF-4 antibody. C, EMSA reactions performed with bacterially produced HNF-3β and a site B probe. One hundred-fold molar excess of competitor oligonucleotide was included as indicated: lane 2, apoAI site B (B); and lane 3, apoAI site A (A). Antibody supershift experiments were performed with preimmune serum (lane 4) or HNF-3β antibody (lane 5).
FIG. 4. Complex formation by HNF-3β and HNF-4 on the enhancer does not entail cooperative binding to DNA. A, EMSA reactions with the apoAl AB probe and recombinant HNF-3β and HNF-4. Lanes 1–4 contain a fixed amount of HNF-3β (1.5 μg) with increasing amounts...
transcription factors implicated in its regulation.2 Thus, consistent with our prior report (11), constructs A.LUC and B.LUC containing sites A and B, respectively, upstream of the LUC reporter were strongly activated by their cognate factors (i.e. HNF-4 and HNF-3β) in CV-1 cells (Fig. 2A). The construct AB.LUC was also activated by either HNF-4 or HNF-3β, albeit at lower (2-3-fold) levels (Fig. 2B). In contrast, AB.LUC activation in the presence of both HNF-4 and HNF-3β was in the 10-fold range, implying a synergistic mode of action of these factors (5, 11). This synergy is dependent on the integrity of both sites A and B, as evidenced by the failure of the mutant constructs XB.LUC and AX.LUC to be fully activated by HNF-4 and HNF-3β (Fig. 2B). Thus, it appears that the reconstituted enhancer activity faithfully mimics the situation in HepG2 cells (Fig. 1B).

What is the molecular basis for this synergy? The inability of HNF-3 and HNF-4 to fully activate transcription through the AB.LUC reporter (Fig. 2, compare A and B) suggests that the activity of each factor on its cognate site is restricted by factors interacting with the other site. For example, an HNF-4 requirement for HNF-3 activity in the AB context may reflect displacement of CV-1-negative factors (e.g. ARP-1/COP-TF) from site A that potentially interferes with the HNF-3 transcriptional activity. However, the inability of HNF-3 to activate from the mutant construct XB.LUC (Fig. 2B) bearing a mutant site A argues for a more complex explanation.

Independent Binding of HNF3 and HNF4 to the Minimal Enhancer—Given the proximity of sites A and B to which HNF-4 and HNF-3 bind (Fig. 1A), we investigated if these factors bound to DNA cooperatively. For this purpose, we determined the relative affinities of HNF-3 and HNF-4 for the minimal enhancer element AB, either singly or in combination, by EMSA using highly purified, bacterially expressed preparations of these factors (Fig. 3A). Each of the proteins was characterized with respect to binding to its cognate site. Recombinant HNF-4 and HNF-3β bound sites A and B, respectively, with high specificity as determined by oligonucleotide competition and antibody supershift experiments (Fig. 3, B and C).

Simultaneous binding of recombinant HNF-3 and HNF-4 to an oligonucleotide probe containing sites A and B in their natural arrangement was then evaluated. Specifically, to a fixed amount of HNF-3β increasing amounts of HNF-4 was added, and binding was analyzed by EMSA (Fig. 4A). A higher order complex displaying a mobility slower than that of the complexes formed by either HNF-3β or HNF-4 alone was apparent. The presence of both factors in the ternary complex (T) was further demonstrated by selective oligonucleotide competitors (lαL-antitrypsin for HNF-4 and transthyretin for HNF-3), which precluded binding of one factor but not the other (Fig. 4A, lanes 5 and 6). Similar results were obtained when HNF-3β concentration was varied against a fixed amount of HNF-4 (data not shown).

To assess if the formation of this ternary complex involves cooperative interactions between HNF-3 and HNF-4, the relative binding affinities of each protein to the AB probe were determined in the absence or presence of the other factor. Formation of the two binary complexes (Fig. 4B), as well as the ternary complex (Fig. 4C), was monitored as a function of

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2 D. C. Harnish, S. Malik, E. Kilbourne, R. Costa, and S. K. Karathanasis, unpublished observations.
Fig. 6. The N-terminal domain of HNF-3β is required for independent and synergistic transcription activation.

Summary of transcriptional activities of HNF-3β and its deletion mutants, shown schematically. Ten ng of vectors expressing HNF-3β and amino- (ΔN-HNF-3β), lacking transactivation domains IV and V or carboxyl-terminal (ΔC-HNF-3β), lacking transactivation domains II and III) deletion variants as indicated were cotransfected with B.LUC or AB.LUC reporter into CV-1 cells. For each cotransfection involving AB.LUC reporter construct, 250 ng of pMT2.HNF-4 vector were also included. The LUC activity levels are depicted as a ratio of mutant to wild-type levels. The transcriptional activities obtained previously (21) for a TTR reporter are included for comparison; absolute transcription levels from site B and TTR-dependent reporters were not compared.

| TRANSACTIVATION DOMAIN | DNA BINDING | TRANSACTIVATION DOMAIN |
|------------------------|-------------|------------------------|
| HNF-3β                 |             |                       |
| ΔC-HNF-3β              |             |                       |
| ΔN-HNF-3β              |             |                       |
| 1-566                  |             | 1-1458                 |
| 1-369                  |             | 1-458                  |
| 153-456                |             | 900                    |

