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The ATF Site Mediates Downregulation of the Cyclin A Gene during Contact Inhibition in Vascular Endothelial Cells

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Contact inhibition mediates monolayer formation and withdrawal from the cell cycle in vascular endothelial cells. In studying the cyclins—key regulators of the cell cycle—in bovine aortic endothelial cells (BAEC), we found that levels of cyclin A mRNA decreased in confluent BAEC despite the presence of 10% fetal calf serum. We then transfected into BAEC a series of plasmids containing various lengths of the human cyclin A 5′ flanking sequence and the luciferase gene. Plasmids containing 3,200, 516, 406, 266, or 133 bp of the human cyclin A promoter directed high luciferase activity in growing but not confluent BAEC. In contrast, a plasmid containing 23 bp of the cyclin A promoter was associated with a 65-fold reduction in activity in growing BAEC, and the promoter activities of this plasmid were identical in both growing and confluent BAEC. Mutation of the activating transcription factor (ATF) consensus sequence at bp −80 to −73 of the cyclin A promoter decreased its activity, indicating the critical role of the ATF site. We identified by gel mobility shift analysis protein complexes that bound to the ATF site in nuclear extracts from growing but not confluent BAEC and identified (with antibodies) ATF-1 as a binding protein in nuclear extracts from growing cells. Also, ATF-1 mRNA levels decreased in confluent BAEC. Taken together, these data suggest that the ATF site and its cognate binding proteins play an important role in the downregulation of cyclin A gene expression during contact inhibition.

The endothelial cell layer is important in thrombosis, thrombolysis, lymphocyte homing, inflammation, modulation of the immune response, and regulation of vascular tone. Growth inhibition and cell cycle withdrawal mediated by contact, characteristic of most normal cell types, are critical for monolayer formation by vascular endothelial cells both in vivo and in vitro. Yet despite extensive research, little is known about the molecular mechanisms of contact inhibition in these cells (2, 17, 29).

The progress of the cell cycle is regulated by the sequential expression of cyclins and the activation of their associated cyclin-dependent kinases (Cdks) (22, 43). p27, a recently identified inhibitor of Cdk2 and Cdk4, is activated in contact-inhibited cells (36, 38, 39, 45). Therefore, the activation of Cdk inhibitors such as p27 may explain in part the process of contact inhibition. It is just as likely, however, that contact inhibition results from downregulation of Cdk activators like the cyclins. Indeed, the cyclin A gene has been identified by differential screening as a gene downregulated at confluence in Mv1Lu cells (40).

Cyclin A associates with Cdk2 in the S phase of the cell cycle and with Cdc2 in the G_2/M phase, and it is required for DNA replication in the S phase (6, 13, 32, 37, 43, 44). Cyclin A can also affect G_1/S transit, as overexpression of cyclin A overcomes the G_1/S block induced by loss of cell adhesion (16). The genomic organization of the human cyclin A gene was published recently (20, 50). The gene contains eight exons spanning 8 kb of DNA (20), and multiple transcription start sites have been identified by nuclease S1 protection analysis. The cyclin A promoter contains potential regulatory elements that include four Sp1 sites, beginning at bp −193, −170, −138, and +118; one activating transcription factor (ATF) site, also termed the cyclic AMP (cAMP)-responsive element (CRE) (5, 15, 25, 52), at bp −80; two sites that overlap the murine G_1/S-specific transcription factor Yi (8, 9), at bp +108 and +112; and two E2F-binding sites (31), at bp +31 and +165. The cyclin A promoter has been shown to be active in proliferating cells in reporter gene transfection experiments in NIH 3T3 (20) and hamster (48) cells. However, the sequences of the positive regulatory elements mediating cyclin A promoter activity, and their cognate DNA-binding proteins, have not been identified.

In this report, we show that cyclin A mRNA is downregulated by contact inhibition in bovine aortic endothelial cells (BAEC) despite the presence of 10% fetal calf serum. To elucidate the mechanisms of this downregulation, we transfected into BAEC a series of reporter plasmids containing various lengths of the cyclin A 5′ flanking sequence and the luciferase gene. We found that the ATF motif is critical for cyclin A promoter activity in growing BAEC and for cyclin A downregulation in confluent BAEC. Using gel mobility shift analysis, we identified specific protein complexes that bind to the ATF site in nuclear extract from growing but not confluent BAEC. We then identified (with antibodies) ATF-1 as a protein that binds to the ATF site in nuclear extracts from growing cells and determined that ATF-1 mRNA is downregulated in confluent BAEC.

