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Multiple Liver-Specific Factors Bind to a 64-bp Element and Activate Apo(a) Gene

Vikas Handa, Mehboob-ul-Hussain, Nirupma Pati, and Uttam Pati[1]
Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

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The high plasma levels of lipoprotein(a) [Lp(a)] are associated with atherosclerosis. The apo(a) gene is responsible for the variance of Lp(a) concentration and its expression is liver-specific. By 5′-deletion analysis, we, in a luciferase gene reporter assay, have identified a 64-bp AT-rich region of upstream apo(a) gene (−703 to −640) that binds to multiple liver-specific factors. The 64 bp *cis*-element contained three dyad symmetry elements (DSEs) that are crucial for synergistic binding to the factors. We have demonstrated that both DSE-2 and -3 together are responsible for factor binding *in vitro,* and for gene activation in liver cells. Further, we have purified one of the UV cross-linked DNA–protein complexes to homogeneity by streptavidin magnetic bead chromatography. The identification of a further upstream negative regulatory region (−1432 to −704) led us to predict that as yet unidentified transcriptional repressor(s) might also repress apo(a) gene transcription.

Key Words: **transcriptional activation; apo(a) gene; dyad symmetry element; *trans*-acting factors; magnetic bead chromatography.**

The plasma Lp(a), a low density lipoprotein-like particle containing one molecule of apo(a) and apoB, varies in human population ranging from <0.1 to >100 mg/dl (1) and its high level has been responsible for myocardial infarction and atherosclerosis (2, 3). Transgenic mice expressing the human apo(a) gene develop fatty streak lesions in response to a diet rich in fat, suggesting causative link between apo(a) gene and atherosclerosis (4). It was reported earlier that an 1.5 kb apo(a) promoter fragment with different sequence variations from two different individuals with high and low Lp(a) level had variable promoter strength (5) whereas 10 other allelic 1.5 kb promoter fragments had comparable promoter activities (6). A liver enriched *trans*-acting factor HNF-1α was shown to bind to +88 to +130 region of apo(a) gene and activated gene transcription (7). An enhancer element within a LINE retrotransposon has been found to be located 18 kb 5′ at the apo(a) promoter whereas an enhancer requiring Sp1 and PPAR1 transcription factors are located 26 kb upstream (8). An apo(a) transcriptional control region (ACR) 20 kb 5′ to the start site also contained an enhancer element responding to the members of Ets transcription factor families (9). Further, a retinoid response element (10) and an estrogen-responsive element (11) have also been identified in the upstream of apo(a) promoter.

The transcription of eukaryotic genes is regulated by synergistic interactions between multiple transcriptional activators and repressors, and this synergy could be ascribed to cooperative binding of factors to specific cis-acting sequences as well as to interactions between proteins that bind to nonadjacent sites and to protein induced DNA binding (12, 13). We recently proposed (14) that high and low plasma Lp(a) levels among individuals might be due to transcriptional synergy between apo(a) gene-specific activators and repressors that might operate in an individual-specific manner. In this report, we have demonstrated that a 64-bp upstream AT-rich region of apo(a) gene (−703 to −640) bind to multiple liver-specific factors, and activate gene transcription, in liver cells. We have purified one of the UV cross-linked DNA–protein complex to homogeneity by biotin–streptavidin magnetic bead system.

EXPERIMENTAL PROCEDURES

Plasmid constructions, cell transfections, and luciferase assays. The 5′-deletion fragments of the upstream region of human apo(a) gene were generated by PCR (96°C, 1.15 min, 51°C, 1.5 min, 72°C, 1.5 min, Mops buffer, pH 7.6, ×30 cycles; Ultima polymerase, Perkin–Elmer) from human DNA, utilizing various sense primers: 5′- *Kpn*I [−1432, GCGGAAA GATTGATACTATGCTT; −716, CCTGGAAGGATTGATATCT; −668, TGTGAGTTCACTAGGAATAA; −639, GAAGATAATAAAAGAT TTTCA; −593, TGGTATAGGATGTGGAGA; −313, CAAGACTAATCAGGAAAGA TGAA], and an antisense primer 5′- *Bgl*II-ATTAGATCT CACTTC CTTATGTTCCATTT, +13. The PCR products were digested with *Bgl*II and *Kpn*I. The digested PCR products were subcloned to PGL2-Basic vectors (Promega, U.S.A.). The constructs with deletion of various DSE were

[1] To whom correspondence and reprint requests should be addressed. Fax: 6198234. E-mail: uttampati@hotvoice.com.

