Two regulatory elements of similar structure and placed in tandem account for the repressive activity of the first intron of the human apolipoprotein A-II gene

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Recent reports indicate that apolipoprotein (apo) A-II, the second most abundant protein of high-density lipoproteins, plays a crucial role in counteracting the beneficial effect of apo A-I against atherogenesis. Transcription of the human apo A-II gene is controlled by an enhancer comprising 14 regulatory elements located upstream of its promoter whereas the first intron of this gene behaves as a silencer. Here we show that two sequence elements account for the repressive activity of this intron and correspond to negative regulatory elements termed NRE I and NRE II. The activity of intron I and the nuclear proteins binding to NRE I and II are encountered in hepatic cells

but not in non-hepatic cells studied here. Both NREs form nucleoprotein complexes of very similar physicochemical characteristics and bind the same or closely related proteins. Site-directed mutagenesis, transient transfection and gel-shift analysis experiments indicate that both NREs exhibit similar structures, being composed of two sites required for maximal activity and optimal binding of transcription factors. Therefore two negative regulatory elements of similar structure and function, placed in tandem, account for the repressive activity of the first intron of the human apo A-II gene. These NREs do not exhibit structural similarity with known NREs of other genes.

INTRODUCTION

Apolipoproteins A-I and A-II (apo A-I and apo A-II) are the most abundant proteins of high-density lipoproteins (HDLs), the plasma levels of which are inversely correlated with the incidence of coronary artery disease. HDLs protect against coronary artery disease by removing excess cholesterol from peripheral tissues and transporting it to the liver, a process termed 'reverse cholesterol transport'. HDLs are heterogeneous and consist mainly of two lipoprotein subfractions containing either only apo A-I (LpA-I) or apo A-I and apo A-II (LpA-I:A-II) (reviewed in [1,2]). Numerous data indicate that LpA-I plays a crucial role in the protection against atherosclerosis (reviewed in [2,3]). In fact the protein composition of HDLs and particularly their apo A-II content is an important marker for the anti-atherogenic properties of HDLs. Some but not all clinical studies suggest that LpA-I:A-II is a less 'anti-atherogenic' lipoprotein particle than LpA-I [4,5] and naturally selected mouse strains with elevated apoA-II levels are prone to the development of atherosclerosis on an atherogenic diet [6]. Furthermore increased atherosclerotic lesion development was observed either in transgenic mice overexpressing mouse apo A-II compared with normal mice or in transgenic mice overexpressing human apo A-I and apo A-II compared with those overexpressing only human apo A-I [7–10]. These results are consistent with several studies *in vitro* suggesting that the efficiency of the LpA-I particle in promoting cholesterol efflux is substantially greater than that of the LpA-I:A-II particle [11–14]. Because it has been shown that the apo A-II production rate is a major factor determining the relative amounts of LpA-I and LpA-I:A-II in normolipidaemic humans [15], it seems of great interest to elucidate the mechanisms regulating the expression of the apo A-II gene.

Human apo A-II is synthesized by the liver and to a much smaller extent by the intestine [2]. This cell-type-specific ex-

pression is governed by a distal enhancer region located between nucleotides –903 and –680 of the human apo A-II gene and containing six regulatory elements [16–18]. The promoter of the human apo A-II gene contains eight additional regulatory elements distributed in the proximal region –126/–33 and in the median region –573/–255. The optimum expression of this promoter requires the synergistic action of factors binding to the proximal and distal regulatory elements [19–21]. We have recently shown that fibrates increase human apolipoprotein A-II expression through activation of the peroxisome proliferator-activated receptor [22]. In addition, the first intron of the human apo A-II gene, which is located in the non-coding region 5' to the start methionine, behaves as a silencer on the thymidine kinase promoter and contains three sequence elements that bind nuclear proteins in DNase I footprinting assays [23]. In this report we show that two of these sequence elements correspond to negative regulatory elements (NREs) of similar structure binding the same transcription factors.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized on a Applied Biosystems apparatus 394-08 and purified by electrophoresis on a 15% (w/v) polyacrylamide slab gel containing 8 M urea.