MATERIALS AND METHODS

Cell culture. BAEC were isolated and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone, Logan, Utah) and antibiotics as described previously (26). BAEC were passaged every 3 to 5 days,

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and cells from passages 5 to 7 were used for the transfection experiments and for the preparation of RNA and nuclear extracts. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, Calif.) and grown in medium 199 containing 20% fetal calf serum (HyClone), 100 μg of heparin (Sigma Chemical, St. Louis, Mo.) per ml, and 50 μg of endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, Mass.) per ml (51). HUVEC were passaged every 4 to 5 days, and cells from passages 4 to 6 were used in these experiments. Nuclear extracts from BAEC and HUVEC were prepared as described by Andrews and Faller (1). Protein concentrations in nuclear extracts were measured by the Bio-Rad (Richmond, Calif.) protein assay system, which is based on the Bradford method (3). Cells were counted in a Coulter apparatus (Coulter Electronics, Hialeah, Fla.).

Cells were plated at a density of about 30,000 cells per cm^2 on a 10-cm-diameter dish (surface area, 55 cm^2) for 72 h. For cells to be in confluent stage, the medium was changed to avoid the effect of deprivation of growth factor and the cells were cultured for an additional 24 h before harvesting. Confluence was confirmed by the cell count (200,000 cells per cm^2). For cells to be in the growing stage, they were trypsinized, transferred from the 10-cm-diameter dish to a 15-cm-diameter dish (surface area, 140 cm^2), and harvested 24 h later.

RNA isolation and Northern (RNA) analysis. Total RNA was extracted from growing BAEC with RNAzol B (Tel-Test, Friendswood, Tex.) by the method of Chomczynski and Sacchi (7). A 1.1-kb bovine cyclin A cDNA fragment was amplified by reverse transcriptase-mediated PCR from bovine total RNA (46). The sequences of the forward (5′ AAGAGGACCAGGAGAATATCAA 3′) and reverse (5′ TTTTCTCTTATTGACTGTTGTGC 3′) primers were derived from the mouse and human cyclin A cDNA sequences reported in the GenBank database. PCR fragments were subcloned, and their sequences were confirmed by the dideoxy-chain termination method (42).

Total RNA samples from BAEC were fractionated on 1.3% formaldehyde-agarose gels and transferred to nitrocellulose filters. The filters were hybridized with a randomly primed ^{32}P-labeled bovine cyclin A probe. The hybridized filters were washed in 30 mM NaCl–3 mM sodium citrate–0.1% sodium dodecyl sulfate at 55°C and autoradiographed on film or stored on phosphor screens for 6 to 8 h (34). To correct for differences in RNA loading, the filters were washed in a 50% formamide solution at 80°C and rehybridized with a radiolabeled 18S oligonucleotide. The filters were scanned, and radioactivity was measured on a PhosphorImager running ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Cloning of the human cyclin A gene and construction of plasmids. A 416-bp human cyclin A cDNA fragment was amplified by reverse transcriptase-mediated PCR. Total RNA was extracted from growing HUVEC. The sequences of the forward (5′ CGTGGACTGGTTAGTTGA 3′) and reverse (5′ ATGGCAAAT ACTTGAGGT 3′) primers were based on the published human cyclin A cDNA sequence (49). The 416-bp fragment was labeled and used to screen a cosmid library prepared from human placenta genomic DNA and the vector SuperCos (Stratagene, La Jolla, Calif.) as described previously (10). Cosmid clones were purified and DNA was prepared according to standard procedures (42). Restriction fragments derived from the cosmid clone containing the human cyclin A gene were ligated into plasmid pSP72 (Promega, Madison, Wis.). Nucleotide sequences were determined by the dideoxy-chain termination method (42). A 3,500-bp *Sma*I-*Sma*I restriction fragment (~bp −3200 to +245) and a 761-bp *Sac*I-*Sma*I fragment (bp −516 to +245) of the human cyclin A gene were cloned into the promoterless luciferase reporter plasmid pGL2 basic (Promega) in the appropriate orientation. To generate reporter plasmids containing increasingly shorter segments of the cyclin A promoter sequence, we performed PCR with the following primers: plasmid −406/+205, forward (5′ AACGTGCCCCAGATTT-TAGACC 3′) and reverse (5′ TGCACTCTGCCCAGCCGAC 3′); plasmid −266/+205, forward (5′ GGACAGCCTCGCTCACTAGGTG 3′) and reverse (5′ TGCACTCTGCCCAGCCGAC 3′); plasmid −133/+205, forward (5′ TGCTCAGTTTCCTTTGGTTTACCC 3′) and reverse (5′ TGCACTCTGCCC AGCCGAC 3′); plasmid −23/+205, forward (5′ AACTGCAAGAACAGCCGC C 3′) and reverse (5′ TGCACTCTGCCCAGCCGAC 3′); and plasmid −133/−2, forward (5′ TGCTCAGTTTCCTTTGGTTTACCC 3′) and reverse (5′ GAGC GGCGGCTGTTCTTGCAGTTC 3′). Plasmid −133/+100 was generated by digesting plasmid −133/+205 with the restriction enzyme *Xho*I. All PCR fragments were subcloned into pGL2 basic.

