

# Regulation of *apoA<sub>1</sub>* gene expression with acidosis: requirement for a transcriptional repressor

M J Haas<sup>1</sup>, D Reinacher<sup>1</sup>, J P Li<sup>1</sup>, N C W Wong<sup>2</sup> and A D Mooradian<sup>1</sup>

<sup>1</sup>Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Saint Louis University School of Medicine, St Louis, Missouri 63110, USA

<sup>2</sup>Endocrine Research Group, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, T2N 4N1, Canada

(Requests for offprints should be addressed to A D Mooradian, Division of Endocrinology, Saint Louis University School of Medicine, 3691 Rutger Street, Suite 101, St Louis, Missouri 63110, USA; Email: mooradad@slu.edu)

## ABSTRACT

Serum apolipoprotein A<sub>1</sub> (apoA<sub>1</sub>) concentration is inversely correlated with the risk of premature atherosclerosis. Serum apoA<sub>1</sub> concentrations are regulated, in part, at the transcriptional level. ApoA<sub>1</sub> mRNA is synthesized primarily in the liver and small intestine, under the direction of a number of signaling molecules and tissue-specific regulatory elements. Previously, we demonstrated that extracellular acidosis suppresses apoA<sub>1</sub> mRNA levels at the level of transcription. Here we demonstrate that intracellular acidosis, in the absence of extracellular pH changes, represses *apoA<sub>1</sub>* promoter activity. Repression occurs through a pH responsive element (pH-RE) located within the *apoA<sub>1</sub>* gene promoter. Acidosis increases the specific DNA binding activity of a putative repressor protein within the immediate 5'-flanking region of the *apoA<sub>1</sub>* gene. The *cis*-element that binds the putative repressor protein contains a negative thyroid hormone

response element (nTRE) located 3' and adjacent to the *apoA<sub>1</sub>* TATA box. Mutation of the nTRE/pH-RE abrogates protein binding and alters the activity of reporter genes controlled by this element. Repression by acidosis did not require *de novo* mRNA and protein synthesis. Inhibition of tyrosine kinase activity and diacylglycerol-stimulated protein kinase C (PKC) signaling pathways with tyrophostin A47 and phorbol myristate acetate, respectively, did not affect the repression of *apoA<sub>1</sub>* promoter activity with acidosis. These results suggest that transcriptional repression of the *apoA<sub>1</sub>* gene by alterations in ambient pH is associated with enhanced DNA binding activity of a repressor protein, through a mechanism which appears to be independent of *de novo* mRNA and protein synthesis, tyrosine kinase activity, or PKC activation.

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## INTRODUCTION

Serum apolipoprotein A<sub>1</sub> (apoA<sub>1</sub>) concentration is inversely related to the risk of developing atherosclerosis (Miller & Miller 1975, The Lipid Research Clinics Coronary Primary Prevention Trial Results 1984). Synthesis and secretion of apoA<sub>1</sub> by hepatocytes and epithelial cells of the small intestine are regulated at both the transcriptional and post-transcriptional levels (Sastry *et al.* 1988, Widom *et al.* 1991, Shah *et al.* 1995). Clearance of cholesterol from the circulation is thought to occur through a process called reverse cholesterol transport (Franceschini *et al.* 1991). Augmenting *apoA<sub>1</sub>* expression through dietary or pharmacologic means

should provide a significant benefit for those with insufficient HDL concentrations. In order to achieve this goal, it is necessary to determine how liver and intestinal cells control *apoA<sub>1</sub>* gene expression.

Transcription of the *apoA<sub>1</sub>* gene is regulated by several hormones and second messengers, including retinoids (Zolfagari & Ross 1994), insulin (Murao *et al.* 1998, Hargrove *et al.* 1999), thyroid hormone (Mooradian *et al.* 1996, Taylor *et al.* 1996b), sex hormones (Patsch *et al.* 1980, Harnish *et al.* 1998), and others (Taylor *et al.* 1996a, Vu-Dac *et al.* 1998, Murao *et al.* 1997). Several of these pathways function to increase the expression of the *apoA<sub>1</sub>* gene, whereas others act to decrease *apoA<sub>1</sub>* gene

expression. For instance, hyperglycemia inhibits *apoA<sub>1</sub>* promoter activity and decreases apoA<sub>1</sub> mRNA levels through an insulin response core element (IRCE) located between nucleotides –425 and –390, relative to the transcriptional start site (Murao *et al.* 1998). This element is also responsible for induction of the *apoA<sub>1</sub>* gene by insulin (Murao *et al.* 1998). Likewise, thyroid hormone can act as either a transcriptional enhancer or, in a more artificial fashion, as a transcriptional repressor (Taylor *et al.* 1996b). The enhancer effect requires the presence of a thyroid hormone response element (TRE) located between nucleotides –208 and –193, in site A (Taylor *et al.* 1996b), whereas the repressor effect is observed only when the TRE in site A is removed from reporter constructs, and requires the presence of a negative TRE (nTRE), AGGTCA, located between nucleotides –25 and –20. In this case, the nTRE, located 3' end adjacent to the *apoA<sub>1</sub>* TATA box, acts as a transcriptional repressor in the presence of the ligand-activated thyroid hormone receptor (THR) (Taylor *et al.* 1996b, Carr & Wong 1994). The relevance of this cis-element in regulating expression of the *apoA<sub>1</sub>* gene *in vivo* is not known.

Expression of the *apoA<sub>1</sub>* gene is repressed at the transcriptional level by acidosis (Haas *et al.* 2000). Acidosis repressed apoA<sub>1</sub> mRNA levels by 25–30%, and *apoA<sub>1</sub>* promoter activity by more than 50%, relative to alkalosis. Repression due to acidosis is observed in HepG2 liver cells, and in Caco-2 cells (Haas *et al.* 2000) – a cell line with the characteristics of an epithelial cell from the small intestine and which expresses the *apoA<sub>1</sub>* gene. In order to understand the mechanism(s) by which acid–base balance may regulate *apoA<sub>1</sub>* gene expression, we mapped the cis-element responsible for repression by acidosis. The results of our analyses indicate that acidosis controls the DNA-binding activity of a factor that binds to nTRE and has the properties of a transcriptional repressor.

## MATERIALS AND METHODS

### Materials

Actinomycin D, acetylcoenzyme A, bafilomycin A1, cycloheximide, nigericin, ouabain, phorbol-myristate acetate (PMA), tyrophostin A47 and valinomycin were purchased from Sigma Chemical Company (St Louis, MO, USA). Lipofectamine was purchased from Life Technologies Inc. (Gaithersburg, MD, USA). New England Nuclear (Boston, MA, USA) was the supplier for [<sup>14</sup>C]chloramphenicol, and [α-<sup>32</sup>P]dCTP was purchased from Amersham-Pharmacia Biotech

(Piscataway, NJ, USA). Tissue culture media and fetal calf serum were purchased from BioWhittaker Inc. (Walkersville, MD, USA). All other chemicals were of reagent quality and were purchased from either Sigma Chemical Company or Fisher Scientific Company (Pittsburgh, PA, USA).

### Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), and penicillin and streptomycin (100 units/ml and 100 µg/ml, respectively). Caco-2 intestinal cells were maintained in Earl's modified essential medium (EMEM) supplemented with 15% FBS, non-essential amino acids, 0.11 mg/ml sodium pyruvate, and penicillin and streptomycin. Cells were housed in a humidified incubator at 37 °C with 5% CO<sub>2</sub>, 95% air.

An acidic environment was established by switching the cells to sodium bicarbonate-deficient DMEM, supplemented with serum and antibiotics as described above. The pH was established with *N*-2-hydroxyethyl piperazine-*N*-2-ethane sulfonic acid (HEPES), adjusted to either 6.7 or 7.9 with KOH. The pH of the culture medium was maintained at either pH 6.7 or pH 7.9 by the addition of HEPES (at pH 6.7 or pH 7.9 respectively) to a final concentration of 25 mM. No HEPES-related toxicity was noted at either pH extreme.

In some experiments, cells were treated with various ionophores or proton pump inhibitors. Bafilomycin A1, nigericin, ouabain and valinomycin were added to final concentrations of 5 µg/ml, 10 µg/ml, 1 mM, and 2 µM respectively, 24 h after transfection.

Cells were exposed for 16–24 h, then harvested and assayed for reporter enzyme activity.