Plasmid construction

Cloning methods were as described previously [24]. Plasmids were purified twice on caesium chloride gradients. The human apo A-II basal promoter flanked by its enhancer (nucleotides –911 to +40, construct P) or flanked by both the enhancer and the first intron (nucleotides –911 to +206, construct P In) were

Abbreviations used: apo, apolipoprotein; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; HDL, high-density lipoprotein; LpA-I, HDL subfraction containing apo A-I; LpA-I:A-II, HDL subfraction containing apo A-I and apo A-II; NRE, negative regulatory element.

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Figure 1 Activity of the apo A-II first intron in various cell lines

CAT activities, determined and normalized for differences in transfection efficiencies as indicated in the Materials and methods section, are expressed relative to the activities of apo A-II enhancer-basal promoter (P construct) (**A**) and thymidine kinase promoter (TK construct) (**B**). P, P In and In P represent the human apo A-II enhancer-basal promoter alone (–911/+40) and flanked by the first intron downstream and upstream respectively; TK, In TK and 2 In TK represent the thymidine kinase promoter alone and flanked by the intron in monomer and dimer respectively. The results shown are averages of at least three independent transfection experiments performed in duplicate with two different DNA preparations, with the standard deviations indicated by error bars.

cloned in the promoterless reporter vector pBLCAT5 [23]. The complete intron I subcloned in plasmid pUC19 was excised by *Hind*III–*Bam*HI digestion, blunt-ended and imported upstream of the enhancer-basal promoter construction P, which was opened and blunt-ended at the *Hind*III site to generate the construct In P. The oligonucleotide corresponding to element I spans nucleotides +49 to +89 and is flanked by a *Hind*III site at the 5' end and *Sph*I and *Pst*I sites at the 3' end, while the oligonucleotide corresponding to element II spans nucleotides +109 to +149 and is flanked by *Sph*I and *Sal*I sites at the 5' and 3' ends respectively. Both oligonucleotides were cloned in the reporter vector pBLCAT4 [25] containing the minimal thymidine kinase promoter (–105 to +51) to generate plasmids I-TK and II-TK respectively. To place elements I and II in tandem upstream of the thymidine kinase promoter (construct I-II-TK), the oligonucleotide corresponding to element II was imported in the plasmid I-TK previously digested with *Sph*I and *Sal*I. The mutated elements I and II were synthesized with *Hind*III and *Sal*I restriction sites at the 5' and 3' ends respectively, and were cloned directly into pBLCAT4 to generate Im1-TK, Im2-TK and so on. Integrity and orientation of the constructions were controlled by sequencing (USB from Amersham or *fmol* sequencing system from Promega).

Cell culture and transient transfection assays

Human hepatoma HepG2 cells, human chorio-carcinoma JEG-3 cells and African green monkey kidney CV-1 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum and containing 4.5 g/l glucose. Rat hepatoma Fa32 cells were grown in the same medium but containing 1 g/l glucose. Cells were transfected by the calcium phosphate method by using 5 µg of reporter plasmids for HepG2 and 10 µg of reporter plasmids for Fa32, CV-1 and JEG-3 cells. To normalize for differences in transfection efficiencies within the cell lines, a CMV-driven βGAL expression plasmid was transfected simultaneously. Chloramphenicolacetyl transferase (CAT) enzyme activity was assayed as in [18] with different amounts of protein depending on the cells transfected, to ensure