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made by digesting the PCR fragments **h**, **I**, **j**, and **k** (see this section, elecromobility shift assay) with *Bgl*II–*Kpn*I and were cloned to PGL2-Pr (Promega, U.S.A.). 2 μg of plasmid and 1 μg of pSV β-galactosidase (Promega, U.S.A.) control plasmid in maintenance medium were transfected to cells by Transfectamine reagent (GIBCO BRL, U.S.A.). After 6 h of transfection, fresh medium was added and cells were harvested after 30 h. The cells were lysed and the luciferase activities were determined using a luminometer (Turner Design, U.S.A.). The β-gal activities were measured using 5 mM *O*-nitrophenol-β-D-galactoside in phosphate buffer. Transfections were carried out in triplicates. To account for transfection efficiency, results were reported as the ratio of the sample to control plasmid activity.

Preparation of nuclear extract. HeLa and HepG2 cells were grown in DMEM at 5% CO_2 level, at 37°C. 1.5×10^7 cells of HeLa and HepG2 were harvested and centrifuged at 250*g* for 10 min. The pellet was washed with phosphate-buffered saline (PBS; 100 mM NaCl, 4.5 mM KCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4) and was centrifuged at 250*g* for 10 min. The pellet was resuspended in 5 volumes of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF), incubated on ice for 10 min, and then centrifuged at 250*g* for 10 min. It was further resuspended in buffer A and Nonidet P-40 was added to a final concentration of 0.05% (v/v). The pellet suspension was homogenized with 20 strokes of a tight-fitting Dounce homogenizer to release the nuclei. The nuclei were pelleted at 250*g* for 10 min and was resuspended in 1 ml buffer C (5 mM Hepes, pH 7.9, 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). NaCl was added to the final concentration of 300 mM and the sample was incubated on ice for 30 min. It was then centrifuged at 24,000*g* for 20 min. All the centrifugations were carried out at 4°C. Aliquots of the supernatant were stored at −70°C.

Electromobility shift and UV cross-linking assay. The probes for the EMSA were generated by PCR as follows. **a**: 5′-[sense, TTGATATC TTATAACATAAT; antisense, TTCCTAGTGAACTCACA; **b**: s, TTGATATC TTATAACATAAT; as, AAGTGAAAATCTTTTA TTATC TTCAAAA; **c**: s, TGTGAGTTCAC TAGGAA; as, AAGTGAAAATCT TTTATTATCTTCAA AA; PCR conditions: **a** & **b**, 94°C, 15 s, 39°C, 30 s, 72°C, 30 s, ×30; **c**, 94°C, 15 s, 43°C, 30 sec, 72°C, 30 sec, ×30; **d** was obtained by digesting **c** with *Apo* I. **h**: s, 5′-*Kpn*I-TTACAGAAAACATGTGA; as, *Bgl*II-AAGTGAAAATCTTTTATCA AAATTTATTT. PCR condition: 94°C,30 sec, 50°C, 15 s, 72°C, 30 s, ×25 cycles, 72°C, 5 min. **I**: s, *Kpn*I-TTACAGAAAACATGT GAGTTCACTAGGAATAATTT GAAGAT; as, 5′-*Bgl*II-AAGTGAAAATCTT TTATTATCTTC. PCR condition: same as **h**. **j**: s, 5′-*Kpn*I-TTACAGAAAACATTC ACTAGGAAT; as, 5′-*Bgl*I-AAGTGAA AATCTTTTATTATCTTCAAATTA TTCCTAG. PCR condition: 94°C, 30 s, 52°C, 15 s, 72°C, 30 s, ′25 cycles, 72°C, 5 m. **k**. s, *Kpn*I-TTACAGAAAACAT TCACTAG GAAT; as, *Bgl*I-ACATAAGTGAATTA TTATCTTCAA. PCR condition: same as **j**. Binding reactions were carried out in a total volume of 15 μl containing 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol (v/v), 0.5 mM DTT, 1 μg poly(dI-dC) as nonspecific competitor DNA molecules and 2.5 μg HepG2 nuclear extract. It was kept on ice for 5 min. ^{32}P-labeled probe (1.5×10^4 cpm) was then added and the mixture was further incubated on ice for 30 min. The reaction mixture was electrophoresed on a 4% polyacrylamide gel (29:1) in 0.5× TBE and 2.4% glycerol at 100 V for 1.5 h, the gel was dried and exposed to autoradiography. The UV cross-linking reaction was carried out after the binding reaction was performed as mentioned in EMSA. The only difference was that body labeled probe was used and each reaction contained 150,000 cpm. The probe was generated by PCR-amplification using [α-^{32}P]-dATP in dNTP mixture instead of cold dATP and the probe was purified by SDS–PAGE followed by crush and soak method. After final incubation on ice for 30 min, the reaction mixture while keeping on ice, was subjected to UV radiations in a UV Stratalinker (Stratagene, U.S.A.) in auto-crosslinking mode for five times. One half of each reaction was digested with DNase I for 30 min at 37°C. The reaction mixtures were then mixed with SDS-loading buffer and separated on SDS–polyacrylamide gels. The gels were visualized by autoradiogaphy.

