E1A dependent up-regulation of c-jun/AP-1 activity

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ABSTRACT

E1A, the early region 1A transcription unit of human adenovirus, exhibits multiple functions that regulate the expression of some cellular genes and promote cell growth and division. We found that E1A stimulated c-jun gene expression at least fifty-fold in rat 3Y1 cells in a serum-independent manner, concomitantly with E1A down-regulation of jun B expression. The E1A-dependent induction of c-jun transcription resulted in increase amount of cJun/AP1. This induction was mediated by the enhancement of the binding activity of the transcription factor cJun/AP1 to an AP1 binding site in the c-jun promoter. Additionally, this induction can be repressed by introducing junB into the cells. Taken collectively, these results suggest that the differential expression of two closely related proteins greatly expands their cellular regulation. Induction of c-jun expression by E1A as well as c-jun autoregulation may amplify the action of E1A during adenovirus infection. Therefore, some of the biological effects of E1A may include mediating the constitutive activation of c-jun, which is important in transcriptional regulation and oncogenic transformation.

INTRODUCTION

The early region 1A (E1A) transcription unit of human adenovirus encodes nuclear phosphoproteins (1,2), which are important in transcriptional regulation and oncogenic transformation (3–6). During the early stages of infection, the E1A 12S and 13S mRNA are the first detectable viral transcripts (7). The products encoded by the E1A transcripts have a pleiotropic effect on the regulation of a selected set of both viral and cellular genes (for review, see ref. 8, 9) and appear to act directly or indirectly through cis-acting promoter or enhancer elements (10–12). E1A activation involves cellular factors and presumably reflects cellular modes of regulation.

Transcription factor AP1 mediates gene induction by phorbol ester tumor promoters (13–15), transforming oncogenes (16,17) and polypeptide hormones (18). AP1 is a complex whose major components are the products of the c-jun and c-fos proto-oncogenes, the cJun and cFos proteins (19–21). cJun is a sequence specific DNA-binding protein recognizing the same sequence motif, termed the TRE, as AP1 (19,22). Two other jun-related genes, termed junB and junD, have been isolated and characterized recently (23–25). These jun-proteins are encoded by different genes, but are clearly related to cJun and bind to the same consensus sequence as cJun/AP1 (26). While cJun/AP1 is an efficient activator of the c-jun and collagenase promoters, which contain a single TRE, JunB and JunD are not efficient activators (27,28). The c-jun gene is positively autoregulated by the binding of its own gene product to an AP1 site in the c-jun promoter (29), and this autoregulation can be repressed by JunB (27). The mRNA levels of c-jun and junB increase rapidly as part of the response of cultured cells to serum, growth factors or tumor promoters (23,30). Along with other 'immediate early' gene products, the cJun/AP1 may play a key role in regulating many genes involved in cellular proliferation (31).

The mechanism by which E1A stimulates transcription is an area of intense investigation. Several studies have suggested that E1A proteins are capable of modulating the activity of cellular transcription factors. In this report, we demonstrate that E1A induces expression of c-jun in a serum-independent manner and down-regulates expression of junB in rat 3Y1 cells. Deletion analysis of rat c-jun promoter and mutation of the AP1 binding site have revealed that the AP-1 binding site is a target for E1A-mediated transcriptional activation of this gene. The consequence of the constitutive activation of c-jun could be a result of the function of E1A mediated activation of cJun/AP1, which is subsequently amplified by the autregulation mechanism in the presence of a low level of the negative regulator JunB. We propose that E1A mediated constitutive activation of c-jun plays an important role in regulating the expression of a set of other genes, such as those in the promotion of cell growth, division and transformation.

MATERIALS AND METHODS

Cell culture and RNA analysis

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO)

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and antibiotics (100 unit/ml penicillin, 50 μg/ml streptomycin). Total RNA (20 μg), prepared by the guanidium isothiocyanate method (32), was separated on a 1.2% agarose gel that contained 6% formaldehyde (33) and transferred to a hybond-N filter (Amersham). The DNA probe was radiolabelled using a random primer kit (Boehringer Mannheim) and [^{32}p]-dCTP (Amersham, 3000μci/mmol). The filters were prehybridized for 5h and hybridized with [^{32}p]-labelled probe for 20h at 42°C in 6×SSC (1×SSC:150mM NaCl, 15mM Sodium citrate, pH7.0), 0.5% Sodium dodecyl sulfate (SDS), 5× Denhardt's solution, 50mM EDTA and 100μg/ml denatured salmon sperm DNA. Filters were washed several times at 65°C in 0.2×SSC and 0.1% SDS, and exposed to RX-film (Fuji film) at −70°C with an intensifying screen.

