ANTICANCER RESEARCH *24*: 1469-1480 (2004)

Characterization of the CDP-Like/CTAS-1 Binding Site in the Okadaic Acid Response Element (OARE) of the Human CDK1(p34^{cdc2}) Promoter

R. CURTIS BIRD and PATRICIA DEINNOCENTES

Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519, U.S.A.

Abstract. *Background: Transcription of CDK1 is induced at the G0/G1-phase of the cell cycle and after okadaic acid treatment and we identified the Site I okadaic acid response element (OARE -944 to -763nt) enhancer in the human CDK1 promoter. Materials and Methods: The OARE region of the CDK1 promoter was characterized for enhancer/repressor activities. Results: Transient transfection of upper and lower Site I subregions suggested enhanced transcription activity was divided between both while mobility shift assays demonstrated sequence-specific protein binding to Site IA. Site IA also formed shift complexes following serum starvation/refeeding and putative transcription factor binding sites clustered in Site IA. Oligonucleotides encoding a consensus CDP transcription factor binding site effectively competed against authentic Site IA in mobility shift assays. Mutation of the CDP-like binding sequence substantially reduced competition. DNaseI footprinting revealed binding at this site. Conclusion: The CDP-like recognition sequence appears to comprise the OARE binding authentic CDP and/or related factors. We termed this site the CDK1 transcription activation sequence-1 (CTAS-1) enhancer of the human CDK1 promoter.*

Throughout development, cells make the transition from quiescence to proliferation and then terminally differentiate based on integrating external and internal proliferation cues (1). Cyclin-dependent kinase 1 (CDK1 or cdc2) is the principle regulatory complex that controls transition checkpoints at the gateway into mitosis that completes each cell cycle (2-4) and may also have a role in G1-phase ensuring against premature entry into the S-phase (5). CDK1 is controlled during the continuous cell cycle principally at the level of post-translational modification and through its association with its cofactors cyclin and the p21/Cip1/Waf1 and p27/KIP1 families of inhibitors to form cell cycle phase-specific CDK-based integration complexes (6,7). These complexes appear to have evolved in structure into divergent subtypes that are responsible for the sequential transition of cells from one cell cycle phase to the next (8).

In contrast to the post-translational regulation exerted during the continuous cell cycle, transcription of CDK1 appears to be the principle regulatory level during the G0/G1-phase transition as CDK1 transcription is induced from very low to high levels and only then resolves to the constitutive levels observed during sequential phases of the continuous cell cycle (9,10). Thus, as cells re-enter the cell cycle from quiescence, the CDK1 gene is regulated primarily by transcription very different from the controls exerted once cells are proliferating and approaching the mitotic checkpoint (9,11).

The CDK1 promoter has been investigated in several species although the majority of the effort has been directed toward the human sequence. In general, as cells re-enter the cell cycle, CDK1 expression rises from low levels to higher constitutive levels as they enter S-phase (12). A proximal region located at -245 to -70 nt has been associated with E2F and Rb protein trans-activators (13-17) in a number of different developmental/proliferative conditions in which the CDK1 gene is principally under transcriptional control. This includes both activation and repression as cells enter the cell cycle or exit and terminally differentiate (18,19). During G2-phase arrest, repression of CDK1-expression from this site also appears to be p53-dependent (20). However, the subtlety and variety of regulatory inputs that have an impact on the CDK1 promoter can not be accounted for by these E2F-related interactions alone. Other sites/factors must exist to explain the complexity of regulation observed under different proliferative and developmental pathways. Two sites have been associated

Correspondence to: R. Curtis Bird, Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519, U.S.A. Tel: 334-844-2707, Fax: 334-844-2652, e-mail: birdric@vetmed.auburn.edu

Key Words: CDK1, enhancer, transcription, cell cycle, proliferation, gene expression.

with the G0/G1 transition and at least one at -20 nt appears to bind a repressor of CDK1 transcription, active during quiescence and bound by the E2F-4/p130 complex in humans (21,22). Repression in this region has been associated with the repressor/R-box or cell cycle-dependent element located at -22 to -2 nt (23,24) and with an E-box in the same region (19). These elements may also be responsible for transcriptional down-regulation of CDK1 in response to DNA damage later in the cell cycle in a p53 and p21/Waf1-dependent manner (22,25). A silencer sequence has been identified in the rat CDK1 promoter at -374 to -360, though homologous sequences do not appear to be evident in the human sequence (17). A larger region just upstream, located at -416 to -186 nt, has also been associated with the initial transcription activation that accompanies re-entry into the cell cycle (9,11).

Several oncogene products have also been demonstrated to transactivate the CDK1 promoter. The oncogene c-*myb* has been shown to be a direct regulator of CDK1 expression, at least in hematopoietic lineages through the c-*myb* responsive element located at -410 to -392 nt in the human gene (26,27). There is a c-*myc* responsive region located within -695 to -245 nt and sequences within this large region also respond to c-*ras* expression and contain a putative repressor region as well as YY1 and NRF1 recognition sequences (28). Such activation has been observed during the G0/G1 transition by ets-2 protein binding in regions -979 to -959, -520 to -500 and -120 to -100 (29). The proximal promoter contains two inverted CCAAT sequences that appear to bind the CBF/NF-Y transcription factor in transformed cells or in response to either SV40 T antigen or adenovirus E1A expression (30-33). This also appears to be the site through which p53 exerts influence over CDK1 expression (34). The same site, including flanking sequences, appears at least partially responsible for down-regulation of CDK1 transcription during myocyte differentiation (35) and in quiescent cells through the activity of the CDF-1 repressor (35-38). It is clear that CDK1 transcription regulation is complex and subject to context with regard to cell type and physiological state or developmental fate. However, it is unclear that all or even most of the regulatory elements have been identified in this promoter.

Difficulty in interpretation of results from the starvation/refeeding model of cell cycle re-entry has led to speculation that it represents events more in line with wound healing reactions than naturally occurring cell cycle re-entry. Such problems in interpretation and complexity have demonstrated the need for models in which components of the cyclin/CDK complex experience relevantly selective activating stimuli. We have characterized the transcription activation of CDK1 expression that occurs during G0/G1-phase transition early in the cell cycle and modeled these events with treatment by the phosphatase 1/2A inhibitor okadaic acid (9,11). Okadaic acid selectively suppresses phosphatase 1/2A activity, resulting in hyper-phosphorylation of cellular proteins possibly enabling the proliferation signal through activation of signal transduction (39,40). This probably perturbs/stimulates signal transduction systems, mimicking the response of selective signaling components that normally respond to growth factor stimulation. The model is typified by rapid induction of CDK1 expression and several additional gene products associated with initiation of the cell cycle (9,11). Although not supportive of continuous cell cycle progression, the okadaic acid model avoids the broad induction of gene expression observed in starvation/refeeding synchrony offering a unique opportunity to investigate these early events (9).

