Direct Physical Interactions between HNF-4 and Sp1 Mediate Synergistic Transactivation of the Apolipoprotein CIII Promoter

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ABSTRACT: We have investigated the mechanism of functional cooperativity between specificity protein 1 (Sp1) and hepatocyte nuclear factor-4 (HNF-4) on the human apolipoprotein CIII (apoCIII) promoter. Cotransfections in *Drosophila* SL2 cells that lack endogenous Sp1 or Sp1-related activities showed that HNF-4 and Sp1 synergistically transactivate the −890/+24 apoCIII promoter up to 150-fold. Synergistic transactivation required the HNF-4 binding site of the apoCIII enhancer. Deletion of part of the Ser/Thr-rich and Gln-rich domain or the C-terminal domain of Sp1 decreased, and deletion of residues 501−610 of Sp1 increased, the functional cooperativity between Sp1 and HNF-4. Physical interactions between the two factors were demonstrated by glutathione *S*-transferase pull-down and co-immunoprecipitation assays. The amino terminal domain of both factors and the carboxy terminal domain of Sp1 contribute to these interactions. Antagonism between HNF-4 and Sp1 was demonstrated on homopolymeric promoters containing multiple binding sites for either factor, suggesting that the synergism between the two factors occurs only when both factors are bound simultaneously to the DNA. The observed physical interactions between Sp1 and HNF-4 in the context of the apoCIII promoter may explain in part their in vitro and in vivo synergism in the transcriptional activation of the apolipoprotein A-I/apoCIII/apolipoprotein A-IV gene cluster.

Previous studies have established that there is a linkage (*1*) and a common regulatory mechanism of the apoA-I/apoCIII/apoA-IV[1] gene cluster (*2*−*11*). Each of the three genes contains hormone response elements (HREs) in its proximal promoter which bind orphan and ligand-dependent nuclear receptors with different specificities (*2*−*5*). Cell culture studies showed that the distal regulatory region of the apoCIII promoter (−790/−590) acts as a common enhancer for the three genes of the cluster (*4*, *6*, *7*). The enhancer also contains two HREs and three Sp1 binding sites (*3*, *6*, *7*). Mutagenesis analysis showed that alterations which prevent the binding of nuclear receptors to HREs diminished the activity of these promoters in cell cultures (*2*−*8*). Mutations in the HREs and some of the Sp1 sites of the enhancer also significantly affected the enhancer activity in cell cultures (*3*, *4*, *6*, *7*). It appears that the hepatic transcription of the human apoCIII gene is regulated by complex interactions between HNF-4 and possibly other nuclear receptors bound to the proximal promoter and distal enhancer sites and Sp1 bound to the enhancer (*3*, *4*, *6*, *7*).

Previous in vivo studies had indicated that the apoCIII enhancer is required for the intestinal expression of the apoA-I, apoCIII, and apoA-IV genes (*11*−*15*). Transgenic mice carrying the wild-type proximal apoA-I and apoCIII promoters in combination with the apoCIII enhancer showed correct intestinal and hepatic expression of the two linked genes in vivo (*14*). When all the HREs of the proximal apoCIII and apoA-I promoters and the HRE of the apoCIII enhancer were mutagenized, the expression of the two linked genes was abolished, indicating that the HREs are essential for the in vivo activity of the promoter/enhancer cluster in all tissues. Similarly, mutagenesis of all three Sp1 sites of the enhancer abolished the expression of the apoCIII gene in all tissues (*14*, *15*). This analysis showed that the HREs and the Sp1 binding sites of the apoCIII enhancer control the intestinal expression of the apoA-I and apoCIII genes and contribute to the hepatic expression of the two genes in vivo (*14*, *15*).

The current study was undertaken to further probe the mechanism responsible for the functional synergism between

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[1] Abbreviations: 9-*cis*-RA, 9-*cis*-retinoic acid; AdML, adenovirus major late promoter; apoA-I, apolipoprotein A-I; apoA-IV, apolipoprotein A-IV; ARP-1, apoA-I regulatory protein 1; ATCC, American tissue culture collection; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; DR1, DR2, etc., direct repeats with 1, 2, etc. nucleotide spacing between the half-repeats; DTT, dithiothreitol; EAR-2, EAR-3, v-erbA-related factor-2 and -3, respectively; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GEMSA, gel electrophoretic mobility shift assay; GST, glutathione *S*-transferase; HNF-4, hepatocyte nuclear factor-4; HRE, hormone response element; HRP, horseradish peroxidase; OPNG, *O*-nitrophenyl galactoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; PMSF, phenylmethylsulfonyl fluoride; PPARα, peroxisome proliferator activated receptor α; RARα, retinoic acid receptor α; RXRα, retinoid X receptor α; Sp1, specificity protein 1; T3, triiodothyronine; T3Rβ, thyroid hormone receptor β; TAFs, TATA box binding protein associated factors; TIFs, transcriptional mediators/intermediary factors; WT, wild type.

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HNF-4 and Sp1 on the human −890/+24 apoCIII promoter/enhancer using the Sp1-deficient *Drosophila* SL2 cell line (*16*). Our findings suggest that the functional synergism may result from direct physical interaction between the two transcription factors bound to the apoCIII enhancer and requires that both HNF-4 and Sp1 are DNA-bound. A model is proposed whereby an HNF-4/Sp1 complex bound on the apoCIII enhancer interacts with HNF-4 and possibly other factors bound to the proximal promoter. Additional interactions of the two complexes with transcriptional coactivators and the basal transcription factors may be responsible for the transcription synergism between HNF-4 and Sp1. Understanding the transcriptional regulatory mechanisms of the apoCIII gene may provide rational approaches to downregulate this gene and control hypertriglyceridemia.

MATERIALS AND METHODS

Materials. Reagents were purchased from the following sources: Sequenase v2 kit from Amersham/USB; reagents for cell culture from GIBCO-BRL (Life Technologies, Inc., Gaithersburg, MD); luciferase assay kit from Promega Corp. (Madison, WI); all oligonucleotides from IMBB (Heraklion, Crete, Greece); monoclonal anti-FLAG M2 antibody from Sigma; polyclonal anti-Sp1 antibody from Santa-Cruz Biotechnology; anti-mouse horseradish peroxidase-conjugated secondary antibody from Chemicon; acetyl CoA, dNTPs, and [^{14}C]chloramphenicol from Amersham/Pharmacia.

Methods. (a) Cell Cultures, Transient Transfections, and CAT and Luciferase Assays. Human hepatoma HepG2 cells and monkey kidney COS-1 and COS-7 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. *Drosophila* SL2 cells (ATCC-1963 CRL) (*16*) were cultured in Schneider's SL2 medium (Life Technologies) supplemented with 10% FBS and penicillin/streptomycin at 25 °C. Transient transfections were performed using the $Ca_3(PO_4)_2$ coprecipitation method (*17*). Chloramphenicol acetyl transferase, β-galactosidase assays were performed as described previously (*18*, *19*). The luciferase activity was determined using the luciferase assay kit (Promega) according to the manufacturer's instructions. Bioiluminescence was monitored using the Turner illuminometer.

(b) Plasmid Constructions. The apoCIII promoter plasmids CIII −890/+24 CAT, CIII-B mut CAT, CIII-I4 mut CAT, CIII AdML CAT, CIII (IM4) AdML CAT, and CIII (IM1) AdML CAT have been described previously (*6*, *7*, *9*). The reporter plasmid −890/+24 CIII luc was constructed by subcloning the −890/+24 apoCIII promoter fragment into the *Sma*I site of vector pGL3-Basic (Promega). The *Drosophila* expression vectors pPacO, pPac-Sp1 83−778 (WT), pPac-Sp1 262−778 (ΔA), pPac-Sp1 83−702 (ΔD), pPac-Sp1 451−778 (Δ501−610) (ΔABC), and pPac-Sp1 Δ501−610 (ΔC) were a generous gift of Dr. Robert Tjian, University of California, Berkeley. Plasmid pPac-HNF-4 was constructed by excising the cDNA encoding the rat HNF-4A from vector pGEX-HNF-4 by *Bam*HI and *Eco*RI, filling the overhang by the Klenow fragment of the DNA polymerase I, and ligating into the blunted *Bam*HI/*Xho*I sites of vector pPacO. For the construction of the GST−Sp1 fusion proteins pGEX-Sp1 83−778, pGEX-Sp1 262−778 (ΔA), and pGEX-Sp1 83−702 (ΔD), the cDNAs encoding the Sp1 forms were excised from the corresponding pPac vectors and were subcloned into the *Eco*RI/*Xho*I or *Bam*HI sites of the pGEX-1ZT vector (Amersham/Pharmacia). The WT or truncated HNF-4 forms 1−465, 1−370, 1−360, 1−339, 1−289, 1−174, 1−128, 128−370, 175−370, and 48−455 as well as the FLAG-tagged HNF-4 forms 1−455 and 1−128 in vector pCDNA1-amp (Invitrogen Corp.) have been described previously (*20*). The pG_6TI-CAT plasmid containing six tandem Sp1 sites in front of the tk minimal promoter and the CAT gene were a generous gift of Dr. G. Gill, Harvard Medical School, Boston. The pG_5B-CAT vector was a generous gift of Dr. G. Mavrothalassitis (University of Crete Medical School, Heraklion, Greece). The $(BA1)_5$-CAT reporter plasmid has been described elsewhere (*21*).