Plasmids

To construct the −4,500/+874c-junCAT reporter, a 4.5kb HindIII-FspI fragment containing the 5′-regulatory region of rat c-jun genomic DNA (34) was cloned into the HindIII site of pICAT, in which NdeI/HindIII fragment of pSVOCAT was replaced by NotI/ClaI fragment from a multiple cloning sites of pBluescript (Stratagene). The other c-jun/CAT reporters were constructed by various deletion fragments from the 5′-regulatory region of the rat c-jun gene and these fragments were inserted into multiple cloning sites of pICAT. pE1A13Sneo (Ad12 E1A 13S cDNA with a neo resistant gene) was kindly given from Dr. K. Shiroki (Tokyo University). pME1A13Sneo is a framshift mutant of E1A generated by deletion of 4 bases at the KpnI site (+88 to +91 coding sequence for E1A) of pE1A13neo (35). The TRE/tkCAT and tkCAT reporters, and RSV-cjun and RSV-junB expression vectors were described previously (13,27,29). CRE/tkCAT was generated by insertion of a synthetic oligonucleotide containing CRE into BamHI-HindIII site of pBLCAT2. In RSV-mJB an 8bp BglII linker was inserted at the AccI site at position +822 of the junB cDNA.

Transfection and CAT assay

Cells were transfected by a calcium phosphate co-precipitation procedure as described previouly (36). Cells were harvested 60h after transfection. As an internal control for the variation in transfection efficiency, 2.5μg of RSV-β-gal was co-transfected with the test plasmid to normalize the CAT assay. Equal amounts of the cell lysates were incubated with 8μci of [^{14}C]chloramphenicol and 4mM acetyl-coenzyme A at 37°C for 30 min in a total volume of 200μl reaction mixture. [^{14}C]chloramphenicol and its acetylated products were separted by thin-layer chromatography and chromatograms were exposed to x-ray film. The conversion of chloramphenicol to the acetylated form was measured using Bio-image analyzer BAS2000 (Fuji film).

DNA binding assay

Nuclear extracts were prepared as described elsewhere (36). Extracts from 3Y1 or E1AY4 cells were incubated on ice with 2μg of poly(dI.dC) in 10mM Hepes, pH8.0, 17% glycerol, 1mM EDTA, 20mM NaCl, 4mM $MgCl_2$, 1mM dithiothreitol and 0.1mM phenylmethylsulfonyl fluoride (PMSF) in a total volume of 20μl. After 10 min. the [^{32}p]-labeled probe and the competitor oligonucleotides were added and the incubation was continued for another 20 min. The reaction mixture was subjected to electrophoresis on a 5% polyacrylamide gel in 0.5× TBE (44.5mM Tris, 44.5mM Boric acid, 1.25mM EDTA). The gel was dried and subjected to autoradiography.

Cell labelling, immunoprecipitation, and gel electrophoresis

Cell proteins were biosynthetically labeled for 3 hrs by addition of l-[^{35}S]methionine (200 μci/ml, Amersham) to subconfluent 10cm dishes of 3Y1 and E1AY cells cultured in methionine-free DMEM containing 2% dialyzed fetal bovine serum. The supernatant of cell lysates were incubated with 10 μl of anti-cjun-peptide antibody (Ab-2, Oncogene Science) at 4°C for 12 hr, and followed by agitation with 20 μl of protein-A-sepharose (Pharmacia) for 2hr. Cell lysates, immunoprecipitation and gel electrophoresis were prepared as previously described (21). Anti-CREBP1 antiserum was kindly given from Dr. Shunsuke Ishii (RIKEN). E1A1 and E1A^{S} are polyclonal anti-E1A13S C-terminal peptide (amino acids 250−265 and 256−265, respectively) antiserum.

RESULTS

Constitutive activation of c-jun by E1A in rat embryonal fibroblast

It was previously reported that AP1-binding activity is induced to significantly higher levels by cAMP in the presence of E1A proteins (37). To determine which of the known jun genes was responsible for the appearance of AP1-binding activity, we analyzed by Northern blot, RNA extracted from rat embryonal fibroblast 3Y1 cells (38) and E1A transformed 3Y1 cells (E1AY,39) with various jun-specific probes. Surprisingly, the steady state level of c-jun transcripts was strongly induced in the E1AY cells ranging from fifty- to one hundred-fold (Figure 1a, lane 2). The same blot was sequentially rehybridized with probes specific for junB and junD. mRNAs for these genes exhibited different expression patterns from that of c-jun. Expression of junB in wild-type 3Y1 cells was relatively high (at least five- to ten-fold higher than c-jun), but was decreased four- to six-fold in the E1AY cells (Figure 1a, lane 4). The third member of the jun family, junD, notably reached a new steady state level of expression in the E1A transformed cells, However, the magnitude of this induction response was much lower than that of c-jun and did not exceed two- to three-fold (Figure 1a, lane 6). To examine whether a CRE/ATF binding protein CREBP1 (53) and an AP1 complex protein c-Fos are regulated by E1A protein, the same blot was also probed for CREBP1 and c-fos expression. Unlike the jun transcripts, no difference of CREBP1 and c-fos transcripts could be detected between wild-type and E1A transformed 3Y1 cells (Figure 1a, lanes 7−10)