We have previously shown that enhancement of transcription activation of the CDK1 gene following okadaic acid treatment is due principally to two upstream regions of the CDK1 promoter (11). The most important appears to be the Site I region and the secondary region appears to be Site II which appears coincident with the E2F/Rb binding site region. Our rationale for investigation of the Site I enhancer sequence has been to examine sub-regions of the sequence to determine where the regulatory enhancer lies and to gather evidence as to its identity.

Materials and Methods

Cell culture and okadaic acid or calyculin treatment. HeLa S3 cells were cultured in α-modified Eagle's essential medium (Gibco BRL, Gaithersburg, MD, USA) with antibiotics (Sigma St. Louis, MO, USA) and 10% fetal bovine serum (Summit, Fort-Collins, CO, USA) as previously described (9,11). Cells were treated with 19 nM okadaic acid (Moana Bioproducts, Honolulu, Hawaii, USA), from a 2.0 μM stock solution, in growth medium or 0.9 nM calyculin A (CalBioChem, La Jolla, CA, USA), from a 2.0 μM stock solution, for 0, 12, 24, or 48 h.

Northern (RNA) blot analysis. Total RNA was extracted from HeLa cells using RNA Stat 60 (Tel-Test, Inc., Friendswood, TX, USA), the RNA pellet was resuspended in diethylpyrocarbonate-treated water, the concentration of RNA was determined by absorbance at 260nm and the samples were stored at -80˚C (41). Ten mg of total RNA from each sample was denatured by incubation in a buffer containing 0.2 mg/ml ethidium bromide, 50% formamide, 2.2 M formaldehyde and 1xMOPS running buffer at 60˚C for 5 min and fractionated by electrophoresis on a 1% agarose gel containing 1xMOPS buffer (41). RNA was transferred by capillary blotting RNA to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) in 3xSSC (1xSSC is 0.15 M NaCl, 15 mM sodium citrate), as previously described (41). Following transfer of the RNA, the membrane was UV crosslinked with 1200 ujoules of UV light (Strategene, Cedar Creek, TX, USA).

Cloned cDNAs. Cloned cDNA fragments encoding CDK1 and CDK2 were obtained from P. Nurse (42) and cyclin A and cyclin B cDNAs were obtained from J. Pines (43,44). cDNA was purified from plasmids using the PEG procedure (45) and were labelled using random primer DNA labeling (Gibco BRL). The membranes

were prehybridized for 2 h at 42˚C and α-[^{32}P]dCTP-labelled probe (3000 μCi/mmol) was hybridized to the transferred RNA in buffer containing 50% formamide, 5xSSC, 20mM sodium phosphate, pH 7.0, 0.5% SDS, 4xDenhardt's reagent (41) and 10mg/ml denatured herring sperm DNA for 18 to 20 h at 42˚C. The membranes were washed at 55˚C in decreasing dilutions of 2xSSC to 0.25xSSC including 0.5% SDS. The membranes were air-dried and quantified on a phosphoimager screen (Molecular Dynamics, Sunnyvale, CA, USA) followed by autoradiography.

Plasmid DNA construct preparation and purification for transfection. Plasmid DNAs containing promoter fragments derived from the human CDK1 promoter encoded in pcdk1-11-CAT (27) and including the Site I region extending from -944 to -763 nt were used to assemble reporter constructs (46). The Site I region was first divided into 2 sub-regions: IA - upstream and IB - downstream. The two subfragments of the Site I region were created by PCR with Site IA extending from -944 to -841 nt and Site IB extending from -847 to -763 nt (47). These sub-regions were cloned by PCR amplification and T/A-cloning into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA, USA). Promoter fragments were then subcloned, at the Bgl II site, into the pCAT-promoter plasmid (Promega, Madison, WI, USA) containing a minimal basal promoter. The PEG procedure was used to purify high quality plasmid DNA required for efficient transfection with modification (45). DNA concentration and purity was measured by absorbance at 260 nm.

Transient cell transfection and CAT reporter assay by the phase extraction method. HeLa cells were transfected with CDK1 promoter/CAT DNA constructs as previously described (11,48). All transient transfections included transfection control plasmid pSV-β-galactosidase. All transient transfection experiments also included replicates transfected with positive control plasmid pRSV-CAT or empty pCATpromoter plasmid as a negative control. CAT assays were performed by the phase extraction assay procedure (65), using [^{3}H]chloramphenicol and butyryl co-enzyme A as substrates as previously described (11,48). Samples were counted in a scintillation counter and the counts obtained were compared to the total counts added to each reaction to calculate the relative uncorrected CAT enzyme activity per sample. Aliquots of transfected cell lysates for non-isotopic detection of β-galactosidase (Galacto-Light Chemiluminescent Reporter Assay, Tropix-Applied Biosystems, Foster City, CA, USA) were thawed on ice and prepared as described by the manufacturer. Light output was proportional to β-galactosidase activity and gene expression level in each sample. β-galactosidase values were used to normalize (correct) relative CAT-assay values for each sample to eliminate variation due to differences in transfection efficiency between samples (11).

Nuclear and whole cell protein extract. Nuclear proteins were extracted from flasks (75-cm^2) of exponentially growing cells (grown to less than 70% confluence) by washing 2X with ice-cold PBS, draining and lysis in 5 packed cell volumes LB (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) and incubation on ice for 10 min followed by centrifugation (45). Cells were resuspended in 3 volumes LB and 0.02 vol 10% NP-40. The cells were homogenized in a chilled Dounce homogenizer 10X followed by centrifugation to collect nuclei. Nuclei were resuspended in 3 volumes NEB (5 mM HEPES, pH 7.9, 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA,

