Overexpression of cJun enables Rat1a cells to grow in an anchorage-independent manner. We used an inducible cJun system under the regulation of doxycycline in Rat1a cells to identify potential cJun target genes necessary for cJun-induced anchorage-independent growth. Induction of cJun results in sustained expression of cyclin A in the non-adherent state with only minimal expression in the absence of cJun. The promoter activity of cyclin A2 was 4 fold higher in Rat1a cells in which cJun expression was induced compared to the control cells. Chromatin immunoprecipitation demonstrated that cJun bound directly to the cyclin A2 promoter. Mutation analysis of the cyclin A2 promoter mapped the cJun regulatory site to an ATF site at position −80. cJun was able to bind to this site both in vitro and in vivo and mutation of this site completely abolished promoter activity. Cyclin A1 was also elevated in cJun overexpressing Rat1a cells, however, cJun did not regulate this gene directly as it did not bind directly to the cyclin A1 promoter. Suppression of cyclin A expression via the introduction of a cyclin A antisense sequences significantly reduced the ability of cJun overexpressing Rat1a cells to grow in an anchorage-independent fashion. Taken together, these results suggest that cyclin A is a target of cJun and is necessary but not sufficient for cJun-induced anchorage independent growth. In addition, we demonstrated that the cytoplasmic oncogenes Ras and Src transcriptionally activated the cyclin A2 promoter via the ATF site at position −80. Using a dominant negative cJun mutant, TAM67, we showed that this transcriptional activation of cyclin A2 requires cJun. Thus, our results suggest that cJun is a mediator of the aberrant cyclin A2 expression associated with Ras/Src-induced transformation.

cJun is a transcription factor that binds to AP-1 (TGAG/CTCA) sites in the promoters of a large number of genes. It binds to these sites as either a homodimer or a heterodimer together with Fos family members (c-Fos, FosB, Fra-1 and Fra-2) or ATF family members (ATF-1-4, CREB1 or CREM1). cJun is a versatile transcription factor and contributes to transformation and tumor aggressiveness (1,2) as well as cell cycle progression (3), differentiation (4) and apoptosis (5) in a tissue and cell-specific manner. It acts in conjunction with Ras to transform primary cells (6) and is essential for transformation of cells by Ras (7). In addition to being necessary for Ras transformation of cells, cJun is also required for transformation induced by c-fos, raf, c-myc, mos and abl (8).

We have demonstrated previously that cJun induces Rat1a cells to grow in an anchorage-independent manner in soft agar (9). Although a number of cJun target genes have recently been identified (10-14) the “downstream genes” which mediate this pathway are yet to be determined.

In transformed cells, cell cycle-dependent proteins are often aberrantly expressed resulting in abnormal cycling through the various phases of the cell cycle. The cyclins (A, B, D, E) and their associated kinases (cdk2, 4, 6) provide a stimulus for progression through the cell cycle while the proteins p16Ink4a, p19ARF, p21, p27, Rb and p53 all function to inhibit cell cycle progression. Cyclins D1, E and A can function as oncogenes when aberrantly expressed and p19ARF, p53,
p21cip1, p27kip1 and Rb are suppressor genes whose reduction, deletion or inactivation causes transformation (15-18).

Two distinct genes encode cyclin A in mammalian cells, CcnA1 and CcnA2 (19-21). CcnA2 which encodes the originally described cyclin A2, is ubiquitously expressed in cultured cells and in various tissues (21,22) whereas the expression of cyclin A1 seems to be testis-specific and restricted to the germ line (21), although it has been detected in leukemic cell lines (19). Cyclin A2 functions during both G1-S and G2-M phases of the cell cycle (23,24) and binds to and activates both Cdk1 and Cdk2 (25-27). Much less is known about cyclin A1, however it has also been reported to be involved in signaling pathways important for cell cycle regulation (28). A number of oncogenes have been shown to regulate the expression of cyclin A. Its expression is induced by c-myc, and is crucial for c-myc mediated transformation Rat1 cells (29-31). Ras induces cyclin A expression in NIH3T3 cells, promotes cyclin E-cdk2 kinase activity and hyperphosphorylation and inactivation of Rb, all of which may contribute leading to Ras-induced transformation (32). Raf induces cyclin A expression as well as that of cyclin D, cyclin E and p21cip1 with a concomitant decrease in p27kip1 expression (33). c-Fos induces cyclin A expression resulting in cellular proliferation (34). Early studies revealed that cJun expression is necessary for G0/G1 transition or G1 progression (35-37). More recently, cJun was found to alter the expression of cell-cycle-related genes such as cyclin D1 (11), p53 (10) and p21cip1 (38). However, there have been few reports on whether the alteration of expression of these cell cycle related proteins by cJun plays a role in cell transformation.

We have previously described a cellular model system where cJun is inducibly expressed under the control of a doxycycline responsive promoter which induces non adherent cell growth (39,40). Using this cJun inducible system, we examined the effect of cJun on cell cycle regulators to investigate the hypothesis that direct regulation of cell cycle genes by c-Jun is necessary for cJun induction of non-adherent cell growth of Rat1a cells. We demonstrate that all cell cycle proteins only cyclin A demonstrated dysregulation under cJun expression. Further, cJun binds to the ATF site in the cyclin A2 promoter and enhanced cyclin A expression under non-adherent growth conditions. cJun also enhanced cyclin A1 expression under these conditions, although it did not interact directly with the cyclin A1 promoter. Inhibition of cyclin A expression resulted in a decrease in the ability of the cells to form colonies in soft agar, however, overexpression of cyclin A alone did not enable the cells to grow in an anchorage-independent manner. Taken together, these results demonstrate that cyclin A2 is a direct target of cJun and is necessary, but not sufficient, for cJun-induced anchorage-independent growth.