(c) Gel Electrophoretic Mobility Shift Assays. Gel electrophoretic mobility shift assays (GEMSAs) were performed as described previously (*21*). Sense and antisense oligonucleotides were annealed to generate the double-stranded oligonucleotide probe and labeled by filling the overhangs with the Klenow fragment of the DNA polymerase I using dCTP (α-^{32}P) as described (*21*). The sequence of the Sp1 consensus binding site oligonucleotide used as a probe is as follows: 5′-ATT CGA TCG GGG CGG GGC GAG C-3′.

(d) In Vitro Transcription−Translation. pCDNA1-amp constructs containing the wild-type HNF-4 and its truncated forms were linearized with *Bam*HI, transcribed in vitro with T7 RNA polymerase, and translated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine using the TnT kit from Promega as recommended by the manufacturer.

(e) GST Protein Interaction Assay. Glutathione−Sepharose 4B beads (Pharmacia) were equilibrated in PBS and mixed with 1 volume of bacterially expressed GST fusion proteins on a rotary shaker for 60 min at 4 °C. The beads were washed three times with 10 volumes of PBS and equilibrated in washing buffer (20 mM Hepes (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.2% NP-40, 2 mM PMSF, 10 μg/mL aprotinine). A 50 μL sample of a 1:1 bead slurry in washing buffer was combined with 5−10 μL of in vitro synthesized proteins in a final volume of 500 μL of washing buffer−10% glycerol (interacting buffer) on a rotary shaker for 90 min at 4 °C. The beads were then washed five times with 20 volumes of washing buffer, and the bound proteins were eluted by boiling in Laemmli SDS−PAGE loading buffer and were subjected to SDS−PAGE. Bound proteins were visualized by autoradiography.

(f) Co-immunoprecipitation−Western Blotting Assays. For co-immunoprecipitation experiments, HepG2 cells were transfected with 17 μg of FLAG plasmids: HNF-4 and C-terminal deletion mutant HNF-4 (1−128). After 40 h, the cells were washed 2× with ice-cold phosphate-buffered saline (PBS) and scraped into TEN buffer (40 mM Tris•HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl). The cells were pelleted by centrifugation at 10000 rpm for 10 min at 4 °C and resuspended in nonidet P-40 lysis buffer (150 mM NaCl, 1% nonidet P-40, 50 mM Tris•HCl (pH 8.0), 1 mM dithiothreitol, 5 mM NaF) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 13 μg/mL aprotinin, and 6.7 μg/mL leupeptin). The lysates were collected by centrifugation at 10000 rpm for 10 min at 4 °C and then precleared by incubation with 30 μL of protein G PLUS-agarose (Santa-Cruz Biotechnology) on a rotating

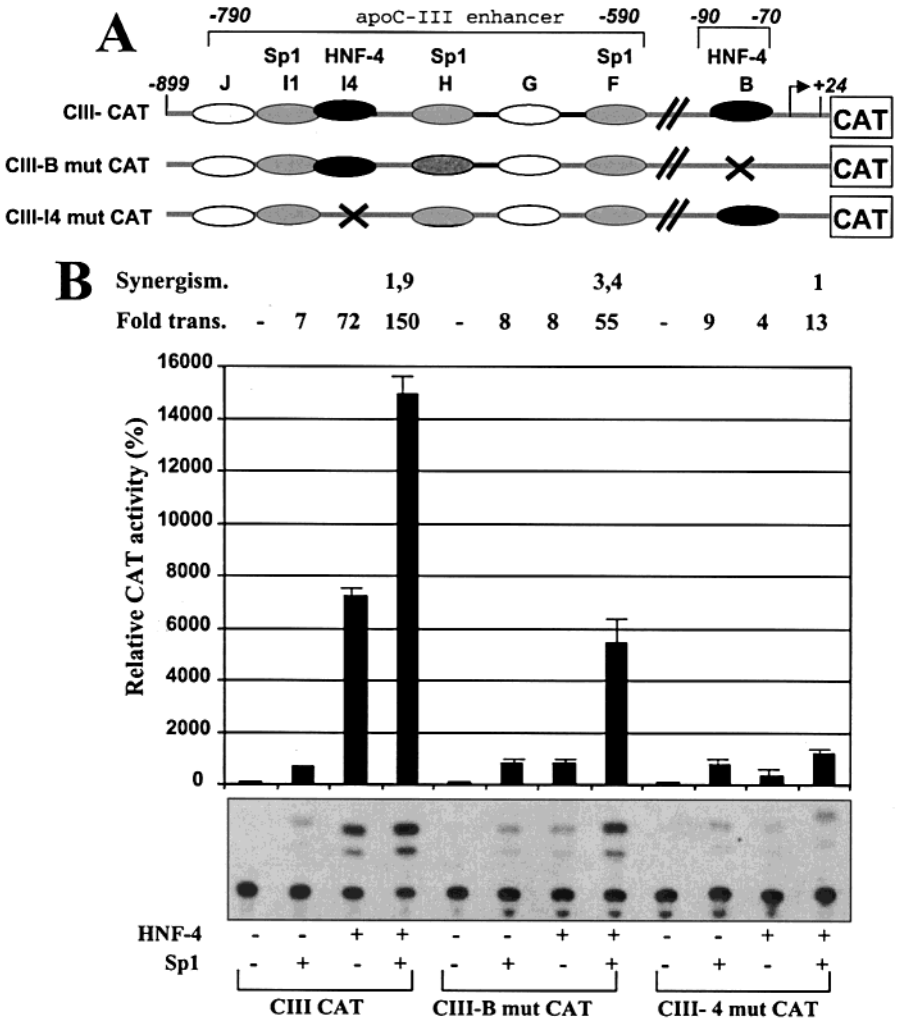

FIGURE 1: Cotransfection experiments in *Drosophila* Schneider SL2 cells showing functional synergism between HNF-4 and Sp1 on the human −890/+24 apoCIII promoter. (A) Schematic representation of the regulatory elements present on the human apoCIII promoter. The positions of the elements relative to the transcription initiation site of the apoCIII gene are shown at the top. The transcription factors HNF-4 and Sp1 that bind to elements CIIIB, CIII-I4 and CIIIF, CIIIH, and CIII-I1, respectively, are also indicated. Mutations in the HNF-4 sites are shown with an "×". (B) shows the contribution of the distal and proximal HNF-4 binding sites to the functional synergism between HNF-4 and Sp1. *Drosophila* SL2 cells were cotransfected with 1 μg of the indicated WT or mutated (−890/+24) apoCIII CAT reporter plasmid, 25 ng of pPac-Sp1, and 50 ng of pPac-HNF-4 plasmids as indicated. The hsp-lacZ plasmid (500 ng) expressing β-galactosidase under the control of the hsp70 promoter was included in each transfection for normalization of transfection variability. CAT assays were performed as described in the Experimental Procedures. The mean values (±SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph.

platform for 3 h at 4°C, followed by 30 s of spin (quick spin) and collection of the supernatant. The supernatants were incubated with 5 μg/mL polyclonal anti-Sp1 antibody (Santa-Cruz Biotechnology) on a rotating platform O/N at 4°C, followed by incubation with 30 μL of protein G PLUS-agarose beads for 3 h. The beads were washed three times with PBS containing 0.02% Tween 20, pelleted by 30 s of spin at 4°C, resuspended in 5× loading buffer, and analyzed on 10% SDS−polyacrylamide gel. The proteins were electrotransferred to Immobilon-P membranes (Millipore). The membranes were probed with 10 μg/mL monoclonal anti-M2 antibody (Sigma) followed by HRP-conjugated anti-mouse antibody and visualized by treatment with enhanced chemiluminescence substrate (Amersham).