the linear conversion of the chloramphenicol to its acetylated form. After normalization for βGAL activity and cellular protein content in the assay mixtures, CAT activity driven by the apo A-II promoter is one-thirtieth as strong in Fa32 cells as in HepG2 cells; in the same way the CAT activities driven by the thymidine kinase promoter were one-thirtieth, one-eighth and one-eleventh as strong in Fa32, JEG-3 and CV-1 cells respectively as that in HepG2 cells.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts from rat liver were prepared as previously described [26]. For cultured cells, nuclei were prepared as described [27] and crude nuclear extracts were prepared as described by Dignam et al. [28]. Nuclear extracts were dialysed in 20 mM Hepes buffer, pH 7.9, containing 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol and 20% (v/v) glycerol. All buffers contained 0.5 mM PMSF and 0.1 mg/ml each of leupeptin, aprotinin and pepstatin. Oligonucleotides were labelled with [α -³²P]dCTP by using the Klenow fragment of DNA polymerase I. Nuclear extracts (4 µg) were incubated for 1 h on ice in the dialysis buffer (final reaction volume 20 µl) in the presence of 3 µg of double-stranded poly(dI,dC)·poly(dI,dC) and 0.2 ng of radiolabelled oligonucleotides. Alternatively a 50–150-fold molar excess of competitor oligonucleotides was added. Nucleoprotein complexes were resolved on a 5% (w/v) polyacrylamide gel in 1 × TAE (6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9).

RESULTS

Cell specificity of the activity of the human apo A-II intron I

In our previous study the silencer activity of apo A-II intron I was demonstrated only on the heterologous thymidine kinase promoter [23]. To ascertain whether this intron can also down-regulate the homologous apo A-II promoter, we checked its

Figure 2 Activity of NRE I and NRE II on the thymidine kinase promoter

The abbreviations TK and In TK are as explained in Figure 1. I TK, II TK and I-II TK represent the footprinted sequences I and II (corresponding to NRE I and NRE II respectively) fused alone or together upstream of the thymidine kinase promoter respectively. CAT activities, determined and normalized for differences in transfection efficiencies as indicated in the Materials and methods section, are expressed relative to the activity of the thymidine kinase promoter. The results shown are averages of at least three independent transfection experiments performed in duplicate with two different DNA preparations, with the standard deviations indicated by error bars.

activity when placed upstream of the human apo A-II enhancer-basal promoter (spanning nucleotides $-911/+40$; construct P) in the construct In P. Comparison of CAT activities driven by constructs P and In P in HepG2 cells (Figure 1A) shows that intron I decreases apo A-II promoter activity at the transcriptional level. The lower capacity for repression when the intron is placed upstream (construct In P) rather than downstream (construct P In) of the apo A-II promoter (Figure 1A, compare P In and In P) might be due to a distance effect in combination with the very strong activity of the hepatic enhancer located between nucleotides -903 and -680 [18]. Alternatively we cannot rule out the possibility that the lower activity of the

construct P In reflects, in addition to its transcriptional activity, a difference in the processing or stability of mRNA.

To determine whether the silencer behaviour of intron I exhibits a cell specificity, we studied its activity in two non-hepatic cell lines: the human chorio-carcinoma cell line JEG-3 and the African green monkey kidney cell line CV-1. These cells were transfected by using the thymidine kinase promoter-driven vectors because the apo A-II promoter is not active in these cell lines. As shown in Figure 1B, intron I has no effect on the activity of the thymidine kinase promoter, either in JEG-3 cells or in CV-1 cells, thus indicating a cell-type specificity of the repressive activity of this intron. In contrast, in addition to the repression previously observed in the human-hepatoma-derived cell line HepG2 [23], the silencer activity of the intron I is also observed in three rat-hepatoma-derived cell lines Fa32 (Figure 1), Morris Mc A-RH7777 and Mc A-RH8994 (results not shown). Interestingly the decrease of activity of the thymidine kinase promoter by intron I (construct In TK) is 3-fold higher in rat hepatoma Fa32 cells than in human hepatoma HepG2 cells and no additive effect is observed in the former cells when intron I is placed in tandem (construct 2 In TK) (Figure 1B).