### Plasmids and transient transfection analysis

Plasmids containing various lengths of the rat *apoA<sub>1</sub>* gene promoter were transfected into both HepG2 and Caco-2 cells for analysis of *apoA<sub>1</sub>* promoter activity. The plasmid pAI.474.CAT contains 467 bp of the rat *apoA<sub>1</sub>* gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene (Murao *et al.* 1998). Reporter constructs with various lengths of the *apoA<sub>1</sub>* promoter, generated using the polymerase chain reaction (PCR) followed by subsequent cloning of the amplified product in the parent CAT construct, were described previously (Taylor *et al.* 1996b, Murao *et al.* 1998). CAT constructs containing deletions within the

apoA<sub>1</sub> 5'-flanking sequence from -7 to -474 (pA1·474.CAT), -425 (pA1·425.CAT), -375 (pA1·375.CAT), -325 (pA1·325.CAT), -186 (pA1·186.CAT), -170 (pA1·170.CAT), -144 (pA1·144.CAT), and -46 bp (pA1·46.CAT) were used as described previously (Taylor *et al.* 1996b, Murao *et al.* 1998). The apoA<sub>1</sub> CAT constructs containing a mutant nTRE, pA1.m474.CAT and pA1.m46.CAT, were described previously (Taylor *et al.* 1996b).

Cells cultured to 80% confluence in six-well plates were transfected with 1 µg of the apoA<sub>1</sub> reporter plasmid and 1 µg of the plasmid pCMV.SPORT-β-Gal (Life Technologies, Inc.) using Lipofectamine. The latter plasmid, containing the β-galactosidase gene driven by the cytomegalovirus (CMV) immediate-early promoter, was used to normalize reporter gene activity to transfection efficiency. After 24 h, the cells were switched to DMEM at a pH of either 6·7 or 7·9. After a further 24 h, the cells were harvested and assayed for CAT activity (Gorman *et al.* 1982).

A portion of the extract was used for determination of β-galactosidase activity (Herbomel *et al.* 1984).

### RNA isolation and northern blotting

ApoA<sub>1</sub> mRNA levels were assessed by northern blotting with a previously characterized apoA<sub>1</sub> cDNA probe (Mooradian *et al.* 1996). Total RNA was prepared from cells cultured at either pH 6·7 or pH 7·9, and treated with either actinomycin D (1 µg/ml) or cycloheximide (CHX) (10 µg/ml) for 24 h, as described previously (Chomczynski & Sacchi 1987). Fifteen micrograms RNA were fractionated on an agarose gel containing 2·2 M formaldehyde, using established procedures (Sambrook *et al.* 1982). The RNA was transferred to a nylon membrane (Hybond, Amersham-Pharmacia Biotech), and hybridized with a cDNA probe specific to the apoA<sub>1</sub> gene. After appropriate exposures were obtained, the nylon membrane was stripped and rehybridized with a second cDNA probe specific for the glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene. The amount of signal present on the autoradiographs was quantified with a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

### ApoA<sub>1</sub> western blotting

ApoA<sub>1</sub> protein concentrations were assessed by western blotting (Towbin *et al.* 1979) with an antibody prepared and characterized in this laboratory (Mooradian *et al.* 1997). HepG2 cells in 25 cm<sup>2</sup>

flasks were cultured under either acidic (pH 6·7) or alkaline (pH 7·9) conditions for 24 h. Cells were washed twice with ice-cold phosphate-buffered saline (PBS, 50 mM sodium phosphate (pH 7·4), 150 mM NaCl) and resuspended in Laemmli sample buffer (Laemmli 1970). Protein concentration was determined with the Bradford protein assay (Bradford 1976) with bovine serum albumin as the standard. Twenty five micrograms protein were used in the analysis.

### Nuclear protein extract preparation and EMSA analysis

Nuclear protein extracts were prepared from cells at 90% confluence, cultured at either pH 6·7 or pH 7·9 for 24 h. Cells were washed three times with ice-cold PBS, and resuspended in 10 ml of nuclear wash buffer (10 mM HEPES (pH 8·0), 5 mM NaCl, 15% sucrose, 1 mM ethylene diaminetetra-acetic acid (EDTA), 0·5% Triton X-100, 1 mM dithiothreitol (DTT), 5 mM MgCl<sub>2</sub>, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and incubated on ice for 10 min. The mixture was underlaid with 5 ml of nuclear wash buffer containing 30% sucrose, but no Triton X-100, and centrifuged at 3000 *g* for 30 min at 5 °C. The nuclei in the pellet were resuspended in 1 ml of a buffer containing 10 mM HEPES (pH 8·0), 500 mM NaCl, 10 mM MgCl<sub>2</sub>, 0·1 mM EDTA, 1 mM DTT, 1 mM PMSF and 5 mM spermidine, and placed on ice for 1 h. The supernatant fraction was obtained by centrifugation at 10 000 *g* for 10 min, and was dialyzed extensively against 20 mM HEPES (pH 7·9), 100 mM KCl, 0·2 mM EDTA, 0·5 mM DTT, 0·5 mM PMSF and 20% glycerol. Protein concentration was determined as previously described (Bradford 1976), with bovine serum albumin as the standard.

The electrophoretic mobility shift assay (EMSA) was performed by incubating 10–15 µg nuclear protein extract with 15 000 c.p.m. of a <sup>32</sup>P-labeled oligonucleotide containing 46 bp of DNA corresponding to the proximal promoter region of the apoA<sub>1</sub> gene. The double-stranded oligonucleotides (5'-\*GCCCTGCCACACACATATATAGGTCA GGGAAGACCTGGACACCCAGACT-3', 5'-TC AGTCTGGGTGTCCAGGTGTTCTTCCCTG ACCTATATATGTGTGTGTGCAGG-3') were annealed and the probe labeled by filling in the 5'-overhang with [α-<sup>32</sup>P]dCTP using DNA polymerase I. The \* denotes which base in the template strand was labeled in the experiments described here. The underlined bases indicate the position of the nTRE (Taylor *et al.* 1996b), and the bold bases indicate the position of the apoA<sub>1</sub> TATA box (Widom *et al.* 1991). Incubation of the

protein extract with the labeled DNA probe was accomplished in a solution containing 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl<sub>2</sub>, 4 mM Tris-(hydroxymethyl)aminomethane hydrochloride (Tris-Cl) (pH 7.9), 0.6 mM EDTA, 0.6 mM DTT and 2 µg of poly(dI-dC)·poly(dI-dC), then placed on ice for 30 min. The mixture was loaded onto a 5% polyacrylamide gel, and subjected to electrophoresis in 0.5 × TBE (1 × TBE is 45 mM Tris base, 32.3 mM boric acid, and 1.25 mM EDTA (pH 8.3)) at room temperature, 200 V, for 90–120 min. The gel was dried and exposed to Hyperfilm (Amersham-Pharmacia Biotech).

Specificity of binding was assessed by adding a 10- and 100-fold molar excess of either an unlabeled double-stranded competitor oligonucleotide consisting of the wild type (WT) 46 bp DNA fragment or the unrelated DNA sequence, Site A (sense strand: 5'-TGAACCCTTGATCCCA-3'). Competition with a 10- and 100-fold molar excess of an unlabeled, double-stranded oligonucleotide containing the *apoA<sub>1</sub>* mutant nTRE (Taylor *et al.* 1996b) (5'-ACACACATATATAGGCTGGGGAAGAAGACCTGG-3') was performed in a similar manner. The underlined bases in the nTRE were altered from the WT sequence, TCA. Unlabeled competitor DNA was added at the time of addition of the probe. The remainder of the assay, including the conditions used for electrophoresis, was identical to the procedure described above.

### Statistical analysis

Changes in reporter gene activity, and apoA<sub>1</sub> protein and mRNA levels were evaluated with the two-tailed Student's *t*-test.  $P < 0.05$  was considered the limit for statistical significance. The results are expressed as means ± S.E.M

## RESULTS

### Effect of acidosis on apoA<sub>1</sub> protein content

We have recently shown that apoA<sub>1</sub> mRNA content and *apoA<sub>1</sub>* promoter activity are suppressed by acidosis in both Caco-2 intestinal cells and HepG2 hepatocytes (Haas *et al.* 2000). To confirm and extend our previous findings, apoA<sub>1</sub> protein content in HepG2 cells was measured by western blotting. The results (Fig. 1) demonstrate that apoA<sub>1</sub> protein concentrations are significantly reduced when cells are cultured under acidic conditions. Quantitation of the results with a densitometer showed that acidosis reduced apoA<sub>1</sub> protein concentrations in HepG2 cells nearly 20%, from 100 ± 2.3% to 80.3 ± 5.4% ( $P < 0.01$ ). In this cell line, apoA<sub>1</sub> mRNA levels

are also reduced by acidosis, by 20% (Haas *et al.* 2000). This suggests that the steady-state apoA<sub>1</sub> protein concentration is likely to be directly related to the decrease in apoA<sub>1</sub> mRNA levels.