E1AY4 cells display a 3-fold shorter doubling time as compared to wild-type 3Y1 cells. In an attempt to determine whether the increased cell proliferation is mediated by E1A due to the induction of cellular oncogenes that most likely regulate the expression of proliferation-related genes (40), we monitored the levels of several such proto-oncogene transcripts, c-myc, c-myb and c-Ha-ras, in 3Y1 and E1AY cells (Figure 1a, lane 9−14). For example the c-Ha-ras was notably induced by E1A. The induction of ras oncogenes may be responsible for initiation of c-jun gene transcription and lead to activation of c-jun/AP1. The fact that the ras oncoprotein stimulates AP1 activity has been reported previously (16,17).

The growth of E1AY4 cells was less-dependent upon serum than that of wild-type 3Y1 cells (data not shown). The level of c-jun mRNA was not strongly decreased under conditions of serum starvation, whereas the levels of junB and fos mRNA were decreased to an undetectable level (Figure 1b). This result indicates that E1A can activate c-jun expression in a serum- and fos-independent manner.

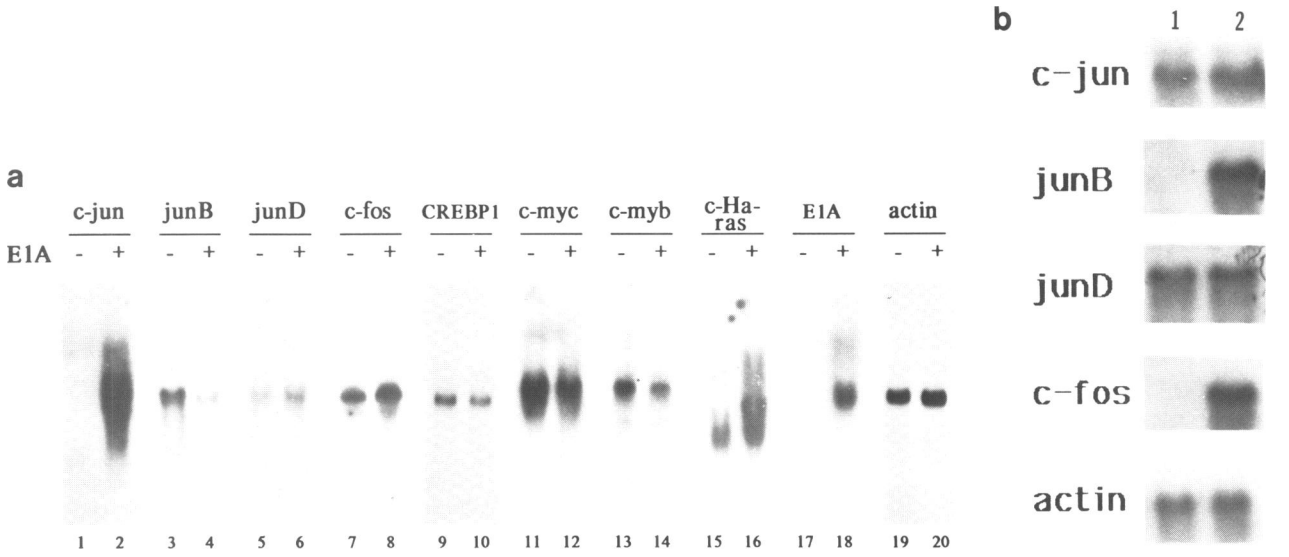

Figure 1. Differential expression of jun genes in E1A transformed rat embryonal fibroblast 3Y1 cells. (a) Total RNA (20μg) 3Y1 (E1A −) or E1AY4 (E1A +) cells were separated on agarose-formaldehyde gels and transfered onto Hybond-N filters. The filters were hybridized with the following specific probes: rat c-jun SacI-FspI fragment (c-jun), rat junB BamHI-SacI fragment (junB), junD probes corresponding to first amino-terminal 93 amino acids of mouse junD (24,25) prepared by polymerase chain reaction (54), v-fos PstI-PvuII fragment (c-fos), 3.7 kb human CREBP1-1 cDNA (CREBP1, 53), human c-myc cDNA (c-myc), v-myb PstI-XbaI fragment (c-myb), v-Ha-ras HindIII fragment (c-Ha-ras), adenovirus type 12 (Ad12) E1A 13S cDNA (E1A) and rat β-actin cDNA (actin). Exposure times were: 12 h for c-jun and c-myc, 20 h for junB, junD, c-Ha-ras and E1A, 60 h for c-fos and c-myb, and 8 h for actin. (b) c-jun expression is serum independent in E1A transformants. E1AY4 cells were maintained in 0.5% serum for 42 h (lane 1) or cells were grown in a standard culture condition with 10% serum (lane 2) before cells were harvested. 20μg RNA samples were analyzed by blot hybridization with c-jun, junB, junD, c-fos, and β-actin specific probes. Exposure times were: 12 h for c-jun, 40 h for junD and c-fos, 72 h for junB, and 10 h for actin.