We also used PCR to mutate the ATF motif in the human cyclin A gene as described previously (27). The ATF consensus sequence (TGACGTCA) in plasmid −266/+205 and plasmid −133/+205 was mutated to TGCCCCCA by inserting a primer with three mismatched base pairs: forward (5′ TGAATGCCCC CAAGGCCGCGAG 3′) and reverse (5′ CTCGCGGCCTTGGGGGCATTC A 3′). The sequences of the mutated plasmids mt −266/+205 and mt −133/+205 were confirmed by the dideoxy-chain termination method.

Transfection and luciferase assays. BAEC were transfected with 15 μg of luciferase construct by the calcium phosphate method as described previously (26). To correct for variability in transfection efficiency, we cotransfected 1 μg of pCMV-βGAL (containing the potent cytomegalovirus enhancer and promoter driving the structural gene coding for β-galactosidase) in all experiments. The luciferase assay and β-galactosidase assay were performed as described previously (4, 26), and the ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity. Each construct was transfected at least three times, and each transfection was done in triplicate. Data for each construct are presented as the mean ± standard error.

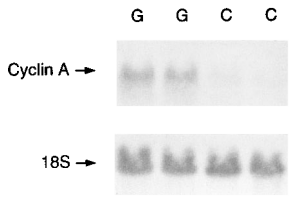

FIG. 1. Downregulation of cyclin A mRNA in confluent BAEC. Total RNA was extracted from growing (G) and confluent (C) BAEC. Northern blot analyses were performed with 20 μg of total RNA per lane, and results from two independent samples of growing and confluent cells are shown. After electrophoresis, the RNA was transferred to nitrocellulose filters, which were hybridized to a ^{32}P-labeled cyclin A probe. The filters were also hybridized with the radiolabeled rRNA oligonucleotide 18S to control for differences in loading.

Gel mobility shift assay. Probes were made from DNA fragments of the human cyclin A gene (−133/−2 probe) or from double-stranded oligonucleotides synthesized according to the sequence of the cyclin A ATF site (bp −84 to −63, 5′ TGAATGACGTCAAGGCCGCGAG 3′). The probes were radiolabeled as described previously (27). A typical binding reaction mixture contained DNA probe at 20,000 cpm, 1 μg of poly(dI-dC)·poly(dI-dC), 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 40 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 3 μg of nuclear extract in a final volume of 25 μl. The reaction mixture was incubated at room temperature for 20 min and analyzed by 5% native polyacrylamide gel electrophoresis in 0.25× TBE buffer (22 mM Tris base, 22 mM boric acid, 0.5 mM EDTA). To determine the specificity of the DNA-protein complexes, we performed competition assays with an unlabeled double-stranded oligonucleotide encoding the wild-type ATF sequence or the mutated ATF sequence. To characterize specific DNA-binding proteins, we incubated nuclear extracts with various antibodies for 12 h at 4°C before the addition of probe. The polyclonal antibodies (Upstate Biotechnology, Inc., Lake Placid, N.Y.) were raised against human ATF-1, ATF-2, Jun, CRE-binding protein (CREB), and phosphorylated CREB.

RESULTS

Downregulation of cyclin A mRNA in confluent BAEC. To determine whether cyclin A mRNA was downregulated in confluent BAEC, we performed Northern analysis with RNA prepared from confluent BAEC or growing BAEC and a bovine cyclin A probe amplified by PCR. Contact inhibition induced by confluence is very consistent in BAEC, and the density of confluent BAEC does not increase above 200,000 cells per cm^2 despite the presence of 10% fetal calf serum. We routinely use this criterion to ascertain the confluence of BAEC. Cyclin A mRNA was readily detectable in growing BAEC (Fig. 1); however, cyclin A mRNA levels were at least fivefold lower in confluent BAEC. In contrast, cyclin D1 mRNA levels did not change significantly in response to confluence (data not shown).