Characterization of the negative elements of the apo A-II intron I

In HepG2 cells, when the intron I sequence elements I and II, which are protected from DNase I digestion by rat liver nuclear extracts [23], were placed upstream of the thymidine kinase promoter, the activity of this promoter decreased to 55% and 60% of the control respectively (Figure 2). This indicates that both sequence elements correspond to negative regulatory elements; these were termed NRE I and NRE II respectively. In HepG2 cells the repressive activity of NRE I and NRE II exhibits an additive effect when they are placed together (construct I-II TK in Figure 2). As with the complete intron, the decrease of the activity of the thymidine kinase promoter by the NRE I and

Figure 3 The nuclear proteins binding to NRE I and NRE II of the first intron of the human apo A-II exhibit a cell-type specificity

The double-stranded oligonucleotides corresponding to (A) NRE I and (B) NRE II were end-labelled as indicated in the Materials and methods section and used as probes in EMSAs. Complexes were formed with the nuclear extracts from: 1, rat hepatoma Fa32 cells; 2, rat liver; 3, human hepatoma HepG2 cells; 4, human chorio-carcinoma JEG-3 cells; 5, African green monkey kidney CV-1 cells. The symbols $-$ and $+$ stand for the absence and the presence of 100-fold molar excess of homologous unlabelled competitors (Comp.). The arrows indicate specific complexes.

Figure 4 NRE I and NRE II of the first intron bind to the same proteins

End-labelled double stranded oligonucleotides corresponding to NRE I and NRE II were complexed with 4 μ g of rat liver nuclear extracts. Cross-competitions were performed with increasing amounts (indicated at the top of the figure) of unlabelled oligonucleotides corresponding to NRE I or NRE II.

NRE II is significantly higher in Fa32 cells than in HepG2 cells: in the former cells the activity of the promoter is reduced to 20 % of control and the additive effect of these elements is not observed (Figure 2).

Because the two negative regulatory elements NRE I and NRE II can account for the silencer activity of the intron, we analysed by EMSA the nuclear proteins binding to these elements. As shown in Figure 3, lanes 2, both NRE I and NRE II form two specific shifted bands with rat liver nuclear extracts: their

formation was competitively prevented by unlabelled oligonucleotides corresponding to NRE I and NRE II respectively but not by unlabelled irrelevant oligonucleotides (site A of human apo A-I or sites J and M of human apo A-II gene; results not shown). When EMSAs were performed with nuclear extracts from various cell lines, only nuclear extracts from rat liver, and from cultured cells where the repressing activity of the intron was observed, yielded these two specific complexes (Figure 3). Indeed, different electrophoretic patterns are observed with JEG-3 and CV-1 nuclear extracts. Therefore there is a good correlation between the cell specificity of the activity of these regulatory elements and the presence of nuclear proteins binding to these elements. It is worth pointing out that the two specific shifted bands obtained with NRE I or with NRE II have identical electrophoretic mobilities, suggesting that they correspond to complexes formed with highly related proteins. To test this hypothesis, mutual cross-competition experiments were performed next (Figure 4). When complexes formed with labelled NRE I were incubated with an excess of unlabelled NRE II as well as unlabelled NRE I, a similar competition was observed. Similarly, an excess of unlabelled NRE I or an excess of unlabelled NRE II are able to compete for the binding of nuclear proteins to labelled NRE II. Altogether these results indicate that the proteins binding to NRE I and II are identical or very similar.

Identification of the sequences involved in the activity of NRE I and NRE II

To determine the sequences involved in the activity of the negative regulatory elements NRE I and NRE II, we

Table 1: Sequences and activities of mutants of negative regulatory elements NRE I and NRE II of the human apo A-II intron I gene.