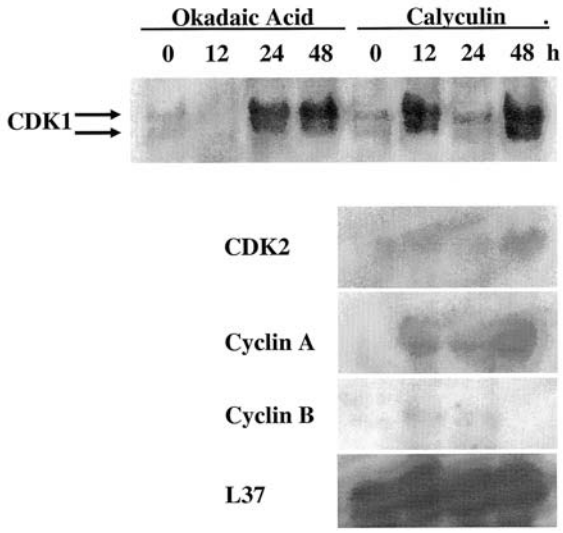
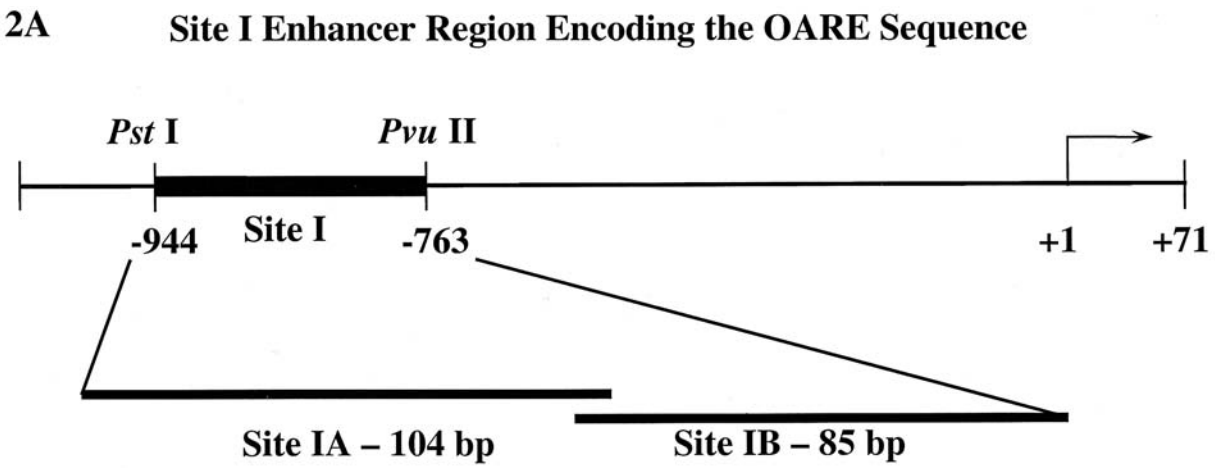
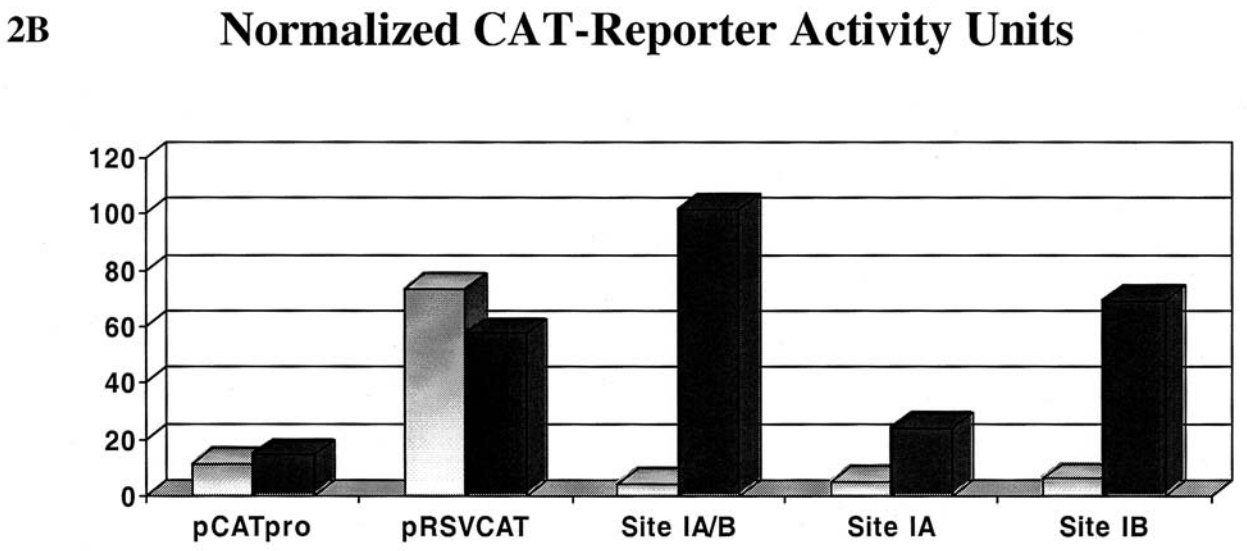


Figure 1. *Selective induction of CDK1, CDK2 and cyclins A and B in exponentially growing HeLa cells. Northern blot hybridization of total RNA (10 mg/lane) isolated from exponentially growing HeLa S3 cells (0 h) or exponentially growing cells treated with okadaic acid (19 nM) or calyculin (0.9 nM) for 12, 24 or 48 h. Messenger RNAs assayed included CDK1, CDK2, cyclin A, cyclin B or constitutively expressed ribosomal protein L37 (64).*

0.5 mM DTT, 0.5 mM PMSF) and then NaCl to 300 mM was added dropwise on ice with mixing. Extraction for 30 min on ice was followed by centrifugation at 14,000xg for 10 min at 4˚C. The protein concentration of the supernatant was determined (Pierce, Rockford, IL, USA) and nuclear extracts frozen in 25-ml aliquots in liquid nitrogen and stored at -85˚C. Alternatively, whole cell extracts were prepared. Flasks (75-cm^2) of exponentially growing cells (grown to less than 70% confluence) were washed twice with ice-cold PBS, drained and lysed in 100 ml EB (20 mM HEPES, pH 7.8, 450 mM NaCl, 0.04 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF) followed by 3 rounds of freeze-thaw (freeze at -85˚C followed by just thawing while incubating at 65˚C). The lysate was centrifuged at 14,000xg for 10 min at 4˚C and the supernatants assayed for protein concentration and stored at -85˚C.