Functional analysis of cyclin A promoter in growing and confluent BAEC. To investigate the mechanisms mediating downregulation of cyclin A gene expression by contact inhibition, we first transfected into BAEC a series of luciferase reporter gene plasmids containing various lengths of the human cyclin A 5′ flanking sequence. Sixteen to twenty hours after transfection, BAEC were trypsinized, transferred from a 10-cm-diameter plate to a 15-cm-diameter plate (to avoid contact inhibition), and harvested 24 h thereafter. Two reporter plasmids, containing bp −3200 to +245 and bp −516 to +245 of the human cyclin A genomic fragment, directed high levels of luciferase activity that were similar to those directed by plasmid pGL2 control (which is driven by the potent simian virus 40 promoter and enhancer) (Fig. 2). Compared with the luciferase activity of plasmid −516/+245, the activities of plasmids −406/+205 and −266/+205 were 80% higher, suggesting

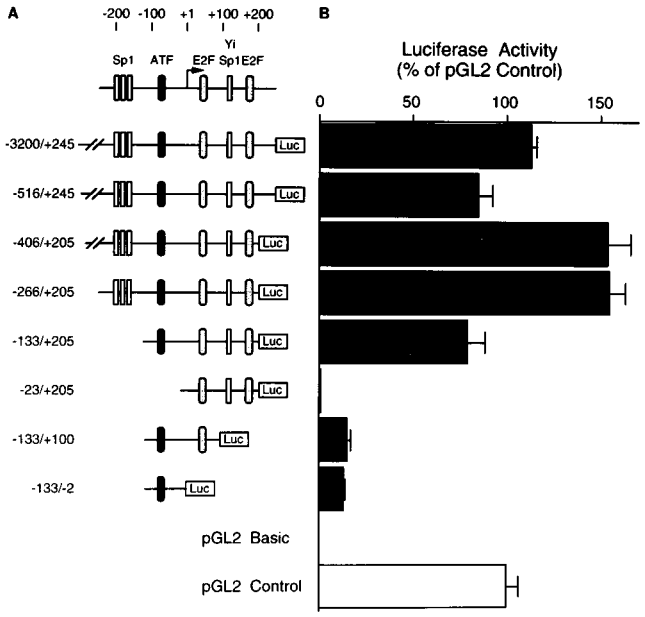

FIG. 2. Deletion analysis of cyclin A promoter activity in growing BAEC. (A) Plasmids containing variable lengths of the 5′ flanking sequence of the cyclin A gene and luciferase (Luc) reporter gene. The transcription start site and *cis*-acting elements are indicated by an arrow and boxes, respectively, as defined by Henglein et al. (20). Each plasmid (15 μg) was transfected into BAEC by the calcium phosphate method. For each construct, the plasmid pCMV-βGAL was cotransfected to correct for differences in transfection efficiency. Luciferase activity and β-galactosidase activities were measured, and relative luciferase units were calculated, as described in Materials and Methods. (B) Luciferase activity (mean ± standard error) of each cyclin A plasmid as a percentage of positive control activity (pGL2 control). The promoterless plasmid pGL2 basic served as a negative control.

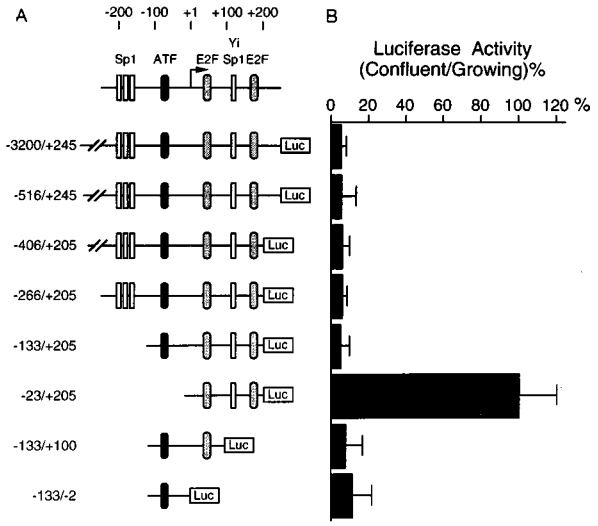

FIG. 3. Downregulation of cyclin A promoter activity in confluent BAEC. (A) BAEC were transfected with the cyclin A plasmids shown in Fig. 2. The transfected BAEC were allowed to become confluent or remain growing. (B) Ratio of luciferase activity in confluent cells to that in growing cells (mean ± standard error).