Coding sequence of E1A is required to induce c-jun expression

Focusing our attention on the constitutive activation of c-jun by E1A, we observed up-regulation of c-jun in all of the tested E1A-transformed cells, but not in a chemical carcinogen induced transformed 3Y1 cell, D303. (Figure 2, Lanes 1−9). It is clear that the induction of c-jun expression in these cells is due to the expression of coding sequences of E1A, regardless of the tumorgenic potential of adenovirus type (type 12 or 5) or the different mRNA species of E1A (12S or 13S). This finding suggests that the induction of c-jun is correlated with the cell transforming activity of E1A (conserved regions 1 and 2), and not simply a result of cellular transformation.

Expression of the E1A 13S product in a G418 selected pool was sufficient to induce c-jun expression in these cells (Figure 2, lanes 10−11). In contrast, the G418 selected pool transfected with a 13S cDNA framshift mutant, pME1A13Sneo (35), did not show stimulation of c-jun expression activity nor did cells transfected with pSV2neo alone (Figure 2, lanes 9,12−13). These results are consistent with a requirement for functional E1A proteins.

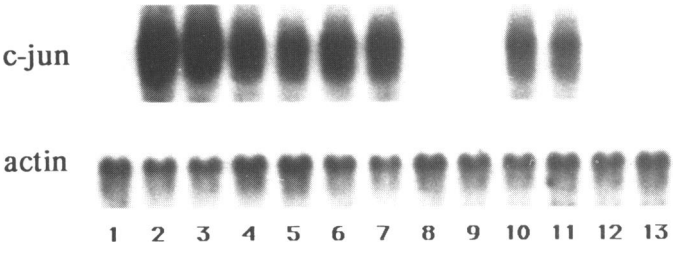

Figure 2. Constitutive activation of c-jun in all E1A transformants. Total RNAs from the following clones (lanes 1−8) and G418-selected pool (lanes 9−13) derived from 3Y1 cells: 1. wild-type 3Y1; 2 and 3, Ad12 E1A transformed cells, E1AY1 and E1AY4, respectively; 4. Ad12 E1A+E1B transformed cells, CYpAdC3; 5. Ad12-E1A 12S cDNA transformed cells, YH12S; 6. Ad12-E1A 13S cDNA transformed cells, YH13S; 7. Ad5-E1A 13S cDNA transformed cells; 8. Nitrosoguanidine-transformed cells, D303; 9. pSV2neo; 10 and 11, pE1A13Sneo; 12 and 13, pME1A13Sneo, a frameshift mutant of pE1A13Sneo generated by deletion of 4 bases at the KpnI site. 20μg of RNA samples were blot hybridized with c-jun and β-actin specific probes.

AP-1 binding site is a target for E1A-mediated transcriptional activation

To further address the question of whether the induction of c-jun expression by E1A is primarily due to transcriptional regulation, we isolated rat c-jun 5′-promoter regions. A reporter construct, −730/+874JunCAT, was co-transfected with an E1A expression vector, pE1A13Sneo or pE1A12Sneo, into rat 3Y1 or mouse F9 cells (Figure 3a). The transient expression of E1A 13S and 12S cDNA led to a significant increase in CAT activity by a factor of 5 to 7 and 3 to 5, respectively.

To assess the role of the various sequence elements in the c-jun promoter in allowing E1A-dependent induction, we employed a variety of rat c-jun promoter constructs fused to the CAT gene in transient transfections. As shown in Figure 4, analysis of 5′ promoter deletions indicated that inducible expression was not affected by deletion to −79, but that there was a sharp reduction to basal level upon further deletion to −62. This would therefore indicate that the AP1 binding site (−63 to −72) is uniquely required for stimulated expression. Mutation of AP1 binding site resulted in severe decreases in E1A-induced transcriptional levels (Figure 3a, 4), further proof that the AP1 binding site is a target for E1A-mediated transcriptional activation. The deletions downstream of the transcription start site did not affect the E1A inducibility of c-jun promoter (Figure 4).