RESULTS

Synergistic Interactions between HNF-4 and Sp1 Transactivate the Human apoCIII Promoter in the Sp1-Deficient SL2 Cells. Synergism Requires the HNF-4 Binding Sites of the apoCIII Enhancer. The human −890/+24 apoCIII promoter/enhancer contains two binding sites for HNF-4 (elements CIIIB and CIII-I4) and three binding sites for Sp1 (elements CIIIF, CIIIH, and CIII-I of the enhancer) (Figure 1A). These sites are essential for the activity of the apoCIII promoter in vitro and in vivo (*6*, *7*, *14*, *15*). We have used *Drosophila*-derived SL2 cells which lack Sp1 or Sp1-related activities (*16*, *22*) to probe the potential synergism between HNF-4 and Sp1 in the transactivation of the apoCIII promoter/enhancer. SL2 cells were transiently cotransfected with the WT or mutated −890/+24 apoCIII CAT reporter constructs (shown in Figure 1A) in the absence or presence of *Drosophila*-specific expression vectors for HNF-4, Sp1, or a combination of the two. As shown in Figure 1B, overexpression of HNF-4 or Sp1 in SL2 cells resulted in a strong transactivation of the apoCIII promoter/enhancer (72-fold and 7-fold, respectively). More importantly, coexpression of HNF-4 and Sp1 resulted in a stronger 150-fold transactivation of the apoCIII promoter/enhancer. This trans-

activation was not additive (72 + 7 = 79-fold), but rather synergistic (150-fold). This finding indicates functional cooperativity between the two factors when they are bound simultaneously to the apoCIII promoter/enhancer. Synergism can be defined as the quotient of the sum of the fold transactivations achieved when the two factors are cotransfected separately by the fold transactivation achieved when the two factors are transfected simultaneously. This quotient, using the WT HNF-4 and Sp1 forms, is approximately 2 (range 1.9−2.2).

To examine whether the observed synergism between HNF-4 and Sp1 requires HNF-4 binding to the proximal or distal HREs present on elements CIIIB and CIII-I4, respectively, or to both, we performed cotransfection experiments in SL2 cells using promoter constructs mutated in either of the two HNF-4 binding sites. Two such constructs were utilized (Figure 1A): Construct CIIIB mut CAT contains the −890/+24 apoCIII promoter/enhancer with nucleotide substitutions in the proximal element CIIIB (−90/−70). As shown previously, this region contains an HRE which serves as a binding site for a number of orphan and ligand-dependent nuclear receptors including HNF-4 (*3*, *7*−*9*). Mutagenesis of this region abolished binding of all nuclear receptors to this site and reduced the hepatic activity of the human apoCIII promoter/enhancer to 5% relative to the control (*9*). Construct CIII-I4 mut CAT (Figure 1A) contains nucleotide substitutions in element CIII-I4 of the apoCIII enhancer, shown previously to bind HNF-4 and other hormone nuclear receptors (*3*, *7*). Mutagenesis in the CIII-I4 element reduced the hepatic activity of the apoCIII promoter/enhancer by 90% (*3*). As shown in Figure 1B, mutagenesis of the proximal HNF-4 site of the CIIIB mut CAT construct reduced the HNF-4-mediated transactivation of the apoCIII promoter/enhancer (8-fold for the mutant promoter/enhancer versus 72-fold for the WT promoter/enhancer), and it had no effect on the Sp1-mediated transactivation. It also reduced the overall transactivation of the apoCIII promoter/enhancer by HNF-4 and Sp1 (55-fold for the mutant promoter/enhancer versus 150-fold for the WT promoter/enhancer). However, the synergism between the two factors was retained (1.9-fold for the WT promoter/enhancer versus 3-fold for the mutant promoter/enhancer). Mutagenesis of the distal CIII-I4 site of the CIII-I4 mut CAT construct again had no effect on Sp1-mediated transactivation, which was 9-fold, but it reduced the HNF-4-mediated transactivation to 4-fold and abolished the synergism between HNF-4 and Sp1 in the transactivation of the apoCIII promoter/enhancer (transactivation by combination of Sp1 and HNF-4 was 13-fold) (Figure 1B). It appears that the apoCIII promoter/enhancer can be transactivated by two types of synergistic interactions: (a) synergistic interactions between HNF-4 molecules bound to the proximal apoCIII promoter and the apoCIII enhancer (Figure 1B, lane 3); (b) synergistic interactions between HNF-4 and Sp1 molecules bound to the enhancer (Figure 1B, lane 8). The overall transactivation of the −890/+24 apoCIII promoter (Figure 1B, lane 4) by combination of the HNF-4 and Sp1 represents approximately the sum of synergistic interactions between HNF-4 and Sp1 molecules bound to the apoCIII enhancer and the HNF-4 molecules bound to the proximal apoCIII promoter and the enhancer (compare lanes 3, 4, and 8 of Figure 1B).

The combined data of Figure 1 indicate that (a) the apoCIII promoter/enhancer in SL-2 cells is activated synergistically by Sp1 and HNF-4, (b) transcriptional synergism between HNF-4 and Sp1 on the apoCIII promoter/enhancer requires the HNF-4 binding site of the apoCIII enhancer, and (c) optimal transactivation of the apoCIII promoter/enhancer appears to be the result of functional interactions between HNF-4 and Sp1 molecules bound to the apoCIII enhancer and HNF-4 molecules bound to the proximal apoCIII promoter.

To study further the mechanism of synergism between Sp1 and HNF-4 on the apoCIII enhancer, three additional constructs were utilized (Figure 2A): Construct CIII AdML CAT (*6*) expresses the reporter CAT gene under the control of the WT apoCIII enhancer region (−790/−590) fused with the minimal adenovirus major late promoter (AdML) region −44/+1. Constructs CIII (IM4) AdML CAT and CIII (IM1) AdML CAT are the same as above except that the apoCIII enhancer contains point mutations in elements CIII-I4 and CIII-I1 of the apoCIII enhancer which bind HNF-4 and Sp1, respectively (*6*, *7*). These constructs were cotransfected into SL2 cells along with expression vectors for Sp1, HNF-4, or a combination of the two factors, and the CAT activity was monitored by CAT assays. As shown in Figure 2B, Sp1 and HNF-4 caused a 46- and approximately 2-fold transactivation of the apoCIII enhancer, respectively, whereas a combination of HNF-4 and Sp1 resulted in 103-fold transactivation. The findings suggest that the combination of the two factors potentiated the activity of the apoCIII enhancer by approximately a factor of 2 (103/47 = 2.2). The mutation in the CIII-I4 element abolished the HNF-4-mediated transactivation of the apoCIII enhancer and reduced the Sp1-mediated transactivation by 74% (12-fold for the mutant versus 46-fold for the WT enhancer). The synergism between HNF-4 and Sp1 was lost, and instead antagonism between HNF-4 and Sp1 was observed in this mutated enhancer. The transactivation by Sp1 alone was 12-fold, and that by combination of Sp1 and HNF-4 was 3.5-fold (Figure 2B). This finding suggests potentially negative interactions between HNF-4 and Sp1 when only one of the two factors, in this case Sp1, binds to the DNA and the other factor, in this case HNF-4, is in excess in the nucleus. These negative effects were confirmed using homopolymeric promoters and are presented later in the Results.

Mutagenesis of one of the three Sp1 binding sites of the apoCIII enhancer (on element CIII-I1) reduced the transactivation of the −890/+24 apoCIII promoter/enhancer by Sp1 (8-fold for the mutant enhancer versus 46-fold for the WT enhancer) and by combination of HNF-4 and Sp1 (39-fold for the mutant enhancer versus 105-fold for the WT enhancer). However, the synergism between HNF-4 and Sp1 was retained (43-fold), possibly due to the presence of two additional Sp1 binding sites (transactivation by Sp1 and HNF-4 is 8- and 1-fold, respectively, and that by a combination of both factors is 39-fold). Previous studies in HepG2 cells also showed that the activity of the −890/+24 apoCIII promoter/enhancer was reduced by 58% when element CIII-I1 was mutated, but its transactivation by HNF-4 was not affected (*6*).