Mobility shift assay. Mobility shift assays were performed, with modification, as previously described (11,49). The Pst I-Pvu II fragment (Site I) of the CDK1 promoter (27) was gel-isolated (Qiagen, GmbH, Hilden, Germany) and labelled with γ-[^{32}P]dATP (3000 μCi/mmol) by dephosphorylation with calf intestine alkaline phosphatase and then phosphorylation with polynucleotide kinase (47). The DNA was precipitated and resuspended in 100 μl of distilled water followed by scintillation counting to determine specific activity (47). HeLa S3 cells were treated for 24 h with okadaic acid (19 nM) or left untreated and nuclear extracts prepared. Labelled DNA (3x10^5 cpm)/protein complexes (1 μg) formed for 30 min at room temperature in 1xBB (2xBB is 500 mM NaCl, 10 mM HEPES, pH 7.9, 150 mM KCl, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 10 mg/ml poly [dI:dC]). Complexes were analyzed on low ionic strength 4.5% polyacrylamide gels (30% acrylamide:0.8% bis-acrylamide) containing 0.2xTBE buffer (1xTBE





is 89 mM Tris-HCl, 89 mM boric acid, 4 mM EDTA, pH 8.0), 0.1% ammonium persulfate and 0.2% TEMED and compared to probe alone. Gels were pre-electrophoresed at 150 V for 1 h with buffer recirculation. Samples were loaded and electrophoresis continued at 150 V for 45 min and 200 V for approximately 1.75 h with buffer recirculation until the bromophenol blue dye reached the bottom of the gel. The gel was dried under vacuum prior to exposure to X-ray film with intensifying screens. Densitometry was used to estimate the quantity of the DNA-binding protein complexes present in each lane.

Transcription factor binding site sequence analysis. Potential transcription factor binding sites within the Site I region were analyzed for homology using the TFSEARCH program (50) to compare query sequences against the TFMATRIX transcription factor binding site profile database in the TRANSFAC databases (GBF-Braunschweig, 51).

Oligonucleotide hybridization. Complimentary oligonucleotides encoding Site IA sub-regions were synthesized and combined (50 μg of each complimentary single-stranded oligonucleotide per 100 μl hybridization reaction mix) in hybridization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 5 mM $MgCl_2$) and allowed to hybridize by cooling from 70˚C to room temperature (~20˚C) overnight.

DNase I footprint analysis. Nuclear extracts from okadaic acid treated and untreated HeLa S3 cells were prepared as described above. The DNA fragment was labelled by random primer DNA labeling (Gibco/BRL). A reaction mix was prepared by combining the protein extract with the α-[^{32}P] dCTP-labelled probe (15000 cpm/lane) to allow binding and equilibration. Subsequently, RNase-free DNase 1 (1 mg/ml, Promega) was added to each reaction and digestion was performed as previously described (47). Increasing

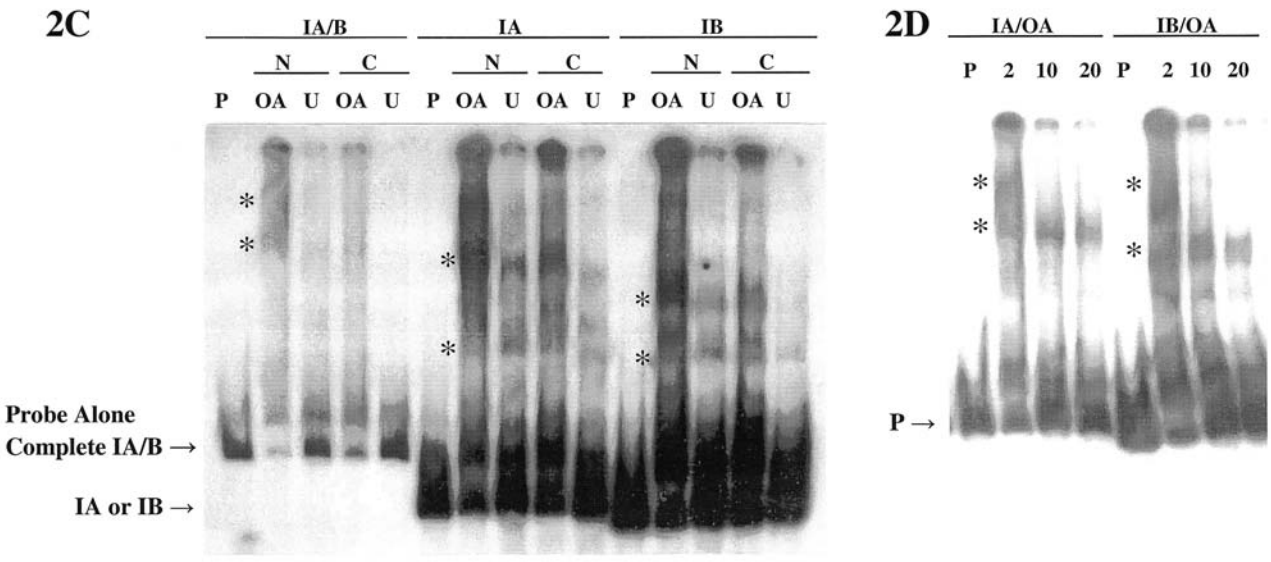


Figure 2. *Characterization of the Site I enhancer. (A) Schematic representation of the Site I enhancer region of the human CDK1 promoter. The Site I promoter region containing the OARE sequence (nt -944 to -763) is bounded by Pst I and Pvu II restriction sites. The Site IA region is the upstream 104 bp and the Site IB region is the downstream 85 bp encompassing the entire Site IA/IB region with a 7 bp overlap. The arrow indicates the +1 base or start of transcription. (B) Okadaic acid-sensitive enhancer activity in the CDK1 Site I enhancer region assayed by transient transfection and CAT-reporter expression. pCATpromoter reporter clones contained 1 copy of Site I in the forward orientation (Site IA/B), or 1 copy of Site IA or Site IB in the forward orientation ahead of the CAT reporter gene. Negative controls were transfected with empty pCATpromoter vector (pCATpro) and positive controls were pRSVCAT. HeLa cells were transiently transfected with equal molar amounts of 1 of these clones and the transfection control plasmid pSV-β-galactosidase. Experiments were performed 3 times in duplicate wells with (dark bars) or without (shaded bars) okadaic acid treatment. Data is expressed as units of CAT reporter activity normalized for β-galactosidase activity. (C) Mobility shift analysis of the Site IA and IB regions of the CDK1 promoter using whole cell and nuclear extracts from okadaic acid-treated and untreated cells. Mobility shift analysis of the complete Site I (IA/B), IA and IB regions of the CDK1 promoter were performed to compare whole cell and nuclear extracts from okadaic acid treated and untreated cells. HeLa S3 cells were treated for 24 h with 19 nM okadaic acid (OA) or left untreated (U) and either nuclear (N) or whole cell (C) protein extracts prepared. Labelled DNA ($3x10^5$ cpm) and 1 mg of protein extract were allowed to form complexes for 30 min at room temperature in 1xBB. Complexes were analyzed by low ionic strength 0.2xTBE polyacrylamide gel electrophoresis and compared to probe alone (arrows). Asterisks indicate the positions of major protein/DNA shift complexes. (D) Poly [dI:dC] titration of nonspecific interactions of nuclear proteins with the Site IA and IB regions of the CDK1 promoter. Mobility shift assays were performed on the Site IA and IB sub-fragment regions using okadaic acid-treated nuclear protein extracts in which the concentration of poly [dI:dC] was 2, 10 or 20 mg/ml as noted. Extracts were compared to probe alone (P). Asterisks indicate the position of major protein/DNA shift complexes.*