Materials and Methods

Cell Lines, Culture Conditions and Antibodies - Rat1a cells expressing either cJun (Rat1a-J4 and Rat1a-HA-Jun) or green fluorescent protein (Rat1a-GFP) in a doxycycline-controlled manner were established from parental Rat1a cells as previously described (39). Culture conditions were as previously described (14,39,40). The following primary antibodies were used: anti-p27KIP1 (K25020; Transduction Laboratories); anti-cJun (Ab-1; Oncogene Research Products); anti-cyclin D1 (sc-752); anti-cyclin E (sc-247); anti-cyclin A (sc-751 and sc-596); anti-cyclin A1 (sc-15383); anti-cyclin B1 (sc-752); anti-p16Ink4 (sc-1661); anti-p19ARF (sc-7403); anti-p16INK4a (sc-7403); anti-p57KIP2 (sc-8298); anti-cJun (sc-45x), anti-ATF1 (sc-270x); anti-ATF2 (sc-187x); anti-ATF3 (sc-188x); anti-ATF4 (sc-200x); anti-CREB1 (sc-186x) and anti-CREML1 (sc-440x) (all Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Western Blotting - Cell lysates from Rat1a-J4, -HA-Jun and -GFP cells grown in the absence or presence of doxycycline (2µg/ml) were prepared by lysing the cells in RIPA buffer (150mM NaCl; 1% Triton X100; 1% deoxycholate; 0.1% SDS; 10mM Tris, pH7.4; 100µg/ml leupeptin; 100µg/ml aprotinin; 10mM PMSF). Western blotting was carried out as previously described (14,40).

RNA analysis - RNA from Rat1a-J4 and GFP cells was analyzed by northern blotting as previously described (14,40). To determine the cyclin A mRNA half life, Rat1a-J4 cells grown non-adherently for 3 days in the presence or absence of 2µg/ml doxycycline were treated with the
transcription inhibitor actinomycin D (10μg/ml). Total RNA was extracted from the cells 0, 2, 4 and 8 hours after addition of actinomycin D. The RNA was subjected to Northern blot analysis for cyclin A as described above. The intensity of the signal was quantified densitometrically.

**Reporter Constructs and Luciferase Assays**

Cyclin A2 promoter luciferase reporter plasmids -7300/+245, -1048/+245, -406/+205, -266/+205, -133/+205 and mtATF-266/+205 containing a mutated ATF site were kind gifts from Dr B Henglein. The cyclinA1 promoter luciferase plasmids pGL3cycA-1299/+145 and pGL3cycA-190/+145 were a kind gift from Dr C Muller and have been previously described (41). pGL3cycA-129/+145 and pGL3cycA-69/+145 were constructed by PCR using pGL3-190cycA-190/+145 as a template with the upstream primers 5'-CCCCTCGAGCGTGCCCTGCCCTTCCCTGC-3' (pGL3cycA-129/+145) and 5'-CCCCTCGAGCCAACCCTGCCCCGCCCTGC-3' (pGL3cycA-69/+145) and the common downstream primer 5'-TTTAAGCTTGCAGCCGGAGCGCCGTCCCAT-3'. The PCR products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and XhoI/HindIII fragments from the resulting clones were then subcloned into pGL3-basic (Promega, Madison, WI). pcDNA3-Erb1 and RSV-v-Src were kind gifts from L. Neckers and U. Rapp respectively. All transfections were carried out in cells grown non-adherently in the absence and presence of 2μg/ml doxycycline as previously described (14).

**Gel Mobility Shift and Supershift Assays**

Nuclear extracts were prepared from Rat1a-J4, cells grown for 3 days under non-adherent conditions in the presence or absence of 2μg/ml doxycycline as described previously (14,43). The DNA fragments used as probes for cyclin A2 were double-stranded oligonucleotides containing the wild-type ATF site (5'-TGAATGACGTCAAGGCCCGAG-3') and a mutated ATF site (5'-TGAATGCCCCCCAGGCGCCGAG-3'), and that for cyclin A1 was a fragment spanning positions –190 to –129 of the cyclin A1 promoter generated by PCR amplification with primers 1.1F (5'-CCCCTCGAGAAGCCCGGCCCTCCTCCAGG C-3') and 1.2R (5'-GCAGGGAAGGCGAGCCACG-3'). All fragments were 5'-end-labeled with T4 polynucleotide kinase and gel mobility shift and supershift assays were carried out as previously described (14).

**Establishment of Cyclin A Overexpressing Rat1a Cells**

The retroviral human cyclin A expression vector BabeHygro2-HCA2 (a kind gift from B. Amati) was used to establish Rat1a cells stably expressing cyclin A. Briefly, 10μg of the retroviral expression vector was transfected into PhoenixA cells, the supernatant harvested and used to infect Rat1a fibroblasts. 48 hours after infection, the cells were serially diluted and clones selected with 200μg/ml hygromycin B (Calbiochem). Rat1a cells able expressing cyclin A in an inducible fashion were also established as follows. An EcoRI fragment from pCEM4 containing the human cyclin A cDNA was cloned into pBlueScript II SK(+/-). A XhoI-NotI fragment from pBlueScript-cyclin A was cloned into the pLRT retroviral vector to construct pLRT-cyclin A. pLRT-cyclin A was transfected into Rat1a cells using the standard calcium phosphate method and clones selected using blasticidin (8μg/ml).

**Colony Forming Assay**

1.0x10^4 cells were plated in triplicate in 6ml of 0.8% agarose (sea plaque) in complete growth medium in the presence or absence of 2μg/ml doxycycline overlaid on a 0.4% agarose base, also in complete growth medium. 3 to 4 weeks after incubation colonies more than 50μm in diameter were counted using an Omnicon 3600 Image Analysis System. The colonies were visualized after staining for 16-24hr with 1.0 mg/ml p-iodonitrotetrazolium violet.

**Kinase Assays**

Cyclin A associated kinase activity in the cJun expressing Rat1a cells was measured using the cdk1/cdc2 Kinase Assay Kit (Upstate Biotechnology, Charlottesville, VA) as described by the manufacturer.
**Cell Proliferation Assays** - cJun expressing Rat1a cells were seeded at 5000 cells per well in 96 well plates in normal growth medium containing 1% methylcellulose (Sigma) in the presence or absence of 2μg/ml doxycycline, and cell growth was measured by MTT assays.