DNase I footprinting assay. The lower strand footprinting was performed with a 233 bp (−716 to −484, antisense-labeled primer 5′-GATCTCCCGAGGTTGTGTCGCTACC CAGGGGTGCA GCT GATAGACACAACCCT). The upper strand footprinting was performed in the following manner. A 410 bp fragment (−995 to −532) was amplified by PCR utilizing two primers (s, 5′-*Kpn*I-ATGGTACCTGAGACATTCTTGCTCTG; as, 5′-TCCCGAGGTTGTGTC GCTACCCAGGGGTGCAGCTGATAGACACAACCCT, PCR condition: 94°C, 15 s, 52 C, 30 s, 72°C, 1 min, ×30 cycles, 72°C, 5 min). The PCR product was digested with Sau3AI to generate a 292 bp fragment (−775 to −484) that was Klenow-filled with radiolabeled dCTP. The reactions were performed with 2 μg poly(dI-dC) for 5 min on ice, in 25 mM Tris–HCl, pH 8.0, 6.25 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 10% glycerol (v/v), 0.5 mM DTT. The ^{32} P-labeled probe (3×10^4 cpm) was then added to the NE in a total volume of 75 μl and incubated on ice for 20 min. After completion of incubation, 75 μl Ca^{2+}/Mg^{2+} solution (5 mM $CaCl_2$ & 10 mM $MgCl_2$) was added and the mixtures were incubated at room temperature for 1 min. RQ1 DNase I (0.04 U) was then added and the sample was incubated at room temperature for 1 min. The digestion was stopped by adding 135 μl stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS and 100 μg/ml yeast tRNA) at 37°C. Following phenol–chloroform extraction and ethanol precipitation, the digested DNA was analyzed on 6% urea–polyacrylamide gel. The gel was then dried and exposed to autoradiography.

Methylation interference assay. A 91 bp fragment containing the positive regulatory region (−706 to −616) was end labeled with [γ-^{32}P]ATP (3×10^5 cpm) and was treated for 3 h with 1% DMS (dimethyl sulfate) in buffer (60 mM NaCl, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM EDTA). The reaction was stopped by addition of 50 μl of cold DMS stop mix (1.5 M sodium acetate, pH 7.0, 1 mM 2-mercaptoethanol) and the DNA was ethanol precipitated with cold ethanol. The probe was redissolved in 200 μl cold 0.3 M sodium acetate (pH 7.0) and ethanol precipitated. The precipitate was washed with 80% ethanol, dried, and redissolved in water to recover the DNA (2×10^4 cpm). The probe was mixed with 40 μl of $HepG_2$ extract for EMSA. Both the bound and the free DNA were separated on 4% PAGE and the bands were cut, following autoradiography. The DNA was recovered by crush and soak method. The DNA was extracted with phenol and chloroform and ethanol precipitated. The methylated DNA was cleaved with piperidine and piperidine was removed by repeated drying and dilution of the samples. The sample was redissolved in water and 2×10^3 cpm was loaded unto 8% sequencing gel. The gel was dried and subjected to autoradiography.

