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Transcriptional regulation of the apolipoprotein A-IV gene involves synergism between a proximal orphan receptor response element and a distant enhancer located in the upstream promoter region of the apolipoprotein C-III gene

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ABSTRACT

Apolipoprotein A-IV expression is limited to intestinal and hepatic cells, suggesting a tissue specific transcriptional regulation of its gene. To investigate the mechanism controlling apo A-IV transcription we have analysed its promoter region by *in vitro* DNA binding and transient transfection experiments. DNase I footprinting analysis of the proximal promoter with rat liver nuclear extracts revealed four protected regions: AIVA (−32 to −22), AIVB (−84 to −42), AIVC (−148 to −92) and AIVD (−274 to −250). Element AIVC which is necessary for maximal promoter activity, binds HNF-4, Arp-1 and Ear-3 with similar affinity in a mutually exclusive manner. HNF-4 transactivated chimeric constructs containing intact AIVC site in the context of either the apo A-IV promoter or the heterologous thymidine kinase minimal promoter, while Arp-1 and Ear-3 repressed this activation. Increasing amounts of HNF-4 alleviated Arp-1 or Ear-3 mediated repression, suggesting that the observed opposing effects is a result of direct competition of these factors for the same recognition site. In transient transfection assays the apo A-IV promoter region (−700 to +10) had a very low activity in cells of hepatic (HepG2) and intestinal (CaCo2) origin. This activity was increased 13 to 18-fold when the upstream elements of the distantly linked apo C-III gene were fused to the proximal promoter. Results obtained with different 5′ and 3′ deletion constructs indicated that the *cis*-acting elements F to J between the nucleotides −500 and −890 of the apo C-III promoter were absolutely necessary to drive maximal enhancement in HepG2 and CaCo2 cells. The apo C-III upstream elements enhanced the activity of the minimal AdML promoter or the apo A-IV site C mutant less efficiently than the intact apo A-IV or AdML promoter constructs containing single HNF-4 sites. The findings suggest that the enhancer effect is mediated by synergistic interactions between the *trans*-acting factors which recognize the apo C-III regulatory elements and HNF-4 which binds to the proximal apo A-IV promoter.

INTRODUCTION

Apolipoprotein A-IV (apo A-IV) is a component of high density lipoproteins (HDL) in several mammalian species but in humans is found mainly in the lipoprotein free (d > 1.21 g/ml) plasma fraction (1,2). Although the precise function of this protein is not known, it has been shown that it promotes cholesterol efflux from adipose cells (3), and activates lecithin-cholesterol acyltransferase (LCAT) using DMPC as a substrate (4). These observations suggest that apo A-IV, similar to apo A-I may play a significant role in the reverse transport of cholesterol. Recently it has been reported that apo A-IV potentiates the activation of lipoprotein lipase by apo C-II (5). In humans, apo A-IV is synthesized primarily in the intestine, and to a lesser degree in the liver (6), where its mRNA levels are affected by hormones (6,7), and dietary fat (8), suggesting that apo A-IV is regulated at least in part at the transcriptional level. An earlier study aimed at the identification of sequences responsible for the tissue specific expression of apo A-IV, indicated that apo A-IV promoter constructs extending either to −893 or −300 nucleotide had approximately 10-fold higher activities in HepG2 and Caco2 cells, as compared to nonhepatic and nonintestinal cells (9). Other studies however suggested that the proximal promoter region (up to −300 nucleotide position) was insufficient to drive transcription in HepG2 or Caco2 cell lines (10), and in transgenic mice (11). When large DNA segments extending to −7700 (11) or −6711 and −3500 (10) nucleotide position from the apo A-IV transcriptional start site were examined, substantial activity could be detected in both experimental systems. In transient transfection experiments the active constructs were transactivated by hepatocye nuclear factor 4 (HNF-4) (10), suggesting that similarly to other apolipoproteins (12), apo A-IV may also be subject to hormone receptor mediated transcriptional regulation.

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Since the gene coding for apo A-IV is located 6.5 kb downstream of the apo C-III gene on the long arm of chromosome 11 (13), these observations raised the possibility that sequences located in the 5′ flanking region of the apo C-III gene may be involved in the control of its transcription.

In this paper we describe the fine mapping of the regulatory regions involved in the hepatic and intestinal transcription of the apo A-IV gene. We report the identification and characterization of a *cis*-acting element in the proximal region of the apo A-IV promoter which is absolutely necessary for its activity. We show that HNF-4 binds to this region and activates transcription, while the recently identified hormone receptor Arp-1 (14), and its homologue Ear-3 (15), act as repressors, by competition with HNF-4 for the same binding site. We also show that transcriptional activation of apo A-IV promoter by HNF-4 requires the presence of enhancer elements which have been mapped in the distantly located apo C-III upstream promoter region. Finally, we present evidence for synergism between HNF-4 and the *trans* acting factors bound to the enhancer.