Arrows indicate the sequences involved in the activity of these regulatory elements. Underlined nucleotides are mutated. Stars stand for sites hypersensitive to DNase I (see text). Only the main strand is presented in bold letters; normal letters correspond to restriction sites. Numbers indicate the positions of the ends of regulatory elements in intron I. The activities of NRE I and NRE II mutants were analysed on the thymidine kinase promoter (TK). CAT activities, determined and normalized for difference in transfection efficiencies as indicated in the Materials and methods section, are expressed as percentages of TK-CAT activity. The results shown are averages of at least three independent transfection experiments performed in duplicate with two different DNA preparations. Abbreviation: n.d., not determined.

	Sequences	CAT activities in HepG2 cells (%)
	<div style="text-align: center;"> 49 $\xrightarrow{\quad\quad\quad}$ * $\xrightarrow{\quad\quad\quad}$ 89 </div>	
Iwt	AGCTTGCAAGATGTGTGAGCAGCATCCAAAGAGGCCTGGGCTGTCGA	54 $\pm$ 3
Im1	AGCTTGCAATTAGTGATAGCAGCATCCAAAGAGGCCTGGGCTGTCGA	60 $\pm$ 5
Im2	AGCTTGCAAGATGTGTGAGCAGCATCCAAAGAGGCCATCTGTGTCGA	65 $\pm$ 2
Im3	AGCTTGCAATTAGTGATAGCAGCATCCAAAGAGGCCATCTGTGTCGA	90 $\pm$ 2
	<div style="text-align: center;"> 109 $\xrightarrow{\quad\quad\quad}$ 149 </div>	
IIwt	AGCTTCAGGTTGGAATGGGCAGCAGGTAGGGAGATCCCTGGGGTTCGA	59 $\pm$ 3
	*	
IIIm2	CAGGTTGGAATGGGCAGCAGGTAGG	n.d.
IIIm1	AGCTTCAGGTTGGAACCAAAGCAGGTAGGGAGATCCCTGGGGTTCGA	83 $\pm$ 2
IIIm3	AGCTTCAGGTTGGAATGGGCAGCAGGTATCGAGTAACCTGGGGTTCGA	92 $\pm$ 4

Figure 5 Effects of mutations of NRE I and NRE II on their binding to nuclear proteins

The labelled probes (indicated at the top of the panels) and rat liver nuclear extracts were incubated as indicated in Figure 3 in the absence (—) or presence (+) of the indicated fold molar excess of wild-type NRE I (**A**) or NRE II (**B**) or their respective mutants. In (**C**) the nucleoprotein complexes formed with NRE I and NRE II are cross-competed for by mutants of NRE II and NRE I respectively.

hypothesized that they bind the same proteins; design of the mutations was therefore conducted by analysis of sequence similarities between these regulatory elements and the positions of sites hypersensitive to DNase I previously detected by DNase I footprinting assays [23]. Single mutations in NRE I (see Table 1) of either the GATGTGTG sequence (mutant Im1) or the TGGGC sequence (mutant Im2) were not sufficient to suppress the repressive effect of wild-type NRE I, whereas simultaneous mutations of both sequences in mutant Im3 resulted in the loss of this repressive activity (Table 1). Interestingly, mutants Im1 and Im2, bearing the mutation of a single sequence, can bind the nuclear proteins because these unlabelled mutants both at a 50- or 150-fold molar excess efficiently competed for the complexes formed with labelled NRE I (Figure 5A, lanes 3, 4, 8 and 9); in contrast, mutant Im3, where both GATGTGTG and TGGGC sequences were simultaneously mutated, did not compete for these complexes, indicating that it no longer binds nuclear proteins (Figure 5A, lanes 5 and 10). These results support those of functional assays (Table 1); therefore the requirement to mutate both sequences to lose nuclear protein binding and the repressive activity of NRE I indicates that this regulatory element is composed of two sites. The first site includes nucleotides +53 to the site hypersensitive to DNase I at position +60, and the second is nucleotides +80 to +84 (Table 1).