DNase 1 dilutions of the stock solution (1:1, 1:2, 1:5, and 1:10) were used to titrate sensitivity of individual sequence regions (data not shown). The 1:2 dilution was determined to provide the optimum level of digestion. The samples were analyzed on a 6% acrylamide sequencing DNA gel along with a sequencing marker to estimate the sizes of the bands (47). The gel was exposed to X-ray film to visualize the DNase I footprints by autoradiography.

Results

Okadaic acid and calyculin induce selective induction of gene expression. We have previously demonstrated that early events in cell cycle re-entry from G0-phase can be modeled in HeLa S3 cells by treatment with the phosphatase 1/2A inhibitor okadaic acid (11). We repeated these experiments using the alternative phosphatase 1/2A inhibitor calyculin to demonstrate that the effects were not due to other activities associated with okadaic acid but were the result of inhibition of phosphatase activity. HeLa cells treated with either 19 nM okadaic acid or 0.9 nM calyculin induced expression of both CDK1 mRNAs (apparent relative size 1.2-1.4 kilobases) in agreement with our previous studies (9,11,13-15,21,23). Calyculin also induced expression of cyclin A and cyclin B to high levels in exponential cells (Figure 1) as previously demonstrated for okadaic acid (9). Neither inhibitor induced expression of CDK2 or ribosomal protein L37 which remained at constitutive levels and served as loading/transfer controls (data shown for calyculin only) which is concordant with the lack of any effects of okadaic acid on GAPDH expression (9). Both protein phosphatase 1/2A inhibitors okadaic acid and calyculin caused similar selective inductions of gene expression, suggesting that these effects were specific to the targeted inhibition of phosphastases 1/2A and not to some other effect of either inhibitor.

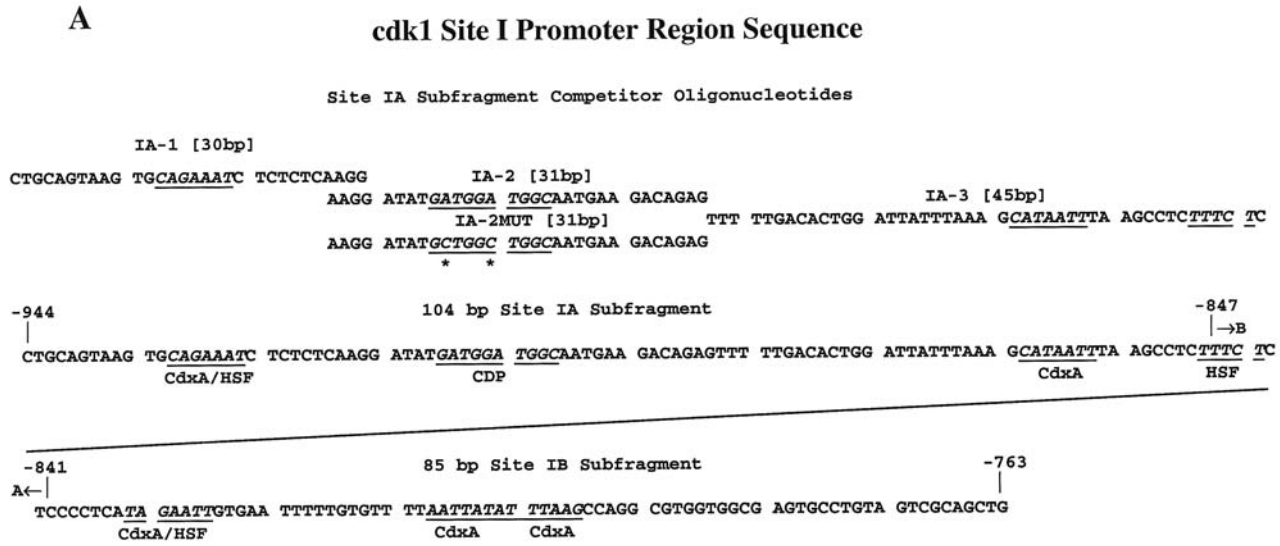


Figure 3. *Analysis of putative transcription factor binding sites in a schematic representation of the Site I enhancer region of the human CDK1 promoter. The Site I region containing the OARE sequence (nt -944 to -763) was divided into the upstream 104 bp Site IA region and the downstream 85 bp Site IB region. Putative transcription factor binding sites identified by consensus (sequence in underlined italics) are noted (see text for details of analysis). Consensus sequences representing multiple putative Cdx homeobox and heat shock factor (HSF) binding sites are noted as is the unique CDP binding site. The set of three single-stranded competitor oligonucleotides encompassing the entire Site IA region are also noted (IA-1, IA-2 and IA-3). The alternative competitor oligonucleotide IA-2M containing two base mutations (identified by *) to the putative CDP binding site is also noted.*

Table I. *CDP/Cut-like homeodomain protein DNA binding sites.*

Putative CDK1 promoter-derived CDP-like enhancer	5'-GATGGATGGC-3' * ↑ **
Consensus CDP binding site†	5'-NATYGATSSS-3'

* - Position of nucleotides conserved in 85-100% of known CDP binding sequences. The two adenosine residues were those altered in the Site IA-2 oligonucleotide to create the Site IA-2M oligonucleotide.
↑ - Position of the only nucleotide in the CDK1 promoter sequence not represented in the most conserved CDP consensus sequence. However, a 'G'-residue like that found in the CDK1 promoter is present at this site in 10% of known CDP-binding sites.
†- All data upon which the consensus sequence was derived were obtained through the TFSEARCH program (50).