MATERIALS AND METHODS

Plasmid constructions

Plasmid AIV-CAT, which contains the 5′ flanking region of the human apo A-IV gene from −700 to +10 nucleotides fused to the chloramphenicol acetyltransferase cDNA, was obtained by inserting the *KpnI/HindIII* fragment of pBS-AIV700, to the same sites of pUC-CAT (16). Different part of the apo C-III promoter were isolated by *BstX*1, or *Bsu*36I, or partial *StuI* digestions of the apo C-III CAT plasmid (16), filled in with the Klenow fragment of DNA polymerase I, followed by *XbaI* digestion. These fragments were ligated to the *XbaI/Asp*718-blunt sites of AIV-CAT, to obtain [CIIIJ-B]AIV-CAT, [CIIIJ-C]AIV-CAT, [CIIIJ-F]AIV-CAT and [CIIIJ-H]AI-CAT, respectively. [CIII I]AIV-CAT and [CIII G]AIV-CAT were obtained by ligation of double stranded oligonucleotides encompassing the −766 to −726 (CIII I) and −669 to −648 (CIII G) region of the apo C-III promoter, into the *SalI* site of AIV-CAT. The inserts of [CIIIH-G]AIV-CAT, [CIIIG-F]AIV-CAT and [CIIIH-F]AIV-CAT were synthesized by the polymerase chain reaction using the appropriate primers, and ligated to the blunt ended *Asp*718 site of AIV-CAT. [CIIIF-J]AIV-CAT was constructed by excising the *XbaI/XhoI* fragment of [CIIIJ-F]AIV-CAT, followed by filling in with the Klenow fragment of DNA polymerase I and ligation back to the parent plasmid. The same fragment was also inserted into the blunt ended *HindIII* site of pML44-CAT (17), to create the [CIIIJ-F]ML44-CAT. Double stranded oligonucleotides AIVC and CIIIB were phosphorylated by polynucleotide kinase and ligated to the *SalI* site of either pML44-CAT or [CIIIJ-F]ML44-CAT. The resulting constructs were designated as [AIVC]ML44-CAT, [CIIIB]ML44-CAT, [CIIIJ-F][AIVC]ML44-CAT and [CIIIJ-F][CIIIB]ML44-CAT, respectively. The $[AIVC]_3$TK-CAT plasmid was constructed as follows: First the *EcoRI/BglII* (−85 to +51) fragment of the HSV-TK-CAT (18) plasmid was blunt ended and ligated to the *SmaI* site of pUC-CAT. Then double stranded AIVC oligonucleotide was inserted to this vector and plasmids containing three concatamerized AIVC oligonucleotides in the sense orientation were selected. [CIII-J-F]AIVCM-CAT was generated by PCR based mutagenesis method using mutated sense and antisense AIVC oligonucleotides as internal primers. The external primers were synthesized from vector sequences located at the 5′ or 3′ adjacent region of AIV-CAT. The amplified products were mixed and subjected to a second amplification using only the external primers. This secondary product was sequentially digested with *Bsu*36I and *HindIII*, and inserted to the same sites of [CIIIJ-F]AIV-CAT. The AIV-Gl(380) plasmid which contains the −700 to −2 nucleotide region of the apo A-IV promoter was obtained by inserting a PCR fragment encompassing this region into the *XbaI/EcoRV* sites of the previously described pGl- (19). pMT-HNF-4, pMT-Arp-1 and pMT-Ear-3 (12,20), were generously provided by Dr J.Ladias. pCB6-HNF-4 was created by subcloning the *EcoRI/HindIII* fragment of pMT-HNF-4, containing the entire coding region of HNF-4, into the *Asp*718/*HindIII* sites of the pCB-6 expression vector (21). All clones were verified by DNA sequencing using T7 polymerase (Sequenase).

In vitro DNA binding assays

Rat liver nuclear extracts were prepared according to Lichsteiner *et al.* (22), with the exception that protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), approtinin (10 μg/ml), pepstatin (1 μg/ml) and leupeptin (1 μg/ml) were included in all buffers. Whole cell extracts from Cos-1 cells were prepared as described in (12). DNase-I footprinting, mobility shift assays and methylation interference assays were performed as previously described (12, 19).

The oligonucleotides used in this study were as follows:

AIVC: 5′ TCGATAGTCTCAGGGTCACAAAAGTCCAAGAGGCC−120
AIVCM: 5′ ACGTAGTCTCAGATCTACAATCTTCCAAGAGGC−121
CIIIB: 5′ TCGAGGTCAGCAGGTGACCTTTGCCCAGCG−67
HNF-1A: 5′ TCGAGGCTGAAGTCCAAAGTTCAGTCCCTTCGC−41
AlbPE: 5′ TCGAGTGTGGTTAATGATCTACAGTTA−48

Cell cultures and tranfections

Monolayer cultures of HepG2 and CaCo2 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% and 20% heat inactivated fetal calf serum, respectively. Twenty four hours before transfections, cells were seeded at 50−60 % confluency. The indicated amounts of various constructs alongside with 3 μg pRSV-β-gal plasmid (24) were introduced to the cells by the calcium phosphate DNA coprecipitation method (25). Forty hours later the cells were harvested and lysed by three consecutive freeze-thaw cycles. Chloramphenicol acetyltransferase activitiy was assayed as described in (26), using constant amounts of proteins. The protein concentrations and incubation times were carefully selected by titration and kinetic experiments to assure that the enzyme reaction was at a linear range. β-galactosidase activity was measured according to (24), and the values were used to normalize variations in the transfection efficiency.

In vitro transcription assays

200 ng AIV-Gl(380) and 100 ng AdML(180) (27) templates supplemented with 300 ng salmon sperm DNA, were incubated with 50 μg protein containing rat liver nuclear extract, in a reaction mixture containing 25 mM Hepes pH 7.9, 6 mM $MgCl_2$, 50 mM KCl, 35 units of RNasin, 0.5 mM dithiothreitol, 0.1 mM 3′-O-methyl-GTP, 0.6 mM ATP and CTP, 0.035 mM UTP, 7 μCi [α^{32}P]UTP (3000 Ci/mmol) and 10 % glycerol, in the presence or absence of 100 ng double stranded competitor oligonucleotides as indicated. The reactions were allowed to proceed for 45 minutes at 30°C and stopped by the addition of 2.5 μl 6 % SDS, 250 mM EDTA 250 mM Tris pH 8.0 and 2.5