The combined data of parts A and B of Figure 2 indicate that HNF-4 binding to element CIII-I4 is necessary for the

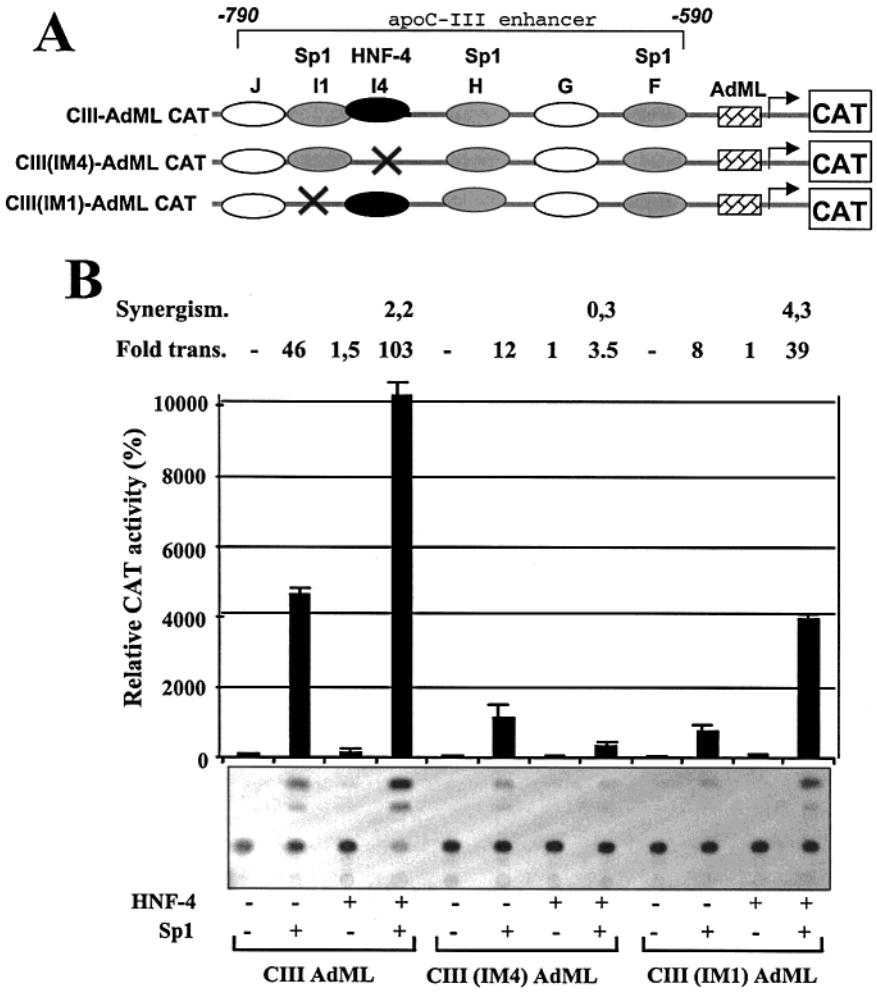

FIGURE 2: Cotransfection experiments in *Drosophila* Schneider SL2 cells showing that functional synergism between HNF-4 and Sp1 requires the regulatory element I4 on the apoCIII enhancer. (A) Schematic representation of the reporter constructs used in the transactivation experiments of (B). Symbols are as in Figure 1A. AdML: Minimal region (−44/+1) of the adenovirus major late promoter. The arrow indicates the transcription initiation site. (B) shows the contribution of the distal HNF-4 binding site on element CIII-I4 to the functional synergism between HNF-4 and Sp1. *Drosophila* SL2 cells were cotransfected with 1 μg of the indicated WT or mutated (−790/−590) apoCIII-AdML-CAT reporter plasmid, 25 ng of pPac-Sp1, and 50 ng of pPac-HNF-4 plasmids as indicated. The hsp-lacZ plasmid (500 ng) expressing β-galactosidase under the control of the hsp70 promoter was included in each transfection for normalization of transfection variability. CAT assays were performed as described in the Experimental Procedures. The mean values (±SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph.

optimal synergistic transactivation of the apoCIII promoter/enhancer by HNF-4 and Sp1.

Domains in Sp1 That Are Required for the Synergism with HNF-4. To identify domains in Sp1 that are required for the synergism with HNF-4, a number of mutant Sp1 forms were utilized (*22*, *23*). These mutants, which are shown schematically in Figure 3B, include amino-terminal, carboxy-terminal, and internal deletions of the Sp1 protein. *Drosophila* expression vectors for these Sp1 mutants were transfected into SL2 cells either alone or in combination with HNF-4, and the activity of the −890/+24 apoCIII promote/enhancer was monitored by luciferase assays. As shown in Figure 3A, deletion of domain A or D of Sp1 reduced, and deletion of domains A, B, and C of Sp1 eliminated, the functional synergism between HNF-4 and Sp1. Interestingly, deletion of only domain C of Sp1 resulted in an Sp1 form that was a more potent transactivator of the apoCIII promoter/enhancer (140-fold transactivation for the mutant versus 5-fold for the WT Sp1) and displayed enhanced synergism with HNF-4 (900-fold for the mutant versus 53-fold for the WT Sp1, which correspond to a synergism of 5,6 and 2,1, respectively). The data presented in Figure 3 suggest that the functional synergism between HNF-4 and Sp1 in the transactivation of the apoCIII promoter/enhancer requires multiple regions of Sp1.

Physical Interactions between HNF-4 and Sp1 in Vitro. Potential physical interactions between HNF-4 and Sp1 were investigated by GST pull-down assays. For this purpose, WT Sp1 or two mutant Sp1 proteins (Sp1 262−778, which lacks domain A, and Sp1 83−702, which lacks domain D) (Figure 4A) were fused with GST, expressed in bacteria, and coupled to glutathione−Sepharose beads. These affinity beads were incubated with in vitro transcribed−translated and ^{35}S-labeled HNF-4 protein. Bound proteins eluted from the column were separated in SDS−PAGE and were detected by autoradiography. As shown in Figure 4B, HNF-4 was retained in the GST−Sp1 83−778 beads but not in the GST beads, indicating specific physical interactions between HNF-4 and Sp1. Deletion of Sp1 domain A or D reduced the affinity of Sp1 for HNF-4, confirming the results of Figure 3 regarding the importance of these Sp1 domains for the synergistic transactivation of the apoCIII promoter by combination of

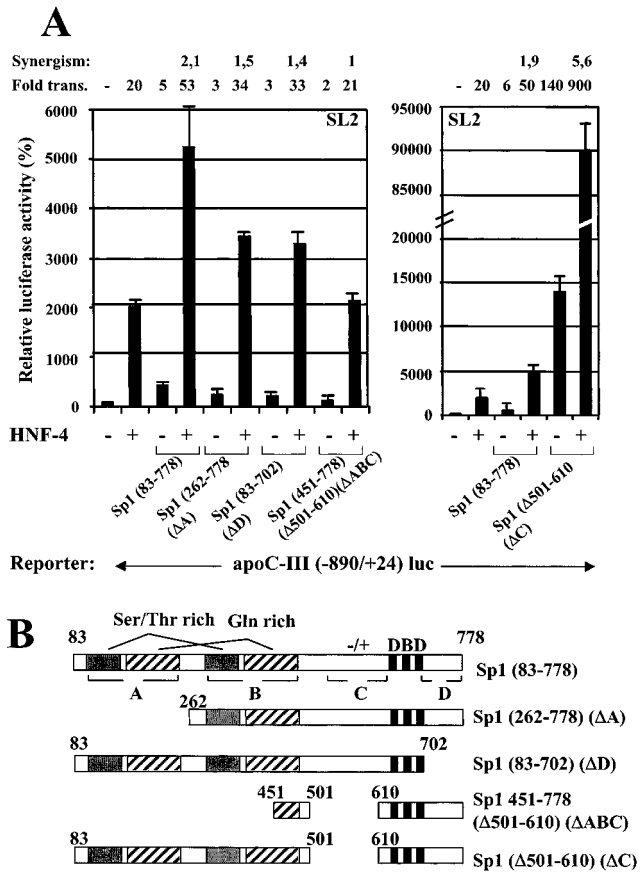

FIGURE 3: Cotransfection experiments in *Drosophila* Schneider SL2 cells using WT HNF-4 and WT and mutant Sp1 forms identified the domains in Sp1 that are required for the functional synergism with HNF-4. (A) *Drosophila* SL2 cells were cotransfected with 1 μg of the −890/+24 apoCIII luc reporter plasmid, 50 ng of pPac-HNF-4, and 25 ng of the WT or mutated pPac-Sp1 plasmids as indicated. The hsp-lacZ plasmid expressing β-galactosidase was included in each transfection for normalization of transfection variability. Luciferase assays were performed as described in the Experimental Procedures. The mean values (±SEM) of at least two independent experiments are shown in the form of a bar graph. (B) Schematic representation of the WT or mutated Sp1 form used in the transactivation experiments shown in (A). The Ser/Thr-rich subdomains of the transactivation domains are shown as gray boxes and the Gln-rich subdomains as stippled boxes. The zinc-finger-containing DNA binding domain (DBD) of Sp1 is shown as three small black squares, and −/+ indicates sequences that modulate the transcriptional activity of Sp1. The regions of Sp1 that are deleted in each mutant are indicated in parentheses.