the presence of silencer elements between bp −516 and −407 or bp +206 and +245. Further deletion of the cyclin A 5′ flanking sequence to 133 bp in plasmid −133/+205, which lacks three of the four Sp1 sites, decreased promoter activity by 45% (relative to the activities of plasmids −406/+205 and −266/+205). Plasmid −23/+205, which contains only 23 bp of the cyclin A 5′ flanking sequence, had a 65-fold-lower luciferase activity in comparison with plasmid −133/+205, indicating the presence of potent positive regulatory elements within bp −133 to −23. Two other plasmids with 3′ deletions, −133/+100 and −133/−2, had luciferase activities five- to sixfold lower than that of plasmid −133/+205. Nonetheless, these two plasmids still directed significant luciferase activity in comparison with the promoterless pGL2 basic. Taken together, the data indicate the presence of positive regulatory elements at bp −266 to −133, −133 to −23, and +100 to +205. Elements within all three areas appear to be necessary for maximal cyclin A promoter activity in growing BAEC; however, elements within bp −133 to −23 may be the most important because deletion of this fragment caused the biggest reduction in promoter activity (Fig. 2; compare −133/+205 with −23/+205).

To determine whether contact inhibition downregulates cyclin A promoter activity through positive elements in the −266/−133, −133/−23, and +100/+205 sequences, we transfected into BAEC the eight plasmids used in the deletion mutant studies described above and harvested cells in the growing or confluent state. For each plasmid, the ratio of luciferase activity in confluent cells versus growing cells was calculated (Fig. 3B). Luciferase activity in confluent BAEC was markedly lower (5 to 8% of that in growing BAEC) for all plasmids containing the cyclin A promoter except plasmid −23/+205. In comparison with the other plasmids, plasmid −23/+205 lacks the sequence within bp −133 to −23 that contains the ATF site (bp −80 to −73). In addition, there was no difference in the luciferase activity of plasmid −23/+205 in confluent and growing BAEC (the ratio of confluent to growing cells is 100.6%; Fig. 3). These data indicate that the sequence between bp −133 and −23 is important in both the downregulation of the cyclin A promoter by contact inhibition in confluent cells and the maintenance of high promoter activity in growing cells. In contrast, the sequences located within bp −266 to −133 and bp +100 to +205 appear to be important only to cyclin A promoter activity in growing cells.

To exclude the possibility that downregulation of cyclin A promoter activity by contact inhibition was the result of a nonspecific suppression of transcription, we also transfected into BAEC a plasmid containing the promoter of endothelin-1 (bp −204 to +150). Endothelin-1 mRNA is expressed in vascular endothelial cells and is not downregulated by confluence. We found that the promoter activity of endothelin-1 in confluent BAEC was no different from that in growing BAEC (data not shown).

Mutation of ATF consensus sequence decreases activity of cyclin A promoter. To determine whether the ATF site (TGACGTCA, bp −80 to −73) is the positive regulatory element within the sequence −133 to −23, we first mutated the ATF consensus sequence in plasmids −266/+205 and −133/+205 to TGCCCCCA to generate the plasmids mt −266/+205 and mt −133/+205 by PCR. The luciferase activities of mt −266/+205 and mt −133/+205 were lower in growing BAEC (Fig. 4). Also, the luciferase activities of these two mutant plasmids in growing BAEC were not significantly different from those in confluent BAEC (data not shown). Thus, the ATF sequence appears to be the positive regulatory element within the −133 to −23 sequence.

Confluence downregulates ATF-binding proteins in BAEC. To characterize the nuclear proteins binding to the positive regulatory element in the sequence −133 to −23, we performed gel mobility shift analysis by using a DNA fragment

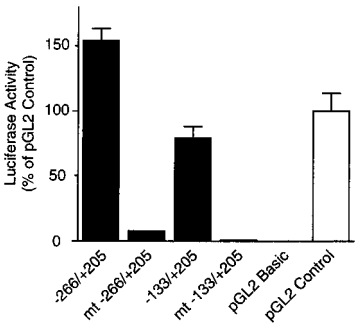

FIG. 4. Mutation of the ATF site markedly decreases the promoter activity of the cyclin A gene. The ATF site in plasmids −266/+205 and −133/+205 was mutated from TGACGTCA to TGCCCCCA by PCR. The wild-type and mutated plasmids were transfected into BAEC, and luciferase activity was measured in growing cells as described in Materials and Methods.