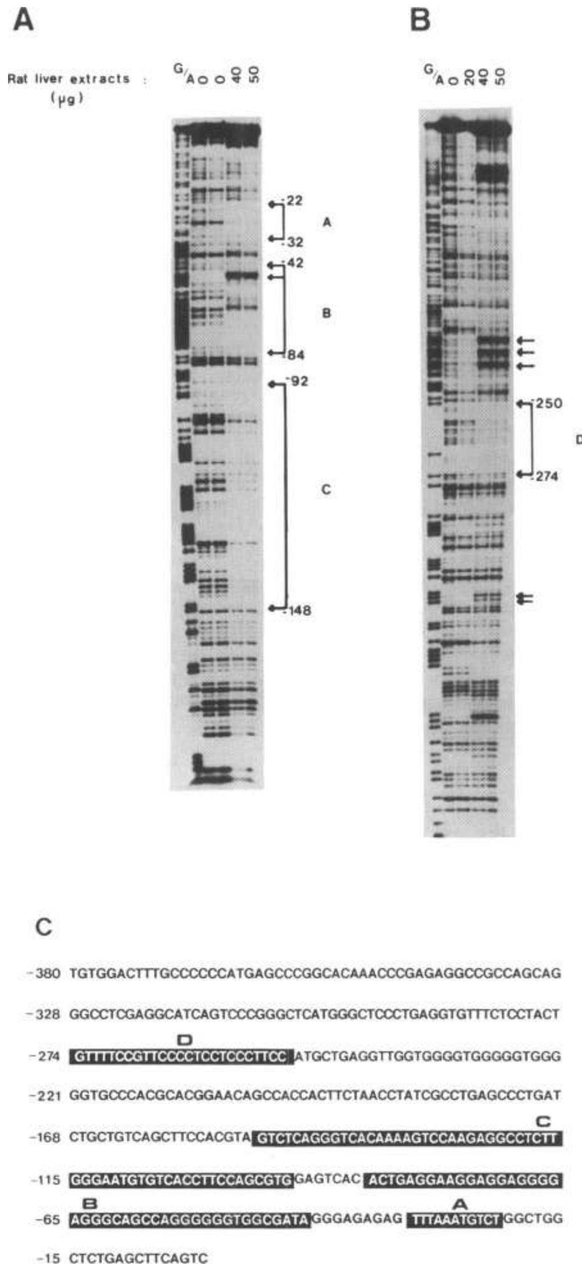


Figure 1. Mapping the nuclear factor binding sites in the proximal Apo A-IV promoter. DNase-I footprint analysis was performed with 5′ end labeled probes containing the −165 to +10 (panel **A**), and −322 to +10 (panel **B**) nucleotide region of the apo A-IV promoter using the indicated amounts of rat liver nuclear extracts. G/A represent Maxam−Gilbert chemical sequencing ladders of the same probes. Panel **C**: Nucleotide sequence of the proximal apo A-IV promoter and summary of the protected regions (boxed).

μl 16 mg/ml proteinase K, 0.5 mg/ml tRNA. The samples were digested at 65°C for 20 minutes, extracted with phenol−chlorophorm, precipitated with ethanol and analysed in 6 % polyacrylamide−8 M urea gels.

RESULTS

Identification of an orphan receptor binding site in the proximal region of apo A-IV promoter

The binding sites of nuclear proteins along the apo A-IV proximal promoter (−300 to +10) were determined by DNase I footprinting using rat liver nuclear extracts. Four protected regions were identified: AIVA (−32 to −22), AIVB (−84 to −42), AIVC (−148 to −92) and AIVD (−274 to −250) (Fig. 1). In addition to the protected regions, these extracts induced at least six DNase I hypersensitive sites in the upper strand at the nucleotide positions −50, −222, −228, −238, 307 and 308 (Fig. 1). Inspection of the sequence at the protected region AIVC (AGGGTCACAAAAGTCCAA), revealed a high degree of similarity with the consensus binding site of the liver enriched transcription factor HNF-4 (28). To asses if indeed HNF-4 and/or other members of the hormone receptor family can interact with this site we performed electrophoretic mobility shift experiments using Cos cell extracts expressing HNF-4, Arp-1 and Ear-3. As shown in Fig. 2. each of these proteins formed a complex with the AIVC oligonucleotide probe. In order to determine the relative binding affinities of these proteins to the AIVC site, we performed mobility shift assays in which constant amounts of protein extracts were titrated with increasing amounts of radiolabeled probe. Scatchard analysis (29) of the data revealed that HNF-4, Arp-1 and Ear-3 bind with similar affinities to the AIVC site. The dissociation constants (Kd) were: 5.5 nM for HNF-4, 4.3 nM for Arp-1 and 7.7 nM for Ear-3 (data not shown). The participation of these orphan receptors in the DNA−protein complex formed with AIVC recognition site with crude rat liver nuclear extracts was evidenced by competition and antibody supershift assays (Fig 3). Strong competition was observed with previously well characterized hormone receptor binding sites from the apo C-III promoter (CIIIB) and HNF-1 promoter (A), but not with mutant AIVC site (AIVCM1) (Fig. 3A). Antibodies raised against HNF-4 which recognize only HNF-4, and COUP-TF which recognize Arp-1 and Ear-3 but not HNF-4, supershifted part of the complex formed on AIVC site (Fig. 3B). A substantial amount of unaltered activity remained when both antibodies were included in the binding reaction, which may indicate that besides these hormone receptors other nuclear factors can also recognize this element.

Methylation interference of nuclear protein binding to the AIVC oligonucleotide probe was observed in the region between −142 and −128. Binding of HNF-4 was affected by methylation of guanine residues at positions −141, −140, −131, in the coding strand and at −138, −136, −129, −128 in the non-coding strand. Interference of Arp-1 and Ear-3 binding was observed by methylation of guanine residues at positions −142, −141, −140, −131 in the coding strand, and at −138 in the non coding strand (Fig. 4). This methylation interference pattern shows that although these factors recognize highly overlapping sites of the AIVC region, they do not make exactly the same contacts, suggesting subtle differences in their DNA binding specificities.

HNF-4 is a dominant activator of the apo A-IV promoter

The function of HNF-4, Arp-1 and Ear-3 on the AIVC element was tested by transient transfection assays in HepG2 cells using a reporter in which three AIVC sites were fused to the herpes simplex virus thymidine kinase minimal promoter ($[AIVC]_3$TK-CAT). HNF-4 activated transcription driven by this chimeric promoter 7-fold, while Arp-1 and Ear-3 repressed this activation (Fig. 5A). These results demonstrate that the AIVC region represents a functional orphan receptor response element.

The involvement of HNF-4 in the activity of the apo A-IV promoter was initially assayed in cell free transcription system. The DNA template containing the −700 to −1 nucleotide region

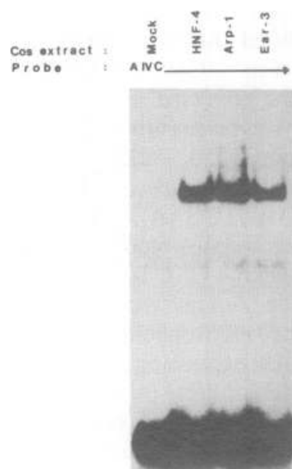


Figure 2. Binding of HNF-4, Arp-1 and Ear-3 to element AIVC. Double stranded oligonucleotide AIVC was labeled and used as a probe in gel electrophoretic mobility shift assay performed with whole cell extracts from mock transfected (M), pMT2-HNF-4 transfected (HNF-4), pMT2-Arp-1 transfected (Arp-1) or pMT2-Ear-3 transfected (Ear-3) Cos-1 cells. Each binding reaction was performed with 5 μg total protein containing cellular extract.

