Modulation of Apolipoprotein D and Apolipoprotein E mRNA Expression by Growth Arrest and Identification of Key Elements in the Promoter*

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Apolipoprotein D (apoD) and apolipoprotein E (apoE) are co-expressed in many tissues, and, in certain neuro-pathological situations, their expression appears to be under coordinate regulation. We have previously shown that apoD gene expression in cultured human fibroblasts is up-regulated when the cells undergo growth arrest. Here, we demonstrate that, starting around day 2 of growth arrest, both apoD and apoE mRNA levels increase between 1.5- and 27-fold in other cell types, including mouse primary fibroblasts and fibroblast-like and human astrocytoma cell lines. To understand the regulatory mechanisms of apoD expression, we have used apoD promoter-luciferase reporter constructs to compare gene expression in growing cells and in cells that have undergone growth arrest. Analysis of gene expression in cells transfected with constructs with deletions and mutations in the apoD promoter and constructs with artificial promoters demonstrated that the region between nucleotides −174 and −4 is fully responsible for the basal gene expression, whereas the region from −558 to −179 is implicated in the induction of apoD expression following growth arrest. Within this region, an alternating purine-pyrimidine stretch and a pair of serum-responsive elements (SRE) were found to be major determinants of growth arrest-induced apoD gene expression. Evidence is also presented that SREs in the apoE promoter may contribute to the up-regulation of apoE gene expression following growth arrest.

Unlike most of the other plasma apolipoproteins whose expression is limited to the liver and/or the intestine, apolipoprotein D (apoD)† and apolipoprotein E (apoE) are expressed in almost all tissues that have been tested (1–9). In plasma, apoE is associated with very low density, intermediate density, and high density lipoproteins, and chylomicrons, and it plays an important role in the metabolism of triglyceride-rich lipoproteins. ApoD, a 29-kDa glycoprotein, is bound to plasma high density lipoproteins in humans and other species (3, 10–14). ApoE is a member of the apolipoprotein gene family whereas apoD is a lipocalin, a superfAMILY whose members are transporters of small hydrophobic ligands (15, 16, for review, see Ref. 17). Although apoD has been shown to bind a number of small molecules, the one or more physiological ligands of apoD have yet to be definitively identified, and it has been proposed that apoD may have multiple tissue-specific, physiological ligands and functions (16, 18–22).

ApoE is expressed within the central nervous system and inheritance of one of the apoE alleles, apoE4, is associated with an increased susceptibility to Alzheimer’s disease. Its gene expression is induced following experimental injury in the peripheral nervous system where apoE is thought to play a role in lipid redistribution during nerve degeneration and regeneration (23). ApoD is also expressed at high levels in the normal central nervous systems of various species (2–6, 24), and its expression can be further increased in pathological conditions. ApoD levels are elevated in the cerebrospinal fluid of patients with Alzheimer’s disease, stroke, meningocencephalitis, motor neuron disease, dementia (25), Niemann-Pick disease (26), and schizophrenia (27). Both an increase in apoD mRNA and immunoreactive protein is detected at sites of regenerating peripheral nerves (23, 28), in kainic acid-lesioned or entorhinal cortex-lesioned rat hippocampus (29, 30) and in brain after traumatic injury (31). In non-neurological pathologies, apoD protein accumulates in advanced stages of prostate cancer (32) and in the cyst fluid from women with breast gross cystic disease (33, 34). In cultured cells, apoD expression can be enhanced by growth arrest and senescence (35) or by other factors such as steroids (36), Interleukin-1α (37), 1,25-dihydroxyvitamin D₃ (38), retinoic acid (38), or 25-hydroxycholesterol (39).

The apoD promoter region and non-coding first exon contain a number of potential regulatory and responsive elements that may be important modulators of apoD mRNA expression (20, 40). Here, we report a functional analysis of the 5'-flanking region of the apoD gene that was designed to identify elements that are important in the induction of apoD gene expression in cells that undergo growth arrest provoked by serum deprivation. Serum deprivation and the ensuing growth arrest is believed to elicit a stress response in cells. We demonstrate that, within the apoD promoter, a pair of serum-responsive elements (SRE) and an alternating purine-pyrimidine (APP) stretch that

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‡ The abbreviations used are: apoD, apolipoprotein D; apoE, apolipoprotein E; AP-1, activating protein 1; APP, alternating purine-pyrimidine stretch; APRE, acute phase-responsive element; ERE, estrogen-responsive element; FSE, fat-specific element; GR, glucocorticoid-responsive element; MOPS, 3-[N-morpholino]propanesulfonic acid; PBE, progesterone-responsive element; SDR, sterol-dependent represor; SRE, serum-responsive element; SRF, serum-responsive factor; DMEM, Dulbecco’s modified Eagle’s medium; UNR, unrelated sequence; TCF, ternary complex factor; EBS, Ets binding site.