Table: Summary of transcriptional activities of HNF-3β and its deletion mutants.

| Domain | Activity (B.LUC) | Activity (AB.LUC) | Activity (TTR) |
|--------|------------------|-------------------|----------------|
| IV     | 1.0              | 1.0               | 1.0            |
| V      | 1.0              | 0.6               | 0.2            |
| BLUC   | 0.3              | 0.2               | 0.4            |

The N-terminal domain of HNF-3β is required for independent and synergistic transcription activation. The oligonucleotide XB containing a mutant site A was used to measure the affinity of HNF-3β, whereas the oligonucleotide AX containing a mutant site B was used to measure the affinity of HNF-4. As shown in Fig. 4, D and E, the competition profiles of the binary complexes formed by each protein, as well as that of their ternary complexes, are virtually indistinguishable. Therefore, synergistic transcriptional activation by HNF-3 and HNF-4 is unlikely to result from cooperative binding to DNA.

Does the natural arrangement of sites A and B in the enhancer (i.e., their proximity and/or stereospecific alignment) contribute to the functional synergy between HNF-3 and HNF-4? To address this issue, spacer-length mutants of the minimal enhancer were generated by introducing half or full helical turns between sites A and B. The mutants were assayed for function by introducing them into LUC reporters (A-0.5-B and A-1.0-B) and for binding by EMSA.

As with the parental construct, a −10-fold synergistic activation was elicited from each construct upon cotransfection with expression vectors for HNF-3β and HNF-4 into CV-1 cells (Fig. 5A). Similar results were observed when these constructs were transiently transfected into HepG2 cells (data not shown). Another construct, AXB.LUC, in which nucleotides between sites A and B were mutated, also behaved like the parent.

EMSA experiments (Fig. 5B) using A-0.5-B and A-1.0-B oligonucleotides as probes also showed that ternary complex formation by HNF-3 and HNF-4 was unaffected by these spacing mutants. Taken together with the finding that HNF-3 and HNF-4 do not bind cooperatively to the enhancer sites, these data suggest that transcriptional synergy operates at a level secondary to factor binding to DNA.

Colocalization of HNF-3β Domains for Independent and Synergistic Transcription Activation—Does HNF-3β acting in its synergistic mode with HNF-4 draw on interactions not normally invoked for unilateral activation through a single cognate site? Multiple HNF-3β transactivation domains have been characterized and shown previously to be required for activation from the transthyretin promoter (14, 21). Therefore, we determined whether the same regions were necessary to activate transcription from apoAI site B or, synergistically with HNF-4, through the minimal element AB. For this purpose, mutant derivatives of HNF-3β in which various domains had been selectively deleted while retaining DNA binding activity (14, 21) were tested in transient cotransfection experiments in CV-1 cells (Fig. 6). As observed for activation via the transthyretin site (21), deletion of the N-terminal HNF-3β conserved domains IV and V abrogated both site B-dependent transactivation (B.LUC) as well as synergistic activation with HNF-4 through the AB.LUC reporter. Deletion of the C-terminal transactivation domains II and III, which abolishes transthyretin promoter activation, had no effect on activity through site B, whereas synergistic activation with HNF-4 via AB was only slightly affected. These data indicate that, although the cognate site may dictate which activation domains (C-terminal or N-terminal) predominate, for a given site qualitatively similar activation interfaces are used by HNF-3β in both its unilateral or synergistic modes.

HNF-3 and HNF-4 Synergy Is Cell Type-specific—Based on the data presented above, it is conceivable that HNF-3β and HNF-4 synergize via a mechanism involving joint interaction with a molecular target leading to enhanced transcription by RNA polymerase II at the apoAI promoter. This could include recruitment of a common intermediary factor (1, 22) or multi-pronged targeting of the basal transcription factors associated with the polymerase (23, 24).

To begin discriminating between these alternatives, we sought to establish the cell type repertoire in which HNF-3β and HNF-4 exhibit synergistic transactivation through the minimal enhancer. We titrated the amount of HNF-4 and HNF-3β expression vectors to determine the saturating amount of each required for activity, respectively, on A.LUC and B.LUC reporters in a variety of non-hepatic cells (Fig. 7, A and B). Next, the AB.LUC construct was monitored for LUC activity upon cotransfection of the saturating amount of HNF-3β and HNF-4 expression vectors. As shown in Fig. 7C, the degree of transcriptional synergy varied for each cell type. In CV-1, 293, and Hela cells, the synergy factor (defined as the difference between the observed activity and the activity expected if the factors functioned additively) was −100, −85, and −30-fold, respectively. By contrast, L cells demonstrated no ability to support HNF-3- and HNF-4-dependent synergy (synergy factor, −0.4). These results indicate that cell type context is an important determinant of whether HNF-3β and HNF-4 can synergize on the apoAI enhancer and, therefore, point to the involvement of additional cell type-specific factor(s) in enhancer activation.