Sp1 and HNF-4. The reduction in the affinity of mutant Sp1 for HNF-4 was greater in the Sp1 mutant that lacks the carboxy terminal domain D as compared to the mutant lacking the amino terminal domain A (Figure 4B).

Regions of HNF-4 Contributing to Physical Association with Sp1 in Vitro. To map domains of the HNF-4 protein that are required for its physical association with Sp1, a number of C-terminal or N-terminal deletion mutants of HNF-4 were utilized in GST pull-down assays. The HNF-4 mutants which are shown schematically in Figure 5A were in vitro transcribed−translated and ^{35}S-labeled and were incubated with GST−Sp1 83−778 affinity beads. As shown in Figure 5B, deletion of the C-terminal region of HNF-4 up to amino acid 289 did not affect its physical association with Sp1. All of the HNF-4 C-terminal deletion mutants tested (1−370, 1−360, 1−339, 1−289) displayed affinity for Sp1 similar to that of the full-length HNF-4 protein (Figure 5B). Further deletion to amino acid 174 or 128 of HNF-4 abolished its binding to the GST−Sp1 beads. Binding of HNF-4 to Sp1 was also abolished by combined deletions of the N-terminal 1−128 or 1−174 domain and the carboxy terminal 371−465 domain. Finally, a combined deletion of the N-terminal domain 1−47 and the C-terminal domain 456−465 of HNF-4 (HNF-4 48−455) did not affect its binding affinity for Sp1. In summary, the data of Figure 5 indicate that, under the in vitro conditions used, the HNF-4−Sp1 interactions require the 49−289 region of HNF-4.

Physical Interactions between HNF-4 and Sp1 in Vivo. To examine the physical association between HNF-4 and Sp1 in vivo, co-immunoprecipitation experiments were performed. For this purpose, an expression vector for HNF-4 tagged at its N-terminus with the FLAG epitope was transiently transfected into HepG2 cells, and extracts from transfected cells were subjected to immunoprecipitation using an antibody against endogenous human Sp1 protein. Immunoprecipitated complexes were analyzed by SDS−PAGE and Western blotting using the anti-FLAG M2 monoclonal antibody. As shown in Figure 6A, HNF-4 was co-immunoprecipitated with endogenous Sp1 present in HepG2 cells, suggesting a direct physical association between the two factors in vivo. In contrast, a truncated HNF-4 form containing residues 1−128 was unable to co-immunoprecipitate with Sp1 under the same conditions (Figure 6B). This result is in agreement with the in vitro binding data of Figure 5B.

Functional Interactions between HNF-4 and Sp1 on the apoCIII Promoter Are Lost by Deletion of the 128−465 Region. The data presented in Figures 5 and 6 indicate that physical interactions between HNF-4 and Sp1 in vitro and in vivo are abolished in HNF-4 mutants lacking the 128−465 region. These results were confirmed by transactivation experiments in SL2 cells. As shown in Figure 7A, an approximately 3,5-fold synergism was observed in the transactivation of the −890/+24 apoCIII promoter by Sp1 and WT HNF-4 or the HNF-4 1−370 mutant. The HNF-4 1−370 mutant lacks domain F of HNF-4 (Figure 7B). These findings are in agreement with the GST pull-down data presented in Figure 5, which established that the C-terminal region 371−465 of HNF-4 is not required for physical interactions with Sp1. In contrast, the synergism between HNF-4 and Sp1 in the transactivation of the apoCIII promoter in SL2 cells was nearly abolished when the HNF-4 1−128 mutant was utilized. This finding is in agreement with the in vitro and in vivo protein−protein interaction data of Figures 5 and 6 and establishes the fact that the N-terminal 1−128 region of HNF-4 is not sufficient for physical or functional interactions with Sp1 but rather at least an additional epitope within the 128−289 region of HNF-4 is required.

Antagonistic Interactions between HNF-4 and Sp1 on Homopolymeric Promoters Consisting of HNF-4 or Sp1 Binding Sites. The data presented in Figures 5 and 6 support a model of synergistic transactivation of the apoCIII promoter by HNF-4 and Sp1 driven by physical and functional interactions between these factors when both are bound to the DNA. To examine whether HNF-4 could have an additional function, i.e., could act as a coactivator of Sp1 in promoters containing only Sp1 binding sites, SL-2 cells were

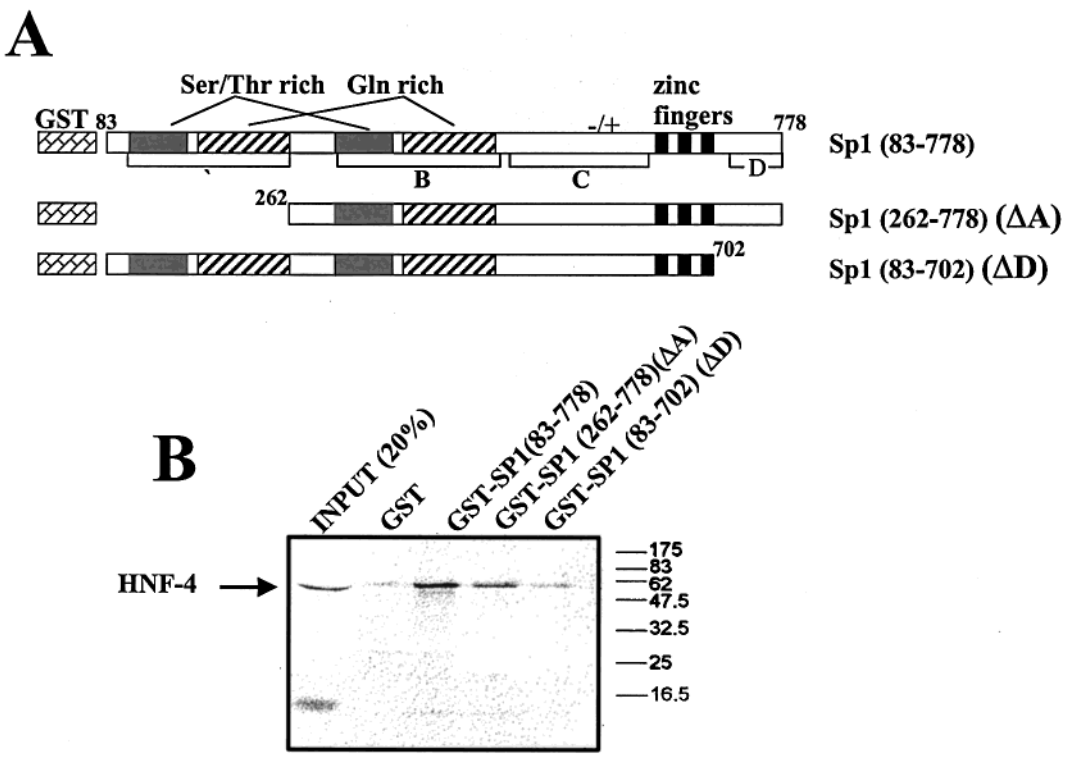

FIGURE 4: GST pull-down assays establish physical interactions between HNF-4 and wild-type and mutant Sp1 forms in vitro. (A) Schematic representation of the GST−Sp1 fusion proteins used for the in vitro interaction assays shown in (B). Abbreviations and symbols are as in Figure 2B. (B) Interaction of GST or GST−Sp1 immobilized on glutathione beads with in vitro transcribed−translated and ^{35}S-labeled HNF-4 protein. The in vitro synthesized HNF-4 protein was allowed to interact with glutathione beads carrying GST alone or the indicated GST−Sp1 fusion proteins, washed thoroughly, resolved by 7% SDS−PAGE, and detected by autoradiography as explained in the Experimental Procedures. Input represents 20% of the [^{35}S]HNF-4 counts which interacted with the GST−Sp1 fusion proteins. Molecular mass markers (expressed in kilodaltons) are shown on the right of the autoradiogram.

transfected with a synthetic reporter construct consisting of the CAT gene under the control of six tandem consensus binding sites for Sp1 (6 × Sp1 CAT) fused with the minimal tk promoter (*23*) in the presence and absence of plasmids expressing Sp1, HNF-4, or both. This analysis showed that Sp1 transactivated the homopolymeric Sp1-driven promoter 10-fold. However, the Sp1-mediated transactivation was totally abolished by HNF-4 (Figure 8A). The finding indicates that HNF-4 negatively affects the transactivation functions of Sp1 that is bound to its cognate sites on the homopolymeric promoter. A similar inhibition of the activity of the CIII (IM_4) AdML chimeric promoter was observed by combination of Sp1 and HNF-4 (Figure 2B). This chimeric promoter can bind Sp1 but cannot bind HNF-4 due to mutations in the HNF-4 binding element I4.