**Chromatin Immunoprecipitation (ChIP)** - Rat1a-HA-Jun cells expressing hemagglutinin (HA) tagged cJun were seeded at a density of 10 x 10⁶ cells in 150mm PolyHeme-coated tissue culture dishes in the presence and absence of 2μg/ml doxycycline and incubated for 4 days at 37°C. Protein-DNA complexes were cross-linked with 1% formaldehyde added directly to the culture medium at room temperature for 15 minutes followed by the addition of 0.125M glycine, pH 2.5 for 5 minutes. Cells were pelleted at 1500rpm for 5 minutes at 4°C and washed once with ice-cold PBS. The cell pellet was resuspended in 300μl lysis buffer (1% SDS, 5mM EDTA, 50mM Tris, pH 8, 100μg/ml aprotinin, 100μg/ml leupeptin and 1mM PMSF) and incubated on ice for 10 minutes. The solution was then sonicated 3 times for 15 seconds each on maximum power and cell debris pelleted by centrifugation for 5 minutes at 13000rpm. Twenty microliters of the soluble chromatin was set aside as the input fraction and the remainder was diluted 1:10 in dilution buffer (1% Triton X-100, 2mM EDTA, 50mM Tris, pH 8, 100μg/ml aprotinin, 100μg/ml leupeptin and 1mM PMSF). The diluted soluble chromatin (1ml) was pre-cleared with 2μg sheared herring sperm DNA, 20μl pre-immune serum and 45μl protein G-agarose beads (50% slurry in 10mM Tris, pH 8, 1mM EDTA) for 2 hours at 4°C and the beads pelleted by centrifugation at 13000rpm for 1 minute. Two micrograms of antibodies to hemagglutinin (71-5500, Zymed Laboratories Inc., San Francisco, CA) or control rabbit IgG (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA) were added and the solution incubated overnight at 4°C. Following this incubation, 45μl protein G-agarose beads and 2μg sheared herring sperm DNA were added and incubated for an additional 1 hour at 4°C. The beads were pelleted by centrifugation and washed sequentially for 10 minutes each with TSE I (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris; pH 8, 150mM NaCl), TSE II (0.1% SDS, 1% triton X-100, 2mM EDTA, 20mM Tris; pH 8, 500mM NaCl), buffer III (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris; pH 8) and TE (10mM Tris; pH 8, 1mM EDTA). DNA was eluted from the beads with 100μl elution buffer (1% SDS, 0.1M NaHCO₃) and the cross links were reversed by incubation at 65°C overnight. DNA was purified using the Qiaquick PCR Purification kit (Qiagen, Valencia, CA) as per the manufacturers instructions. One microliter of the ChIP DNA was amplified by real time PCR with primers cycA-F (5’- CCTCAGGCTCCCGCTGTAAGATTCC-3’) and cycA-R (5’- TCAAGTACCGCGACTATTGAATAT-3’) on an iCycler Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules, CA) with the Quantitect SYBR Green PCR Kit (Qiagen Inc., Valencia, CA) as per the manufacturers instructions. The fold change was calculated using the 2^ΔΔCT method as previously described (44).

**RESULTS**

cJun maintains the expression of cyclin A in nonadherent Rat1a cells - Overexpression of cJun in Rat1a cells results in non-adherent growth (14,39,40). We hypothesized that this cJun-mediated non-adherent growth might be the result of direct regulation by cJun of cell cycle genes. Western blot analysis was performed in order to determine the levels of various cell cycle-related proteins. The level of cyclin A in Rat1a-J4 cells grown for 3 days under non-adherent conditions in the presence of doxycycline was increased compared to cells grown in the absence of doxycycline or Rat1a-GFP cells (figure 1A). The levels of cyclins D1, E and B1, on the other hand, remained unchanged between the cells overexpressing cJun and control cells. (figure 1A). Expression of p16Ink4a, p19ARF and p57Kip2 remained the same between the uninduced and induced conditions. Similar results were obtained in another Rat1a clone (Rat1a-J2) overexpressing cJun (see supplemental information).

Cyclin A-Dependent Kinase Activity is Increased in Rat1a Cells Overexpressing cJun - To determine whether this increase in cyclin A expression was accompanied by a concomitant increase in cyclin A dependent kinase activity, extracts from Rat1a-
J4 cells grown in the absence or presence of doxycycline were assayed for cyclin A associated kinase activity. Cyclin A associated kinase activity was approximately 3.5 fold higher in the cJun overexpressing Rat1a cells than in the control cells (figure 1B), confirming that the increase in cyclin A expression was accompanied by an increase in its associated kinase activity.

cJun Enhances Expression of Cyclin A at a Transcriptional Level - Time-course experiments showed that the cyclin A protein levels in the control Rat1a cells decreased approximately 2.5 fold over time when the cells were grown in suspension, however when cJun was induced in these cells, cyclin A levels were sustained (figure 2A). This pattern of expression was also observed at the transcriptional level. Northern blot analysis showed that cyclin A mRNA in the cJun overexpressing cells remained elevated compared to cells grown in the uninduced state (figure 2B). In the control Rat1a-GFP cells, cyclin A mRNA rapidly decreased when the cells were grown under non-adherent conditions (data not shown). Analysis of the cyclin A mRNA half life confirmed that the increase in cyclin A expression was transcriptional rather than due to increased stability of the mRNA (figure 3). No difference in cyclin A mRNA half lives was observed between Rat1a-J4 cells in the uninduced and induced states, with both having a half life of approximately 30 minutes (figure 3). A similar result was obtained using the other cJun overexpressing Rat1a clone, Rat1a-J2 (see supplemental information) suggesting that these results are not due to clonal variation.