The AP1 binding site in the c-jun promoter diverges from the consensus sequence found in the collagenase promoter by a single

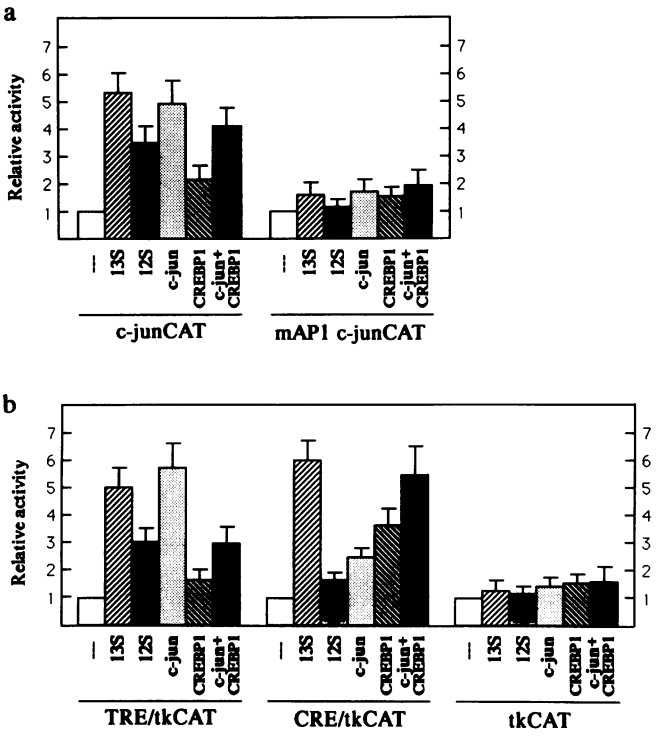

Figure 3. (a) Trans-activation of c-jun promoter-CAT fusion gene by E1A, cJun and CREBP1. 3Y1 cells were co-transfected with 2 μg of −730/+874c-junCAT or −730/+874(mAP1)c-junCAT and 5 μg of indicated expression vectors (p12H13S, p12H12S, RSVc-jun and RSVCREBP1). (b) TRE and CRE differ in E1A-dependent transactivation. TRE/tkCAT, CRE/tkCAT or tkCAT were co-transfected with indicated expression plasmids into 3Y1 cells. Cells were harvested 60 h after transfection and cell lysates were analyzed for CAT activity.

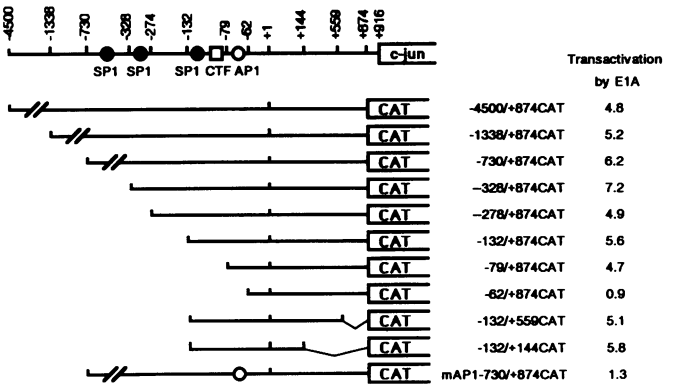

Figure 4. The E1A responsive element in the rat c-jun promoter maps to the TRE element. On the top is an extended map of the rat c-jun promoter, indicating locations of various protein binding sites. Deletion mutants, as depicted schematically below the wild-type −4500/+874CAT construct, were assayed by co-transfection into 3Y1 cells with or without the E1A-expressing plasmid pE1A13Sneo. Cell extracts were assayed for CAT activity. The numbers refer to the 5'and 3' ends of jun sequence. −79/+874 contains the promoter region from position −79 to +874, in which the high-affinity binding site for AP1 (5'-GTGACATCA-3') is included. mAP1 (5'-GATCCATCA-3') −730/+874c-junCAT, in which the AP1-binding site is inactivated by site-directed mutagenesis. The trans-activation by E1A refers to the ratio of CAT enzyme activity in E1A-expressing cells to that in non-E1A-expressing cells and are averages of three different experiments.

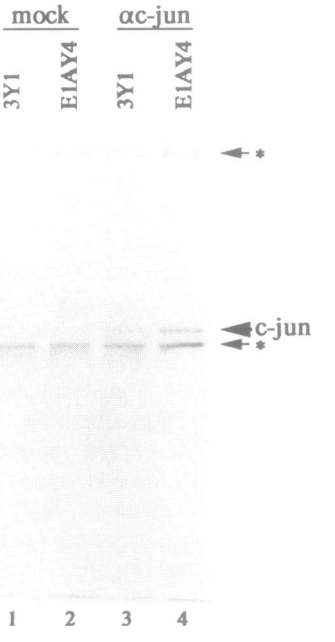

Figure 5. Immunoprecipitation of cJun protein from [^{35}S]methionine labelled 3Y1 or E1AY4 cells. Cell lysates were immunoprecipitated with either anti-c-jun-peptide antiserum or preimmune rabbit serum. The immune complexes were resolved on 10% SDS-polyacrylamide gels. Arrowhead marks the position of cJun protein. Non-specific bands were indicated by a small arrow with asterisk.