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is predicted to adopt a Z-DNA conformation, and are important regulators of apoD expression in cells in growth arrest. In addition, elements within non-coding exon 1 have an inhibitory effect on apoD gene expression. The complexity of the regulation of apoD gene expression may reflect the multifunctional nature of the apoD protein.

EXPERIMENTAL PROCEDURES

Materials—The media and supplements used for cell culture were obtained from Invitrogen. Estradiol, used in the MCF7 adenocarcinoma cell line culture, was kindly provided by Dr. Jacques Simard (Molecular Endocrinology Laboratory, Centre Hospitalier de l’Universite Laule Research Center, Quebec). The Total RNA Extraction kit was from Qiagen (RNeasy total RNA kit, Qiagen Inc., Chatsworth, CA). Radiolabeled nucleotides ([32P]dATP, 3000 Ci/mmol) were from ICN and Hybond-N nylon membranes for Northern blots from Amersham Biosciences, Inc. as were the restriction endonucleases.

Cell Culture and Growth Arrest Induction—Primary murine fibroblasts were derived from C57/Bl and BALB/c mice embryos. Cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin G (100 units/ml), streptomycin (100 μg/ml), and 5% CO₂ humidified atmosphere and were fed every 2 days with fresh medium. Growth arrest was achieved by allowing fibroblasts (after 5–10 passages) to reach confluence (this point was called day 0 post confluence).

Immortalized cell lines were obtained from ATCC, Rockville, MD (except when mentioned), and grown in the following media: mouse NIH/3T3 cells and COS cells, DMEM-10% calf serum; human 293 cells, (except when mentioned), and grown in the following media: mouse DMEM-10% calf serum; humanastrocytoma U373MG cells, RPMI/1640 containing 5% FCS; mouse NIH/3T3 cells and COS cells, DMEM-10% calf serum; human 293 cells, (except when mentioned), and grown in the following media: mouse DMEM-10% calf serum; human breast cancer ZR75-1 cells (obtained from Dr. J. Simard), DMEM/F12K-5% fetal calf serum supplemented with 10 μM sodium pyruvate, 2 mM t-glutamine, and 1 μM estradiol; human astrocytoma U373MG cells, RPMI/1640–10% fetal calf serum; human breast cancer ZR75–1 cells (obtained by J. Simard), phenol red-free RPMI/1640–10% fetal calf serum. All cells were grown at 37 °C in a 5% CO₂ humidified atmosphere.

PCR-32P-labeled apoD, apoE, or β-actin cDNAs) at 42 °C in hybridizing solution, containing 50% formamide, 1 M NaCl, 0.5% SDS, 10% dextran sulfate, and 3× Denhardt’s reagent. Blots were rinsed for 20 min in 2× SSC at room temperature (two rinses), washed at 60 °C in 1× SSC, 0.1% SDS for 45 min, then rinsed in SSC at room temperature before exposure. Membranes were exposed overnight to Kodak XAR-5 X-Omat films at −80 °C with an intensifying screen (Coronex Lighting Plus, E.I. du Pont de Nemours and Co., Wilmington, DE). Autoradiographs were scanned on a Personal Densitometer from Molecular Dynamics (Sunnynvale, CA), and optical densities were measured. For each autoradiography, the serum concentration was reduced from 10 to 0.2% (or 5 to 0.1% of serum) was renewed. This time point was considered as day 0 of serum starvation. When cells reached 80% of confluence, the serum concentration was reduced from 10 to 0.2% (or 5 to 0.1% of serum) was renewed. This time point was considered as day 0 of serum starvation. Cells were kept in this medium and harvested at 0, 2, 4, 7, 10, 14, and 21 days post serum starvation when possible.

RNA Extraction, Northern, and Slot Blot Analysis—For each time point, cells were trypsinized, rapidly frozen in liquid nitrogen, and kept at −80 °C until all time points for a cell line were collected. For Northern blots, total RNA was extracted and denatured in formaldehyde/formamide and electrophoresed in 1% agarose gel containing MOPS (20 mM) and formaldehyde (17%) (adapted from Ref. 41). Integrity of RNA was controlled by staining the gel with ethidium bromide. Nucleic acids were subjected to serum starvation. When cells reached 80% of confluence, the serum concentration was reduced from 10 to 0.2% (or 5 to 0.1% of serum) was renewed. This time point was considered as day 0 of serum starvation. Cells were kept in this medium and harvested at 0, 2, 4, 7, 10, 14, and 21 days post serum starvation when possible.