Since there are 2 distinct cyclin A genes in mammals, CcnA1 and CcnA2, encoding cyclin A1 and cyclin A2 respectively, we analyzed our Rat1a cells to determine whether cJun expression enhanced cyclin A1 as well. Western blot analysis using an antibody specific to cyclin A1 showed that it’s expression was enhanced in Rat1a-J4 cells grown under non-adherent conditions (figure 4). Furthermore, the pattern and time course of induction was similar for that observed for cyclin A2 (compare figures 2A and 4).

The ATF Site in the Cyclin A2 Promoter is Necessary for its Activation in Nonadherent Rat1a Cells Overexpressing cJun - The cyclin A2 promoter contains numerous regulatory elements including a variant AP-1 site (at position –279), consensus ATF (-80), Sp1 (-197, -173 and –142) and E2F sites (+31 and +165) (45). To determine which element in the promoter of cyclin A2 contributed to cJun-mediated expression of cyclin A, luciferase assays using the full-length cyclin A2 promoter and various 5’ deletion and mutant promoter constructs linked to a luciferase reporter gene were performed (figure 5). Luciferase assays using the 7.5 kb DNA fragment of the cyclin A2 promoter showed that the promoter activity was approximately 2-3 fold higher in the Rat1a-J4 cells overexpressing cJun compared to that in the control cells. Deletion of the regions between -7300 and -133 showed no significant decrease in this enhanced promoter activity (figure 5). Mutation of the ATF site at position –80 in the –266/+205 deletion construct though, completely abolished the increase in promoter activity observed in this construct containing an intact ATF site in Rat1a-J4 cells overexpressing cJun (p< 0.05) (figure 5). Thus, the ATF site is crucial for cjun-mediated transcriptional activation of cyclin A2 in Rat1a-J4 cells grown under non-adherent conditions. Similar results were obtained using Rat1a-J2 cells (see supplemental information).

cJun Binds Directly to the ATF Site in the Cyclin A2 Promoter - To determine whether cJun binds directly to the cyclin A2 promoter at the ATF site at position -80, gel mobility shift assays were performed using an end-labeled oligonucleotide containing the wild-type ATF site in the promoter of cyclin A2 (figure 6). Specific DNA-protein complexes are indicated on figure 6 (*). An additional complex was formed by the nuclear extracts from the non-adherent cells induced to express cJun as compared to the control cells (see asterisks in lanes 1 and 11 in figure 6). Incubation of the nuclear extracts with unlabelled wild type oligo resulted in a significant loss of binding, whereas incubation with a mutated oligo did not alter binding of the complexes to the DNA, suggesting that the complexes formed were specific (figure 6). In addition, a supershifted complex was observed when the nuclear extracts from the cJun overexpressing cells were incubated with an antibody to ATF2 (♦ in figure 6). This supershifted complex was absent in the control
cells. No altered supershift pattern from the uninduced state was observed with antibodies against, ATF1, ATF3, ATF4, CREB1 and CREM1 (figure 6). These results indicate that cJun binds directly to the ATF site in the cyclin A2 promoter in conjunction with ATF2. Similar results were observed with nuclear extracts from Rat1a-J2 cells (see supplemental information).

To confirm that cJun interacts directly with the cyclin A2 promoter in vivo, chromatin immunoprecipitation (ChIP) assays with an antibody against hemagglutinin (HA) were performed using Rat1a cells stably transfected with HA-tagged cJun grown non-adherently for 4 days in the presence and absence of 2µg/ml doxycycline, and primers flanking the ATF site (figure 5) (this clone was used since use of a cJun antibody for ChiP analysis did not yield consistent results). Western blotting using antibodies to cyclin A2 and hemagglutinin (to detect the HA-tagged cJun) confirmed that the expression of cyclin A2 and cJun in these cells was comparable to that in Rat1a-J4 and J2 cells (figure 7A). cJun is bound to the cyclin A2 promoter and in the presence of doxycycline, this binding is enhanced (figure 7B). There was approximately a 2-3 fold (p < 0.05) induction of binding at this site when cJun expression was induced, as determined by quantitative real time PCR (figure 7C).

cJun Indirectly Regulates the Cyclin A1 Promoter
- The cyclin A1 promoter has a putative AP-1 binding site at position –231 to –223 (46). To determine whether cJun was also able to activate transcription from the cyclin A1 promoter, Rat1a-J4 cells were transfected with a cyclin A1 promoter luciferase construct spanning positions –1299/+145. cJun activated transcription from this construct in cells grown under non-adherent conditions (figure 8A). Deletion of –1299 to –190 did not affect promoter activity, suggesting that cJun-induction of this promoter does not occur through the putative AP-1 binding site. Deletion of –190 to –129 resulted in a significant decrease in promoter activity (figure 8A). A further deletion of –190 to –69 did not result in any further decrease in promoter activity (figure 8A). Although the –129/+145 and –69/+145 deletion constructs resulted in a significant loss in promoter activity, they still showed some responsiveness to cJun, although it was not as pronounced as the –190/+145 construct. These results suggest that elements between –190 and –129 of the cyclin A1 promoter are required for cJun-induced cyclin A1 expression in Rat1a cells, and further elements between –129 and +145 may also play a role. Since there are no AP-1 or ATF binding sites within these regions, these results suggest that cJun is acting indirectly on this promoter.

To determine whether cJun binds to the cyclin A1 promoter, electrophoretic mobility shift assays were performed using a fragment spanning –190 to –129 of the cyclin A1 promoter and nuclear extracts from Rat1a-J4 cells grown non-adherently for 3 days. A single complex was formed which was enhanced when the cells were induced to express cJun (figure 8B). This complex did not contain cJun since addition of an anti-cJun antibody did not result in a supershift, suggesting that cJun is not a component of this complex (figure 8B). These results, together with the luciferase assays, suggest that cJun indirectly enhances expression of cyclin A1.