encoding bp −133 to −2 of the cyclin A gene as the probe and nuclear extract prepared from growing or confluent BAEC. Incubation of nuclear extract from growing BAEC with the −133/−2 probe resulted in two specific protein-DNA complexes, A and B (Fig. 5), that were abolished by a 100-fold molar excess of identical nonradiolabeled DNA. A DNA fragment encoding the human cyclin A ATF consensus sequence, but not one encoding the mutated ATF sequence, abolished complex A. Only complex B was present when the probe was incubated with nuclear extract from confluent BAEC, and an excess of unlabeled probe −133/−2 abolished the complex. These data indicate the presence of ATF-binding nuclear proteins in growing but not confluent BAEC. We have also used

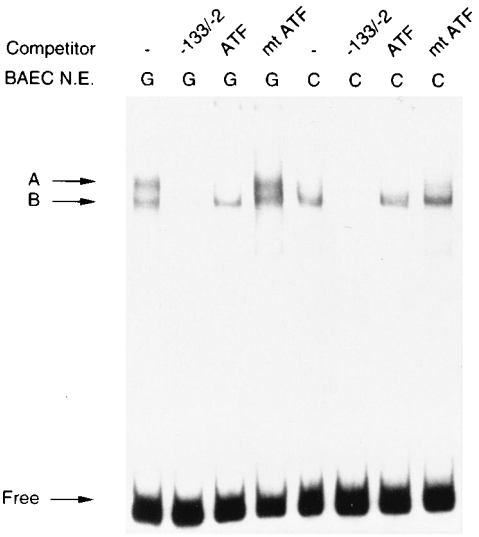

FIG. 5. Downregulation of ATF-binding proteins by confluence in BAEC. Gel mobility shift assays were performed with a ^{32}P-labeled DNA fragment encoding bp −132 to −2 of the human cyclin A gene and nuclear extracts (N.E.) from growing (G) or confluent (C) BAEC. Incubation of the probe (20,000 cpm) with nuclear extract from growing BAEC resulted in two DNA-protein complexes, A and B. The specificity of complex A and complex B binding was confirmed by the fact that a 100-fold molar excess of unlabeled DNA fragment bp −133/−2 abolished both. The 22-bp double-stranded oligonucleotide containing the ATF site (−80 to −73) of the cyclin A promoter (ATF) prevented formation of complex A only. The oligonucleotide with a mutated ATF site (mt ATF) did not compete for binding to complex A. Incubation of the −132/−2 probe with nuclear extracts from confluent BAEC resulted in the formation of complex B only.

DNA fragments encoding bp −23 to +205 of the cyclin A gene as probes in gel mobility shift analysis and observed no difference in the binding patterns of nuclear extracts from growing and confluent BAEC (data not shown). Taken together, these data indicate the ATF site as the most important (if not the only) element regulating cyclin A promoter activity during contact inhibition. However, we cannot exclude elements more 5′ to bp −3200 of the cyclin A gene.

The binding studies described above were performed with a large probe (bp −133 to −2). To further characterize the ATF-binding proteins, we performed gel mobility shift analysis with the smaller, 22-bp probe encoding the ATF consensus sequence. Incubation of nuclear extract from growing BAEC with the 22-bp probe resulted in three specific DNA-protein complexes, X, Y, and Z (Fig. 6A). The three complexes were abolished by an unlabeled oligonucleotide containing the ATF site but not by an oligonucleotide containing a mutated ATF site. In contrast, complex X was not visible, and the density of complexes Y and Z diminished, when the probe was incubated with nuclear extract from confluent BAEC (Fig. 6A). The disappearance of complexes A (Fig. 5) and X (Fig. 6A) in response to confluence indicates that they contain similar, if not identical, nuclear proteins.

To identify the ATF-binding proteins present in complexes X, Y, and Z, we performed gel mobility shift analysis in conjunction with antibodies. Because the antibodies had been raised against human antigens, we used in this set of experiments nuclear extract from growing HUVEC. Three complexes identical to the X, Y, and Z complexes found in BAEC were found in HUVEC (Fig. 6B). Because the ATF site has been shown to bind homo- or heterodimers of proteins of the CREB, ATF, and c-Jun families, we preincubated the nuclear extract with antibodies to ATF-1, ATF-2, c-Jun, CREB, and phosphorylated CREB. Preincubation of growing HUVEC nuclear extract with the antibody to ATF-1 abolished complex X and diminished the density of complexes Y and Z. In contrast, antibodies to ATF-2, c-Jun, and phosphorylated CREB had no effect on the density of these complexes. The anti-CREB antibody completely abolished complexes Y and Z and produced a supershifted DNA-protein-antibody complex (indicated by an asterisk in Fig. 6B). These results suggest the presence of ATF-1 in complexes X, Y, and Z and CREB in complexes Y and Z.