base-pair insertion rendering it more similar to a CREB binding site (41,42). The question arises as to whether a specific TRE sequence is necessary for E1A stimulation, or possibly, that CREB/ATF family members bind to this site and activate c-jun expression. The results presented in Figure 3 support the assumption that the TRE element is a regulatory target for E1A control. Co-transfection of pE1A13Sneo with a −730/+874c-junCAT, TRE/tkCAT or CRE/tkCAT resulted in a large stimulation in CAT activity in 3Y1 and F9 cells. However, co-transfection of pE1A12Sneo with a reporter gene as described above resulted in only modest activation of c-junCAT and TRE/tkCAT activity as compared to E1A 13S cDNA. This result suggests that c-junTRE and collagenase TRE are differentially regulated from CRE by E1A. In contrast to the transfection of tkCAT, lacking both a TRE and a CRE, into either cell type 3Y1 or F9, no response was observed due to E1A (Figure 3b). CREBP1/ATF2 mediates transcription by E1A protein has been reported (43). It has also been demonstrated that cJun-CREBP1 complexes have a high affinity for CRE and a low affinity for the AP1 site (44). To test the possibility that homodimer CREBP1 or heterodimer cJun/CREBP1 activates c-junCAT, we co-transfected CREBP1 and c-jun expression vectors or combinations of both with various reporter genes as described above. We found that CREBP1 and cJun-CREBP1 complexes have trans-activation activity through c-junTRE, collagenase TRE and CRE/ATF site. However, it has higher activity mediated through the CRE/ATF site. Interestingly, CREBP1 represses c-Jun dependent activation of both c-jun and collagenase TRE. This repression may be due to the formation of cJun-CREBP1 complexes with a lower affinity to TRE. We conclude that the AP1 binding site in the c-jun promoter is functioning more like a TRE than a CRE. The TRE element in the c-jun promoter is a target for E1A trans-activation and is not needed in conjunction with other targets.

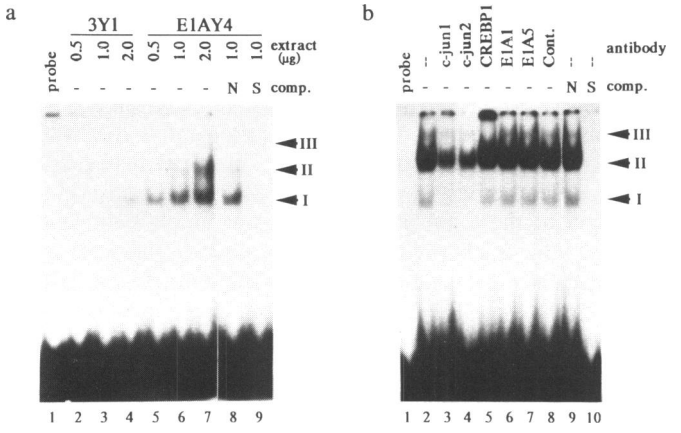

Figure 6. (a) E1A-dependent induction of AP-1 binding activity. A double stranded oligonucleotides (5′-atgttataaagcaTGAGTCAgacacctctctggct-3′) was ^{32}p-end-labeled and used as a probe in a gel mobility shift assay. After incubation of the probe with the indicated increased amount of nuclear extracts (μg) from 3Y1 or E1AY4 cells, the reaction mixture was subjected to electophoresis on a 5% polyacrylamide gel. The specificity of the binding complex was determined by competition with a 50-fold excess of non-radiolabelled mutated AP-1 oligonucleotides (5′-agcttgAATCTCAgaagct-3′, lane 8) or wild-type AP-1 oligonucleotides (5′-agcttgTGAGTCAgaagct-3′, lane 9). In the experiments shown, the free probe has been electrophoresed to the bottom of the gel. (b) The antibody interferes with the specificity of the binding complex. The DNA probe was incubated with 4 μg of E1AY4 nuclear extracts (lane 2) and indicated antibodies (lanes 3−8). Lanes 3 and 4, anti-c-jun-peptide antiserum, Ab-1 (c-jun1) and Ab-2 (c-jun2), respectively; Lane 5, anti-CREBP1 antiserum; Lanes 6 and 7, adenovirus type 12 E1A polyclonal antibodies, E1A1 and E1A5, respectively; lane 9, rabbit preimmune serum. Lanes 9 and 10 were performed by competition with wild type (S) or mutated AP1 (N) oligonucleotides as described above.