Purification of DNA–protein complex. A biotinylated fragment spanning the region −703 to −652 (**a**) was generated by using sense, 5′-Biotin–CAACCCGGGCTGGAAGGATTGATATCT; and antisense, 5′-TTCCTAGTGAACTCACA with PCR conditions: 94°C, 15 s, 43°C, 30 s, 72°C, 30 s, ×30 cycles, 72°C, 5 min. The binding reaction of EMSA using the biotinylated fragment was scaled up 30-fold and after the incubation period, the reaction mixture was UV cross-linked in UV Stratalinker. The streptavidin magnetic beads (Dynal) were resuspended in the scaled up binding reaction and the suspension was gently agitated on a roller–shaker for 30 min at room temperature for coupling of biotinylated DNA to the magnetic beads. The magnetic bead streptavidin–biotin complex was then separated with the help of a strong magnet and the supernatant was removed. The pellet was washed with T_{10} E_1, 0.1% SDS in T_{10} E_1 and 1.0% SDS in T_{10} E_1 for 5 min each, subsequently. The biotin-streptavidin binding was broken by applying 7 M urea in 1× SDS–PAGE loading solution to the pellet and boiling it for 15 min to release the purified biotinylated DNA–protein complex. The released complex was separated on SDS–polyacrylamide gel and visualized by silver staining. To distinguish the complex from free probe in gel, a ^{32}P-radiolabeled UV cross-linked complex was mixed with the unlabeled scaled up reaction mixture (15).

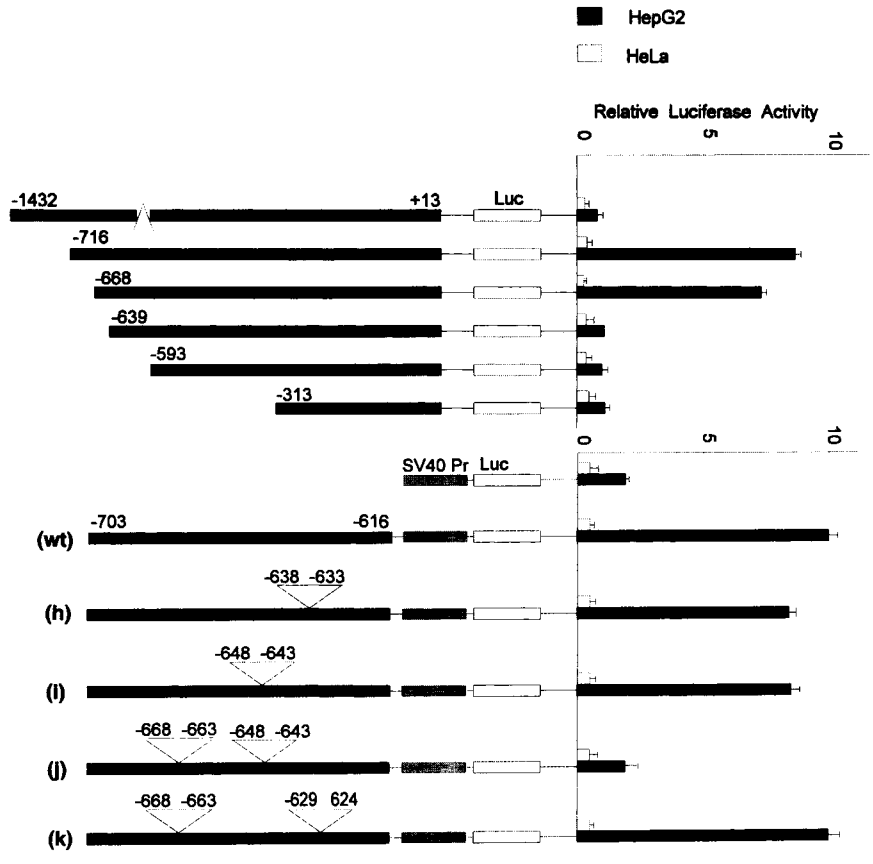

FIG. 1. A 64-bp positive regulatory region is located in (−703 to −640) region of apo(a) promoter. The black and the white bars represent the relative luciferase gene expression levels in HepG2 and in HeLa cells, respectively. The plasmid constructs containing the fragments from −716 to +13 and −668 to +13 show high luciferase expression in HepG2 cells indicating that the positive regulatory region is between −716 to −640. In HeLa cells, all the constructs show basal activity. The construct **j** (−703 to −616) with a simultaneous deletion of DSE-2 and -3 shows deactivation of transcription from SV40 promoter. Results are the mean and standard error of the mean of three independent transfections.