### Effect of cation pump inhibitors and ionophores on *apoA<sub>1</sub>* promoter activity

To determine whether the changes in *apoA<sub>1</sub>* promoter activity with acidosis (Haas *et al.* 2000) that we had observed could be reproduced with various chemicals known to reduce intracellular pH directly or indirectly, the effect of various cation pump inhibitors and ionophores on *apoA<sub>1</sub>* promoter activity was evaluated. Changes in intracellular Na<sup>+</sup> and K<sup>+</sup> concentration results in changes in intracellular pH as a result of the activity of a Na<sup>+</sup>/H<sup>+</sup> antiporter (Aronson 1985, Krapf *et al.* 1991). In order to determine if changes in intracellular Na<sup>+</sup>/K<sup>+</sup> concentration can affect *apoA<sub>1</sub>* promoter activity, we assessed what the effects the Na<sup>+</sup>/K<sup>+</sup> ATPase inhibitor ouabain, and the ionophores nigericin and valinomycin, may have on *apoA<sub>1</sub>* promoter activity. HepG2 and Caco-2 cells were transfected with the plasmids pA1.474.CAT and pCMV.SPORT.β-gal, and after 24 h, the culture media was switched to DMEM at pH 6.7 or pH 7.9. Ouabain (1 mM), nigericin (10 µg/ml), or valinomycin (2 µM) were added to cells in pH 7.9 culture media. After 24 h, the cells were harvested and assayed for CAT and β-galactosidase activity. The results (Table 1) clearly show that each effector was as potent at inhibiting CAT activity as acidosis itself. In this experiment, acidosis repressed *apoA<sub>1</sub>* promoter activity to 32.8% in HepG2 cells ( $P < 0.05$ ). Nigericin, ouabain, and valinomycin repressed *apoA<sub>1</sub>* promoter activity to 57.7% ( $P < 0.01$ ), 45.3% ( $P < 0.01$ ), and 37.9% ( $P < 0.05$ ) of controls respectively. Similar results were obtained with valinomycin and nigericin in Caco-2 cells. Additional experiments with bafilomycin A1-treated Caco-2 cells showed a similar reduction in *apoA<sub>1</sub>* promoter activity (Table 1). Bafilomycin A1, a potent inhibitor of the vacuolar H<sup>+</sup>/ATPase (Harada *et al.* 1997, Keeling *et al.* 1997), prevents endosome acidification and decreases cytosolic pH. Caco-2 cells transfected with the plasmid pA1.474.CAT and treated with bafilomycin A (5 µg/ml) showed decreased *apoA<sub>1</sub>* promoter activity to 57.8% relative to untreated cells (Table 1). These effects were accomplished without changes in extracellular pH, as assessed by measurements of media pH (data not shown). These results suggest that changes in intracellular pH through pharmacological means, in the absence of changes in extracellular pH, repress *apoA<sub>1</sub>* promoter activity to an extent similar to that produced by extracellular acidosis.

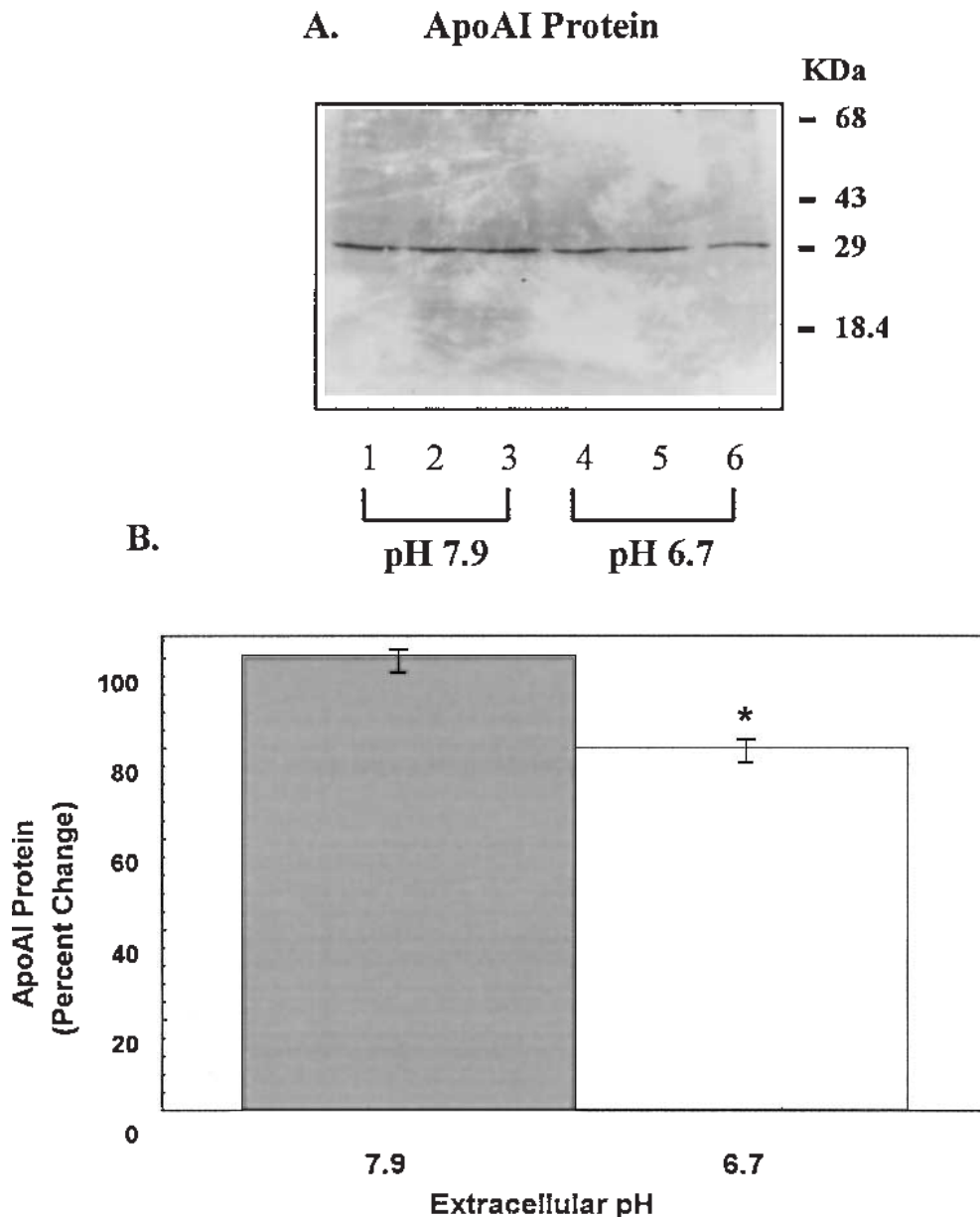


FIGURE 1. Effect of acidosis on steady-state apoA<sub>1</sub> protein concentrations. A representative western blot analysis (A) to examine the effect of acidosis on apoA<sub>1</sub> protein concentrations was performed with protein extracts prepared from HepG2 cells cultured at either pH 7.9 (lanes 1–3) or pH 6.7 (lanes 4–6) for 24 h. The signal from various blots was quantified using a scanning laser densitometer and the results are summarized in panel B. Acidosis decreased apoA<sub>1</sub> protein concentrations nearly 20%, relative to alkalosis.  $n=6$  in each group. \* $P<0.01$ .