Table I

| Promoter | Name in this study | SRE sequence | Position | Orientation |
|----------|-------------------|--------------|----------|-------------|
| Apo D    | SRE1              | CCCTGCGGTTG  | −1132 to −1123 | (−)         |
|          | SRE2              | CCAATAGTG    | −502 to −493  | (+)         |
| Apo E    | SRE1              | CCACCTGAAG   | −474 to −465  | (+)         |
|          | SRE2              | CCACGCTTGG   | −560 to −551  | (+)         |
|          | SRE3              | CCCACTGTGG   | −489 to −470  | (−)         |
|          | SRE4              | CACACCTGG    | −254 to −245  | (+)         |
|          | SRE5              | CCCAGACTGG   | −242 to −233  | (−)         |
|          | SRE6              | CCCCTTCCGG   | −198 to −189  | (−)         |
ACT CGC GAA TAG AGA GAG AGA GAG TA) also included a segment that was complementary to sequences upstream of the APP, whereas the oligonucleotide (ATT CGC GAG TGT TAA CAA AAC AAT ATC TCA TT) included a segment that was complementary to a sequence that was downstream of the APP. The two vector-specific primers were the reverse and universal primers of pBluescript. Double and triple mutants were constructed by the same technique using sequential mutagenesis. All of the luciferase reporter constructs were sequenced to confirm that the desired mutation was present and that no other alterations had occurred.

DNA Sequencing—Nucleotide sequencing was performed on single-stranded plasmid templates using the Sanger method (44) and T7 polymerase enzyme as recommended by the suppliers (Amersham Biosciences, Inc.).

Transfection and Transient Expression—NIH/3T3 and 293 cells were transfected by the calcium phosphate-DNA coprecipitation method (45) with sterile plasmids (10 μg total; 9 μg of the tested plasmid plus 1 μg of the control plasmid pRSV[GAL]). Twenty-four hours later, cells were rinsed twice with phosphate-buffered saline and culture medium was changed for medium supplemented with 10% or 0% serum. Cells were harvested 4 days after transfection for luciferase and β-galactosidase assays. A time-course analysis showed that this delay was optimal for apol mRNA expression analysis (see Figs. 1 and 2).

Luciferase and β-Galactosidase Assays—Cells were washed with phosphate-buffered saline before adding 100 μl of Tris-HCl, 0.25% x, pH 7.8. Cells were then scraped with a cell lifter, transferred into micro-centrifuge tubes, and lysed by three cycles of freeze-thawing as described previously (46). After centrifugation at 12,000 × g for 5 min, lysates were stored at −20 °C. Luciferase assays were performed with a Wallac 1404 luminometer using the conditions and buffers recommended by the Luciferase assay system (Promega). β-Galactosidase assays were done as follows: each sample (30 μl) was adjusted to a final concentration of 1 mM MgCl₂, 45 mM β-mercaptoethanol, 0.88 mg/ml o-phenyl-β-d-galactopyranoside, and 0.1 mM sodium phosphate (pH 7.5) and incubated at 37 °C for 30 min. Reactions were stopped by the addition of Na₂CO₃ to a final concentration of 625 mM, and the optical density was read at 420 nm (42). The plasmid pSV2LUC was included as a control promoter. The results were standardized by calculating promoter activity relative to that of the co-transfected internal control plasmid pRSV[GAL]. Each value is the average of at least three independent experiments performed in triplicate.

Electrophoretic Mobility Shift Assays—50 ng of sense oligonucleotide was 5’-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP and annealed with 200 ng of the complementary oligonucleotide. Nuclear extracts from growing or from 4-day serum-starved NIH/3T3 fibroblasts were prepared according to the method of Dignam et al. (47). Nuclear extracts were analyzed by gel shift assays as described previously (48). 5 μg of nuclear extract were added to 0.8 ng of the labeled double-stranded oligonucleotide, and, after a 20-min incubation at room temperature, the mixture was run on a 6% acrylamide, non-denaturing gel in 0.5× TBE, at 150 V for 90 min. The dried gels were autoradiographed on Kodak X-Omat films. For competition assays, a 25-fold excess (20 ng) of cold double-stranded oligonucleotide was added before addition of the nuclear extract. The sense strand sequences of the oligonucleotides used are: SRE1, TGA CTC CCA TTA GTG GAG TCA G; SRE2, CAT GTT CCA CTT CAG GAA ATG A; c-fos, SRE, GGA TGT CCA TAT TAG GAC ATC TGC; UNR (unrelated sequence), CCA AAG AGG ATA TCT GTA ATA AGC AG. The mutated SRE1 and SRE2 oligonucleotides, called mSRE1 and mSRE2, respectively, are identical to those used for the mutagenesis (section here above).