Transient transfection analysis. Our previous mobility shift analysis identified the entire Site I promoter region of the human CDK1 promoter extending from the *Pst* I site at -944 to the *Pvu* II site at -763 basepairs (bp) upstream from the start of transcription (11). We divided the 180 bp Site I region into two halves – Site IA (upstream 104 bp) and Site IB (downstream 85 bp) encompassing a 7 bp overlap (Figure 2A). The entire Site I region can now be described as Site IA/IB. The entire Site IA/IB region, as well as the Site IA and Site IB subfragments, were inserted into CAT-reporter/minimal promoter constructs and transfected into HeLa cells to test the ability of each fragment to act as an enhancer in the presence or absence of okadaic acid (Figure 2B). In the absence of okadaic acid, Site I sequences all elicited a small (50-60%) but reproducible repression of basal expression from the pCATpromoter construct. However, okadaic acid treatment elicited clear enhancement of transcription from all three Site I region clones with the strongest reaction elicited by the complete Site I region (25.6-fold above untreated). Both Site IA (4.7-fold above untreated) and Site IB (11.4-fold above untreated) subfragments also elicited enhancement of reporter activity in the presence of okadaic acid. All Site I-derived fragments enhanced reporter expression well above negative controls, in the presence of okadaic acid, to levels comparable to or exceeding those achieved with pRSVCAT-positive control plasmids. Only expression from reporter constructs containing the Site I derived fragments were induced by okadaic acid treatment as this treatment had no effect on control constructs. This data clearly demonstrates that in these cells okadaic acid response through enhancement of CDK1 expression resided in both upstream and downstream halves of the CDK1 Site I promoter region and that these sequences could function independently.

Mobility shift analysis of the Site I region. Mobility shift analysis of the Site I Region demonstrated that 2 major species of protein/DNA complexes formed with Site IA/IB,

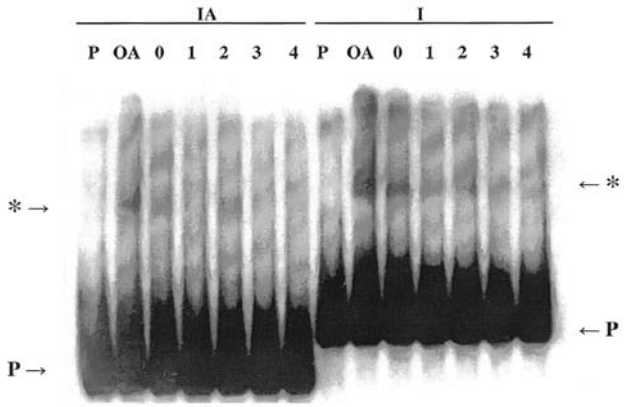


Figure 4. *Comparison of mobility shift interactions between nuclear proteins from okadaic acid-treated and starved/refed cells with the Site I and Site IA regions of the CDK1 promoter. HeLa cells were grown to approximately 70-80% confluence in medium containing 10% FBS in α-MEM and then medium was switched to starvation medium containing 0.5% FBS in α-MEM for 2 days. Medium containing 10% FBS in a-MEM was added back and nuclear extracts were made from the cells at 0, 1, 2, 3 and 4 h after refeeding as noted. Mobility shift assays using either the Site I or IA DNA fragments were performed. Extracts were compared to each probe alone (P). Asterisks indicate the position of protein/DNA shift complexes.*

IA or IB DNA fragments (Figure 2C). Complexes were observed using either nuclear or whole cell protein extracts. However, nuclear extracts contained higher apparent concentrations of sequence-specific DNA binding proteins per mg. All of the fractions contained some DNA binding activity but okadaic acid treated extracts consistently contained much higher DNA binding activity than untreated extracts. In all cases that included extract, both high molecular weight bands were observed (Figure 2C).

Competition mobility shift assays against non-sequence specific competitor molecules composed of poly [dI:dC] were used to determine which of the two complexes recovered were binding due to sequence-specific interactions with labelled probe molecules (Figure 2D). Incubation of the labelled probe with increasing concentrations of poly [dI:dC] prior to addition of the nuclear extract effectively competed binding to the larger of the two complexes. Binding to the lower molecular weight complex was essentially unaffected by addition of poly [dI:dC] up to 10 μg/reaction except for a slight reduction in the amount of complex recovered using the IB fragment at the highest levels of non-specific competitor employed (20 μg/reaction). All subsequent mobility shift reactions were performed in the presence of 10 μg/reaction of poly [dI:dC]. These results suggested that detectable sequence-specific binding activity was encoded in both the Site IA and IB subfragments.