### Localization of the pH responsive element (pH-RE)

As the affect of acidosis on expression of the *apoA<sub>1</sub>* mRNA and protein is due to inhibition of *apoA<sub>1</sub>* promoter activity (Haas *et al.* 2000), we wished to determine what *cis*-element is necessary for mediat-

ing this response. CAT reporter constructs containing 474, 425, 375, 325, 186, 170, 144, and 46 bp of 5'-flanking sequence of the *apoA<sub>1</sub>* promoter region (Fig. 2) were transfected into both HepG2 and Caco-2 cells. After 24 h, the cells were switched to bicarbonate-deficient DMEM adjusted to either pH 6.7 or 7.9 by the addition of HEPES. CAT activity

TABLE 1. Effect of ion perturbation on apoA<sub>1</sub> promoter activity. HepG2 and Caco-2 cells were transfected with the plasmids pA1-474.CAT and pCMV.SPORT.β-gal. After 24 h, the media were switched to DMEM at either pH 6.7 or 7.9, at which time bafilomycin (Bafilo., 5 µg/ml), nigericin (10 µg/ml), ouabain (1 mM), and valinomycin (Valino., 2 µM) were added to cells containing pH 7.9 DMEM. After 24 h, cells were harvested and assayed for CAT and β-galactosidase activity. In all cases, bafilomycin, nigericin, ouabain, and valinomycin treatment repressed apoA<sub>1</sub> promoter activity significantly

Treatment/pH	HepG2		Caco-2	
	CAT activity	Percent change	CAT activity	Percent change
C/6.7	39.8 ± 1.9	-32.8†	5.6 ± 1.1	-72.8†
C/7.9	59.3 ± 2.5	—	20.6 ± 2.3	—
Bafilo./7.9	N.D.	N.D.	8.7 ± 2.4	-57.8†
Nigericin/7.9	25.1 ± 1.7	-57.7†	0.7 ± 0.2	-96.9†
Ouabain/7.9	32.4 ± 2.2	-45.3†	N.D.	N.D.
Valino./7.9	36.8 ± 2.0	-37.9†	2.5 ± 0.8	-87.9†

C, untreated cells; N.D., not determined; †*P*<0.01 relative to C/pH 7.9, *n*=4.

in HepG2 and Caco-2 cells cultured at pH 6.7 for 24 h decreased 45–46% relative to the activity observed in cells at pH 7.9 (Fig. 2) for the pA1-474.CAT plasmid. All of the other apoA<sub>1</sub> deletion constructs were repressed to a similar degree by acidosis (Fig. 2). In all cases, the decrease was statistically significant. These results suggest that a pH-RE is located in the 46 bp DNA fragment immediately adjacent to the transcriptional start site.

### Effect of acidosis on protein–DNA binding activity

In order to determine if the effect of acidosis on the apoA<sub>1</sub> promoter is due to modulation of the activity of a DNA binding protein, we performed EMSAs with nuclear protein extracts prepared from HepG2 and Caco-2 cells cultured at either pH 6.7 or pH 7.9 for 24 h. The probe used in the gel shift assay consisted of a double-stranded oligonucleotide containing the region of the apoA<sub>1</sub> promoter from -48 to +5bp, relative to the transcriptional start site (+1). This fragment includes the region present in the smallest CAT construct (Fig. 2) used in the pH-RE localization experiments described above. One major protein–DNA complex was observed in HepG2 cells at both pH 6.7 and pH 7.9 (Fig. 3). However, the amount of complex formation in the extracts from the cells maintained at pH 6.7 was increased relative to the amount of complex

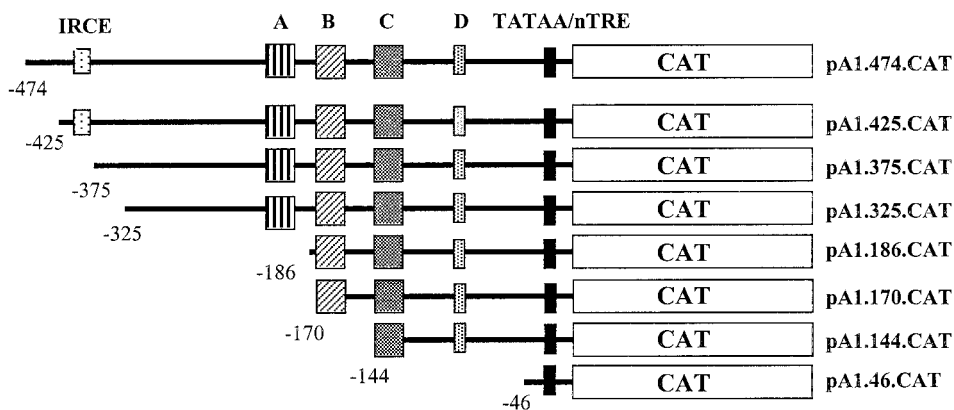
formation with extracts prepared from cells at pH 7.9 (compare lanes 2 and 3 with lanes 4 and 5). The amount of complex formation was equivalent in identically treated cells (compare lanes 2 and 3 with lanes 4 and 5), suggesting that the degree of variability in complex formation is small. Quantitation of the results by densitometry showed that complex formation was increased by 30.1 ± 3.9% with acidosis. This finding suggests that acidosis may increase the DNA-binding activity of a transcriptional repressor protein. Similar results were obtained with nuclear protein extracts prepared from Caco-2 cells (Fig. 3) maintained under either acidic (lane 6) or alkaline conditions (lane 7). These results suggest that acidosis affects apoA<sub>1</sub> gene expression in a similar manner in both cell types.

Gel shift assays with probes containing the apoA<sub>1</sub> IRCE (Murao *et al.* 1998) and site A suggest that protein–pH-RE binding is real, because the amount of complex formation with these probes did not show change with acidosis (data not shown).

DNA–protein binding was not suppressed by the addition of an unlabeled oligonucleotide containing the apoA<sub>1</sub> site A region (Fig. 4, lanes 7 and 8), but was completely suppressed when the homologous oligonucleotide containing the pH-RE was added (Fig. 4, lanes 3 and 4). These results support the conclusion that binding of the pH-responsive factor in HepG2 cells to the pH-RE is specific. A similar specificity profile was obtained using nuclear protein extracts prepared from Caco-2 cells (data not shown).

### Effect of pH-RE mutation on pH-RE activity

The region of the apoA<sub>1</sub> promoter which contains the pH-RE activity and is able to bind the putative repressor protein contains a consensus nTRE motif located immediately adjacent to the apoA<sub>1</sub> TATA element (Carr & Wong 1994, Taylor *et al.* 1996a,b). Therefore, we investigated whether or not mutation of the nTRE would affect the ability of acidosis or alkalosis to affect reporter gene expression. Two different constructs were used, one of which contained a mutant nTRE in the context of the full-length apoA<sub>1</sub> promoter (pA1.m474.CAT), the other of which contains the mutant nTRE within only 39 bp of the apoA<sub>1</sub> 5'-flanking region (Fig. 5) (pA1.m46.CAT). In both cases, the central nucleotides within the WT nTRE, TCA, were replaced with CTG. When Hep G2 cells were transfected with the plasmid pA1-474.CAT and subjected to acidic culture conditions for 24 h, CAT activity decreased to 81.6% relative to CAT activity at



Cell Line	HepG2	Caco-2
Plasmid	Percent Change	Percent Change
pA1.474.CAT	-46.8 <sup>†</sup>	-45.1 <sup>‡</sup>
pA1.425.CAT	-55.9 <sup>‡</sup>	-63.0 <sup>‡</sup>
pA1.375.CAT	-38.9 <sup>†</sup>	-60.9 <sup>‡</sup>
pA1.325.CAT	-55.2 <sup>‡</sup>	-51.4 <sup>‡</sup>
pA1.186.CAT	-45.2 <sup>‡</sup>	-36.2 <sup>†</sup>
pA1.170.CAT	-63.7 <sup>‡</sup>	-61.9 <sup>‡</sup>
pA1.144.CAT	-56.8 <sup>‡</sup>	-49.5 <sup>‡</sup>
pA1.46.CAT	-64.5 <sup>‡</sup>	-50.6 <sup>‡</sup>

FIGURE 2. Mapping the pH-RE. The location of the pH-RE in the rat *apoA<sub>1</sub>* gene promoter was mapped using transient transfection analysis with CAT reporter genes containing deletions within the promoter. The location of the IRCE, sites A, B, C, D, the TATAA box/nTRE, and the CAT coding region are indicated. HepG2 and Caco-2 cells were transfected with the indicated *apoA<sub>1</sub>* reporter plasmid and the plasmid pCMV.SPORT.β-gal, and after 24 h, the medium was switched to DMEM at pH either 6·7 or 7·9. After 24 h, cells were harvested and assayed for CAT and β-galactosidase activity. CAT activity was normalized to β-galactosidase activity. The percent decrease in reporter gene activity from cells cultured at pH 7·9 relative to pH 6·7 is indicated in the table for both HepG2 and Caco-2 cells. Although somewhat variable, repression was observed in all of the reporter plasmids examined, suggesting that the pH-RE resides in the proximal promoter region of the *apoA<sub>1</sub>* gene. <sup>†</sup> $P < 0.05$ , <sup>‡</sup> $P < 0.01$ ;  $n = 3$ .

pH 7·9 (Fig. 5). However, CAT activity HepG2 from cells transfected with the plasmid pA1.m474.CAT, containing the TCA→CTG mutation, was suppressed by acidosis to 77·2% relative to CAT activity at pH 7·9. Although this difference was statistically significant ( $P < 0.01$ ), the lack of complete abrogation of acidosis-related suppression indicates that the nTRE is not the sole pH-RE in the *apoA<sub>1</sub>* promoter.