RESULTS

ApoD and ApoE mRNA Expression in Growth-arrested Cell Lines—The expression of both apoD and apoE was first ana-
lyzed in primary cell lines. Primary mouse embryonic fibroblast cultures from BALB/c and C57/Bl mice were collected in growing conditions (50% of confluence), and at different times after they reached confluence. For both strains, apoD mRNA expression was very low in growing cells and reached a peak at day 4 of growth arrest (Fig. 1). There was no apoE mRNA expression in sparse culture and detectable expression appeared at day 4 in BALB/c and in C57/Bl primary fibroblasts, although apoE expression was much lower in the latter.

Immortalized cell lines were also tested for apoD and apoE expression in growth arrest. In murine NIH/3T3 fibroblasts (Fig. 2), the expression pattern was very similar to that of the primary mouse fibroblasts, reaching a peak at day 10 with a 15-fold induction. ApoE expression was quite similar to that observed in C57/Bl fibroblasts. Several other cell lines were also tested but, to better quantify apoD and apoE mRNA expression, total RNA from various cell lines were slot-blotted and expression of apoD and apoE relative to actin gene expression was measured on a densitometer (Fig. 2). In ATCC 293 human fibroblast-like cells, both apoD and apoE expression increased continuously to reach a 5-fold induction by day 21 of growth arrest. In COS cells (Fig. 2), apoD gene expression increased by 3- and 12-fold at 4 and 10 days, respectively, after growth arrest. ApoE expression in COS cells showed a similar response to serum deprivation.

We also measured mRNA expression in several cancer cell lines. ApoD expression has been detected in mammary carcinomas (34), in prostate cancers (32), and in the cyst fluid from women with breast gross cystic disease (49). In cell lines, apoD expression was modulated by estrogens, androgens, 25-hydroxycholesterol, steroids, interleukins, retinoic acid, and 1,25-dihydroxyvitamin D3 (36–39, for review see Ref. 17). We were therefore interested in testing the effect of serum starvation on some of the cells used in these studies. Expression levels were measured during growth arrest in U373MG, a human astrocytoma, in MCF7 and ZR-75–1, two human breast cancer cell lines of epithelial origin, and in LNCaP, a prostate cancer cell line. In U373MG, expression of both apoD and apoE was modulated by growth arrest. Four days after serum deprivation, apoD mRNA levels increased 12- and 27-fold at day 21 and apoE mRNA levels increased 7.5-fold at day 4 and 20-fold at day 21 (Fig. 2). A totally different pattern of expression was observed in the breast cancer cell lines. In MCF7 cells, apoD gene expression was inhibited following growth arrest and was completely abolished by day 10 (Fig. 2). However, apoE mRNA expression was induced by growth arrest as in the other cell lines tested with an 8-fold induction at day 14. The ZR75–1 breast cancer cell line and LNCaP prostate cancer cells revealed no significant variation in apoD or apoE expression after growth arrest (Fig. 2).

Analysis of the Promoter Region and Effect of the Non-coding Exon 1

Fig. 3. Sequence of, and potential regulatory elements within, the 5′-flanking region and exon 1 of the apoD gene. The exon 1 sequence is presented in italics. AP-1 and AP-2, activation proteins 1 and 2; APRE2 and 3, acute phase-responsive elements 2 and 3; CP2, erythroid cell nuclear factor; E47, recognized by TAL-1; E2F, early adenovirus transcription factor; EBP4, Z-DNA binding proteins of bZIP family; ERE, estrogen-responsive elements; FSE, fat-specific element; GRE, glucocorticoid-responsive element; IK-1, Ikaros-1; IRF-1, interferon regulatory factor; MZF-1, myeloid zinc finger protein; MRE, metal-response element; NF-K-B, nuclear factor kappa B; PRE, progesterone-responsive element; RFX, X-box binding protein; ROR1, steroid hormone nuclear receptor (retinoic acid) alpha1; SDR, sterol-dependent repressor; STAT, ligand-activated transcription factor; STRE, stress response element; TAL-1, T-cell acute lymphoblastic leukemia; TpIE, transforming growth factor β1 inhibitory element; TRE, thyroid-hormone response element.
the expression of apoD in fibroblast cultures in growth arrest, we constructed mutants by making progressive deletions from the 5′-end starting at −2128 in the sequence. Each construct was made with and without the 66-bp non-coding exon 1 in the pXP2 expression vector that encodes the luciferase gene. Exon 1 contains a transforming growth factor-β inhibitory element, an element that has been identified as a transcription inhibitor in other genes. Each construct was co-transfected in 293, 293T, and NIH/3T3 cells with a construct containing the β-galactosidase gene. After transfection, the cells were cultured in either serum-containing medium or serum-free medium. In these conditions, both serum deprivation and achievement of confluence contribute to growth arrest. The luciferase activity was analyzed after 4 days, and the results were normalized for the β-galactosidase activity.