The sequence TGGGC, which is also present in NRE II at nucleotides +119 to +123, was mutated to yield mutant IIm1,

and the sequence GATCTCCC, placed on the complementary strand and ending at the DNase I hypersensitive site at nucleotide +131 [23], was mutated to yield mutant IIm3. In addition this latter sequence is lacking in the truncated fragment corresponding to mutant IIm2 (see Table 1). The transcriptional activities of mutants IIm1 and IIm3 are presented in Table 1: mutation of the TGGGC sequence only partly suppresses the repressive activity of NRE II, whereas mutant IIm3 has lost its repressive activity. In addition, in EMSA at 150-fold molar excess, mutants IIm1 and IIm2, with a single unaltered sequence, retain their protein binding capacity because they compete, although slightly less efficiently than wild-type NRE II, for the complexes formed with labelled NRE II (Figure 5B, lanes 2–4 and 7–9 for competitions at 150-fold and 50-fold molar excesses respectively), a behaviour reminiscent of that observed with NRE I mutants (Figure 5A). In contrast, mutant IIm3 does not compete for the complexes formed on NRE II (Figure 5B, lane 5). Even if mutation of only one site in NRE II (mutant IIm3) is sufficient to suppress DNA binding and transcriptional repressing activities, results obtained with the other NRE II mutants indicate that, similarly to the NRE I, NRE II consists of two sites. Furthermore the very close relationship between the nucleoprotein complexes formed with both NREs is confirmed by the identical behaviours of mutants of NRE II and NRE I towards complexes formed with NRE I and II respectively as shown by cross-competition experiments (Figure 5C). The observation that, in contrast with NRE I,

mutation of a single site in NRE II (mutant IIm3) is sufficient to suppress complex formation and activity is most probably due to the difference in distance and orientation between the sites within NREs I and II: the sites are 19 bp apart in element I but 8 bp apart in element II.

DISCUSSION

The results in this paper demonstrate that the first intron of the human apo AII gene decreases the activity of its own promoter at the transcriptional level, even when placed upstream of the very strong enhancer spanning nucleotides -903/-680 in this gene. This extends our previous observations showing that this intron acts as a silencer on the thymidine kinase promoter [23]. Furthermore we have localized the silencer activity of the first intron to the two sequences spanning over nucleotides +49 to +89 and +109 to +149, termed NRE I and NRE II respectively. Our results also show that the silencer activity and the nuclear proteins binding to these elements are encountered in hepatoma-derived cells but not in human chorio-carcinoma JEG-3 cells nor in African green monkey kidney CV-1 cells.

NRE I and NRE II of the first intron exhibit the same repressive activity and form complexes of identical electrophoretic mobilities, which are mutually cross-competed for by these elements, thus indicating that these regulatory elements bind the same or closely related proteins. In addition our results show that both NRE I and NRE II comprise two sites that are required for their maximal activity. The site TGGGC is identical in both NREs, whereas the second site differs in sequence at its 3' end and in distance and orientation relative to the former site. Nevertheless, its 3' end coincides with a site that is strongly hypersensitive to DNase I in both NREs [23]. In addition all the mutants of NRE I and NRE II exhibit the same competition behaviour on complexes formed on both NRE I and NRE II (Figure 5C). In agreement with these data indicating a close relationship between the nucleoprotein complexes formed on these NREs are their identical behaviours when gel-shift assays are performed in the presence of univalent (Na^+) or bivalent (Mg^{2+}) cations, or chemicals such as the chelator of bivalent cations EDTA, the reducing agent dithiothreitol, and urea (results not shown). Therefore, despite the above-mentioned differences, it seems that NRE I and NRE II correspond to elements of similar structure and function, placed in tandem in the first intron of the human apo A-II gene.