Different results were obtained with the *apoA<sub>1</sub>*-CAT reporter construct containing only 39 bp of 5'-flanking DNA (Fig. 5). When HepG2 cells were transfected with pA1.m46.CAT and cultured at either pH 6·7 or 7·9, no statistically significant differences in CAT activity were observed in HepG2 cells (5·1% acetylation at pH 7·9 compared with 4·9% acetylation at pH 6·7,  $P > 0.05$ ) or in Caco-2 cells (3·9% at pH 7·9 compared with 3·5% at

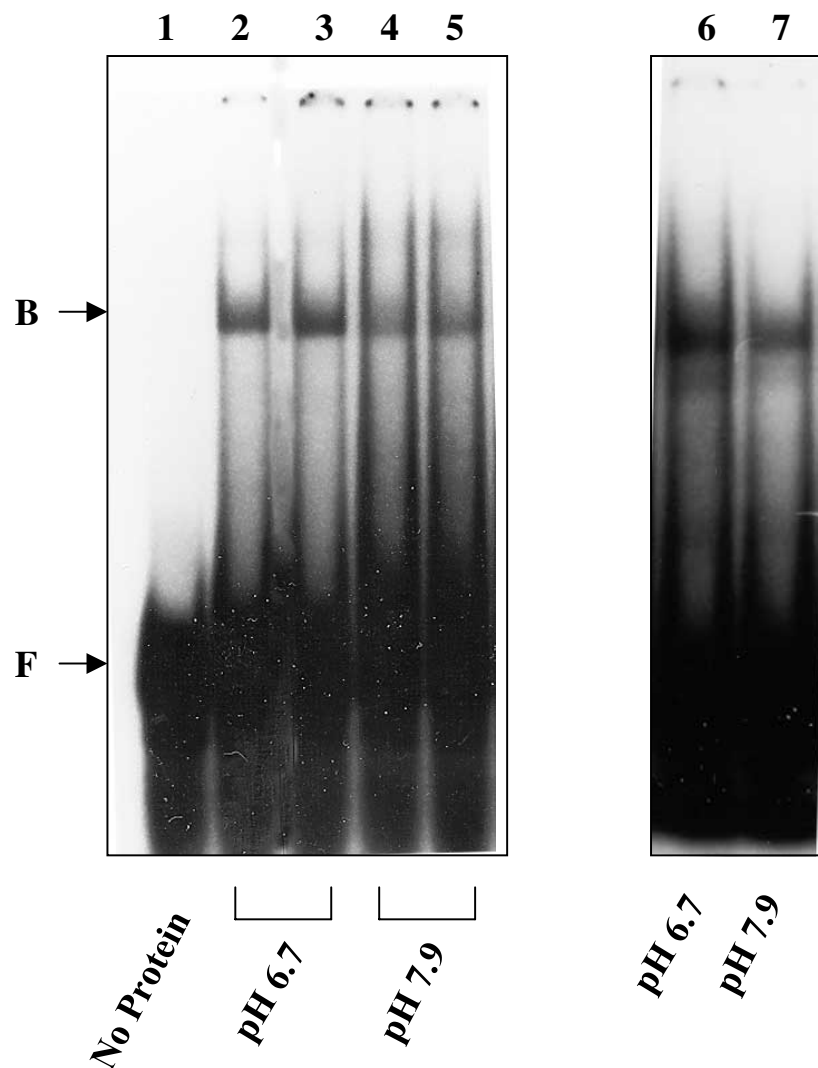


FIGURE 3. Effect of acidosis on factor binding to the 46 bp DNA fragment. EMSAs were used to examine the effect of acidosis on protein–DNA binding. The probe, consisting of the 46 bp DNA fragment to which the pH-RE was localized, was incubated with either 15 or 10  $\mu$ g of nuclear extract protein from HepG2 or Caco-2 cells respectively, cultured at either pH 6.7 or pH 7.9 for 24 h. Increased protein–DNA binding can be observed in extracts prepared from cells cultured at pH 6.7 (lanes 2 and 3), relative to extracts prepared from cells cultured at pH 7.9 (lanes 4 and 5). Increased protein–DNA binding was also observed in cell extracts prepared from Caco-2 cells at pH 6.7 (lane 6) relative to pH 7.9 (lane 7). Migration of the bound (B) and free (F)  $^{32}$ P-labeled DNA probe is indicated. The experiment was repeated three times.

pH 6.7,  $P>0.05$ ). Taken together, these experiments indicate that the previously described nTRE may also act as a pH-RE. However, there must be additional cis-acting elements within the promoter that can also function as pH-REs when the nTRE/pH-RE is mutated.

#### Effect of pH-RE mutation on protein–pH-RE binding

Because mutation of the pH-RE appeared to be at least partly involved in the repressive effect of acidosis on the *apoA<sub>1</sub>* promoter, we next determined



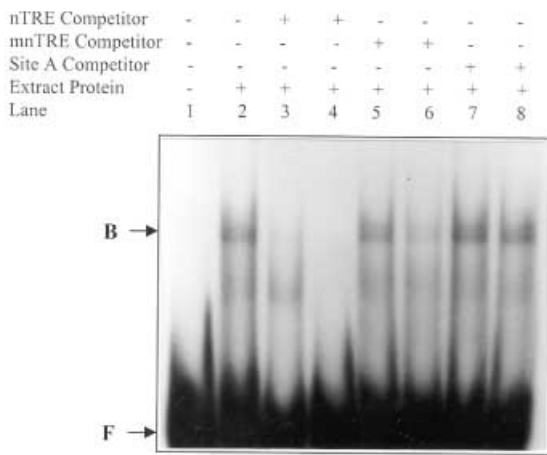


FIGURE 4. Specificity of pH-RE-protein binding. The specificity of pH-RE-protein binding was examined with an EMSA competition experiment, using the 46 bp DNA fragment as a probe and a nuclear extract from HepG2 cells cultured in pH 6.7 DMEM for 24 h. Lane 1, no nuclear extract protein; lanes 2–8, 15 µg nuclear extract; lanes 3 and 4, 10- and 100-fold molar excess of the unlabeled 46 bp DNA fragment; lanes 5 and 6, 10- and 100-fold molar excess of the unlabeled mutant pH-RE; lanes 7 and 8, 10- and 100-fold molar excess of the unlabeled site A oligonucleotide. The oligonucleotide containing site A was unable to compete for factor binding, whereas the unlabeled 46 bp DNA fragment was capable of doing so. The mutant pH-RE was not able to compete as effectively as the WT 46 bp fragment. Migration of the bound (B) and free (F) <sup>32</sup>P-labeled DNA probe is indicated. The experiment was repeated three times.

whether or not the same nucleotide changes also effect pH-RE-protein binding. Double-stranded oligonucleotides containing the TCA→CTG substitution were synthesized, annealed, and used as competitors in the gel shift assay shown in Fig. 4. Inclusion of this oligonucleotide in the binding reaction displaced protein from the <sup>32</sup>P-labeled probe less effectively than the WT sequence. These results indicate that the mutated pH-RE binds to nuclear protein(s) with less affinity than the WT sequence, suggesting that a transcriptional repressor may be involved in the acidosis-related downregulation of the apoA<sub>1</sub> promoter.