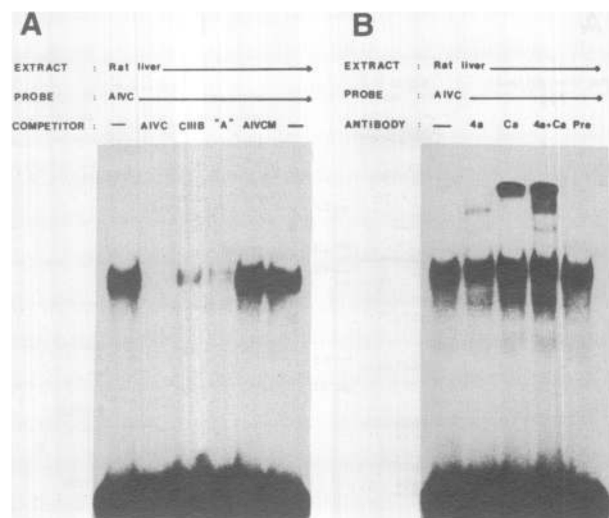


Figure 3. The presence of nuclear hormone receptors in the DNA–protein complex formed on AIVC site using rat liver nuclear proteins. Gel electrophoretic mobility shift assays were performed with rat liver nuclear extracts (10 μg total protein per lane), in the presence or absence of 100-fold molar excess double stranded cold competitor oligonucleotides AIVC, CIIIB, 'A' and AIVCM (panel A), or antiserum raised against HNF-4 (4a) and COUP-TF (Ca) or both (4a+Ca) or preimmune serum (pre) (panel **B**).

of the apo A-IV gene fused to a −380 bp G-free casette was incubated with transcriptionally active rat liver nuclear extract in the presence or absence of excess double stranded competitor oligonucleotides AIVC, CIIIB and 'A'. AIVC and CIIIB are binding sites of both HNF-4 and Arp-1, while site 'A' is able to bind only HNF-4 with high affinity (19). As shown in Fig. 5B the apo A-IV promoter (−700 to −1) could efficiently drive transcription of the reporter cassette *in vitro*, as compared to the AdML promoter which was used as internal control. This activity was reduced to the basal levels when the different HNF-4 binding sites were used as competitors, suggesting that binding of HNF-4 to AIVC site is absolutely necessary for the activity of the apo A-IV promoter.

The upstream (−890 to −500) regulatory elements of the apo C-III gene enhance apo A-IV promoter activity in HepG2 and Caco2 cells

Further analysis of the apo A-IV regulatory region was pursued in transient transfection assays. Initially a reporter construct containing the apo A-IV promoter region from −700 to +10 nucleotide position (apo A-IV-CAT) was tested in HepG2 and Caco2 cells. Surprisingly, we observed a very weak promoter activity in both cell lines (approximately 1−1.5% of the activity obtained with RSV-CAT). In order to identify any possible *in vivo* acting negative element, progressive 5′ deletion mutants up to the nucleotide position −160 were tested. None of these mutants exhibited higher activity (data not shown). These findings show that in contrast to the *in vitro* situation, the −700 to +10 nucleotide region of the apo A-IV gene is not sufficient to drive transcription *in vivo*, and additional regulatory regions may be required for its activity. In search of such type of sequences we found that the apo C-III promoter region (−890 to −65), which is situated approximately 6500 bp upstream of the apo A-IV transcriptional start site, enhanced the transcription driven by the proximal apo A-IV promoter 9 to 10-fold in both HepG2 and Caco2 cells (Fig. 6 lanes 1−2). In order to localize the minimal sequences required for this enhancer activity we have linked different 3′ and 5′ deleted fragments of the apo C-III promoter upstream to the apo A-IV regulatory region. The results obtained from transient transfection experiments of these constructs suggest that the sequences located between the −890 to −500 region of the apo C-III gene exhibited the most marked effect (17.8 and 11.8 fold induction in HepG2 and Caco2 cells respectively), and they were sufficient and necessary for the full enhancer activity in both HepG-2 and Caco2 cells (Fig. 6 lanes 1−4). Essentially the same level of induction was observed when this region was fused to the apo A-IV promoter in the opposite orientation (Fig. 6 lane 6). Further deletions sharply decreased or abolished the enhancer activity (Fig. 6 lanes 7−12). Cotransfection with an HNF-4 expression vector further stimulated the activity of the active enhancer constructs (up to 156-fold and 50-fold above the basal levels in HepG2 and Caco2 cells respectively Fig. 6 lanes 1−5). Since none of the reporters except [CIIIJ-B]AIV-CAT contained an HNF-4 binding site on the apo C-III region, we suspected that HNF-4 exerted its effect through binding to the apo AIVC site, and the mechanism of action of the apo C-III enhancer may involve a functional synergism. To test this hypothesis, we introduced a mutation to the AIVC region that completely abolished HNF-4 binding. As shown in Fig. 6 lane 5 the apoC-III upstream elements enhanced this mutant promoter at a greatly reduced level (25 to 30% of the wild type). More importantly, HNF-4 could not transactivate this mutant promoter, suggesting that the enhancer functions by inducing HNF-4 dependent transcription driven by the proximal apo A-IV promoter.

Independent evidence for this notion was provided by transfection experiments using chimeric reporter constructs containing dissected parts of regulatory regions in front of the AdML minimal promoter. The apo C-III upstream elements enhanced approximately 5-fold the activity of the AdML promoter (Fig. 7 lanes 1−2). As expected, HNF-4 did not transactivate these constructs. Single copies of HNF-4 binding sites from the

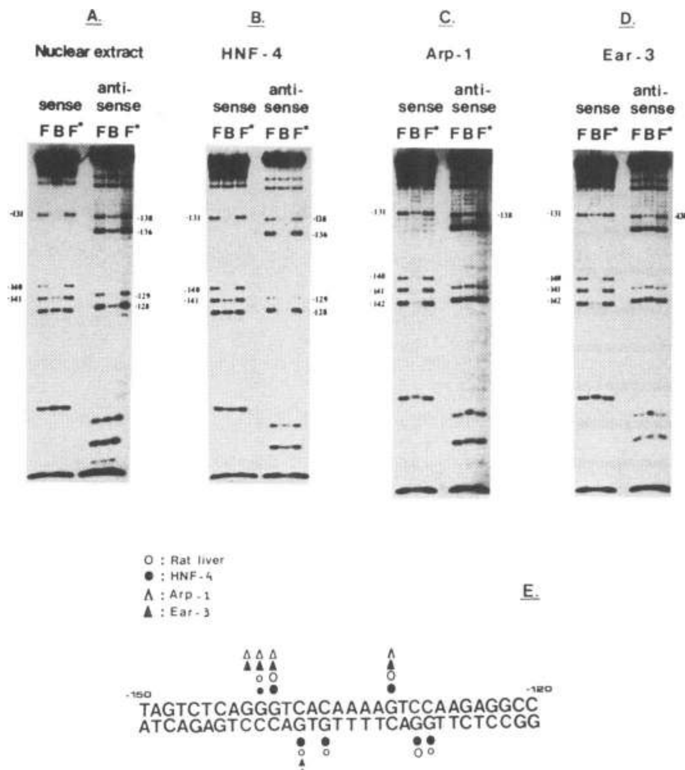


Figure 4. Methylation interference pattern of protein–DNA complexes obtained with the AIVC site. DNA binding reactions were performed with partially methylated double stranded AIVC oligonucleotide labeled at the 5′ end of the coding strand (sense) or the 5′ end of the noncoding strand (antisense) using rat liver nuclear extract (panel **A**), or whole cell extracts from Cos-1 cells transfected with pMT2-HNF-4 (panel **B**), pMT2-Arp-1 (panel **C**) and pMT2-Ear-3 (panel **D**). F, free DNA; B, bound DNA and F*, free DNA recovered after binding. The positions of methylated G residues which interferred with binding are indicated by numbers and summarized in panel **E**. Stronger or weaker interference is indicated by different size of the symbols.