With the first construct (−2128 to −4), containing the entire promoter region, growth arrest resulted in a 6-fold induction of promoter activity (Fig. 4A). Induction was also observed with the −1676 to −1, −1176 to −1, and −558 to −1 constructs but was lost when the sequence −558 to −179 was deleted, which suggests that major regulatory elements are located in this region. Very similar results were obtained with 293, 293T, and NIH/3T3 cells. Region −179 to −4 seems to contain the minimal promoter activity, and no major differences were observed in luciferase activity between growing conditions and growth arrest in cells that were transfected with the −179 to −4 construct. Also, region −32 to −4, which contains only the TATA box gives very low levels of activity. The effect of exon 1 on gene expression was consistent but relatively minor, with the notable exception of the −1176 construct that showed an important reduction (4-fold; p < 0.0001) of expression both in the presence and absence of serum.

To further analyze the exon 1 inhibitory effect, the −4 to +71 fragment was cloned in both orientation between the SV40 early promoter and the luciferase gene (Fig. 4C). A linker of the same length was also cloned in the same position to compensate for the spacing effect between the promoter and the reporter gene. Luciferase activity was measured 5 days after transfection in growing cells. Similar results were obtained in growth-arrested cells (results not shown). As can be seen in Fig. 4C, the construct containing exon 1 in the sense orientation showed a 4-fold decrease of promoter activity as had been observed for the human promoter (construct −1176). This effect is not due to the spacing but to the exon 1 itself, because replacing the exon sequence by a linker of identical length does not reduce the SV40 promoter activity. Also, the inhibitory effect seems to be associated with the exon sequence, because the antisense construct also shows a 4-fold decrease of promoter activity. It is interesting to note that the most important reduction of activity was observed in the −1176 construct only.
suggesting that some elements present between −1176 and −558 could interact with the exon 1 but only when upstream sequences are absent.

**Mapping of the Functional Elements Associated with the Growth Arrest**—Results of Fig. 4B revealed a severe loss of promoter activity both in presence of serum and in growth arrest when the region −558 to −179 was removed. Region −179/−4 does not have any remaining promoter inducibility. If we look at the 3-fold induction for the first six constructs in Fig. 5, it seems that the primary determinants for the induction of expression during growth arrest are located between nucleotides −558 and −179 with a 27-fold induction. However, the promoter activity of the four first constructs in the absence of serum is quite similar, and the differences in the magnitude of induction (6- to 27-fold) are due mainly to variation of the activity in the presence of serum (Fig. 5). The region −558 to −179 contains several potential regulatory sequences, including an AP1 site, two serum-responsive elements (SRE), an alternating purine-pyrimidine stretch (APP), three steroid-responsive elements (progesterone (P), estrogen (E), and glucocorticoid (G)), and two acute phase-responsive elements (Fig. 3). The SREs could be considered as prime candidate elements for the induction of apoD expression by serum deprivation, because this sequence has been clearly associated with cell growth in other models (50).