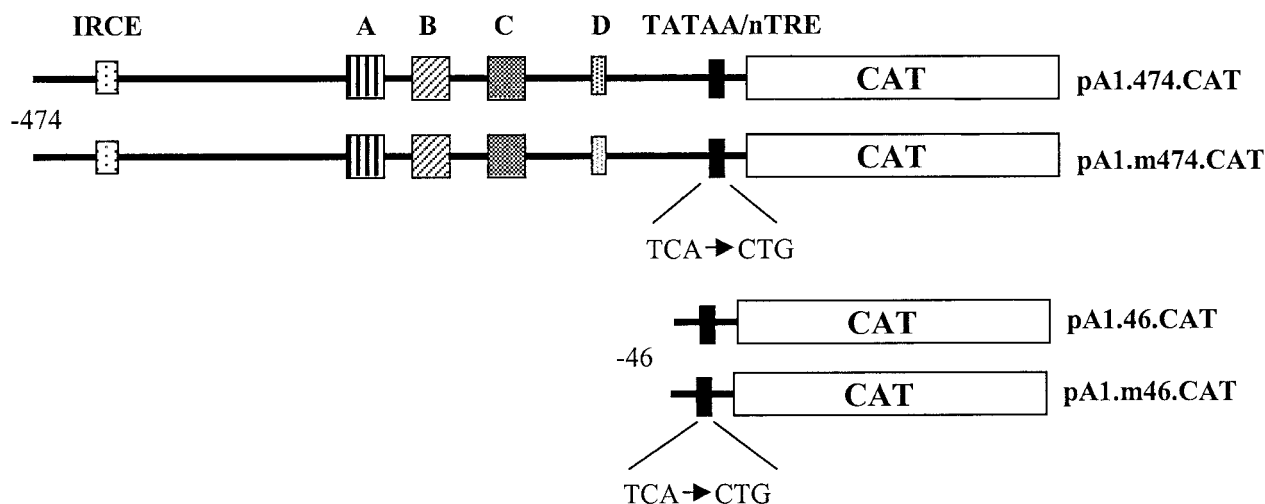
#### Effect of RNA and protein synthesis inhibitors on repression of the apoA<sub>1</sub> gene by acidosis

In order to determine if either *de novo* RNA or protein synthesis are required for repression of the apoA<sub>1</sub> gene by acidosis, HepG2 cells were placed in culture medium at pH 7.9 or at pH 6.7 and treated with either 1 µg/ml actinomycin D or 10 µg/ml

cycloheximide (CHX). At these doses, cell survival was 94.3% (actinomycin D) and 95.6% (CHX) relative to cells at pH 6.7 (98.5%), as assayed by trypan blue exclusion. After 24 h, RNA was harvested from the cells and northern blotting was performed with the apoA<sub>1</sub> cDNA probe. Acidosis repressed apoA<sub>1</sub> mRNA levels by 50% (Fig. 6) ( $P < 0.05$ ). Addition of either actinomycin D or CHX when the medium was switched to pH 6.7 failed to inhibit the repression normally observed with acidosis; even in the presence of actinomycin D and CHX, acidosis repressed apoA<sub>1</sub> mRNA levels to 42% and 51% of control respectively. These results suggest that repression of the apoA<sub>1</sub> gene by acidosis does not require *de novo* mRNA and protein synthesis.

#### Effect of PMA and tyroprostin A47 on repression of the apoA<sub>1</sub> promoter by acidosis

As inhibition of mRNA and protein synthesis did not prevent the repressive effect of acidosis on apoA<sub>1</sub> mRNA levels, we examined two potential post-transcriptional mechanisms by which acidosis may affect apoA<sub>1</sub> promoter activity: protein kinase C and tyrosine kinase activation. Protein kinase C is a possible mediator for intracellular signaling by acidosis (Moolenaar *et al.* 1984, Nishizuka 1984, Williams & Howard 1994). In order to determine if changes in diacylglycerol-stimulated protein kinase C (PKC) isozymes mediate the repression of the apoA<sub>1</sub> promoter by acidosis, two approaches were taken. In one set of experiments, PMA was added immediately after transfection with pA1.474.CAT, to a final concentration of 400 nM. After 24 h, the medium was replaced with bicarbonate-deficient DMEM adjusted to either pH 6.7 or 7.9 with HEPES. PMA was again added to the appropriate wells to a final concentration of 400 nM. After a further 24 h, the cells were harvested and assayed for both CAT and β-galactosidase activity. Under these conditions, PMA has been reported to downregulate PKC activity (Nishizuka 1986, Yamaji *et al.* 1994). The results (Table 2) indicate that changes in PMA-stimulated PKC isoforms using a high-dose, chronic treatment with PMA failed to alter CAT activity significantly (54.5% decrease in CAT activity in the solvent-treated controls compared with a 50.6% decrease in CAT activity in the PMA-treated HepG2 cells). Similar results were obtained in Caco-2 cells (Table 2). However, basal CAT activity was repressed by PMA-treatment alone in HepG2 cells (from 26.6% to 8.1% in cells at pH 7.9 and from 12.1% to 4.0% in cells at pH 6.7), but not in Caco-2 cells. These results suggest that basal apoA<sub>1</sub> promoter activity is controlled in HepG2 cells, at least in part, by



Cell Line	HepG2	HepG2	Caco-2	Caco-2
Plasmid / pH	CAT Activity	Percent Change	CAT Activity	Percent Change
pA1.474.CAT / 6.7	4.7±2.3	-81.6 <sup>†</sup>	7.9±1.8	-85.7 <sup>†</sup>
pA1.474.CAT / 7.9	25.7±2.3	--	55.3±3.2	--
pA1.m474.CAT / 6.7	3.3±0.0	-77.2 <sup>†</sup>	14.0±3.4	-73.2 <sup>†</sup>
pA1.m474.CAT / 7.9	14.5±1.9	--	52.3±1.7	--
pA1.46.CAT / 6.7	2.6±0.7	-61.2 <sup>†</sup>	2.2±0.6	-57.7 <sup>†</sup>
pA1.46.CAT / 7.9	6.7±0.4	---	5.2±0.4	---
pA1.m46.CAT / 6.7	4.9±0.1	-3.9	3.5±0.2	-10.3
pA1.m46.CAT / 7.9	5.1±0.6	--	3.9±0.4	--

FIGURE 5. Constructs used in elucidating the effect of mutating the pH-RE on repression of the *apoA*<sub>1</sub> promoter by acidosis. HepG2 and Caco-2 cells were transfected with reporter constructs containing either the WT pH-RE, or a mutant pH-RE. After 24 h, the media pH was adjusted to either pH 6.7 or pH 7.9. After another 24 h, CAT activity was assessed. The mutant pH-RE is present in two contexts, either within the full-length 474 bp *apoA*<sub>1</sub> promoter or within the plasmid containing the minimal, 39 bp *apoA*<sub>1</sub> promoter DNA fragment. Acidosis repressed CAT activity from the plasmid pA1.m474.CAT. However, acidosis did not significantly repress CAT activity from the plasmid pA1.m46.CAT. CAT activity in mock transfected cells is less than 0.5%. The percent [<sup>14</sup>C]chloramphenicol conversion without lysate is 0.09%. <sup>†</sup>*P*<0.01 relative to pH 7.9; *n*=5.

protein kinase C. However, downregulation of protein kinase C by chronic exposure to high concentrations of PMA did not alter the ability of acidosis to repress the *apoA*<sub>1</sub> promoter.

In the second set of experiments, PMA was added to the cells 24 h after transfection with pA1.474.CAT, at a final dose of 100 nM. Under these conditions, PMA activates diacylglycerol-stimulated PKC isozymes (Nishizuka 1986, Yamaji *et al.* 1994). After a further 16 h, the cells were

harvested and assayed for CAT and β-galactosidase activity. As shown in Table 2, PKC activation using this regimen inhibited basal CAT activity. CAT activity decreased from 26.6% to 7.1% in the cells at pH 7.9, while at pH 6.7, basal CAT activity dropped from 12.1% to 4.6%. However, repression of the *apoA*<sub>1</sub> promoter with acidosis was still observed. CAT activity was repressed to 35.2% with acidosis in the PMA-treated cells, compared with 54.5% in the solvent-treated controls. This

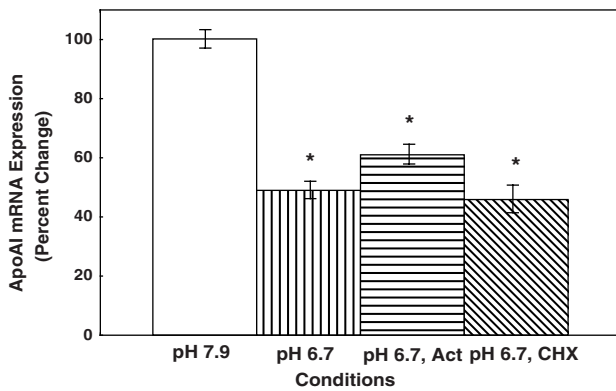


FIGURE 6. Effect of RNA and protein synthesis inhibitors on apoA<sub>1</sub> mRNA expression with acidosis. Total RNA prepared from HepG2 cells maintained at either pH 7.9 or pH 6.7 with or without 1 µg/ml actinomycin D (Act) or 10 µg/ml cycloheximide (CHX). Northern blots were hybridized with the apoA<sub>1</sub> cDNA probe, stripped and hybridized with a probe specific for G3 PDH to normalize apoA<sub>1</sub> mRNA expression levels. The 0.9 kb apoA<sub>1</sub> mRNA band and the 1.2 kb G3 PDH band were quantified by densitometry. Percent changes in apoA<sub>1</sub> mRNA expression are shown, relative to the level of G3 PDH expression. Addition of actinomycin D and CHX had no effect on the ability of acidosis to repress apoA<sub>1</sub> mRNA levels. \* $P < 0.01$  compared with pH 7.9. Other comparisons did not achieve statistical significance.  $n = 3$ .

difference did not achieve statistical significance. Similar results were also obtained with Caco-2 cells (Table 2).