Expression of Cyclin A is Necessary but not Sufficient for Anchorage-Independent Growth of Rat1a Cells Overexpressing cJun - Previous reports have shown that overexpression of cyclin A allows several untransformed cell lines, to grow in an anchorage-independent manner (29,47). Thus, to determine whether overexpression of cyclin A2 allows Rat1a cells to grow in a non-adherent manner, we infected Rat1a cells with a retroviral vector containing the cyclin A2 cDNA. Cyclin A levels in the resulting infected clones were comparable to those in the non-adherent Rat1a-J2 and Rat1a-J4 cells induced to express cJun (figure 9A). These clones failed to proliferate in suspension (data not shown) and furthermore, failed to form colonies in soft agarose (figure 9B). Similar experiments were performed using the BabeHygro2-HCA2 cyclin A expression vector after selection of clones in hygromycin B and similar results were obtained (data not shown). These data indicate that overexpression of cyclin A alone is not sufficient to allow Rat1a cells to grow in an anchorage-independent fashion. Inhibition of cyclin A expression, however, resulted in a reduction in anchorage-independent growth. Transfection of a cyclin A antisense construct into Rat1a-J4 cells reduced the number of colonies formed in soft agarose after induction.
of cJun (figure 10A) and also decreased their ability to grow in suspension (figure 10B). Western blot analysis confirmed that expression of cyclin A was reduced by the antisense construct (figure 10C). Taken together, these results imply that cyclin A is necessary, but not sufficient, for cJun-mediated anchorage independent growth.

The Cytoplasmic Oncogenes Ras and Src Transcriptionally Activate Cyclin A2 via the ATF Site (-80) and this is Dependent on cJun - The cJun/AP-1 complex is known to be critical for mediating the downstream effects of many cytoplasmic oncogenes. Since Ras and Src are well characterized cytoplasmic oncogenes known to transform fibroblasts (48,49) and induce non adherent cell growth (32,50,51), we tested whether Ras and Src would transcriptionally activate the cyclin A2 promoter. Transient transfection of Rat1a cells with the -266/+205 cyclin A2 promoter construct and an activated Ha-Ras (12V) expression construct resulted in a 2-3 fold increase (p < 0.05) in promoter activity as compared to control cells (figure 11A). This increase in promoter activity in the presence of activated Ras was not observed when the –266/+205 cyclin A2 promoter construct containing the mutated ATF site was used (figure 11A). In order to determine whether cJun is required for this Ras-induced increase in cyclin A2 promoter activity, Rat1a cells were transiently co-transfected with the –266/+205 cyclin A2 promoter construct and an activated Ha-Ras (12V) expression construct resulted in a 2-3 fold increase (p < 0.05) in promoter activity as compared to control cells (figure 11A). This increase in promoter activity in the presence of activated Ras was not observed when the –266/+205 cyclin A2 promoter construct containing the mutated ATF site was used (figure 11A). In order to determine whether cJun is required for this Ras-induced increase in cyclin A2 promoter activity, Rat1a cells were transiently co-transfected with the –266/+205 cyclin A2 promoter construct and activated Ras together with TAM67, a dominant negative cJun. The enhanced cyclin A2 promoter activity observed in the presence of activated Ras was abolished by TAM67, but not by the CMV vector control (figure 11B). A similar increase in cyclin A2 promoter activity (p < 0.01) was observed when Src was co-transfected with the cyclin A2 promoter (figure 11C). cJun was also required for this Src-induced cyclin A2 promoter activity since the increase was blocked (p < 0.05) by TAM67 (figure 11D). Co-transfection of the cyclin A2 promoter with the EGF receptor (EGFR) also resulted in an increase in promoter activity (figure 11E) although this was not statistically significant. This increase was also blocked by TAM67 (figure 11F). One possible reason for the marginal increase observed with EGFR is that Rat1a cells already express EGFR and any additional EGFR would not result in a significant increase in activation of this receptor. These results suggest that upregulation of cyclin A2 by the cytoplasmic oncogenes Ras and Src requires the ATF site (-80) in the cyclin A2 promoter and is dependent on cJun.

DISCUSSION

We have previously reported that aberrant expression of human cJun was sufficient to allow Rat1a cells to grow in an anchorage-independent fashion (9). The mechanism(s) by which cJun induces non-adherent growth of Rat1a cells remains unknown. The selective regulation of “downstream” target genes is critical for this process.(14,39,40). The data presented in this paper show that cyclin A is one of the mediators of this process. Overexpression of cJun resulted in an increase in cyclin A expression in Rat1a cells grown in suspension which was accompanied by a concomitant increase in cyclin A associated kinase activity. This cJun-mediated increase in cyclin A expression was due to both direct and indirect induction of cyclin A2 and cyclin A1, respectively.

There is substantial evidence supporting a critical role for cyclin A in cellular transformation and human malignancies. Elevated levels of cyclin A2 have been found in various tumor models as well as human tumors (52-55). Several studies have also showed that deregulated expression of cyclin A is associated with oncogenic transformation and anchorage-independent growth (56,57). Cyclin A is a target of adhesion-dependent signals, and consistent with this, adhesion-independent expression of cyclin A is sufficient for anchorage-independent cell growth (47,50). Furthermore, elevated cyclin A expression is also associated with c-myc-dependent transformation of Rat1a cells (29). Deregulated cyclin A expression is also thought to be a crucial factor for transformation mediated by several oncoproteins such as Ras and HPV E7 (32,58). The mechanism(s) by which these oncogenes and signal transduction pathways deregulate cyclin A2 expression remains unknown. There has been no evidence thus far, however, that cyclin A is a direct target gene of these oncogenes during transformation. Multiple signaling pathways contribute to Ras-induced cell transformation (32). Our results demonstrate that cyclin A2 is a direct
target of cJun with the ATF site (-80) being a crucial regulatory element. Our results also show that this cJun-induced cyclin A2 expression via the ATF site at position –80 is necessary for the aberrant cyclin A2 expression associated with Ras/Src-induced transformation.