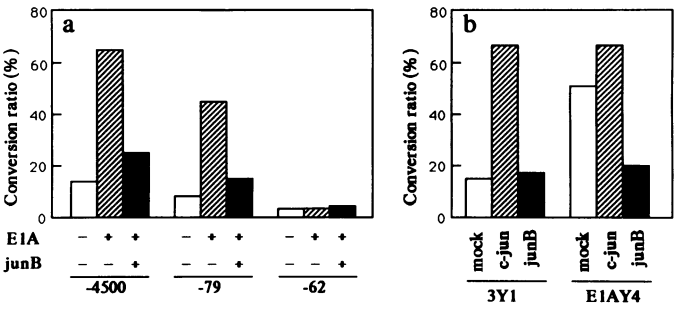

Figure 7. (a) The c-jun promoter is responsive for E1A-mediated transcriptional activation and this activation is repressed by overexpression of junB. Two μg of rat c-jun promoter-CAT gene plasmids −4,5000/+874c-junCAT, −79/+874c-junCAT, and −62/+873c-junCAT was co-transfected with 10 μg of PUC18, 5μg of pE1Aneo and 5 μg of PUC18, or 5 μg of pE1Aneo and 5 μg of pRSVjunB into 3Y1 cells. Cells were harvested 60 h after transfection. Cell extracts were incubated with [^{14}C]-chloramphenicol, and the reaction products were separted by thin-layer chromatography to determine CAT enzyme activity (percent acetylation). (b) JunB inhibits the E1A-dependent trans-activation of c-jun through the AP-1 binding site. 3Y1 and E1AY4 cells were transfected with 2 μg of −79/+874c-junCAT with 5 μg PUC18, 5 μg pRSVc-jun or pRSVjunB. Cells were harvested 60 h after transfection and analyzed for CAT activity (percent acetylation).

E1A-dependent enhancement of AP1 binding and increase in cJun/AP1 synthesis

To extend the studies of the induction of c-jun RNA levels, immunoprecipitations of this protein were performed. Both wild type and E1A transformed cells were labeled with [^{35}S]methionine and immunoprecipitated with polyclonal rabbit anti-cjun-peptide antiserum. Five fold differences in the levels of AP1 were seen in the presence of E1A proteins (Figure 5). This result suggested that E1A up-regulation of c-jun activity results in increases of cJun/AP1 synthesis.

To determine whether the *in vivo* inducibility of the c-jun promoter in the presence of E1A would be reflected *in vitro*, we assayed the DNA-binding activity of nuclear extracts from 3Y1 and E1AY4 cells with respect to the TRE in the human collagenase gene promoter *in vitro*. As shown in Figure 6a, gel retardation assays revealed one (I) and three (I,II and III) AP-1 binding complexes with 3Y1 and E1A transformant E1AY4 nuclear extracts, respectively. The specificity of binding complex was indicated by competition with a 50-fold excess of non-radiolabelled mutated AP1 (N) or wild-type AP1 (S) oligonucleotide probes (Figure 6a, lanes 8,9). Nuclear extracts from E1AY4 cells had a 5- to 10-fold increase of DNA-binding activity to the TRE as compared to the binding activity from wild type cell extracts. To further identify the specific TRE binding protein(s), a specific antibody interference of mobility shift was performed. Addition of two anti-cjun antibodies resulted in eliminatation of complex I and reduction of complex II formation (Figure 6b). Furthermore, addition of anti-CREBP1 and E1A antibodies had no effect on the gel mobility shift (Figure 6b). These results provide the evidence that cJun/AP1 is a TRE binding protein which mediates E1A dependent activation of c-jun expression.

JunB inhibits the E1A-dependent trans-activation of c-jun

One possible mechanism for the induction of c-jun in E1AY cells is the decrease in levels of the negative regulator JunB (see Figure 1a). To examine this possibility, we compared the effects of transient expression of junB and E1A on c-jun promoter and deletion mutant CAT-activities in 3Y1 cells. As shown in Figure 7a, the E1A-dependent activations of −4,500/+874c-junCAT and −79/+874c-junCAT are inhibited by junB expression. More directly, to test whether the transient expression of junB could repress a trans-activation caused by the E1A-dependent constitutively activated c-jun, we co-transfected a junB expression vector with −79/+874c-junCAT reporter gene in 3Y1 and E1AY4 cells. As shown in Figure 7b, overexpression of junB led to repression of the c-jun promoter in E1AY4 cells. This repression did not occur in wild-type 3Y1 cells. A JunB framshift mutant, RSV-mJB, failed to repress E1A-dependent constitutive activation of c-jun (data not shown) indicating that repression by RSV-junB is not due to competition for transcription factors shared by the RSV promoters. These results indicate that the expression of c-jun might be repressed by the junB gene product in wild-type cells, and that the repression would be released through a decrease in the level of the repressor JunB in E1AY cells, resulting in the constitutive activation of c-jun expression.

DISCUSSION

Differential expression of c-jun and junB in E1A transformants

Our results show that c-jun is expressed at very low levels in the wild-type 3Y1 cells, while its steady state mRNA levels are increased more than fifty-fold when the cells are transformed by E1A (Figure 1a, lane 2). In contrast, the junB gene is expressed at high levels in the wild-type cells and is reduced 4- to 6-fold in the E1A transformed cells (Figure 1a, lane 4). We investigated the actual involvement of the junB gene product in controlling

c-jun promoter activity in E1A-transformed cells. As shown in Figure 7b, introduction of junB into E1A-transformed cells resulted in a significant repression of c-jun promoter activity. These data strongly suggest that the differential expression of c-jun/junB is one of the crucial controlling elements to modulate the cellular regulation mediated by cJun/AP1. In E1A transformed cells, repression of c-jun promoter activity by JunB would be released, resulting in the constitutive activation of c-jun. Thus, JunB acts as a repressor of the c-jun gene. This observation is consistent with our previous finding and other reports (27,45). The controlled mechanism of junB expression by E1A is also at the transcriptional level (Kitabayashi et al. unpublished data)