RESULTS AND DISCUSSION

A 64-bp AT-Region of Apo(a) Gene (−703 to −640) Activated Gene Transcription in Liver Cells

We first transiently transfected a plasmid construct containing an entire 1.4 kb apo(a) promoter fragment (−1432 to +13) and two deleted fragments of the 5′-flanking region (−703 to +13, and −313 to +13) both in HepG2 and HeLa cells, and conducted luciferase reporter gene assay. In HepG2 cells, a basal promoter activity was observed with both the1.4 kb (−1432 to +13) and 326 bp (−313 to +13) fragments whereas the luciferase activity displayed by the region (−703 to +13) was 11.3-fold higher than the activity observed with both the above fragments. The lack of luciferase activity in HeLa cells suggested that the transcriptional up-regulation was liver specific. To further narrow down the activating region, we repeated the luciferase gene assay utilizing sequential ~50 bp 5′-deletion constructs (−668 to +13; −639 to +13; −593 to +13) (Fig. 1). The activity with the fragment (−668 to +13) was 9.0-fold higher than the basal activity that was observed with the other two constructs (−639 to +13, −593 to +13). This result demonstrated that a strong positive regulatory region was located at −703 to −640 region of apo(a) gene. The loss of the luciferase activity with an entire 1.4 kb promoter in comparison to the activity shown by the fragment (−703 to +13) suggested that a negative regulatory element might be located in the region (−704 to −1432). We reasoned that the transcriptional activation of the luciferase gene in HepG2 cells by a 64 bp DNA element (−703 to −640) could be due to the HepG2-specific nuclear factors binding to this region.

Multiple Hepato-Specific Factors Bind to AT-Rich Region (−703 to −616) That Contained Multiple DSEs

DNA elements with dyad symmetry have been shown to bind transcription factors and cause transcriptional regulation (16). The nearly palindromic regions can switch between cruciform and linear duplex