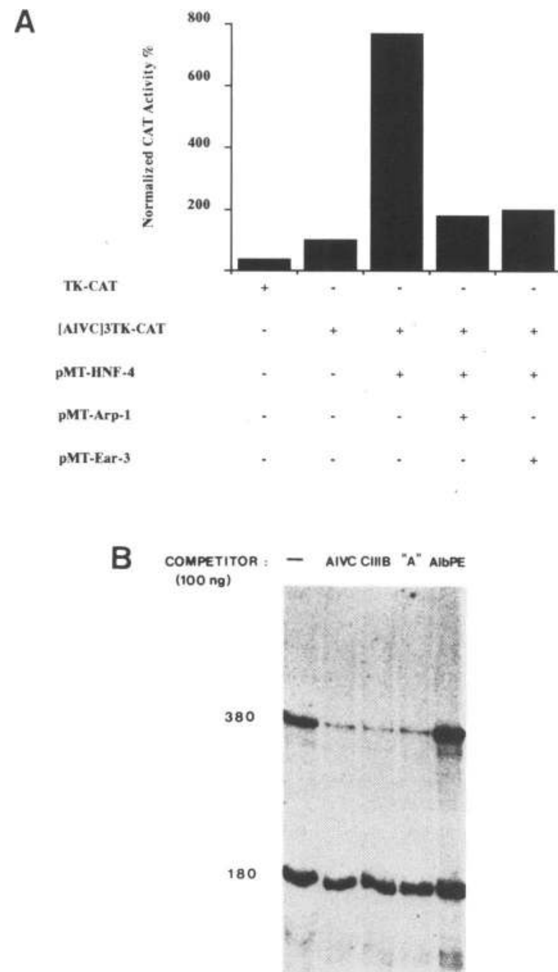


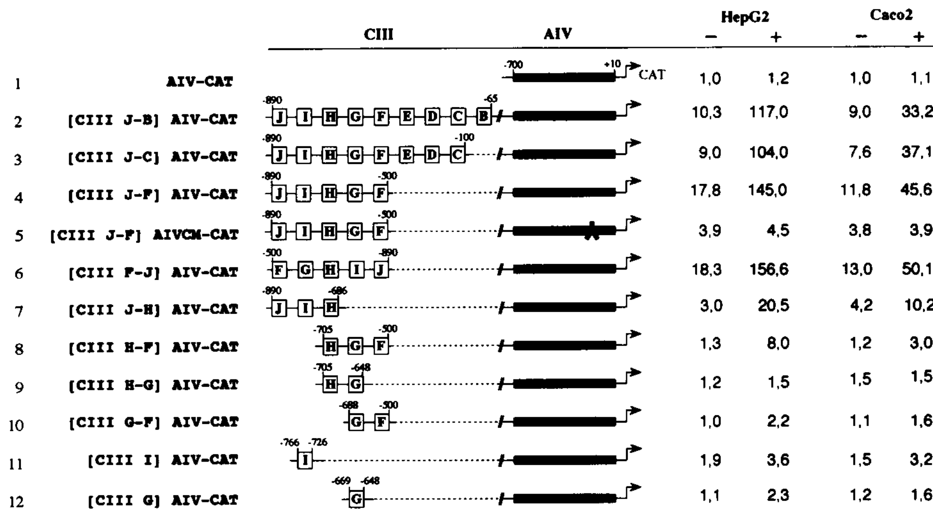
Figure 5. Demonstration that the AIVC site represents a functional hormone receptor response element and it is required for apo A-IV promoter activity. Panel **A**: HepG2 cells were transfected with 2 μgs of TK-CAT or $[AIVC]_3$TK-CAT reporter plasmids in the presence (+) or absence (−) of 2 μgs of pMT2-HNF-4, pMT2-Arp-1 and pMT2-Ear-3 expression vectors. The graphs represent mean values of normalized CAT activities from at least five independent experiments with two different plasmid preparations. The data are expressed as the percentage of normalized CAT activity obtained with $[AIVC]_3$TK-CAT. Panel **B**: The AIV-Gl(380) template was incubated for *in vitro* transcription with rat liver nuclear extracts in the presence or absence of 100 ng double stranded cold competitor oligonucleotides as indicated. The transcription signals from the test template (380) and the AdML(180) internal control template (180) is indicated.

apo A-IV (AIVC) and the apo C-III promoter (CIIIB), increased at a low or insignificant degree AdML promoter activity in both HepG2 and Caco2 cells (Fig. 7 lanes 3–4). Cotransfection with HNF-4 induced this activity 3 to 5 fold above basal levels. On the other hand the transcriptional activity of both single HNF-4 binding site containing constructs ([CIIIB]ML44-CAT and [AIVC]ML44-CAT) were dramatically increased by the apo C-III upstream elements (Fig 7. lanes 5–6). The 23 and 34 fold increase in HepG2, and the 15 and 30-fold increase observed in Caco2 cells, represent a 3 to 4-fold induction above the sum obtained by either regulatory sequence alone, suggesting synergistic interactions between nuclear proteins binding to the apo C-III enhancer and HNF-4. A more profound difference was observed in cotransfection experiments with an HNF-4 expression vector. HNF-4 transactivated the enhancer-AIVC and enhancer-CIIIB containing constructs 148 to 198-fold above basal levels in HepG2 and 118 to 192 fold in Caco2 cells respectively (Fig. 7. lanes 5–6). These values represent a 14 to 18-fold increase above the sum of the activity obtained by the vectors containing either element alone in HNF-4 transfected cells.

Taken together, these data suggest that the apo C-III enhancer acts by a mechanism which strongly potentiates HNF-4 mediated transcription from downstream promoter elements.