Cyclin A1 is also activated by oncogenes and its overexpression has been associated with the transformed phenotype (59). c-Myb activates the cyclin A1 promoter and induces cyclin A1 expression (59). Overexpression of cyclin A1 has also been observed in acute myelocytic leukemias (60,61), testicular germ cell tumors (62) and is thought to play a role in breast cancer (63). These studies support a role for cyclin A1 in transformation, and our results suggest that it may be one of the target genes that play a role in cJun-induced anchorage independent growth in Rat1a cells. Our results suggest that rather than acting on the putative AP-1 site in the cyclin A1 promoter, cJun indirectly enhances expression of this gene through transcription factor binding sites downstream of the AP-1 site (figure 8). We and others have shown that cJun can indirectly regulate gene expression by interacting with other transcription factors (39,64). Previous work from our laboratory has shown that cJun enhances stathmin promoter activity through 2 E2F sites (39). Other studies have shown that both cJun and vJun increase E2F activity by deregulating phosphorylation of the retinoblastoma protein (10,65). The human cyclin A1 5’ upstream region contains an E2F site between position +142 and +145 (41), a region encompassed by our promoter constructs (figure 8A). Activation of E2F by cJun and its consequent interaction with the cyclin A1 promoter may, in part, be responsible for cJun-induced activation of the cyclin A1 promoter. In addition, the region spanning –190 and –129 which has enhanced binding of a complex in cJun overexpressing cells (figure 8) contains putative binding sites for numerous transcription factors including, c-Rel, Elk-1, Oct-1 and NF-kB, and cJun may indirectly activate the cyclin A1 promoter by enhancing expression of one of these transcription factors.

Several studies have shown that cyclin A2 is a target gene for other family members of the AP-1 transcriptional complex such as cFos (34,66) and ATF (67,68) in other cell types including chondrocytes and osteoblasts, as well as CREM and CREB (69). These studies also showed that the E2F site in the cyclin A2 promoter was required for this cFos-induced activation. In our current study, the cJun-induced increase in cyclin A2 expression was due to direct binding of cJun to the ATF site in the cyclin A2 proximal promoter. Furthermore, induction of cJun resulted in a change in the composition of the AP-1 complex binding to the ATF site. Previous studies have shown that cJun:Fra2 dimers increase cyclin A expression in NIH 3T3 cells by binding to the CRE element in the cyclin A promoter (70), and these cells are capable of growing under conditions of serum deprivation. Our results indicate that cJun dimerizes with ATF2 and interacts with the ATF site in the cyclin A2 promoter to increase its expression in anchorage independent growth (figures 6 and 7). Taken together, these data suggest that depending on the cell system used, different AP-1 dimers may be involved in the activation of cyclin A during cJun induced non adherent cell growth, and consequently different biologic phenotypes may result.

AP-1-induced cellular transformation may be mediated by different members of the Jun proteins. We have shown that the different Jun family members induce differential non-adherent cell growth of Rat1a cells (40). Thus it can be expected that some of the mechanisms and pathways of transformation brought about by the different Jun proteins will be shared. This is true for cyclin A2 whose induction parallels the degree of non-adherent cell growth induced by the different Jun family members (data not shown). Additional studies from other laboratories support this result. JunB has recently been shown to enhance mouse cyclin A expression via the CRE element in the cyclin A promoter (71). This result is not surprising since it has been shown that Jun B can substitute for cJun with respect to development as well as gene transcription and cell proliferation (72). Thus, although the exact phenotypes of cJun and JunB overexpression are not identical, there may be some common aspects to the mechanisms of transformation induced by these 2 proteins.

Ectopic expression of cyclin A in our Rat1a cells did not result in anchorage-independent growth. Previous studies have shown that overexpression of cyclin A is sufficient for
anchorage-independent growth of NRK and Rat1a cells (29,47). The reasons for this discrepancy between these published reports and our data are not clear, but may include a difference in cyclin A expression vectors and systems used to establish the cyclin A overexpressing cell lines in the different studies. We used 2 different retroviral vectors (BabeHygro2-HCA2 and pLRT-cyclin A) and the levels of cyclin A expression achieved were comparable to those observed in the cJun overexpressing Rat1a cells. Although the level of cyclin A expression in the stable cyclin A clones was comparable with those obtained in the cJun overexpressing cells, other factors induced by cJun in these cells may contribute to anchorage-independent growth and alleviate the need for such a high level of cyclin A. We have previously shown that both stathmin and HMGI/Y are necessary for cJun-induced anchorage independent growth in Rat1a cells, but alone are not sufficient to induce this phenotype (14,39). Thus it is likely that a set of genes is required to fully recapitulate anchorage-independent growth observed in cJun overexpressing Rat1a cells.

cJun forms a variety of dimers with either itself or other members of the AP-1 family, resulting in complexes with different sequence specificities. These complexes bind to various sites in the promoters of numerous genes, making it difficult to identify specific transformation relevant genes. In all likelihood, a number of target genes are necessary for cJun-induced biology. Using our inducible Rat1a cell system we have recently identified HMG-I/Y as a direct cJun target gene that is necessary, but not sufficient for anchorage independent growth (14). In this study, we have identified cyclin A2 as another cJun target gene that is also necessary, but not sufficient for cJun-mediated anchorage independent growth. It is likely this inducible system will identify additional cJun induced genes which are necessary for non-adherent cell growth.

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**FOOTNOTES**

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**FIGURE LEGENDS**

Fig. 1. Expression of various cell cycle regulators after cJun induction in nonadherent Rat1a-J4 cells. (A) Exponentially growing Rat1a-J4 cells were plated at a density of 1.5x10^6 per 150mm PolyHEMA-coated dish in the absence (-) or presence (+) of doxycycline (2µg/ml) and incubated for 3 days. The cells were lysed and the protein extracts subjected to Western blotting using antibodies against cyclin D1, cyclin E, cyclin A, cyclin B1, p16Ink4a, p19ARF, p27kip1, p57kip2, cJun and β-Tubulin. Rat1a-GFP cells expressing GFP were used as negative control. (B) Whole cell extracts were prepared from Rat1a-J4 cells grown in the absence (-) or presence (+) of doxycycline (2µg/ml) and their histone H1 phosphorylating
activities determined in vitro. Kinase activity is represented as the amount of radioactive phosphate incorporated into histone H1/min. Error bars represent SD.