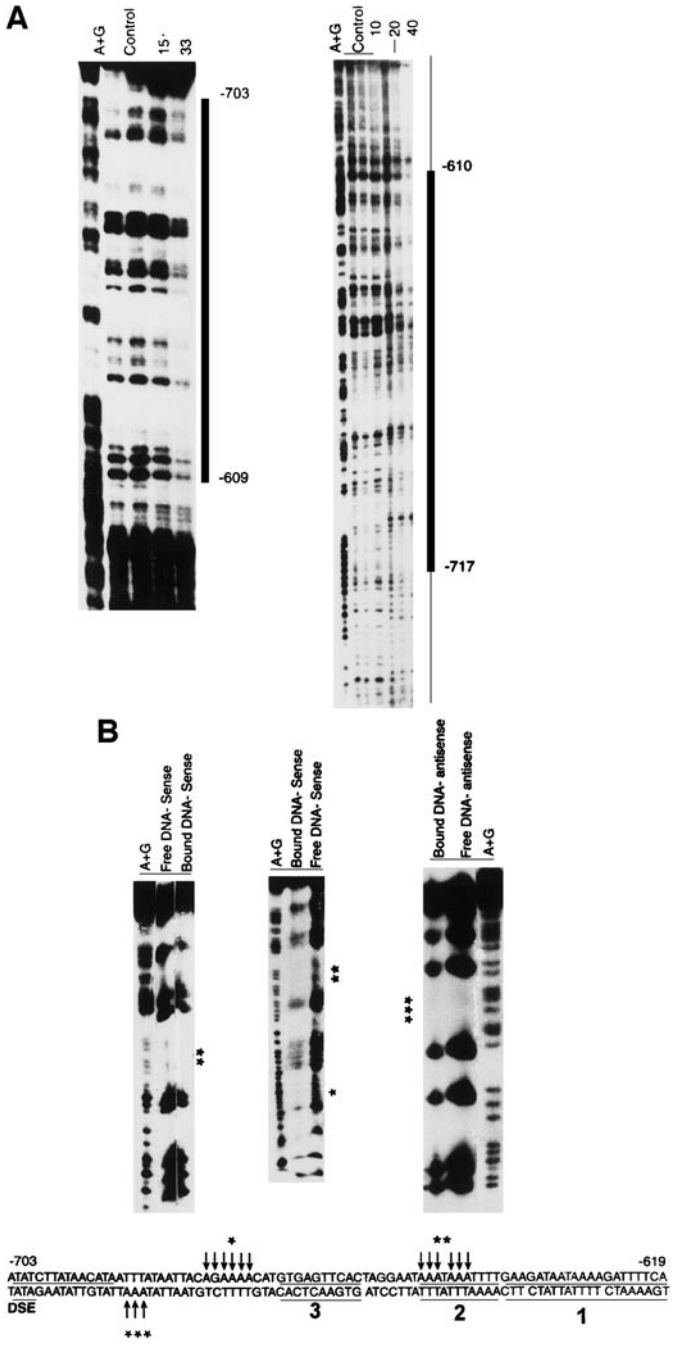

FIG. 2. DNase I protection and methyl interference of −717 to −609 region of the apo(a) promoter by HepG2 nuclear extract. (A) DNase I footprinting assay: A single and continuous footprint was obtained for the lower strand between −703 and −609 spanning a 95-bp region with the probe labeled at −484, the proximal end. The upper strand footprint shows a continuous footprint between −717 to −610 with the probe labeled at −775, the distal end. Left lanes in both the panels show A + G Maxam–Gilbert purine ladder, control lanes contain naked DNA digested with the DNase I, and right lanes contain DNase I-digested DNA after incubation with an increasing amounts of HepG2 NE. (B) Methyl interference assay. The interference pattern of the sense and antisense DNA sequences that are bound to the factors was compared with the methylation pattern of the respective free DNA. Free DNA lanes represent the total profile of methylation reactivity. Binding sites (*) are observed as the absence of bands in the bound samples corresponding to the positions in the free DNA lane where methylation interferes with binding. The first and the second panels show the early and late run of the complex on PAGE in order to visualize the two interference sites (single and double *). The third panel demonstrates a footprinting (three *) on the antisense strand. The interference sites are marked on the DNA below.

structures although a cruciform creates an alternative binding site which may have different affinity for the binding protein. Three dyad symmetry elements, DSE1 (−619 to −640), DSE2 (−641 to −658), and DSE3 (−658 to −667), were identified in the 64 region that might be involved in up-regulating gene transcription.

We examined the interaction of nuclear proteins isolated from HepG2 cells by DNase I footprint analysis, utilizing both the upper and the lower strands. We first utilized a 233 bp fragment (−716 to −484, antisense labeled) for DNase I footprinting of the lower strand. The lower strand footprint demonstrated a clear and single footprint between −703 and −609 spanning a 95 bp region. The upper strand footprinting was done with a 292 bp fragment (−775 to −484). A single footprint was also obtained that was spanned over a 108 bp region (Fig. 2A). A broad footprint region was mapped to be between −717 and −610.

Methyl interference assay was performed on the binding region to identify the number of contact points and to map the location of the *trans*-acting factors binding to the *cis*-element. Three distinct contact points were observed at −686 position on antisense stand and at −671 and −647 positions on sense strand. The contact point at −647 lies in DSE-2 (Fig. 2B). The recognition of factors to the *cis*-element over a broad region is in agreement with the DNase I footprinting result.

To identify the factors that bind to the positive regulatory element we utilized electromobility shift assay with three PCR-generated fragments, **a** (55 bp, −706 to −652), **b** (88 bp, −703 to −616), and **c** (53 bp, −668 to −616) (Figs. 3A and 3B), and NE from HepG2 and HeLa cells. The probes **a**, **b**, and **c**, were shown to bind to factors in HepG2 NE, and failed to bind to any factors in HeLa NE (Figs. 3A and 3B). Four bands with **b**, two bands with **a**, and three bands with **c**, were visualized. As **b** showed four bands and the combined gel shift bands with **a** and **c** were five, we assumed that three factors aA1, aA2, and aA3 bind to **c** and the fourth factor aA4 along with one of the above factors binds to **a**, as **a** and **c** shared a 17 bp common region. To determine the tentative molecular weight of the factors, **a** and **c** were, independently, UV cross-linked to the HepG2 nuclear extracts. Two bands of 97 and 112 kDa with probe **a** and two bands of 73 and 103 kDa with probe **c**, were observed (Fig. 3D). This result is in agreement with the EMSA results that showed four bands. To determine whether the factors could bind to