Compared with HepG2 cells, in Fa32 cells we have observed a greater effect of intron I and of its two negative regulatory elements I and II on the thymidine kinase promoter and a lack of cumulative effects of either the whole intron I placed in tandem or NRE I and NRE II in combination (Figures 1B and 2). It is unlikely that this difference of repression is due to a higher expression in Fa32 cells of the negative transcription factor(s) binding to NRE I and NRE II, because equal amounts of nuclear proteins from both cells yielded a similar intensity of the shifted bands formed with NRE I or NRE II (compare lanes 1 and 3 in Figures 3A and 3B). A more probable explanation would be linked to the much higher strength of the activity of the thymidine kinase promoter in HepG2 cells compared with that in Fa32 cells: in the former the activity of this promoter is therefore only partly repressed by a single copy of the intron and a more pronounced repression can be obtained with two copies of the intron (Figure 1B). A similar cumulative effect is observed in HepG2 cells with two NREs (I-II-TK construct) compared with the result obtained with one NRE (I-TK construct) (Figure 2). In contrast, in Fa32 cells, where the thymidine kinase promoter is

much weaker, full repression is accomplished by a single copy of NRE.

Although the thermolability of the nucleoprotein complexes hampered further investigation of the exact nature of the protein complexes forming the two shifted bands, from our results we can exclude the possibility that the two shifted bands correspond to two complexes formed independently by binding of different proteins on each site of NRE I and NRE II, because they are simultaneously displaced by competition with the mutants bearing a single mutated site (mutants Im1, Im2, IIm1 and IIm2). We have observed that in several EMSAs repeated under the same conditions and with the same nuclear extract preparation, the relative amounts of the two complexes varied from one experiment to the other (example given with NRE I in Figure 5A, lanes 1 and 6, and with NRE II in Figure 5B, lanes 1 and 6). This finding excludes the possibility that the two shifted bands reflect either the formation of two complexes involving protein isoforms with the same DNA binding domain but different molecular sizes, or the formation of two complexes differing by an accessory factor present only in the more slowly migrating complex. Indeed, in such cases the amount of these putative isoforms or accessory factor is constant in a given preparation of nuclear extract and consequently the various complexes formed should remain unchanged. It is also unlikely that, despite the presence of protease inhibitors, a specific proteolysis occurs, because the lowest size complex is not systematically enhanced in a set of experiments. The two shifted bands might reflect an equilibrium between protein multimeric forms, as observed with the prokaryotic DNA binding protein MC1 [29], or eukaryotic factors such as the protein Sp1 [30] and the RZR nuclear receptor [31]. We have observed in EMSAs that decreasing amounts of nuclear extracts do not favour the smallest complex (results not shown), thus indicating that, in contrast with the above-mentioned proteins, the proteins bound to the NRE I and II of intron I do not co-operatively form a putative multimer. However, we cannot rule out a non-co-operative multimerization of these proteins. Therefore the available data cannot give a clear interpretation of the presence of the two shifted bands observed with the negative regulatory elements of this intron.

The 3' ends of NRE I and II are GC-rich, a property reminiscent of the binding site of protein Sp1, but complexes formed with NREs I and II are not competed for by a double-stranded oligonucleotide corresponding to the binding site of this transcription factor, which excludes the involvement of protein Sp1 in the complexes we have observed. Multiple silencer elements exhibiting a cumulative effect and probably binding the same proteins have already been described in the chicken vimentin gene [32]. Silencers consisting of several negative elements have also been found in several genes [33-35] but the NREs identified previously do not consist of two sites that are required for both maximal binding of nuclear proteins and optimal activity and that present sequence similarity with NREs I and II in the apo AII intron I [21,32-43]. Therefore the apo A-II intron I NREs seem to have an original structure.

Substantial evidence indicates that the presence of apo A-II in lipoprotein particles counteracts the protective effect of apo A-I against atherosclerosis development. Furthermore it has been recently shown that the rate of apo A-II production is a major factor determining the relative amounts of LpA-I and LpA-I:A-II HDL subclasses in normolipidaemic humans [15], suggesting that a decrease in apo A-II synthesis can improve the anti-atherogenic profile of HDLs. The characterization of negative regulatory elements might therefore provide a means to down-regulate the apo A-II gene transcription in the liver, which could be of use to protect humans against atherogenesis.

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