To further analyze the importance of the region −558 to −179 of the promoter, we created a series of apoD promoter-reporter constructs that contained internal deletions in this region (Fig. 5). The results demonstrate the complexity of the regulation of apoD expression and likely reflect the abundance of regulatory elements within the apoD promoter. As expected, deletion of the −558/−179 region abolished induction while minimal promoter activity was retained. Larger deletions (Δ−799/−179 and Δ−1052/−179) resulted in reduced promoter activity in the presence or absence of serum. Internal deletions within the −558 to −179 region had variable effects. Dividing the region in two parts with deletions (Δ−558/−352 and Δ−352/−179) reduced the magnitude of induction to 2- and 3-fold, respectively. This would imply that more than one regulatory element contributes to induction of apoD gene expression following growth arrest. The reduced induction with the Δ−352/−179 construct suggests a possible participation of the APP. Deletions −473/−191 and −525/−310 showed an intermediate behavior. The Δ−473/−191 lacks the APP sequence and one SRE, whereas the Δ−525/−310 retains the APP but lacks the two SRE sequences. To refine the analysis, PCR-amplified portions of the apoD promoter were cloned upstream of the minimal−179/−4 promoter construct (Fig. 5). Again, the constructs were transfected into NIH/3T3 cells that were then placed in the presence or absence of serum. The results are consistent with those presented above and suggest an important role for the APP as a regulator (construct −266/−51). However, the addition of the APP-containing PCR fragment to the −179 to −4 basal promoter does not restore the entire -fold induction observed with the region −558 to −179. It is probable that other elements such as the SRE and/or the AP-1 may contribute to the full promoter induction.

**Importance of SRE and APP Regions**—Because the previous results seem to point out the importance of the APP and the SREs in the apoD induction in growth arrest, we decided to mutate the two SREs present in the region −558 to −179 and also to delete the long stretch of alternating purine-pyrimi
Modulation of ApoD and ApoE mRNA Expression

SRE element. Each value represents the mean.

Luciferase activity was determined under conditions of cell growth (78). The addition of three and five SRE in front of the minimal promoter resulted in an increased -fold induction of 13 and 17, respectively, suggesting an additive effect of this element. Also, the orientation of the SRE has no or little effect on the promoter activity (5SREas, Fig. 6B), and such elements are present in the two orientations both in the apoD and apoE promoters. The addition of one or two APP stretches had variable effects depending on the orientation of the sequence. In the sense orientation, the magnitude of the induction increased to 7.4- and 16.7-fold, respectively, again suggesting a copy-number additive effect. However, when the APP units were placed in the opposite orientation, either head to head or tail to tail, the promoter activity in growth-arrested conditions was greatly reduced. Because both SRE and APP elements are present in the apoD promoter, we have analyzed combinations of the elements for their capacity to induce apoD expression under growth-arrested conditions. A combination of three or five SREs with one APP showed inductions of 17- and 21.5-fold, respectively. Surprisingly, if the five SRE are placed in the opposite orientation, the induction drops to 5-fold suggesting an orientation-dependent synergism between the SRE and the APP. This dependence on orientation is lost, however, if the APP is moved upstream of the SRE (compare P-5SRE with Pas5SRE, Fig. 6B). A duplication of the APP sequence in the presence of five SREs was also dependent upon the APP orientation because we obtained the highest induction (26-fold) with a construct containing SSRE followed by two APP in the sense orientation and a much lower induction (6-fold) when the 2-APP sequences were in the opposite orientation.

SRE Elements in the ApoE Promoter—We have shown that

Fig. 6A, site-directed mutagenesis and deletion of the SRE and APP regions. Either or both of the SRE elements were mutated and/or the APP tract was deleted in the -558/4 apoD promoter-reporter construct and the plasmids were assayed for luciferase activity after transfection into NIH/3T3 cells. Gray and black bars represent luciferase activity in growing and growth-arrested cells, respectively. Induction is the ratio of luciferase activity of cells in growth arrest to that in growing cells. Letters at the right of the induction numbers represent statistical significance; different letters indicate statistically different -fold induction (p < 0.003). B, importance of the number and orientation of the SRE and APP regions in the apoD promoter. Different copy numbers of SRE (arrowheads) and APP (P) elements (wave), in sense and antisense (as) orientations, were placed in front of the -179/4 apoD promoter-reporter construct. After transfection of the plasmids into NIH/3T3 cells, luciferase activity was determined under conditions of cell growth (gray bars) or cell arrest (black bars). C, importance of SREs in the apoE promoter. NIH/3T3 cells were transfected with -620/14 (with 5 SREs) and -365/14 (3 SREs) apoE promoter-reporter constructs and luciferase activity was determined under conditions of cell growth (gray bars) or cell arrest (black bars) and compared with cells that had been transfected with the pSSRE-luc plasmid or with -179/4 apoD promoter-reporter constructs that contained either three or five copies of the SRE element. Each value represents the mean ± S.E. of at least three experiments performed in triplicate.
apoE expression is, as that of apoD, induced in growth arrest. Interestingly, five SRE elements are present in the apoE promoter within the first 620 bp of the promoter whereas no alternating purine-pyrimidine stretch is found. A construct containing the 620 bp of the apoE promoter upstream of the luciferase reporter gene showed a 12-fold induction of expression in growth arrest conditions, similar to that observed with the commercial construct containing a basic promoter joined to five SRE tandem repeats (Fig. 6C). The removal of the sequence −620 to −366 that contains two SRE elements caused a decrease of induction in apoE expression to 3-fold. Thus, the SREs in the apoE promoter could contribute to the induction of apoE gene expression that occurs following growth arrest, although we cannot rule out the possibility that other regulatory elements were also removed.