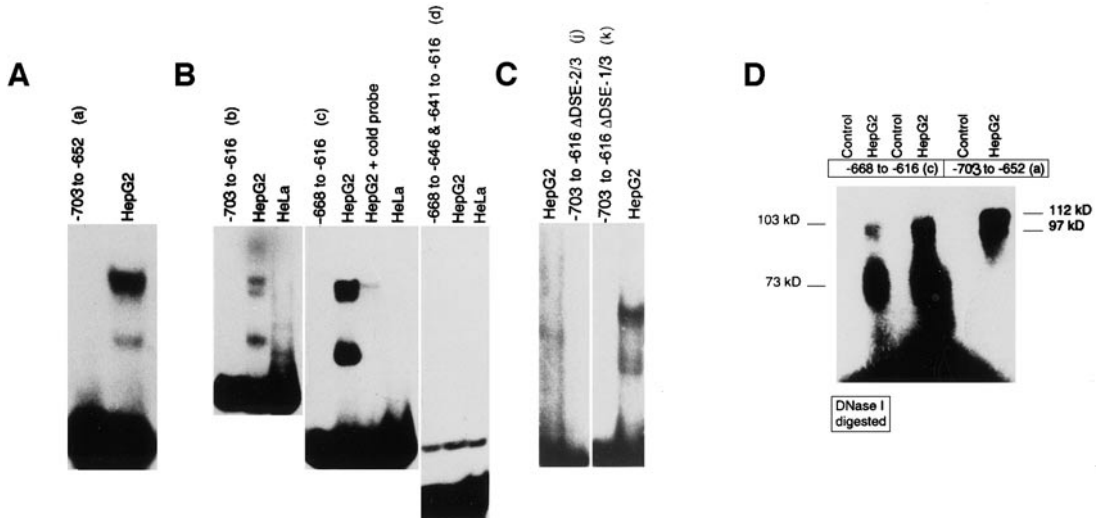

FIG. 3. Gel mobility shift and UV cross-linking assay. (A) EMSA of probe **a** with HepG2 NE. First lane is control lane and second lane shows binding reaction. (B) EMSA of probes **b**, **c**, and **d**. Left lanes for each set are control probes **b**, **c**, and **d**, respectively, the corresponding right lanes are probes incubated with either HepG2 or HeLa NE. Third lane of **c** shows cold competition in binding reaction with HepG2 NE using unlabeled probe **c** in 30× M excess. The end-labeled probes were generated by hot PCR utilizing the sense and antisense primers. (C) Lane 2 is control and lane 1 is the binding reaction with probe **j**. Lane 3 is control and lane 4 is the binding reaction with probe **k**. (D) UV cross-linking assay. Two bands of 73 and 103 kDa were visualized with **c** and two bands of 97 and 112 kDa were visualized with **a**. First and second lanes show UV cross-linked products of **c** in the absence and in the presence of HepG2 NE, digested with DNase I whereas third and fourth lanes have same reaction without DNase I digestion. The fifth and sixth lanes show UV cross-linked products of **a** in the absence and presence of HepG2 NE.

any of the four DSE, independently, we synthesized each of them with an additional flanking 4 bp at both ends, and performed EMSA. All of the four synthetic fragments failed to bind to any of the factors in EMSA.

Deletion of DSE-2 and -3 Relieves Protein Binding and Aborts Transcriptional Activation from SV40 Promoter

To determine the role of various DSEs in recognizing the nuclear factors, we deleted either DSE-1 or DSE-2 alone or along with DSE-3 from the positive regulatory region and performed EMSA with the deleted fragments. The deletion of DSE-1 or DSE-2 alone as well as a simultaneous deletion of DSE-1 and DSE–3 did not disrupt protein binding. However, a simultaneous deletion of DSE-2 and DSE-3 resulted in complete loss of protein binding (Fig. 3C). Further, to correlate whether the role of protein binding to the 64 bp fragment has any consequence to gene transcription and to determine whether the positive regulatory region could activate gene transcription from a heterologous promoter, we fused the WT fragment (−703 to −616) to SV40 promoter ahead of luciferase reporter (PGL2-Pr vector, Promega) gene and conducted transient transfection both in HepG2 and in HeLa cells. We also sub-cloned all the above deleted fragments to PGL-2 Pr vector and transfected them into either HepG2 or HeLa cells, and measured the luciferase activity. In HepG2 cells, the constructs with deleted DSE-1 or DSE-2, and the construct with a deletion of both DSE-1 and -3, did not show any significant loss of transcriptional activation when it was compared with the wild type construct (−703 to −616). However, a deactivation of transcription into basal level was observed with the construct that has simultaneous deletion of both DSE-2 and DSE-3 (Fig. 1).