It is known that deregulation of human c-jun expression can participate in malignant transformation of normal mammalian cells (31). On the other hand, overexpression of junB inhibits the c-jun transforming activity (45). Our results regarding the importance of differential expression of c-jun and junB repeatedly suggest that the constitutive activation of c-jun is favorable for cell transformation. This up-regulation of c-jun in E1A transformants results in activation of a set of other genes, which are involved in the promotion of cellular proliferation.

cJun/AP-1 involved in E1A trans-activation

It was unclear whether the c-jun promoter, previously shown to contain TRE, CAAT and SP1 sequences as regulatory elements, was a direct target for regulation by E1A during 3Y1 cell transformation (47). Here we report that the minimum sequence requirement for E1A-dependent transactivation is clearly localized to the AP-1 binding site of rat c-jun promoter acting as a TRE not a CRE. Mutation of the AP1 binding site resulted in severe decreases in E1A-induced transcriptional levels (Figure 3a, 4). Futhermore, immunoprecipitation of AP1 from both 3Y1 and E1AY cells indicated that the amounts of AP1 protein increased 4−5 fold in the E1A transformed cells. The evidence of the specific antibody effect on the mobility shift (Figure 6b) prove that cJun/AP1 is a TRE binding protein which mediates E1A dependent activation of c-jun expression.

Recently, it was demonstrated that levels of junB and c-fos mRNAs were modestly induced by cyclic AMP alone and strongly induced by cyclic AMP plus E1A. However, these increases were not detected by introducing E1A alone (37). In contrast, we found strongly up-regulated c-jun and down-regulated junB in E1A transformants. The reasons for these disparate results are unknown. A possible explanation however, may be due to differences in the cell-types used in the studies. Indeed, Van Dam et al. (46) reported recently that the regulation of the junB gene is dependant upon cell type and transforming adenovirus serotype. A recent report by Offringa et al. (47) suggests that E1A represses trancsription of the collagenase gene via the phorbol ester responsive element, TRE. However, they also demonstrated that E1A activates AP-1 activity in the presence or absence of TPA with either 1×junTRE-CAT or −1100/+740jun-CAT construct (46,47). It is clear that the reponses to E1A-action in HeLa tk^- cells vary from gene to gene even though the action occurs via the same E1A-dependent target DNA element. In addition, Hela cells contain a papilloma virus E7-like protein which may inhibit E1A mediated trans-activation. This could be the reason why we did not observe the strong trans-activation in HeLa cells in either case using a −4,500/+874cjunCAT or −73/+63COL.CAT construct as a reporter gene (data not shown).

Mechanism of E1A-mediated transcriptional activation

Mechanisms of c-jun constitutive activation by E1A have not been fully elucidated, it is quite clear that efficient induction requires the integrity of the AP1 binding site within the c-jun promoter. The possible explanation for this requirement is that initially modification of an existing AP1 or AP1 like activity occurs, and this modified activity then mediates the initial transcriptional activation of the c-jun gene and leads to amplification of the response. One might predict that, once induced, this gene would be permanently activated or overexpressed in the presence of low levels of repressor. The analysis of F9 cell clones in which human cJun and avian vJun are constitutively expressed from the RSV promoter revealed activation of the endogenous mouse c-jun gene, providing strong support for this autoregulation model (28). This autoregulation mechanism may involve modification of pre-existing AP1 activity. E1A might modulate the phosphorylation state of the AP-1 transcription factor, either directly or indirectly. Therefore, the transcriptional activity would be associated with changing DNA binding affinity of modifed AP1. This model is supported by reports that E1A can affect the phosphorylation of E2F, E4F, and TFIIIC transcription factors mediated by E1A induced cellular kinase (48−50).

Constitutive expression of cJun or vJun in F9 cells leads to activation of differentiation inducible genes such as Endo B (K18), tissue plasminogen activator (tPA), and TROMA-1 antigen (28). Analysis of the tPA and Endo B gene has identified AP1 binding sites within their differentiation response elements (51,52). By extrapolation we suggest that constitutive activation of c-jun promotes cell proliferation. Indeed, a number of genes encoding growth regulatory molecules affecting proliferation and differentiation of early embryonic cells have been shown to possess a TRE element. The differential expression of TRE binding proteins during cell transformation mediated by E1A is therefore likely linked to the regulation of these genes. Hence, at least part of the effects of E1A on the rat embryonal fibroblast cells are likely to be mediated by a cascade of transcriptional activities, in which cJun may play one of the final roles through direct binding and activation of various regulatory genes whose expression is induced during this process.

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