**Fig. 2.** cJun enhances cyclin A expression in Rat1a-J4 cells grown under non-adherent conditions. Rat1a-J4 and GFP cells were grown in suspension in the absence (-) or presence (+) of 2µg/ml doxycycline. Proteins and RNA were extracted at the indicated times and analysed for cyclin A expression by Western (A) or Northern (B) blotting respectively.

**Fig. 3.** The cyclin A2 mRNA half life remains unchanged after induction of cJun. Rat1a-J4 cells were maintained under non-adherent growth conditions for 3 days in the absence (-) or presence (+) of 2µg/ml doxycycline after which actinomycin D (10µg/ml) was added for the indicated times. RNA was then isolated and subjected to Northern blot analysis. The intensity of the bands was quantitated densitometrically.

**Fig. 4.** cJun enhances cyclin A1 expression. Proteins were isolated from Rat1a-GFP and J4 cells grown in suspension for the indicated times in the absence (-) or presence (+) of 2µg/ml doxycycline and analyzed for cyclin A1 expression by western blotting with a cyclin A1 specific antibody.

**Fig. 5.** cJun activates the cyclin A2 promoter. Rat1a-J4 cells were grown in the absence or presence of 2µg/ml doxycycline and transfected with the indicated cyclin A2 reporter constructs and incubated for 4 days non-adherently after which luciferase activity was determined. The luciferase activities are reported relative to the uninduced cells after normalizing the luciferase signals to the signal obtained from the co-transfected Renilla luciferase. The figure represents the mean ± SD of at least three independent experiments performed in duplicate.

**Fig. 6.** cJun binding at the cyclin A2 promoter. Electrophoretic mobility shift assay using the ATF sequence of the cyclin A2 promoter as a probe with nuclear extracts prepared from Rat1a-J4 cells grown for 3 days in the absence (-) or presence (+) of 2µg/ml doxycycline, incubated with various antibodies. Competition with the unlabelled wild-type ATF sequence (wt ATF) confirmed the specificity of the DNA-protein complexes, whereas competition with an unlabelled mutated ATF sequence (mut ATF) failed to cause a reduction in complex formation. (*) indicates the specific DNA/protein complexes and (♦) indicates the additional supershifted complex observed by incubation with an ATF2 antibody in the nuclear extracts from the cJun overexpressing cells.

**Fig. 7.** cJun binds to the cyclin A2 promoter in vivo. Protein extracts were prepared from Rat1a-HA-Jun cells grown non-adherently for 3 days in the absence (-) and presence (+) of 2µg/ml doxycycline and analyzed by western blotting for the expression of cyclin A2 and hemagluttinin-tagged cJun (A). β-tubulin was used as a control for protein loading. (B), chromatin immunoprecipitation (ChIP) using soluble chromatin prepared from Rat1a-HA-Jun cells incubated non-adherently for 4 days in the absence (-) and presence (+) of 2µg/ml doxycycline. The soluble chromatin was immunoprecipitated using antibodies to HA and a control IgG and PCR was performed with primers spanning the ATF site in the cyclin A2 promoter. (C), the increase in binding between the uninduced and induced states was determined by Quantitative Real Time PCR after correcting for the PCR products obtained using the control IgG.

**Fig. 8.** cJun indirectly activates the cyclin A1 promoter. (A), Rat1a-J4 cells were transfected with the indicated cyclin A1 promoter luciferase constructs and incubated for 48 hours under non-adherent conditions. Results represent relative luciferase activity after normalizing to Renilla luciferase to correct for transfection efficiency. The figure represents the mean ± SD of triplicate experiments. (B), gel mobility shift assay using a labeled probe spanning from −190 to −129 of the cyclin A1 promoter and
nuclear extracts from Rat1a-J4 cells grown non-adherently for 3 days in the absence (-) and presence (+) of 2µg/ml doxycycline. The arrow represents the DNA-protein complex formed. This complex was not supershifted when the extracts were incubated with an antibody to cJun (compare lanes 1 and 2, and lanes 3 and 4).

**Fig. 9.** Overexpression of cyclin A2 alone is not sufficient to confer anchorage-independent growth on Rat1a cells. Rat1a cells were infected with the retroviral cyclin A expression vector pLRT-cyclin A and infected cells were selected with blasticidin to obtain stable clones. (A), pools and clones were subjected to Western blot analysis after 3 days treatment with 2µg/ml doxycycline to determine the level of cyclin A expression. (B), soft agarose colony formation of Rat1a cells stably expressing cyclin A (pLRT-cyclin A) compared to Rat1a-J4 cells.

**Fig. 10.** Antisense cyclin A2 suppresses non-adherent growth and colony formation of Rat1a-J4 cells. Rat1a-J4 cells were co-transfected with a retroviral vector expressing a region of the 3’ end of the cyclin A mRNA in an antisense orientation with pSV2-Neo, and a number of clones were selected in G418. (A), Rat1a-J4 cells and a number of clones expressing the antisense construct were grown for 14 days in soft agarose in the presence or absence of 2µg/ml doxycycline, after which the number of colonies formed was counted. The colony size is shown on the right of the figure. (B), cells were also grown in PolyHEMA-coated dishes in the absence or presence of doxycycline, and cell growth determined at the indicated times by MTT assay. (C), protein lysates from Rat1a-J4 cells and the indicated cyclin A antisense clones grown for 7 days in suspension in the presence or absence of doxycycline were analysed by Western blotting for cyclin A. Relative expression of cyclin A is represented in the bar graph after normalization to β-Tubulin expression.