Purification of a 43.5 kDa trans-Acting Factor by Streptavidin Magnetic Beads

To purify the *trans*-acting factors, we utilized streptavidin magnetic bead system. The biotinylated **a** was first complexed with magnetic bead and the matrix was utilized to purify the trans-acting factors. No proteins could be eluted from the column that could be detected by silver staining. Alternatively, the biotinylated DNA-protein complex from an EMSA reaction was exposed to UV radiation and the UV cross-linked complex was purified utilizing streptavidin magnetic beads. The purified complex was released from the column and was analyzed by SDS–PAGE. When compared with the control lane, a protein band of size 43.5 kDa was detected (Fig. 4). To confirm that the band was not of the DNA probe, the gel was dried and exposed to an X-ray film to detect two bands of single and double stranded DNA probes, that was distinct from the silver-stained band.

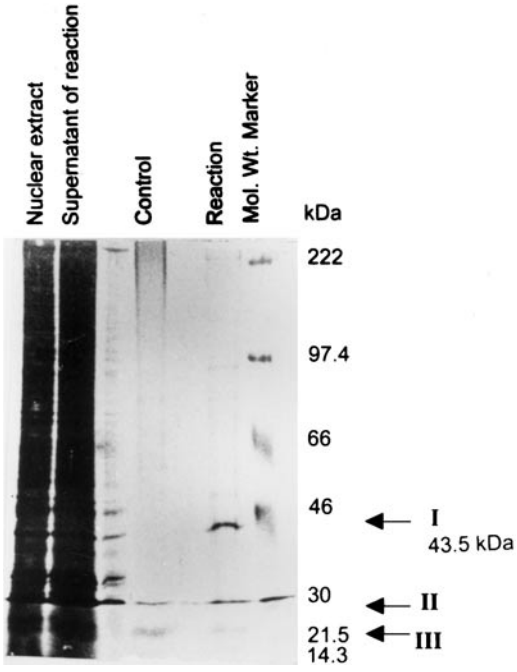

FIG. 4. Purification of cross-linked DNA–protein complex by magnetic bead chromatography. The SDS–PAGE of purified UV cross-linked DNA–protein complex of **a** and its binding protein(s). Lanes 1 and 2 are NE and the supernatant of the UV crosslinked reaction mixture, respectively. Both the control and the reaction lanes show the presence of DNA bands (**II** and **III**). The reaction lane clearly shows a silver-stained 43.5 kDa band of the DNA–protein complex (not present in the control) which was purified by biotin–streptavidin magnetic bead system. The mol wt marker is on the extreme right lane.

Synergistic Binding of Factors to the 64-bp cis-Elements

It was further observed that **c** digested with *Apo*I (resulting in two fragments $\mathbf{d}_1$ and $\mathbf{d}_2$ containing DSE-3 and DSE-1 & -2, respectively) did not show any binding (Fig. 3B). We concluded that the *trans*-factors were binding to the *cis*-element in a cooperative manner and were not capable of independent binding. In a South-Western blot assay none of the probes **a**, **b**, and **c**, could bind to the factors. On one hand a 64 bp region split into four fragments that contained each of the four DSEs did not bind to HepG2 nuclear extract containing all the *trans*-factors together. On the other hand the *trans*-factors separated on SDS–PAGE failed to bind to the intact **a** fragment containing all three DSEs. It might be possible that the DNA–protein complexes involved cooperative binding between the factors taking into consideration of the broad protection region on the DNA as it was evinced from the DNase I and methyl radical footprinting results.

Our finding that multiple factors could recognize multiple *cis*-elements in regulating gene expression is in support of the recent reports. The transcriptional activation of the sulfur amino acid pathway in yeast depends upon the activators Met4p, whose function to tether it to the DNA requires different combinations of the auxiliary factors (17), whereas transcriptional activation of the MET16 gene arises through the assembly of a complex comprising three different subunits (18). The synergistic activation of transcription via protein–protein interactions has also been reported in the fibroblast growth factor 4 (19), leutinizing hormone gene (20), and *Drosophila* sgs-4 gene (21). HMG1 was also found to interact with HOX proteins and enhances their DNA binding and transcriptional activation (22). We have identified four hepato-specific factors that binds to a 64 bp positive regulatory element of apo(a) gene that activated gene transcription in HepG2 cells. The presence of a negative regulatory element upstream of the 64 bp activating region raises the possibility that an as yet unidentified transcriptional repressor might also repress apo(a) gene transcription. The cloning of the genes encoding these factors will throw light into the complexity of apo(a) gene regulation.

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