In order to determine if tyrosine kinase activity is important in repression of the apoA<sub>1</sub> promoter with acidosis, HepG2 and Caco-2 cells were transfected with the apoA<sub>1</sub> reporter plasmid pA1.474.CAT and, after 24 h, treated with either 10 or 100 µM tyroprostin A47 for another 24 h. Tyroprostin A47 is a potent inhibitor of tyrosine kinase activity (Levitzi *et al.* 1991). Tyroprostin A47 did not repress basal apoA<sub>1</sub> promoter activity at either pH 6.7 or 7.9, nor did it significantly affect repression of the apoA<sub>1</sub> promoter by acidosis (Table 3). CAT activity was repressed to 44.2% in the control HepG2 cells, compared with 32.5% in cells treated with 0.5 µM tyroprostin A47. This difference was not statistically significant. CAT activity was repressed to 41.4% of control when HepG2 cells were treated with a higher concentration of tyroprostin A47 (5.0 µM). Similar results were obtained with Caco-2 cells (Table 3). These results indicate that repression of apoA<sub>1</sub> promoter activity by acidosis is independent of tyrosine kinase activity.

TABLE 2. Effect of phorbol myristate acetate (PMA) on regulation of the apoA<sub>1</sub> promoter by acidosis. HepG2 and Caco-2 cells were transfected with the plasmids pA1.474.CAT and pCMV.SPORT.β-gal, and after 24 h, the media was switched to DMEM at either pH 6.7 or pH 7.9. PMA was added immediately to one group of cells to a final concentration of 400 nM (P400). After 24 h, PMA was added to another group at a final concentration of 100 nM (P100), while an equivalent volume of the DMSO solvent was added to the control group (D). After 24 h, the cells were harvested and assayed for CAT and β-galactosidase activity. CAT activity was normalized to β-galactosidase activity. The results are presented as percent change from activity observed at pH 7.9 (set to 100%). Acidosis repressed apoA<sub>1</sub> promoter activity in the presence of 100 nM PMA. Likewise, exposure to 400 nM PMA for 48 h also had no effect on the ability of acidosis to repress apoA<sub>1</sub> gene expression

Treatment/pH	HepG2		Caco-2	
	CAT activity	Percent change	CAT activity	Percent change
D/6.7	12.1 ± 5.6	-54.5‡	1.9 ± 0.8	-81.5‡
D/7.9	26.6 ± 4.2	—	10.3 ± 0.8	—
P100/6.7	4.6 ± 0.7	-35.2‡	2.0 ± 0.5	-74.0‡
P100/7.9	7.1 ± 0.8	—	7.7 ± 0.3	—
P400/6.7	4.0 ± 0.7	-50.6‡	1.1 ± 0.1	-84.9‡
P400/7.9	8.1 ± 0.4	—	7.3 ± 1.4	—

† $P < 0.05$ ; ‡ $P < 0.01$ , relative to pH 7.9,  $n = 3$ .

TABLE 3. Effect of inhibiting tyrosine kinase activity on regulation of the apoA<sub>1</sub> promoter by acidosis. HepG2 cells were transfected with the plasmids pA1.474.CAT and pCMV.SPORT.β-gal, and after 24 h, the media were switched to DMEM at either pH 6.7 or pH 7.9. Tyroprostin A47 was added to a final concentration of either 10 or 100 µM (10T and 100T respectively). After 24 h, the cells were harvested and assayed for CAT and β-galactosidase activity. CAT activity was normalized to β-galactosidase activity. The results are presented as percent change from activity at pH 7.9 (set to 100%). Addition of tyroprostin A47 had no significant effect on CAT activity (normalized to β-galactosidase activity) when cells were cultured at pH 6.7, relative to pH 7.9

Treatment/pH	HepG2		Caco-2	
	CAT activity	Percent change	CAT activity	Percent change
C/6.7	29.0 ± 0.2	-44.2‡	8.6 ± 0.2	-39.4‡
C/7.9	52.0 ± 0.5	—	14.2 ± 4.0	—
10T/6.7	30.2 ± 0.2	-32.5‡	4.8 ± 0.7	-59.3‡
10T/7.9	44.8 ± 0.4	—	11.8 ± 3.1	—
100T/6.7	30.1 ± 0.5	-41.4‡	6.2 ± 2.3	-56.9‡
100T/7.9	51.4 ± 1.7	—	14.4 ± 0.5	—

† $P < 0.05$ ; ‡ $P < 0.01$ , relative to pH 7.9,  $n = 3$ .

## DISCUSSION

ApoA<sub>1</sub> mRNA levels are repressed 25–30% in HepG2 and Caco-2 cells maintained in acidic culture media (pH 6.7), relative to cells maintained in alkaline medium (pH 7.9) (Haas *et al.* 2000). Repression occurred at the level of transcription, as assessed by transient transfection analysis with reporter gene constructs containing the rat *apoA<sub>1</sub>* promoter (Haas *et al.* 2000). The highest level of CAT activity observed in cells maintained at pH 7.9 gradually decreased by 50–60% when the pH of the culture medium was decreased sequentially to pH 6.7 (Haas *et al.* 2000). Furthermore, these effects of acidosis were not observed with CAT activity driven by the SV40 virus early region promoter, suggesting that protein translation is not affected by changes in pH (Haas *et al.* 2000).

Acidosis also suppressed steady-state apoA<sub>1</sub> protein levels in HepG2 cells (Fig. 1). This observation suggests that the reduction in *apoA<sub>1</sub>* promoter activity and mRNA concentration is reflected at the protein level. This level of reduction is comparable to the 25–30% decrease in apoA<sub>1</sub> mRNA levels we reported previously (Haas *et al.* 2000). Importantly, this result also indicates that the protein synthesis capacity of the HepG2 cell line we used is not drastically affected by the change in pH. This finding is important, because Yamaji *et al.* (1994) reported that protein synthesis was suppressed by acidosis in some, but not all cell lines they examined.

Changes in Na<sup>+</sup> and K<sup>+</sup> concentration and distribution by pharmacological means can alter the activity of the membrane-bound Na<sup>+</sup>/H<sup>+</sup> antiporter, resulting in a reduction in intracellular pH. Activity of this ion pump is thought to be necessary for the alkalization observed in cells observed with growth factor stimulation (Boron 1986, Moolenaar 1986). Nucleus–cytosol pH gradients may also be determined in part by a K<sup>+</sup>/H<sup>+</sup> exchanger (Masuda *et al.* 1998). The addition of ouabain, a Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor, and the monovalent cation ionophores nigericin and valinomycin repressed *apoA<sub>1</sub>* promoter activity (Table 1). Thus alterations in intracellular pH, in the absence of changes in extracellular pH, cause repression of *apoA<sub>1</sub>* promoter activity. This finding is important, because the release of carrier-bound molecules and growth factor activation may be increased when culture media containing FBS are acidified. Thus the observation that changes in intracellular pH independent of media pH repress *apoA<sub>1</sub>* promoter activity suggests that there may be an intracellular pH sensor that is capable of regulating *apoA<sub>1</sub>* promoter activity. However, it is also possible

that use of ouabain, nigericin and valinomycin may also have unrelated effects on *apoA<sub>1</sub>* promoter activity.