It is noteworthy that the blocking effect of the anti-ATF-1 antibody on complexes X, Y, and Z in growing cells (Fig. 6B) is similar to the inhibitory effect of contact inhibition in confluent cells (Fig. 6A). To support this conclusion, we confirmed that ATF-1 mRNA levels decreased in confluent BAEC (Fig. 7). In contrast, ATF-2, c-Jun, and CREB mRNA levels did not decrease in confluent BAEC, suggesting that this downregulation of ATF-1 mRNA levels is not due to a universal inhibition of gene transcription. Taken together, these data suggest that the ATF site and its cognate binding proteins play an important role in the downregulation of cyclin A gene expression during contact inhibition.

DISCUSSION

In this report, we show that levels of cyclin A mRNA decrease markedly in response to contact inhibition in vascular endothelial cells. This observation is consistent with an earlier report in which cyclin A was identified by differential screening as one of the downregulated genes in confluent Mv1Lu cells (40). Downregulation during confluence of cyclin A mRNA (Fig. 1) but not that of other cyclins such as D1 (data not shown) also suggests that cyclin A, which is important in the

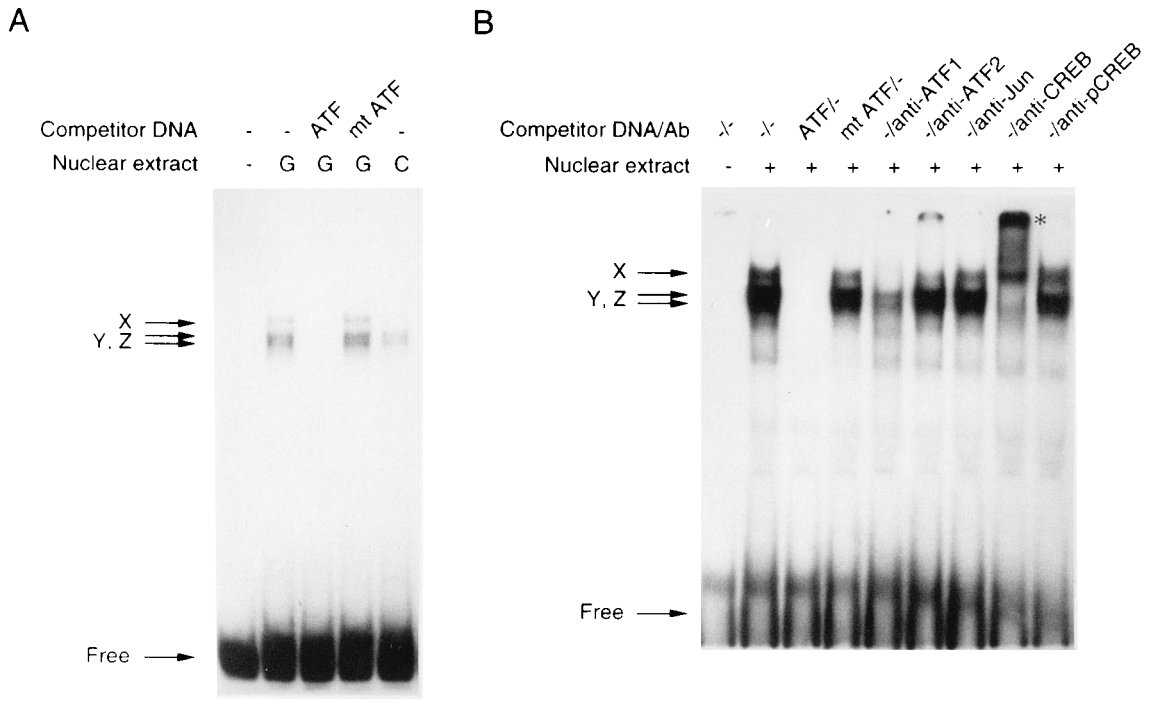

FIG. 6. ATF-1 and CREB are the specific nuclear proteins that bind to the ATF site of the cyclin A promoter in growing endothelial cells. (A) Gel mobility shift assays were performed with a double-stranded, ^{32}P-labeled, 22-bp oligonucleotide containing the ATF site of the cyclin A promoter. Addition of nuclear extracts from growing (G) BAEC resulted in three retarded bands, X, Y, and Z. These three bands were abolished by an unlabeled 22-bp oligonucleotide containing the ATF site (ATF) but not by one containing a mutated ATF site (mt ATF). In contrast, incubation with nuclear extract from confluent (C) BAEC revealed only the two lower bands, Y and Z. (B) Same assay as in panel A. Addition of nuclear extracts from growing HUVEC resulted in three retarded bands, X, Y, and Z. The three bands were abolished by the addition of ATF but not by the addition of mt ATF. Incubation of nuclear extracts with anti-ATF-1, anti-ATF-2, anti-Jun, anti-CREB, and anti-phosphorylated CREB (pCREB) antibodies (Ab) prior to the reaction produced supershifted bands. The anti-ATF-1 antibody erased band X and decreased the intensity of bands Y and Z. However, the anti-ATF-2 and anti-Jun antibodies did not affect the bands. The anti-CREB antibody completely supershifted bands Y and Z.