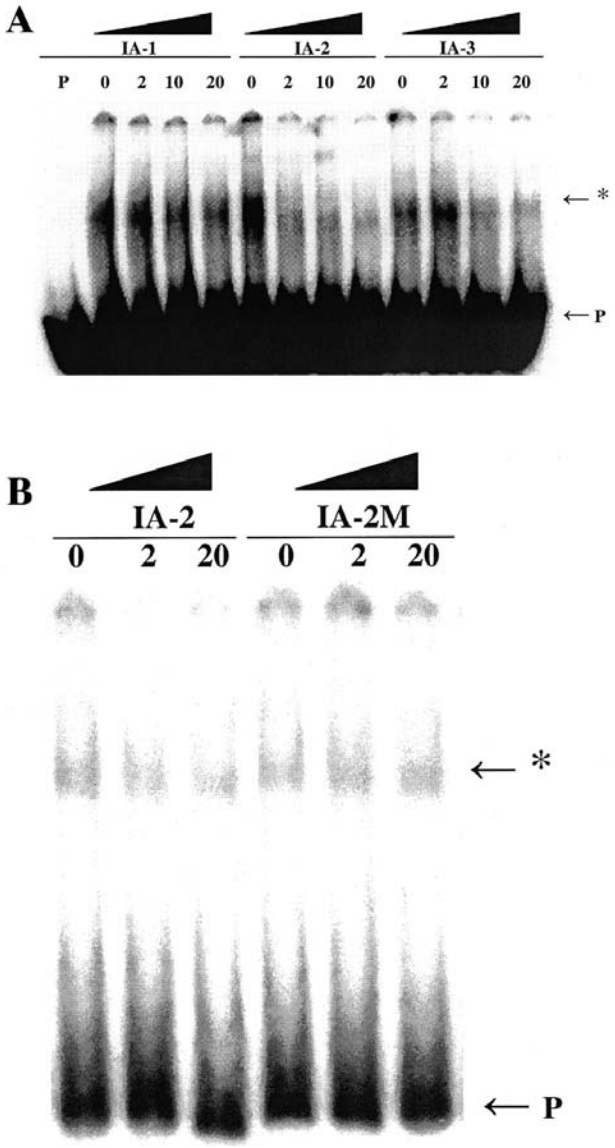


Figure 5. *Identification of the binding subregion within the Site I enhancer. (A) Site IA competition mobility shift assays in the presence of ds-oligonucleotide competitors. Site IA competition mobility shift assays were performed after pre-incubating nuclear protein extracts with an increasing mass of Site IA-1, IA-2 or IA-3 ds-oligonucleotide competitors. The competing unlabelled ds-oligonucleotides were added to mobility shift reactions at 0, 2, 10, or 20 μg/ml and incubated 10 min at room temperature prior to addition of the labelled Site IA probe. Extracts were compared to Site IA probe alone (P) and with complexes formed with nuclear extracts from okadaic acid-treated cells in the absence of competitor oligonucleotide (0). Asterisk indicates the position of protein/DNA shift complexes. (B) Site IA competition mobility shift assays in the presence of Site IA-2 or IA-2M ds-oligonucleotide competitors. Site IA competition mobility shift assays were performed after pre-incubating okadaic acid-treated nuclear protein extracts with an increasing mass of appropriate ds-oligonucleotide competitors at 0, 2, or 20 μg/ml as noted prior to labelled Site IA probe addition. Extracts were compared to Site IA probe in the absence of competitor oligonucleotide (0). Asterisk indicates the position of protein/DNA shift complexes.*

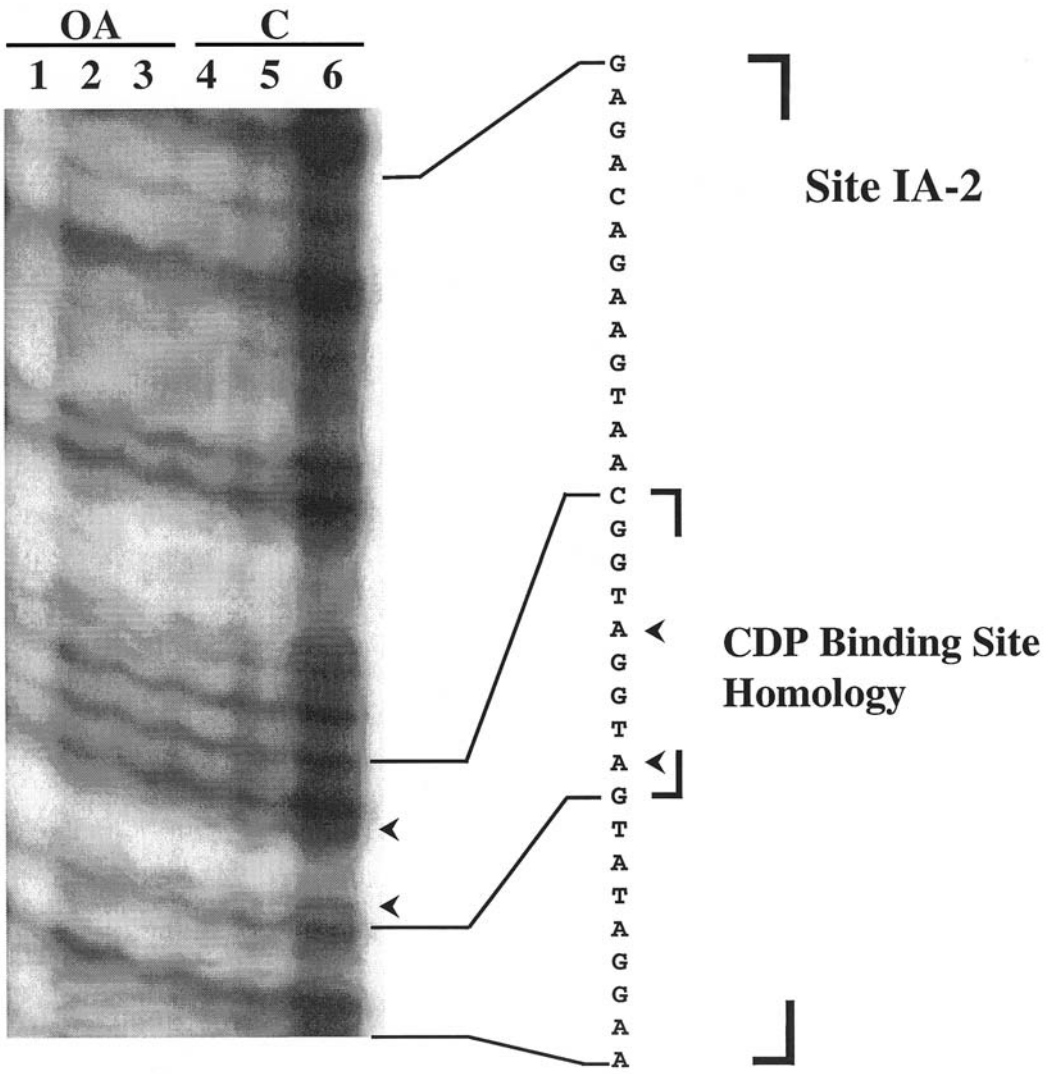


Figure 6. *DNase I footprinting assay of the Site IA-2 promoter region. Site IA DNA fragments were labelled and allowed to react with nuclear extracts from okadaic acid-treated (OA) or control untreated (C) cells. Increasing amounts of nuclear extract were added (1, 3, or 6 μl of extract in sample numbers 1, 2, or 3 for okadaic acid treatment and sample numbers 4, 5, or 6 for control, respectively). Complexes formed were then digested with limiting amounts of DNase I to produce a sequence ladder. Fragment size was compared to sequence standards of known size. Outer brackets define the limits of the Site IA-2 DNA sequence and inner brackets define the site homologous to the CDP binding sequence. Arrows indicate locations corresponding to the two mutated adenosine bases within the CDP binding sequence that were altered in the Site IA-2M oligonucleotide.*

Sequence analysis of the Site I region and competition mobility shift analysis. Analysis of potential transcription factor binding sites within the Site IA/IB region was performed using the TFSEARCH program (50) to search for homologies within this region among known transcription factor binding sites. The TFSEARCH program seeks highly correlated sequences by comparing query fragments against the TFMATRIX transcription factor binding site database in the TRANSFAC databases (51). Matches are compared and displayed based on declining homology and base-specific variation encountered within each example sequence. This analysis revealed several potential transcription factor binding sites within the Site I region (Figure 3). Distributed over the entire Site I region, these included 5 regions with homology to the CdxA homeobox transcription factor binding sequence and 3 regions homologous to heat shock factor binding sites, some of which were coincident between the two sequences. Additionally, there was one copy of the CDP cut-like homeobox protein binding site located in the Site IA region. All of these sequences were 90% identical, or higher, when compared to consensus binding sequences and were within the range of sequence variations known to

function for these transcription factors. Because the CDP sequence was unique to the Site IA fragment it was selected for further experimental analysis (Table I).