Estrogen Regulation of the apoAI Minimal Enhancer—Since HNF-3 and HNF-4 play a dominant role in the transcription activation of the apoAI enhancer and given that estrogen modulates apoAI expression (25–27), we tested whether their activity alone or together could be influenced by the estrogen receptor (ER) in the presence of estrogen. For this purpose, the
The activity of the AB.LUC reporter in CV-1 cells (Fig. 8A) was monitored upon cotransfection with HNF-3β, HNF-4, and ER in the presence of 17β-estradiol. Without cotransfected HNF-3β and HNF-4, the ER (in the absence or presence of 17β-estradiol) had negligible effects on the activity of the AB.LUC reporter. By contrast, the residual activation by HNF-4 on this reporter (see also Fig. 2B) was elevated by 5-fold in the presence of the ER in a ligand-dependent fashion. No effect on HNF-3β activity was observed. As evident from the mutant reporter constructs AX.LUC and XB.LUC (Fig. 8B), the estrogen enhancement of HNF-4 activity was not only dependent upon site A but also on site B. Activation levels of the site A reporter A.LUC in the presence of HNF-4 were not enhanced by estrogen and ER (data not shown). Moreover, the ER further enhanced the activity of HNF-3 and HNF-4 on the minimal enhancer by about 2-fold (Fig. 8A). As expected, this effect was also dependent upon factor binding to sites A and B since no enhancement of HNF-3 and HNF-4 synergy was elicited from the mutant reporter constructs AX.LUC and XB.LUC (Fig. 8B). These results suggest that liganded ER influences the synergy between HNF-3β and HNF-4 by modulating the structure or activity of the multiprotein complex on the minimal apoAI enhancer.

**DISCUSSION**

Early models for transcriptional synergy among distinct cis-acting modules (28) postulated that synergy may result from cooperative binding of the transcription factors to their respective cognate sites (29). Alternatively, multiple secondary interactions of enhancer-bound factors with targets in the core transcription machinery could result in transcriptional synergy (23, 24, 30, 31). The latter could occur directly, involving compo-
ponents of the RNA polymerase II-specific basal transcriptional factors (including the TATA box-binding protein-associated factors; Refs. 32 and 33) or indirectly, through adaptors and/or coactivators that serve to bridge the enhancer and core complexes (1, 34).

The present data unequivocally rule out cooperative DNA binding of HNF-3 and HNF-4 as a contributing factor for the synergistic transactivation of the apoAI enhancer. Therefore, it is likely that these factors synergize at a secondary level wherein protein-protein interactions with the transcription machinery are exclusively used. Similar conclusions have also been reached by other studies investigating the molecular basis of transcriptional synergy (35–37). However, our observation that the cell type repertoire in which this synergy operates is rather limited raises the possibility that the factor responsible for this synergy is unlikely to be one of the ubiquitous basal transcriptional factors or the TATA box-binding protein-associated factors. Cell type-restricted coactivators have been described recently (38, 39) and are thus plausible candidates for factors through which HNF-3 and HNF-4 might channel their combined transactivation potential. Therefore, we propose that an intermediary factor normally present in liver cells is recruited to the enhancer and core transcription complexes when both HNF-3 and HNF-4 occupy their binding sites but not with either of them occupying their cognate sites individually (Fig. 2B). In this way, this factor, in addition to simply being an adaptor molecule, could also function to integrate the signals regulating the primary transcription factors (HNF-3 and HNF-4).

This observation also adds another level at which apoAI tissue restriction is enforced. Thus, it is not expressed in some extrahepatic tissues because they lack the primary transcription factors, and in others, because the intermediary factor is absent. It also follows that the expression through the enhancer could be modulated by regulating the activity of this factor in addition to regulation of the primary transcription factors HNF-3 and HNF-4.

Indeed, our results raise the possibility that estrogen may be among the physiologically relevant signals using such a pathway to modulate apoAI expression. Although the estrogen response is primarily mediated by site A-bound HNF-4 (Fig. 8A), it is interesting that estrogen stimulation of HNF-4 activity (in the absence of cotransfected HNF-3) also required an intact site B (Fig. 8B). This may be due to a DNA-binding factor present in CV-1 cells that can substitute for HNF-3 or, less likely, to site B-dependent allosteric influences in HNF-4 activity. Thus, since estrogen stimulates apoAI enhancer activity through a mechanism that relies on factors binding to both sites A (HNF-4) and B (either HNF-3 or a CV-1 factor), it could, in accord with our suggestion, influence an intermediary factor responsible for the synergy. Although this could be a secondary effect of the liganded ER (i.e. through induction of an alternative intermediary factor) an intriguing interpretation is that the receptor per se facilitates communication of the enhancer-bound HNF-4 (and of HNF-3 in collaboration with HNF-4; Fig. 8) with the basal transcription complex. Since no consensus estrogen response element is discernible in the apoAI minimal enhancer, this is reminiscent of the involvement of the ER in activating the brain creatine kinase gene promoter (40) or the fos-jun complex at the ovalbumin gene promoter (41). As in our model, the ER is thought to be tethered to the nucleoprotein complex solely by protein-protein interactions as in these examples.

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