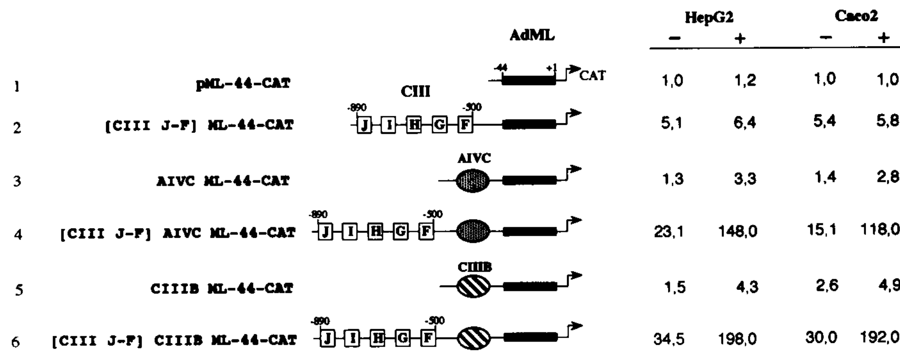
Antagonistic effects of Arp-1 or Ear-3 and HNF-4 on apo AIV promoter

We have shown in the previous sections that transcriptional activation of the apo A-IV promoter by HNF-4 depends on the AIVC element, which is also recognized by Arp-1 and Ear-3. These factors had an opposing effect on a homopolimeric promoter containing three AIVC sites, raising the possibility that HNF-4, Arp-1 and Ear-3 may regulate in the opposite direction the apo A-IV promoter by competing for the same binding site. To address this question we have performed cotransfection experiments using the [CIIIJ-F]AIV-CAT reporter and different combinations of HNF-4 and Arp-1 or Ear-3 expression vectors. As seen in Fig 8. both Arp-1 and Ear-3 repressed transcription of this promoter to the level of 13 to 15 % of the control. This repression was alleviated by increasing amounts of HNF-4, while



	Construct	HepG2 −	HepG2 +	Caco2 −	Caco2 +
1	AIV-CAT	1,0	1,2	1,0	1,1
2	[CIII J-B] AIV-CAT	10,3	117,0	9,0	33,2
3	[CIII J-C] AIV-CAT	9,0	104,0	7,6	37,1
4	[CIII J-F] AIV-CAT	17,8	145,0	11,8	45,6
5	[CIII J-F] AIVCM-CAT	3,9	4,5	3,8	3,9
6	[CIII F-J] AIV-CAT	18,3	156,6	13,0	50,1
7	[CIII J-H] AIV-CAT	3,0	20,5	4,2	10,2
8	[CIII H-F] AIV-CAT	1,3	8,0	1,2	3,0
9	[CIII H-G] AIV-CAT	1,2	1,5	1,5	1,5
10	[CIII G-F] AIV-CAT	1,0	2,2	1,1	1,6
11	[CIII I] AIV-CAT	1,9	3,6	1,5	3,2
12	[CIII G] AIV-CAT	1,1	2,3	1,2	1,6

Figure 6. The upstream regulatory elements of the apo C-III gene enhance apo A-IV promoter activity in HepG2 and Caco2 cell lines. HepG2 and Caco2 cells were transfected with 2μgs of reporter plasmids shown in the left side in the presence (+) or absence (−) of 2μg pCB-HNF-4 expression vector. The numbers represent mean values of normalized CAT activities with less than 10% standard deviation, from five independent experiments with at least two different plasmid preparations. The data are presented relative to the activity obtained with AIV-CAT.



	Construct	HepG2 −	HepG2 +	Caco2 −	Caco2 +
1	pML-44-CAT	1,0	1,2	1,0	1,0
2	[CIII J-F] ML-44-CAT	5,1	6,4	5,4	5,8
3	AIVC ML-44-CAT	1,3	3,3	1,4	2,8
4	[CIII J-F] AIVC ML-44-CAT	23,1	148,0	15,1	118,0
5	CIIIB ML-44-CAT	1,5	4,3	2,6	4,9
6	[CIII J-F] CIIIB ML-44-CAT	34,5	198,0	30,0	192,0

Figure 7. The upstream regulatory elements of the apo C-III gene enhance HNF-4 dependent transcription driven by chimeric promoter constructs containing single HNF-4 binding sites. HepG2 and Caco2 cells were transfected with 2 μgs of the reporter plasmids shown in the left side, in the presence (+) or absence (−) of 2 μg pCB-HNF-4 expression vector. The numbers represent mean values of normalized CAT activities with less than 10% standard deviation, from four independent experiments. The data are presented relative to the activity obtained with pML-44-CAT.

increasing amounts of Arp-1 or Ear-3 abolished HNF-4 mediated transactivation. Since Arp-1 and Ear-3 did not affect the same reporter containing a mutated AIVC site (Fig. 8C), we conclude that their negative effect is excerted by competition with HNF-4 for the same bindig site.

DISCUSSION

In order to elucidate the molecular basis of the tissue specific expression of the apo A-IV gene we have isolated and characterized its promoter region. Alignment of the 5′ flanking sequences of the apo A-IV gene derived from different species, revealed a remarkable conservation extending from nucleotide −167 to −77 (9). Within this region we identified a *cis*-acting element between nucleotides −148 and −92 (AIVC), which plays an important role in the transcriptional regulation of the apo A-IV gene. Part of this element can recognize HNF-4, as well as other members of the hormone receptor superfamily like Arp-1 and Ear-3. HNF-4 strongly activated, while Arp-1 and Ear-3 repressed transcription driven by the apo A-IV promoter. Several lines of evidence presented in this study suggest that the opposing transcriptional effects between HNF-4 and Arp-1 or Ear-3, may be excerted via direct competition for binding to AIVC site. First, all three factors bind to this element with similar affinities in a mutually exclusive manner. Second, transcription driven by the homopolymeric AIVC site containing heterologous promoter was affected in the same direction. Third, these factors did not influence the activity of the apo A-IV promoter containing mutated AIVC site. Since HNF-4, Arp-1 and Ear-3 are present in the same cells in the liver and intestine (20), it seems possible that similarly to other apolipoproteins (12,20), transcription of the apo A-IV gene is also dependent, at least in part, on the relative intracellular concentrations of these hormone receptors. Since HNF-4, Arp-1 and Ear-3 are members of the same hormone receptor family, the possibility exists that they may respond to as yet unidentified ligands. Such ligands, may for instance be the byproducts of intracellular lipid metabolism, and may modulate positively or negatively the expression of the apo