Bafilomycin A1 inhibits endosomal acidification through its ability specifically to inhibit the vacuolar H<sup>+</sup>/ATPase pump (Forgacs 1989, Chinni & Shisheva 1999). Our results show that bafilomycin A1 represses *apoA<sub>1</sub>* promoter activity (Table 1). Whether or not this effect is due to inhibition of endosomal acidification or to a fluctuation in cytosolic pH remains to be determined. Bafilomycin A1 can mimic the effect of insulin on the translocation of glucose transporter 4 to the plasma membrane in 3T3-L1 adipocytes (Chinni & Shisheva 1999). In HepG2 cells, however, insulin, has a positive effect on *apoA<sub>1</sub>* promoter activity (Murao *et al.* 1998). These results suggest that bafilomycin A1 and insulin function through unrelated mechanisms to regulate *apoA<sub>1</sub>* promoter activity in HepG2 cells.

Transient transfection analysis with reporter genes containing deleted segments of the *apoA<sub>1</sub>* promoter were used to determine if a *cis*-element responsive to changes in pH could be identified. Deletion of the *apoA<sub>1</sub>* insulin-responsive core element (IRCE) had no effect on the ability of pH to repress reporter gene activity (Fig. 2). Subsequent deletions of sites A, B, and sites C and D together, also had no effect on repression of *apoA<sub>1</sub>* promoter activity (Fig. 2). This finding was unexpected, as two binding sites for the transcription factor Egr-1 exist in site A of the *apoA<sub>1</sub>* promoter (Kilbourne *et al.* 1995). Egr-1 is an immediate-early, zinc-binding transcription factor (Madden & Rauscher 1993). Egr-1 expression and activity can be induced by mitogenic signals (Sukhatme *et al.* 1988, Lim *et al.* 1987), atherosclerosis (McCaffrey *et al.* 2000) and cellular stress (Santiago *et al.* 1999, Yan *et al.* 1999). Egr-1 acts as a transcriptional repressor of the murine deaminase promoter (Ackerman *et al.* 1991) and the rat malic enzyme promoter (Barroso & Santisteban 1999). Of interest, Yamaji *et al.* (1994) have previously shown that Egr-1 expression is induced by acidosis in the MCT kidney epithelial cell line as early as 15 min after the change in media pH. Therefore, our results indicate that EGR-1 is probably not the major transcriptional repressor responsible for the effect of acidosis on the *apoA<sub>1</sub>* promoter. However, it may have some role in *apoA<sub>1</sub>* repression with acidosis, because more than one element may be present in the *apoA<sub>1</sub>* promoter.

Because repression was noted with the smallest promoter fragment analyzed (–46 bp), we examined this region for the presence of protein–DNA interactions. Taylor *et al.* (1996b) identified an

nTRE 3' end adjacent to the apoA<sub>1</sub> TATA box. This element consists of an nTRE capable of binding only a THR $\alpha$  monomer (Taylor *et al.* 1996b). Alone, the nTRE is capable of suppressing reporter gene activity, while in the context of the full apoA<sub>1</sub> promoter it loses this ability. In fact, site A contains a fully functional TRE that is capable of responding positively to thyroid hormone (Taylor *et al.* 1996b).

A gel shift probe containing the apoA<sub>1</sub> TATA box and nTRE formed a protein–DNA complex with nuclear extract proteins prepared from both HepG2 and Caco-2 cells cultured at pH 7.9 for 24 h (Fig. 3). The amount of complex formed increased in cells maintained at pH 6.7 for 24 h (Fig. 3). These results indicate that acidosis enhances binding of a putative repressor protein to a pH-RE within this DNA fragment. Furthermore, competition experiments with WT and mutant nTRE oligonucleotides indicated that protein–DNA binding requires the intact nTRE (Fig. 4). Addition of the WT nTRE oligonucleotide at 25-fold molar excess did not compete for factor binding (Fig. 4), although addition of the competitor at a 100-fold molar excess did compete to a small extent (Fig. 4). These results suggest that factor binding may be influenced, stabilized, or both, by factors adjacent to it. A likely candidate for this interaction may be the factors capable of binding the apoA<sub>1</sub> TATA box located immediately adjacent to the nTRE. Mutational analysis of this element, however, would probably yield results difficult to interpret, because this element is probably important in basal promoter activity. This finding was confirmed by analyzing the effect of mutations within the nTRE/pH-RE on reporter gene activity. Changing the core TCA sequence within the pH-RE/nTRE to CTG reduced pH-RE protein binding (Fig. 4) and alleviated the repressive effect of acidosis on the apoA<sub>1</sub> promoter construct, pA1.474.CAT (Fig. 5). However, mutation of the nTRE in this context did not abolish the effect of acidosis on promoter activity (Fig. 5). This observation suggests that, although nTRE is a pH-RE, it is not the sole pH-responsive element within the apoA<sub>1</sub> promoter. Additional pH-REs within the promoter are functional when the nTRE is mutated.

Acidosis has been shown to modulate several intracellular signaling modalities, including PKC activation (Moolenaar *et al.* 1984, Moolenaar 1986). Therefore, it was somewhat surprising that PMA did not affect the ability of acidosis to repress apoA<sub>1</sub> promoter activity (Table 2). It is possible that repression by acidosis requires more than one signaling pathway activated by acidosis, one of which may involve PKC. However, our

results with PMA indicate that perturbation of PKC activity affects basal apoA<sub>1</sub> expression (Table 2). Similar results were recently demonstrated by Zheng *et al.* (2000), who showed that PKC modulates apoA<sub>1</sub> promoter through the IRCE and the transcription factor SP1.

Inhibition of tyrosine kinase activity with tyro-phostin A47 had no effect on the ability of acidosis to repress apoA<sub>1</sub> promoter activity (Table 3). Repression of the apoA<sub>1</sub> promoter with acidosis occurred to the same extent in the presence of both 10 and 100  $\mu$ g/ml tyro-phostin A47 in both HepG2 and Caco-2 cells (Table 3). These results are somewhat in contrast to those reported by Yamaji *et al.* (1994) who found that tyrosine kinase activity was necessary for acidosis to stimulate immediately gene expression in renal epithelial cells.

The precise identity of this transcription factor is not known. However, within the nTRE/pH-RE there are two DNA–protein binding motifs (Quandt *et al.* 1995). The sequence TGAC is part of the core sequence recognized by AP1. AP1 activity is acutely regulated by oxidative stress (Arrigo 1999, Diamond *et al.* 1999, Rao *et al.* 1999), where it may be necessary for upregulating the expression of genes necessary for DNA repair and protection against the carcinogenic effects of oxidative by-products of metabolism (Xanthoudakis & Curran 1996, Primiano *et al.* 1997).

When combined with the glucocorticoid receptor on a composite glucocorticoid response element, AP1 can act as a repressor (Diamond *et al.* 1990, Miner & Yamamoto 1992). By itself on such an element, AP1 activity is stimulated by phorbol esters (Mordacq & Linzer 1989). However, in our studies PMA did not significantly influence the ability of acidosis to repress apoA<sub>1</sub> promoter activity (Table 2). Furthermore, glucocorticoids have a stimulatory effect on the apoA<sub>1</sub> promoter (Saladin *et al.* 1996, Hargrove *et al.* 1999), mediated through a glucocorticoid response element located in site A (Taylor *et al.* 1996a). These findings, although inconclusive, taken together suggest that AP1 is probably not the transcriptional repressor involved in mediating the effect of acidosis on the apoA<sub>1</sub> promoter.

The core sequence ACCT is also a binding site for the  $\delta$ -crystallin enhancer binding protein,  $\delta$ -EF1 (Sekido *et al.* 1994).  $\delta$ -EF1 inhibits gene expression by true transcriptional repression, and by displacing positive-acting DNA-binding transcription factors (Sekido *et al.* 1997).  $\delta$ -EF1 binds both to the CACCT site within the  $\delta$ -crystallin minimal enhancer, where it prevents binding of the overlapping transcriptional activator  $\delta$ -EF3 (Sekido *et al.* 1994), and to the E2-box sequence CACCTG (Sekido *et al.* 1994), where it may inhibit the

activity of basic region helix-loop-helix-containing transcription factors.  $\delta$ -EF1 was expressed in most tissues examined (Sekido *et al.* 1996) and appears to be involved in regulating T-cell development (Higashi *et al.* 1997). Future experiments should determine whether or not  $\delta$ -EF1 expression or activity, or both, is influenced by acidosis, and whether it is capable of binding the *apoA<sub>1</sub>* pH-RE.

The present studies show that the acidosis-related reduction in *apoA<sub>1</sub>* promoter activity is also reproduced in the presence of various agents known to alter intracellular pH. The changes observed in promoter activity are mediated by a transcriptional repressor. Future experiments should examine the precise identity of this repressor.

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