**Fig. 11.** Activation of the cyclin A promoter by the Ras signaling pathway occurs via the ATF site. Rat1a cells were transiently co-transfected with the indicated cyclin A promoter reporter constructs and expression vectors for (A) activated HA-Ras (12V), (C), v-Src and (E) EGFR and incubated under non-adherent growth conditions for 48 hours. Results represent relative luciferase activity after normalizing to renilla luciferase to correct for transfection efficiency. Rat1a cells were transiently co-transfected with the -266/+205 cyclin A promoter reporter construct and expression constructs for (B) activated HA-Ras (12V), (D) v-Src and (F) EGFR together with either CMV vector or CMV-TAM67 and incubated for 48 hours under non-adherent growth conditions. Luciferase activity was determined as described above.
**Figure 1**

### A

|          | Rat1-GFP | Rat1a-J4 | Rat1-GFP | Rat1a-J4 |
|----------|----------|----------|----------|----------|
| Dox      | —        | +        | —        | +        |
| cJun     |          |          |          |          |
| Cyclin D1|          |          |          |          |
| Cyclin E |          |          |          |          |
| Cyclin A |          |          |          |          |
| Cyclin B1|          |          |          |          |

### B

![Kinase Activity Graph](chart.png)
Figure 2

A

| Time (days) | 1 | 2 | 3 |
|-------------|---|---|---|
| Dox | - | + | - | + | - | + |

Cyclin A2

β-Tubulin

B

| Time | 8hr | 1day | 3day |
|------|-----|------|------|
| Dox | - | + | - | + | - | + |

Cyclin A

28S

18S
Figure 3

| Time (hr) | Rat1a-J4 (-) | Rat1a-J4 (+) |
|----------|--------------|--------------|
| 0        |              |              |
| 2        |              |              |
| 4        |              |              |
| 8        |              |              |
Figure 4

|          | GFP 3day | J4 1day | J4 2day | J4 3day | Dox | Cyclin A1 | β-Tubulin |
|----------|----------|---------|---------|---------|-----|-----------|-----------|
|          | -        | -       | -       | -       | -   |           |           |
|          | +        | +       | +       | +       | +   |           |           |
Figure 5

Relative Luciferase Activity
Figure 6

Rat1a-J4 (-) Rat1a-J4 (+)

| Condition                  | Rat1a-J4 (-) | Rat1a-J4 (+) |
|----------------------------|--------------|--------------|
| Nuclear extract only       |              |              |
| +wt ATF oligo              |              |              |
| +mut ATF oligo             |              |              |
| +anti-c-Jun                |              |              |
| +anti-ATF1                 |              |              |
| +anti-ATF2                 |              |              |
| +anti-ATF3                 |              |              |
| +anti-CREB1                |              |              |
| +anti-CREM1                |              |              |

* indicates significant differences.
Figure 7

A

- + 2µg/ml Dox

Cyclin A2

HA

β-Tubulin

B

- + 2µg/ml Dox

Anti HA

Control IgG

Input

C

Fold Induction

Dox (-)  Dox (+)
Figure 8

A

B
Figure 9

A

|     | Rat1a-J4 | 2-B4 | 3-D1 | 3-C4 |
|-----|---------|------|------|------|
| Dox | —       | -    | +    | -    |
| Cyclin A | — — —        | + + +     | —         | + + |

pLRT-cyclin A clones

B

| pLRT-cyclin A  | pLRT-cyclin A  | Rat1a-J4 |
| pool          | clone          |          |
| Dox-          | Dox-           | Dox-     |
| Dox+          | Dox+           | Dox+     |
Figure 10

A

![Bar chart showing the number of colonies for different cell lines with and without Dox.](image)

B

![Line graph showing relative cell growth over time for different cell lines with and without Dox.](image)

C

![Graphs showing relative expression of Cyclin A and β-Tubulin for different cell lines.](image)
Expression of various cell cycle regulators after cJun induction in non-adherent Rat1a-J2 cells. Exponentially growing Rat1a-J2 cells were plated in PolyHEME-coated dishes in the absence (-) or presence (+) of doxycycline (2\(\mu\)g/ml) and incubated for 3 days. The cells were lysed and the protein extracts subjected to western blotting using antibodies against cyclin D1, cyclin E, cyclin A, cyclin B1, p16Ink4a, p19ARF, p27kip1, p57kip2, cJun and \(\beta\)-tubulin. Rat1a-GFP cells expressing GFP were used as a negative control.
c-Jun enhances cyclin A expression in Rat1a-J2 cells grown under non-adherent conditions. Rat1a-J2 cells were grown in suspension in the absence (-) or presence (+) of 2µg/ml doxycycline. Proteins and RNA were extracted at the indicated times and analyzed for cyclin A expression by western (A) or northern (B) blotting respectively.
The cyclin A2 mRNA half life remains unchanged after induction of cJun. Rat1-J2 cells were maintained under non-adherent growth conditions for 3 days in the absence (-) or presence (+) of 2µg/ml doxycycline after which actinomycin D (10µg/ml) was added for the indicated times. RNA was isolated and subjected to northern blot analysis. The intensity of the bands was quantitated densitometrically.
cJun activates the cyclin A2 promoter. Rat1a-J2 and -GFP cells were grown in the absence and presence of 2μg/ml doxycycline and transfected with the indicated cyclin A2 reporter constructs. And incubated for 4 days non-adherently after which luciferase activity was determined. The luciferase activities are reported relative to the uninduced cells after normalizing the luciferase signals to the signal obtained from the co-transfected Renilla luciferase. The figure represents the mean ± SD of at least three independent experiments performed in duplicate.
**cJun binding at the cyclin A2 promoter.** Electrophoretic mobility shift assay using the ATF sequence of the cyclin A2 promoter as a probe with nuclear extracts prepared from Rat1a-J2 cells grown for 3 days in the absence (-) or presence (+) of 2µg/ml doxycycline, incubated with various antibodies. Competition with the unlabelled wild-type ATF sequence (wt ATF) confirmed the specificity of the DNA-protein complexes, whereas competition with an unlabelled mutated ATF sequence (mut ATF) failed to cause a reduction in complex formation. (*) indicates the specific DNA/protein complexes and (♦) indicates the additional supershifted complex observed by incubation with an ATF2 antibody in the nuclear extracts from the cJun overexpressing cells.