G_1/S transition and the S and G_2/M phases of the cell cycle (16, 32), may be a target for contact inhibition. The cyclin A promoter has been shown to be active in proliferating NIH 3T3 and hamster cells (20, 48), and the activity of the cyclin A promoter but not the c-*fos* promoter has been shown to decrease in a hamster cell line carrying a mutation of TAF 250 (48). However, the exact sequences of the positive regulatory elements and their cognate DNA-binding proteins have not been identified.

Our reporter gene transfection experiments identified positive regulatory elements important for cyclin A promoter activity in growing BAEC at bp −266 to −133 (containing three Sp1 sites), bp −133 to −23 (containing an ATF site), and bp +100 to +205 (containing two overlapping Yi sites, one Sp1 site, and one E2F site) (Fig. 2). The promoter activities of plasmid −133/+205 (lacking three of the four Sp1 sites) and plasmid −133/−2 (lacking the Yi, Sp1, and E2F sites) were also downregulated by contact inhibition. Although it has been reported that the Yi and the E2F sites are important in the regulation of cell cycle-dependent gene expression (8, 9, 30, 31), our results indicate that these sequences do not appear to be important in suppressing cyclin A gene expression during contact inhibition (Fig. 3). In contrast, our observations suggest that the ATF site within bp −133 to −23 of the human cyclin A gene is important in the downregulation of the cyclin A promoter by contact inhibition (Fig. 3 and 4).

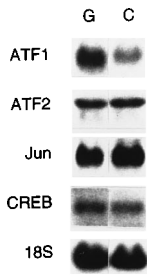

FIG. 7. Downregulation of ATF-1 mRNA in confluent BAEC. Total RNA was extracted from growing (G) and confluent (C) BAEC. Northern blot analyses were performed with 20 μg of total RNA per lane, and the results from two independent samples of growing and confluent cells are shown. After electrophoresis, the RNA was transferred to nitrocellulose filters, which were hybridized sequentially to a ^{32}P-labeled ATF-1 probe (19), an ATF-2 probe (19), a c-Jun probe (27), and a CREB probe (14). The filters were also hybridized with the radiolabeled rRNA oligonucleotide 18S to correct for differences in loading.

ATF sites are present in many viral and cellular promoters (5, 15, 25, 52). Three families of leucine zipper proteins—CREB, ATF, and c-Fos/c-Jun—have been shown to activate or inhibit gene expression by binding to ATF sites as homo- or heterodimers (11, 15, 25, 33, 47, 52). As a consequence of heterodimer formation, transcriptional cross-talk can be expected to occur between different signaling pathways (35). For example, in addition to homodimers, CREB and ATF-1 form heterodimers both in vitro and in vivo (21, 23). Both CREB and ATF-1 can be activated by cAMP and regulate expression of cAMP-responsive genes (14, 41). However, ATF-1 and other members of the ATF family also mediate cAMP-independent transcription of genes containing ATF sites (18, 19, 28).

Although members of the CREB/ATF family have been implicated in the regulation of growth (12, 24), evidence for a direct link between members of the CREB/ATF family and regulators of the cell cycle has not been available. Our results indicate that nuclear proteins binding to the cyclin A ATF site are downregulated by contact inhibition (Fig. 5 and 6A). Using specific antibodies in conjunction with gel mobility shift analysis, we identified ATF-1 as one such protein (Fig. 6B). This identification is further supported by our observation that the mRNA levels of ATF-1 decrease after contact inhibition (Fig. 7). ATF-1 could bind to the cyclin A ATF site as a homodimer, or as a heterodimer with CREB or another unidentified nuclear protein, to regulate the activity of the cyclin A promoter. In any case, our observations establish a direct link between members of the ATF/CREB family and the regulators of the cell cycle. Furthermore, elucidation of the mechanisms downregulating ATF-1 gene expression will provide important insight into the signaling pathways of contact inhibition.

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