In a reverse experiment, SL2 cells were transfected with a synthetic reporter construct consisting of five HNF-4 binding sites in front of a minimal promoter (−35/+1) derived from the apoB gene (5 × HNF-4 −35/+1 apoB CAT)), along with expression vectors for Sp1, HNF-4, or a combination of the two. This analysis showed that HNF-4 transactivated this synthetic promoter 7-fold, whereas overexpression of Sp1 alone had no effect. Combination of HNF-4 and Sp1 reduced the HNF-4-mediated transactivation by 57% (Figure 8B).

To investigate whether overexpression of HNF-4 affected binding of Sp1 to its cognate sites, a gel electropheretic mobility shift assay was performed. For this experiment, SL2 cells were transfected with expression vectors for Sp1 along with HNF-4 or an empty vector, and extracts from transfected cells were asayed in GEMSA using as the probe a double-stranded oligonucleotide containing a consensus binding site for Sp1 (*22*−*24*). As shown in Figure 8C, overexpression of HNF-4 had no effect on the formation or the mobility of the complexes between Sp1 and the DNA probe. The finding indicates that, under the experimental conditions of the GEMSA assay, tertiary complexes between the Sp1 probe, Sp1, and HNF-4 cannot be detected and that HNF-4 does not affect the DNA binding of Sp1 to its cognate site on the Sp1 probe.

The data presented in Figures 1, 2, and 8 indicate that synergistic interactions between HNF-4 and Sp1 occur only when both factors are bound simultaneously to the apoCIII promoter/enhancer. In promoters containing only Sp1 (Figures 2 and 8A) or only HNF-4 (Figure 8B) binding sites, combination of Sp1 and HNF-4 results in transcriptional repression. The observed negative interactions between Sp1 and HNF-4 may occur either due to competition for common basal or auxiliary transcription factors or due to the formation of nonproductive HNF-4/Sp1 complexes which hinder the activation functions of the two factors.

Figure 9 is a schematic representation of the proposed mechanism of transactivation of the apoCIII promoter/enhancer by HNF-4 and Sp1, which is explained further in the Discussion.

DISCUSSION

The human apoCIII promoter contains a set of four proximal (CIIIA−CIIID) and six distal (CIIIE−CIIIJ) regu-

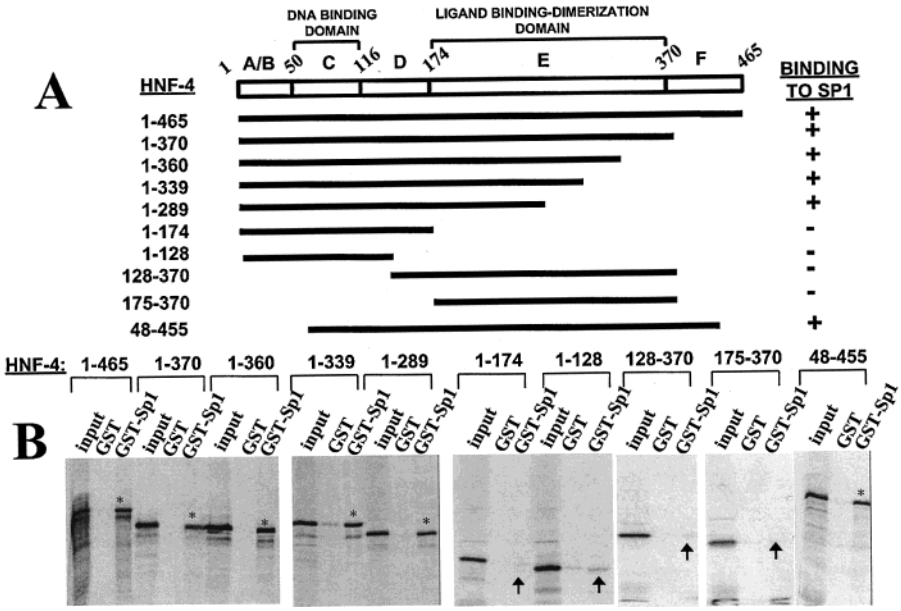

FIGURE 5: GST pull-down experiments using mutant HNF-4 forms established the domains in HNF-4 that mediate physical interactions with WT Sp1. (A) Schematic representation of the various WT or mutant HNF-4 proteins used for the pull-down assays shown in (B). Domains A−F of HNF-4 are shown at the top. The various mutated HNF-4 forms are represented by solid bars. The ability of each HNF-4 form to physically interact with GST−Sp1 is indicated with a plus or a minus symbol. This information is derived from the GST pull-down analyses shown in (B). (B) Interaction of in vitro transcribed−translated and ^{35}S-labeled HNF-4 proteins with GST or GST−Sp1 83−778 immobilized on glutathione beads. The in vitro synthesized HNF-4 proteins were allowed to interact with glutathione beads carrying GST or GST−Sp1 fusion proteins, washed thoroughly, resolved by 7% SDS−PAGE, and detected by autoradiography as described in the Experimental Procedures. Input represents 10% of the transcription−translation product that was utilized in the binding reactions. The numbers on top of the autoradiograms refer to the mutated HNF-4 forms of (A). Sp1−HNF-4 interactions are indicated by asterisks. Lack of Sp1−HNF-4 interactions are indicated by upward arrows.

latory elements between nucleotides −792 and −25 (*9*). Element CIIIB of the proximal promoter contains an HRE with a DR1 spacing and strongly binds HNF-4, ARP-1, EAR-2, EAR-3, and heterodimers of RXRα with RARα and less efficiently binds homodimers of RARα and heterodimers of RXRα with T3Rβ or PPARα (*3*−*8*). Mutations at this site nearly abolished the promoter activity in HepG2 and CaCo-2 cells (*6*, *9*). The distal apoCIII promoter, which acts as a transcriptional enhancer (*7*, *9*), contains three binding sites for the ubiquitous transcription factor Sp1, which recognizes the regulatory elements CIIIF, CIIIH, and CIII-I. The 3′ region of element CIII-I (nucleotides −736/−714) was designated element CIII-I4 and contains an HNF-4 binding site (*7*). This element contains an HRE with a DR1 spacing and strongly binds HNF-4, ARP-1, EAR-3, and RXRα/RARα heterodimers and less efficiently binds RXRα/T3Rβ heterodimers (*3*).

In vitro mutagenesis established that the strength of the apoCIII promoter/enhancer cluster was reduced by 90−95% in HepG2 and CaCo-2 cells by mutations in the proximal and distal HREs. The promoter strength was also reduced by 85−90% by a single mutation in the Sp1 site of element CIIIH or by deletion of two of the three Sp1 binding sites (*6*, *25*).

These observations highlighted the importance of HNF-4 and Sp1 for the function of the apoCIII promoter/enhancer and pointed to synergistic interactions between these factors on the transactivation of the apoCIII promoter/enhancer. The vital role of HNF-4 and Sp1 and their synergism in the transactivation of the apoCIII promoter/enhancer were also established by in vivo experiments in transgenic mice, which showed that mutations in all three Sp1 sites abolished the

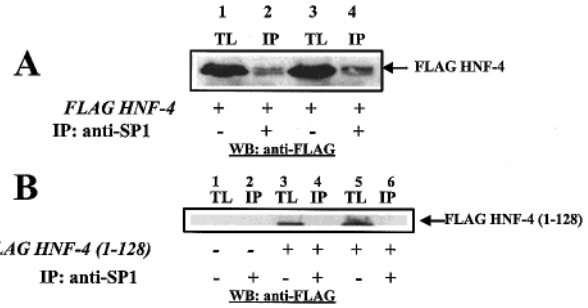

FIGURE 6: Co-immunoprecipitation of WT HNF-4 with Sp1 expressed in mammalian cells. (A) shows that wild-type HNF-4 coprecipitates with Sp1 from transiently transfected HepG2 cells. (B) shows that the truncated HNF-4 form containing residues 1−128 does not co-immunoprecipitate with Sp1. HepG2 cells were transiently transfected with 17 μg of a FLAG-tagged HNF-4 or FLAG-tagged HNF-4 1−128 expression vector. Forty hours post-transfection, cell lysates were immunoprecipitated with antibodies against endogenous Sp1, resolved by 10% SDS−PAGE, transferred to membranes, and probed with anti-FLAG M2 antibody prior to detection by enhanced chemiluminescence as described in the Experimental Procedures. (A) shows detection of HNF-4 protein in the total cell lysates (TL) and the immunoprecipitates (IP). All lanes contain samples from HepG2 cells transfected with 17 μg of wild-type HNF-4. Lanes 1 and 3 are total lysates without immunoprecipitation; lanes 2 and 4 are extracts from HepG2 cells immunoprecipitated with polyclonal antibody against endogenous Sp1. (B) shows the detection of HNF-4 1−128 protein in the total cell lysates but not in the immunoprecipitates. All lanes contain samples from HepG2 cells transfected with 17 μg of HNF-4 1−128. Lanes 3 and 5 are total lysates without immunoprecipitation. Lanes 2, 4, and 6 are extracts from HepG2 cells immunoprecipitated with polyclonal antibody against endogenous Sp1. Lane 1 contains extracts from untransfected HepG2 cells. The symbols indicate the following: TL, total lysates; IP: anti-Sp1, immunoprecipitation with polyclonal anti-Sp1 antibodies; WB: anti-FLAG, probing the Immobilon-p membranes with anti-FLAG antibodies.