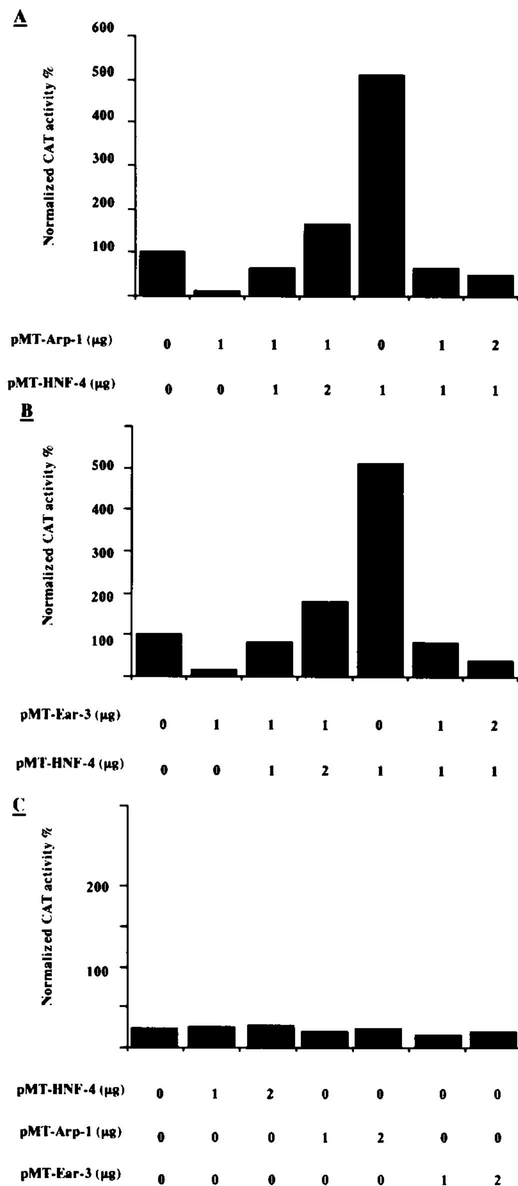


Figure 8. Antagonistic effects of HNF-4 and Arp-1 or Ear-3 on apo A-IV transcription. HepG2 cells were transfected with 2 µgs of [CIIIJ-F] AIV-CAT (panel **A** and **B**) or 2 µgs of [CIIIJ-F] AIVCM-CAT containing mutated AIVC site (panel **C**) alongside with the indicated amounts of expression plasmids. The graphs represent mean values of normalized CAT activities with less than 10% standard deviation from three independent experiments. The data are expressed as the percentage of normalized CAT activity obtained with [CIIIJ-F] AIV-CAT. Note the different scale of the ordinate in panel C!

A-IV gene according to the metabolic state of the cell. The common regulatory effects of HNF-4 and its family members on the transcription of several apolipoprotein genes (e.g. apo A-1, apo A-II, apo A-IV, apo B, and apo C-III) (12, 20), may be conceivable with the theme relating this subset of nuclear factors in the accomplishment of a specific metabolic goal. This speculation however is contradicted by the important regulatory function of HNF-4 and Arp-1 on a large number of functionally unrelated genes, including pyruvate kinase (30), coagulation factor IX (31), transferrin (32), transthyretin (33), retinol binding protein II (34), ornithine transcarbamylase (35), and ovalbumine (36). Another explanation for the phenomenon, would assume the evolution of apolipoprotein genes from a common ancestor, through a series of deletion, translocation and duplication events, that accidentally positioned or preserved a dominant hormone receptor response element in their regulatory region. Consistent with this hypothesis are the phylogenetic studies proposing that apo C-III, apo A-I, apo A-II, apo A-IV, and apo E have evolved by sequential duplication and transposition events of an ancestor of apo C-I gene (37).

Although the 5′ flanking sequences of the human apo A-IV gene extending up to −700 nucleotide were sufficient to drive transcription *in vitro*, this was not the case *in vivo*. This apparently controversial observation can be resolved by taking into consideration the biological complexity of the experimental system. In the *in vitro* system the availability of regulatory proteins at optimal concentrations in the nuclear extracts is the only rate limiting factor in the formation of transcriptionally active complex. In contrast, DNA introduced into living cells is assembled into higher order chromatine structure, and therefore transcription depends on more complex nuclear events, which may necessiate the presence of additional regulatory elements and factors. Precedent cases for the requirement of such extra regulatory elements to obtain maximal transcriptional activity *in vivo* but not *in vitro*, have been described. Well documented examples are the α1-antitrypsin (38), and human transferrin promoters (39).

The results presented in this study show that sequences located within the regulatory region of the distantly linked apo C-III gene are necessary for apo A-IV promoter activity *in vivo*. The minimal region required for the induction was located between −890 and −500 nucleotide positions of the apo C-III gene. This region enhanced transcription driven by the apo A-IV and the heterologous AdML promoters in an orientation independent fashion, which is characteristic of a typical enhancer. Since the respective binding sites in the reporters used in this study are brought much closer to each other than their position on the chromosome, we can only indirectly suggest that the apo C-III upstream region is functioning as a physiological enhancer for the apo A-IV gene. However, the extrapolation of our results to the *in vivo* situation seems to be valid in the light of the recent finding, that only a large DNA segment extending up to −7700 nucleotide position from the transcriptional start site was able to drive apo A-IV transcription in transgenic animals (11), since this construct included the apo C-III promoter region which is located approximately at a −6500 bp distance. Similar results were obtained by transient transfection experiments in HepG2 cells (10), although in the latter case a shorter DNA construct extending up to −3500 nucleotide position had also some activity, suggesting the existence of a second weaker enhancer.

The localization of elements enhancing the apo A-IV promoter, in the upstream regulatory region of the apo C-III gene, is interesting in the light of recent findings, which suggest that this region may function as a common enhancer for all three physically linked apolipoprotein genes in the apo A-I/apo C-III/apo A-IV cluster. Similarly to apo A-IV, transcription driven by the proximal apo C-III (12, Talianidis *et al.*, in preparation), and apo A-I (40) promoter was greatly enhanced by this locus. Analogous function have been described for the locus control region of the β-globin cluster (41), or the distal regulatory region of albumin and α-fetoprotein genes (42). In both cases a common

enhancer region orchestrates the transcription of distinct but physically linked downstream genes.