Factors Binding SRE and APP Regions—To assess the activity of the SRE and APP sequences prior to and following growth arrest, we performed mobility shift assays with SRE1, SRE2, and the APP oligonucleotides. Nuclear extracts from growing or from 4-day-arrested mouse fibroblasts were incubated with oligonucleotides containing the SRE or APP sequences (Fig. 7). The APP region that contributes to the induction of apoD expression during growth arrest did not seem to bind any factor in our electrophoretic mobility shift assays (data not shown). With nuclear extracts isolated from growing cells, SRE1 and SRE2 probes gave three retarded bands (A, B, and D). All three bands were competed with a 25-fold excess of the cold SRE1 or SRE2 oligonucleotide. The binding was also fully competed with the c-fos SRE oligonucleotide. In contrast, the SRE1 and SRE2 oligonucleotides, mutated in the SRE site, as well as an unrelated oligonucleotide (UNR) were unable to abolish the binding.

With nuclear extracts isolated from growth-arrested cells, the same bands A, B, and D were detected. Again, cold SRE1, SRE2, and the c-fos SRE oligonucleotides were effective competitors whereas the mutated SRE and the UNR oligonucleotides did not compete. However, band B was much less intense than that observed with the extracts from growing cells and an additional band (band C) with a faster mobility was present.

DISCUSSION

We have previously shown that apoD expression is induced when cultured human fibroblasts enter a quiescent or senescent state (35) and, in a number of situations, apoD expression is absent in proliferating cells and is induced in cells that undergo growth arrest. In some aspects, growth arrest, such as that provoked by serum deprivation, may be considered as a stress response for growing cells. In exponentially non-transformed growing cells, withdrawal of serum causes a fraction of the culture to undergo apoptosis. After an initial wave of apoptosis, the remainder of the culture successfully enters G2/G1 but does not enter into S phase (51–53). ApoD expression is induced in several stress conditions, and a role as an acute-phase protein has been proposed (17). Thus, the increased apoD expression that follows growth arrest may represent a component of a stress response to serum deprivation. Here, we have extended our previous studies with human fibroblasts (35) to show that growth arrest also induces apoD expression in primary mouse fibroblasts and in a number of cell lines, including NIH/3T3 murine fibroblasts, ATCC 293 human fibroblast-like cells, COS transformed monkey kidney cells, and U373MG human astrocytoma cells. ApoE expression is also increased in these cells by serum starvation, which is interesting in view of the apparent coordinate control of expression of apoD and apoE in a number of pathophysiological situations. In contrast, apoD expression is not induced by growth arrest in MCF-7 andZR75–1 human breast cancer cells nor in LNCap human prostate cancer cells. In the case of the MCF-7 cells, there is a dissociation of the response of apoD and apoE to serum starvation with an inhibition of apoD gene expression and an induction of apoE gene expression. ApoD expression in some cell lines is modulated by estrogens, and the presence of estradiol that was used to supplement the media of the human breast and prostate cancer cells may have masked the effect of growth arrest on apoD expression.

Analysis of constructs with progressive deletions from the 5′ terminus of the apoD promoter revealed that the region between nucleotides −179 and −4 is responsible for the basal gene expression, whereas the region from −558 to −179 is implicated in the modulation of apoD expression following growth arrest with little contribution of sequences further upstream. Inclusion in the constructs of the non-coding exon 1, which contains a transforming growth factor-β inhibitory element, had an inhibitory effect on transcriptional activity in the presence and the absence of serum, and this was particularly apparent with the −1176/−4 construct. Regulatory elements within non-coding exons have been identified in other genes (54). Introduction of internal deletions within the apoD promoter confirmed the importance of the region −558 to −179 in the response of apoD expression to growth arrest. Moreover, it is probable that two or more distinct elements within the region participate in this regulation as deletion of either the sequences −558 to −352 or −352 to −179 largely eliminates the serum-starvation-induced apoD expression. The addition of the APP element restored about half of the activity (Δ−525/−310 and Δ−266/−51) suggesting a role for this DNA element. When the APP was deleted from the −588/−4 construct, the induction of expression by growth arrest was reduced by about 80%. The response of cells transfected with the Δ−676/−471, Δ−548/−321, and Δ−558/−352 constructs to growth arrest suggests a potential role for the two SRE elements located at −502 and −474. However, mutation of one or both SREs in the −558/−4 construct, with or without deletion of the APP, had only a minor effect on inducibility of expression.