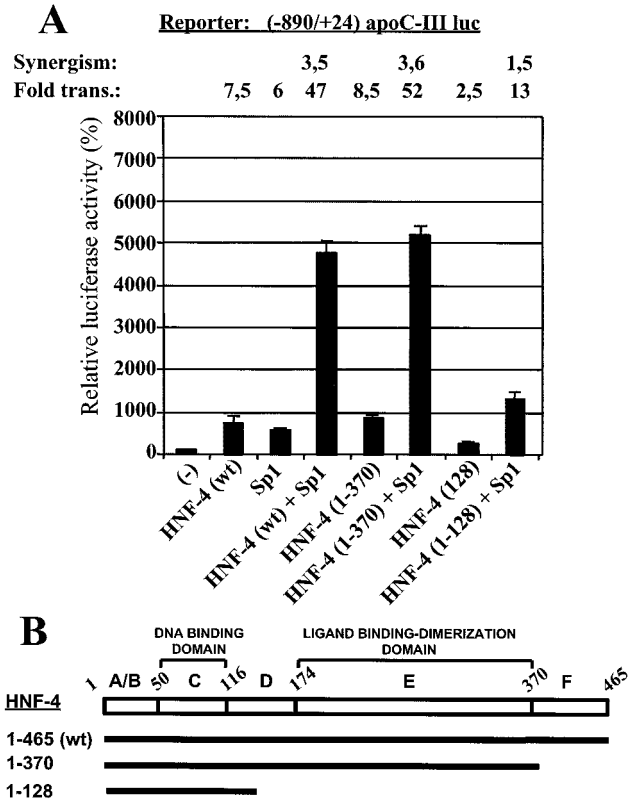

FIGURE 7: Functional interactions between HNF-4 and Sp1 on the apoC-III promoter are lost by deletion of the 128−465 region. (A) *Drosophila* SL2 cells were cotransfected with 1 μg of the −890/+24 apoCIII luc reporter plasmid, 7 μg of the WT and mutant pCDNA-amp HNF-4 expression vectors, and 25 ng of the WT pPac-Sp1 plasmids as indicated. The hsp-lacZ plasmid expressing β-galactosidase was included in each transfection for normalization of transfection variability. Luciferase assays were performed as described in the Experimental Procedures. The mean values (±SEM) of at least two independent experiments are shown in the form of a bar graph. (B) Schematic representation of the WT or mutant HNF-4 forms used in the transactivation experiments shown in (A). The symbols are as in Figure 5A.

expression of the apoCIII gene in all tissues (*14*, *15*). Selective mutagenesis of the HNF-4 binding site of the enhancer abolished the intestinal expression and reduced the hepatic expression of the apoCIII gene to 2% of that of the WT control. These findings established that the binding of HNF-4 to the apoCIII enhancer is essential for the intestinal expression and greatly enhances the hepatic expression of this gene (*14*, *15*).

The objective of the current study was to probe the mechanism through which the synergism between HNF-4 and Sp1 molecules bound to the proximal promoter and apoCIII enhancer comes about. To accomplish this objective, we performed cotransfection assays in *Drosophila* SL2 cells, which lack Sp1, enabling assessment of the effect of Sp1 and/or HNF-4 on the transactivation of the apoCIII promoter/enhancer. This analysis established that both Sp1 and HNF-4 can transactivate the −890/+24 apoCIII promoter/enhancer and that their effect on the promoter/enhancer activity is not additive but synergistic. Most important, however, was the observation that the synergistic transactivation of the apoCIII promoter/enhancer is retained when the proximal HNF-4 binding site is mutated, and is lost when the distal HNF-4 binding is mutated, indicating that the synergistic transactivation of the apoCIII promoter/enhancer by HNF-4 and Sp1 requires the HNF-4 binding site of the apoCIII enhancer. The observed 55-fold synergistic transactivation of the −890/+24 apoCIII promoter mutated at the proximal HNF-4 binding site (CIII-B mut CAT construct of Figure 1A) reflects synergistic interactions between HNF-4 and Sp1 bound to the apoCIII enhancer. In addition, since SL-2 cells do not have Sp1, the observed 72-fold transactivation of the WT −890/+24 apoCIII promoter by HNF-4 (CIII CAT construct of Figure 1A) represents synergism between HNF-4 bound to the proximal promoter and the distal enhancer sites. Finally, the observed 150-fold transactivation of the WT −890/+24 apoCIII promoter by a combination of Sp1 and HNF-4 represents the sum of these synergistic interactions. It is interesting that the sum of synergistic transactivations due to HNF-4−HNF-4 interactions (72-fold) and Sp1−HNF-4 interactions (55-fold) is 127. This value approximates the 150-fold synergistic transactivation of the WT apoCIII promoter due to the overall functional interactions among Sp1 and HNF-4 molecules bound to the apoCIII promoter and enhancer.

Utilization of various Sp1 mutants enabled us to show that Sp1 mutants lacking part of the glutamine-rich activation domain A (1−262) or the carboxy-terminal domain D (702−778) reduced the HNF-4-mediated transactivation of the apoCIII promoter/enhancer, whereas an Sp1 mutant lacking the domain C (501−620) displayed greatly increased transactivation ability. The synergistic effect of the WT Sp1 and HNF-4 on the transactivation of the apoCIII promoter/enhancer was 2-fold, and that of the ΔC Sp1 mutant was 5.6-fold. Sp1 mutants lacking the domains ABC (Δ1−451 and Δ501−610) did not show any synergistic transactivation. These findings indicate that the functional interactions between Sp1 and HNF-4 in the transactivation of the apoCIII promoter/enhancer depend, at least partially, on the Ser/Thr-rich and Gln-rich and carboxy-terminal domains of Sp1. It is possible that deletion of the central domain C (501−610) facilitates the physical and/or functional interactions of this mutant Sp1 form with HNF-4 or with other transcriptional activators and thus increases the transactivation of the apoCIII promoter/enhancer.

In vivo and in vitro experiments established that the activity of the apoCIII promoter/enhancer is lost by elimination of all the Sp1 binding sites (*6*, *14*). Thus, we rationalized that the binding of Sp1 to the enhancer and ensuing protein−protein interactions may enhance the transactivation functions of the HNF-4Sp1 complex. Such interactions between HNF-4 and Sp1 were indeed established by in vivo co-immunoprecipitation and in vitro GST pull-down experiments using wild-type and mutated HNF-4 and Sp1 forms. This analysis showed that the N-terminal 49−289 region of HNF-4 contributes to the physical interactions between Sp1 and HNF-4. The strength of the HNF-4−Sp1 interactions was somehow reduced by deleting the N-terminal Ser/Thr- and Gln-rich region A (1−261) of Sp1 and drastically reduced by deleting the C-terminal region D (703−778) of Sp1.