With respect to the mechanism of action of the apo C-III enhancer we propose that a functional interplay between the factors which recognize this region, and HNF-4 which binds to the proximal apo A-IV promoter is responsible for high level transcription. This notion is strongly supported by the finding that the apo C-III enhancer induced the mutated HNF-4 binding site containing proximal apo A-IV promoter only at a very reduced rate. In addition, the apo C-III enhancer sequences induced transcription driven by single HNF-4 binding sites containing minimal promoter constructs much more efficiently, than the ones lacking the HNF-4 sites. These findings indicate that the observed effect is due to synergistic interactions between the factors which bind to the apo C-III enhancer and HNF-4 which binds to the proximal apo A-IV promoter. The requirement of synergistic interactions between HNF-4 and other factors for high level activation has been observed in several other regulatory regions. Well documented cases include synergism of HNF-4 with C/EBP on the apo B (43), or with HNF-1 on the L-type pyruvate kinase (30), or with CREB on the tyrosine aminotransferase promoters (44). The molecular basis for the 'crosstalk' of HNF-4 with these varied assortment of structurally unrelated factors remains to be investigated.

In humans and in transgenic mice apo A-IV expression is mainly observed in intestinal cells and to a lesser extent in the liver. In contrast, we observed similar enhancer dependent apo A-IV promoter activity in cell lines of both hepatic (HepG2) and intestinal (Caco2) origin. Since HepG2 cells express gene products characteristic to the fetal liver, rather than the adult phenotype (45), this difference may reflect distinct expression patterns of the apo A-IV gene during development. Nevertheless, we can not exclude the possibility that posttranscriptional mechanism(s) affecting RNA stability differently in the adult liver and intestine, may be responsible for the observed pattern of apo A-IV messenger RNA in these tissues.

In conclusion, our results show that transcription of the apo A-IV gene is controlled by HNF-4, which binds to its proximal promoter region. In this promoter context HNF-4 alone is not an efficient activator *in vivo*, and requires the presence of factors which recognize enhancer elements located in the upstream promoter region of the distantly linked apo C-III gene. The functional synergism observed between HNF-4 and the factors binding to the apo C-III enhancer, may represent a common mechanism by which these sequences influence the expression of the tandemly linked Apo A-I, apo C-III and apo A-IV genes.

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REFERENCES

1. Bisgaier, C. L., *et al.* (1985) J. Lipid Res. **26**, 11–25.
2. Uterman, G., and Beisiegel, U. (1979) Eur. J. Biochem. **99**, 333–343.
3. Steinmetz, A., *et al.* (1990) J. Biol. Chem. **265**, 7859–7863.
4. Steinmetz, A., and Uterman, G. (1985) J. Biol. Chem. **260**, 2258–2264.
5. Goldberg, I. J., *et al.* (1990) J. Biol. Chem. **265**, 4266–4272.
6. Karathanasis, S. K., Yunis, I., and Zannis, V. I. (1986) Biochemistry **25**, 3962–3970.
7. Staels, B., *et al.* (1990) Endocrinology **126**, 2153–2163.
8. Apfelbaum, T. F., Davidson, N. O., and Glickman, R. M. (1987) Am. J. Physiol. **252**, 662–666.
9. Elshourbagy, N. A., *et al.* (1987) J. Biol. Chem. **262**, 7973–7981.
10. Ochoa, A., Bovard-Houppermans, S., and Zakin, M. M. (1993) Biochim. Biophys. Acta **1210**, 41–47.
11. Lauer, S. J., *et al.* (1991) Circulation **84**, 1390.
12. Ladias, J. A. A., *et al.* (1992) J. Biol. Chem. **267**, 15849–15860.
13. Karathanasis, S. K. (1985) Proc. Natl. Acad. Sci. USA **82**, 6374–6378.
14. Ladias, J. A. A., and Karathanasis, S. K. (1991) Science **251**, 561–565.
15. Wang, L. H., *et al.* (1989) Nature **340**, 163–166.
16. Ogami, K., *et al.* (1990) J. Biol. Chem. **265**, 9808–9815.
17. Ham, J., *et al.* (1991) EMBO J. **10**, 2931–2940.
18. Cato, A. C. B., *et al.* (1986) EMBO J. **5**, 2237–2240.
19. Kritis, A. A., *et al.* (1993) Nucl. Acids. Res. **21**, 5882–5889.
20. Mietus-Snyder, M., *et al.* (1992) Mol. Cell. Biol. **12**, 1708–1718.
21. Anderson, S., *et al.* (1989) J. Biol. Chem. **264**, 8222–8229.
22. Lichsteiner, S., and Schibler, U. (1989) Cell **57**, 1179–1187.
23. Sambrook, J., Fritsch, J. F., and Maniatis, T. (1989) Molecular cloning: Laboratory manual, 2nd ed. Cold Spring Harbor Press, New York.
24. Edlund, T., *et al.* (1985) Science **230**, 912–916.
25. Graham, F. L., and Van der Eb, A. J. (1973) Virology **52**, 456–467.
26. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. **2**, 1044–1051.
27. Sawadago, M., and Roeder, R. G. (1985) Cell **43**, 165–175.
28. Sladek, F. M., *et al.* (1990) Genes Dev. **4**, 2353–2365.
29. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. **51**, 660–672.
30. Guerra, M. M. D., *et al.* (1993) Mol. Cell. Biol. **13**, 7725–7733.
31. Rejnen, M. F., *et al.* (1992) Proc. Natl. Acad. Sci. USA **89**, 6300–6303.
32. Shaeffer, E., *et al.* (1993) **268**, 23399–23408.
33. Costa, R. H., and Grayson, D. R. (1991) Nucl. Acids Res. **19**, 4139–4145.
34. Nakshatri, H., and Chambon, P. (1994) J. Biol. Chem. **269**, 890–902.
35. Kimura, A., *et al.* (1993) J. Biol. Chem. **268**, 11125–11133.
36. Tsai, S. Y., *et al.* (1987) Cell **50**, 701–709.
37. Luo, C. C., *et al.* (1986) J. Mol. Biol. **187**, 325–340.
38. Monaci, P., Nicosia, A., and Cortese, R. (1988) EMBO J. **7**, 2075–2087.
39. Mendelzon, D., Boissier, F., and Zakin, M. M. (1990) Nucl. Acids Res. **18**, 5717–5721.
40. Walsh, A., *et al.* (1993) J. Lipid Res. **34**, 617–623.
41. Orkin, S. H. (1990) Cell **63**, 665–662.
42. Godbout, R., Ingrim, R., and Tilghman, S. M. (1986) Mol. Cell. Biol. **6**, 477–487.
43. Metzger, S., *et al.* (1993) J. Biol. Chem. **268**, 16831–16838.
44. Nitsch, D., Boshart, M., and Schutz, G. (1993) Proc. Natl. Acad. Sci. USA **90**, 5479–5483.
45. Rollier, A., *et al.* (1993) Mol Biol. Cell. **4**, 56–69.