The abundance of regulatory elements in the proximal apoD promoter complicates the interpretation of the results of exper-
ments in which regions of the promoter are deleted. To study, in isolation, the role of the SRE and APP elements in the growth arrest induction of apoD gene expression, one or more copies of the SRE and/or APP elements were placed upstream of the −179/−4 minimal functional promoter. Constructs that contained either SREs or APPs showed increased expression of the reporter when transfected cells were deprived of serum. The magnitude of the induction was a function of the number of copies of the individual elements in the promoter, and, when placed in tandem, SREs and APPs gave additive effects. Although the SREs and APPs were functional in both sense and antisense orientations, induction was sensitive to the relative orientation of individual elements within the promoter when multiple copies were present. These results provide additional support for a role of the APP sequence in the induction of apoD expression following growth arrest. The role of the SREs in this phenomenon is less clear. When placed upstream of the minimal apoD promoter, SREs confer growth arrest-induced transcriptional activation, whereas deletion of SRE elements in the −558/−4 construct had minimal effects on induction. It would appear, therefore, that the contribution of the SREs to the induction depends on their context within the apoD promoter.

The apoD APP sequence consists of 25 alternating purine-pyrimidine pairs that are dominated by d(CA) pairs and is directly preceded by a track of 14 pyrimidines. Because these characteristics correspond to established criteria for Z-DNA formation (55–61), it is probable that the APP element in the apoD promoter can form Z-DNA. Z-DNA conformations and APP sequences have been associated with both activation (62–65) and repression (66–70) of gene expression in mammalian cells. It has been proposed that Z-DNA structures may modulate transcription by providing binding sites for regulatory proteins that may recognize the Z-DNA conformation rather than a specific sequence (71–73), by assuring the optimal spatial separation between successive RNA polymerases (74), by determining the orientation of different transcription factors on the DNA helix (75), or by altering internucleosomal DNA helical twist in chromatin (62). The mechanism by which the APP in apoD promoter regulates gene expression following growth arrest remains to be determined. Although the electrophoretic mobility shift assays on the APP region did not show any complex formation, the conditions of ionic strength may not have been optimal for Z-DNA formation. It is unlikely that Z-DNA plays a role in apoE gene expression, because no APP sequences were identified in the 30-kbp of the 5′ promoter. Constructs that contained either SREs or APPs showed increased expression of the reporter when transfected cells were deprived of serum. The magnitude of the induction was a function of the number of copies of the individual elements in the promoter, and, when placed in tandem, SREs and APPs gave additive effects. Although the SREs and APPs were functional in both sense and antisense orientations, induction was sensitive to the relative orientation of individual elements within the promoter when multiple copies were present. These results provide additional support for a role of the APP sequence in the induction of apoD expression following growth arrest. The role of the SREs in this phenomenon is less clear. When placed upstream of the minimal apoD promoter, SREs confer growth arrest-induced transcriptional activation, whereas deletion of SRE elements in the −558/−4 construct had minimal effects on induction. It would appear, therefore, that the contribution of the SREs to the induction depends on their context within the apoD promoter.

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Several studies have demonstrated that SREs can be the site of regulation of both induction and repression events in serum-stimulated quiescent fibroblasts (76, for review see Ref. 77). It has been shown that the serum-responsive factor (SRF) binds to the SRE in the c-fos promoter as a homodimer (78). Binding of the SRF then allows the binding of a second protein, initially allows other factors such as stress proteins to bind to the SRE and transactivate expression (Band C). SREs could also contribute to the induction of apoE expression following growth arrest as deletion of a part of the promoter that includes the two most distal SRE results in a reduction of promoter activity (Fig. 6C).

Here we have identified elements within the apoD promoter that contribute to the activation of transcription that occurs when cells enter growth arrest following serum deprivation. It will now be important to identify the proteins that are involved in the transactivation and to determine if these elements are involved in the induction of apoD gene expression that occurs in neuropathological situations and following experimental injury in the peripheral and central nervous systems.

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