Protein−protein interactions which lead to transcriptional activation or repression usually require binding of both proteins to the DNA. In a few cases, transactivation or repression may involve protein−protein interactions with only one factor bound to the DNA and another attached to the DNA-bound factor (*26*, *27*). This, for instance, has been observed in the case of HNF-1 and apoCII promoters, where

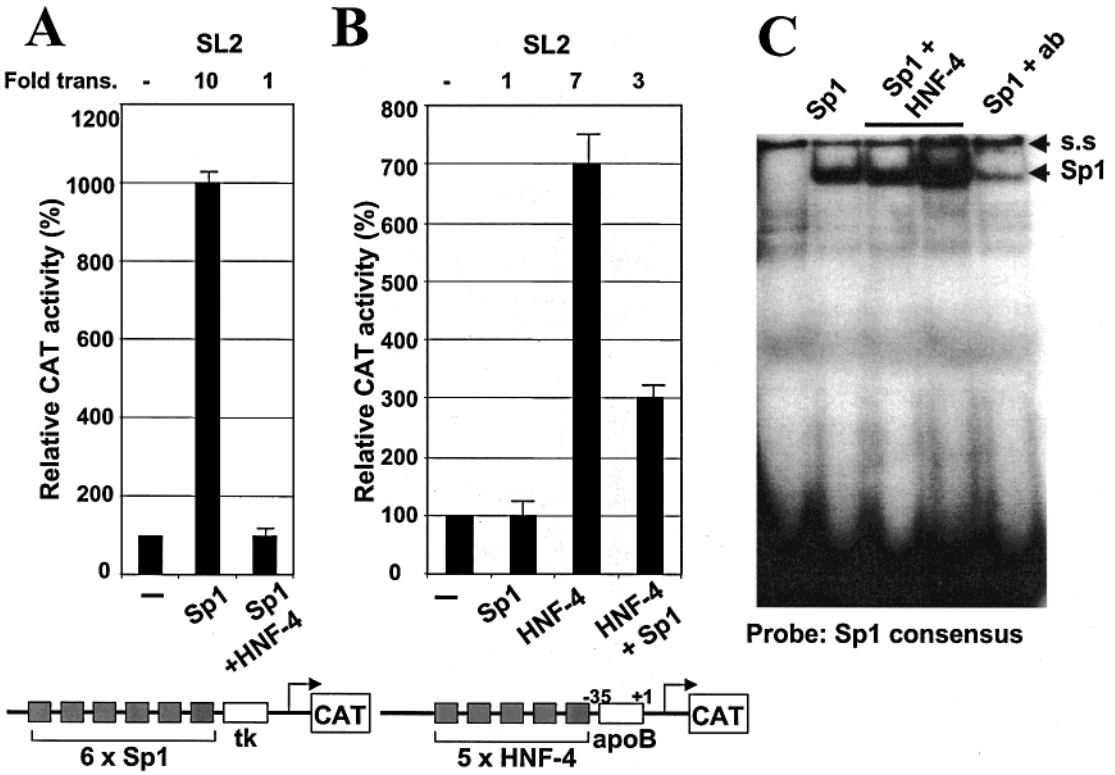

FIGURE 8: Cotransfection and control assays in *Drosophila* IL2 cells using homopolymeric promoters consisting exclusively of HNF-4 or Sp1 binding sites along with HNF-4 or Sp1 expression plasmids showing antagonism between Sp1 bound to DNA and free HNF-4 and vice versa. (A) Cotransfection in SL2 cells of a homopolymeric promoter consisting of six Sp1 binding sites along with HNF-4- and Sp1-expressing plasmids. SL2 cells were cotransfected with the 6 × Sp1 tk CAT reporter construct (2 μg) (shown at the bottom) along with hsp-lacZ (500 ng), pPac-Sp1 (25 ng), and pPac-HNF-4 (50 ng) as indicated. Forty hours post-transfection, cell lysates from the transfected cells were assayed for β-galactosidase and CAT activity as described in the Experimental Procedures. The normalized mean values (±SEM) of CAT activity from at least two independent experiments performed in duplicate are shown in the form of a bar graph. (B) Cotransfections in SL2 cells of a homopolymeric promoter consisting of five HNF-4 binding sites in front of the minimal apoB promoter along with Sp1- or HNF-4-expressing plasmids. SL2 cells were cotransfected with the 5 × HNF-4 apoB CAT reporter construct (2 μg) (shown at the bottom) along with hsp-lacZ (500 ng), pPac-Sp1 (25 ng), and pPac-HNF-4 (50 ng) as indicated. Forty hours post-transfection, cell lysates from the transfected cells were assayed for β-galactosidase and CAT activity as described in the Experimental Procedures. The normalized mean values (±SEM) of CAT activity from at least two independent experiments performed in duplicate are shown in the form of a bar graph. (A) and (B) establish that interaction of Sp1 bound to DNA with HNF-4 that is not bound to DNA and vice versa is associated with transcriptional repression. (C) Control gel electrophoretic mobility shift assay showing that HNF-4 does not prevent binding of Sp1 to its cognate DNA site. SL2 cells (6×10^6 cells) in 100 mm Petri dishes were transfected with pPac-Sp1 (5 μg) alone or along with pPac-HNF-4 (5 μg). Forty hours post-transfection, total cell lysates from SL2 transfected cultures were generated and analyzed in gel electrophoretic mobility shift assays using as a probe a double-stranded oligonucleotide containing a consensus Sp1 binding element (see the Experimental Procedures). A monoclonal antibody for Sp1 was also included in one of the binding reactions. The positions of the Sp1 and ab/Sp1 (s.s) nucleoprotein complexes are shown by arrows. s.s = supershift.

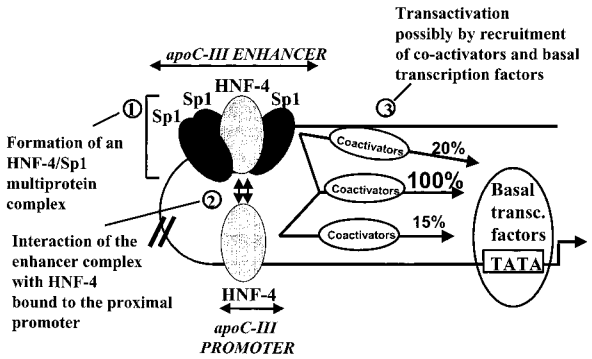

FIGURE 9: Schematic representation of putative interactions of the HNF-4/Sp1 complex bound to the apoCIII enhancer with HNF-4 and possibly other factors bound to the proximal promoter and transcriptional coactivators which lead to synergistic transactivation of the apoCIII promoter/enhancer cluster. See the text for details.

HNF-4 binds to unique sites and ARP-1 potentiates the activity of HNF-4 via protein−protein interactions (*26*, *27*). In the current study using minimal promoters containing either multiple Sp1 or multiple HNF-4 binding sites, we established that when Sp1 is bound to the promoter, excess HNF-4 inhibits the Sp1-mediated transactivation of the homopolymeric promoter. The inhibition is not the result of interference of HNF-4 with the binding of Sp1 to its cognate sites. Similarly, when HNF-4 is bound to the promoter, excess Sp1 diminishes the HNF-4-mediated transactivation of the homopolymeric promoter. This inhibition may represent nonproductive complex formation by the two proteins that cannot be detected by GEMSA. These protein−protein interactions may reduce the transactivation functions of the two factors. Alternatively, the inhibition may represent squelching of common transcriptional coactivators required for the functions of Sp1 and HNF-4 (*28*−*32*).

On the basis of these observations, a model is proposed that is consistent with the transactivation properties of the apoCIII promoter/enhancer by a combination of Sp1 and HNF-4. The essential features of this model, which is shown schematically in Figure 9, are (a) the formation of a

multiprotein complex consisting of HNF-4, Sp1, and possibly other factors on the apoCIII enhancer, (b) formation of a proximal promoter complex involving HNF-4 and possibly other factors bound to the proximal promoter, and (c) interaction of the two complexes with transcriptional coactivators which remodel the chromatin structure and enhance gene transcription (*28*−*32*). This model is consistent with the Sp1/HNF-4 protein−protein interactions documented in Figure 5. This model is also consistent with in vivo studies which demonstrated that the apoCIII enhancer has the ability to activate the hepatic transcription of the apoA-I gene in the absence of a functional proximal promoter (*14*, *15*). Independent transcriptional activity may be observed by recruitment of coactivators by the HNF-4 bound to the proximal apoCIII promoter. A similar mechanism applies for the activation of the apoA-I promoter/apoCIII enhancer cluster in vivo (*14*, *15*). When both the proximal apoA-I promoter and the apoCIII enhancer are intact, the hepatic transcription of the apoA-I gene increases 5−7-fold over the levels achieved by either the promoter or the enhancer alone (*14*, *15*). This transcriptional synergism, documented by in vivo studies, suggests additional protein−protein interactions of the multiprotein HNF-4/Sp1 complex bound to the enhancer with HNF-4 and possibly other factors bound to the proximal promoter. These protein−protein interactions may then elicit allosteric effects which increase their transactivation properties. The prediction of the model is that mutations in either Sp1 or HNF-4 which diminish these protein−protein interactions will abolish or severely affect the apoCIII promoter/enhancer activity in vitro as well as the transcription of the apoCIII gene in transgenic mice.

The transcriptional regulatory mechanisms which emerge from this and similar studies may provide rational approaches for reducing the plasma levels of atherogenic hypertriglyceride-rich lipoprotein particles in humans that are enriched in apoCIII, thus providing protection from atherosclerosis.

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