Okadaic acid treatment and starvation/refeeding produce Site I and IA mobility shift complexes. To determine if the okadaic acid model-induced enhancement of DNA binding activity was indeed a model of G1 phase re-entry, a comparison was sought between okadaic acid-treated cells and cells which had been starved for serum (0.5% v/v FBS in α-MEM for 2 days) and then refed with complete growth media (Figure 4). Starvation/refeeding induced formation of Site I and Site IA complexes indistinguishable from those present in okadaic acid treated cell extracts. Compared to untreated/starved extracts, there was a small reduction and then an enhancement in the amount of complex recovered peaking approximately 2 h after serum addition. This similarity of binding with both complete Site I and Site IA fragments suggests that okadaic acid-treated cells were activating CDK1 Site I binding events associated with re-entry into G1-phase in starvation/refeeding.

Sequence-specific competition mobility shift analysis of the Site IA region. The putative CDK1 CDP binding site identified contains only 1 variant base in 10 (G instead of Y at position 4) compared to the consensus CDP binding sequence, though nearly 10% of examples of the CDP sites known do contain G at this site in complete agreement with the human CDK1 sequence. Thus, in approximately 10% of cases, the CDP site is perfectly homologous with known sites and this is the only unique transcription factor binding site identified within the Site IA region. Because this fragment was still 103 bp in length, efforts to further define the sub-region within the Site IA region required subdivision of this fragment into 3 sub-regions: IA-1, IA-2 and IA-3 (Figure 3). Double-stranded oligonucleotides were synthesized as single-stranded molecules which were hybridized to completion to create double-stranded competitors. One additional double-stranded oligonucleotide was constructed, IA-2M, that contained 2 A/T → C/G mutations at 2 of the 3 most highly conserved locations in the putative CDP site (85% A at position 2 and 100% A at position 6 in known examples of CDP binding sites, Table I). These double-stranded molecules were used as unlabelled competitors in mobility shift assays with the Site IA fragment (Figure 5A). Even at 20 μg/ml, the IA-1 competitor could not compete away all complexes formed. The IA-3 competitor did have an inhibitory effect on complex formation, reducing the amount of complex recovered, but only at the 10 and 20 μg/ml concentrations. In contrast, the IA-2 competitor reduced the amount of complex recovered to nearly background density at all concentrations suggesting a higher effective competitive affinity. This suggests that the 31 bp of the IA-2 oligonucleotide contained the strongest complex binding DNA recognition sequence associated with the Site IA region.

The Site IA-2 region also contained the CDP-transcription factor binding site homology and this was the only recognizable homology to a known transcription factor binding site identified in the Site IA-2 region. To verify that the CDP site within the Site IA-2 region was responsible for the binding observed, the mutated Site IA-2M double-stranded oligonucleotide was employed as a competitor in a Site IA mobility shift assay and compared to the competitive binding of DNA fragments encoding authentic Site IA-2 (Figure 5B). Even at 20 mg/ml, the IA-2M competitor had little effect on the amounts of Site IA complex recovered comparable to control samples without competitor. In contrast, wild-type Site IA-2 competitor reduced Site IA binding by more than half demonstrating effective competition for bound proteins. This suggests that the IA-2 subfragment, containing the putative CDP binding site, is responsible for the binding observed and, further, that disruption of the CDP sequence at two conserved nucleotide locations greatly reduces the effectiveness of competition for binding to the Site IA fragment.

DNase I footprint analysis. To further analyze the binding of proteins to the Site IA region, DNase I footprint analysis was performed. Several regions of protection were consistently evident in these assays within the Site IA-2 sub-region in both okadaic acid-treated and control reactions (Figure 6). These included the region corresponding to the CDP binding site homology within the Site IA-2 region. Nuclear extracts from okadaic acid-treated cells as well as untreated control cells demonstrated protection of the CDP binding site homology region. Protection of the Site IA-2 region even in untreated extracts was consistent with our results from mobility shift assays which also revealed a reduction, but not an absence, of binding in extracts from untreated control cells.

Discussion

We previously demonstrated induction of CDK1 transcription by okadaic acid and this has allowed identification of an okadaic acid response element (OARE) enhancer in the human CDK1 promoter. The OARE is positioned in the Site I region located between -944 and -763 nucleotides upstream from the start of transcription in HeLa S3 cells (9,11). Mobility shift analysis of the Site IA and IB DNA subfragments subsequently demonstrated the formation of sequence-specific complexes that were associated with both subfragments. This data revealed Site I as a complex region with multiple binding sites exhibiting inducible enhancer and possibly repressor activities. Further analysis of this region has resulted in the tentative identification of a putative CDP binding site as part of the binding complex active in this model of re-entry into the cell cycle from quiescence. Competition mobility shift analysis of the Site IA DNA fragment has demonstrated formation of okadaic acid inducible sequence-

specific complexes that can only be effectively competed away by Site IA-2 competitor oligonucleotides. Mutations at 2 sites located in a putative cut-like homeodomain DNA binding protein (CAAT displacement protein or CDP) markedly reduced competitive activity of the IA-2M oligonucleotide suggesting that this sequence is involved in complex formation. Mobility shift analysis of the Site I and IA DNA fragments has also demonstrated the formation of sequence-specific complexes of similar molecular weight in starved and refed cells. This confirms that the okadaic acid model of early re-entry events in the cell cycle includes some of the DNA binding events that accompany G1-phase reentry after stimulation of quiescent cells with serum.

We have presented evidence of complex regulation in the Site I region including the potential for multiple binding sites and inducible enhancer activity as well as repressor activities. A transcriptional repressor of CDK1 expression in normal differentiating keratinocytes has also been localized to -949 to -722 nt of the human CDK1 promoter, which is essentially the same region in which we have identified HeLa cells (3). Although the region is large there is ample sequence to encode multiple binding sites within the greater than 220 bp we have both identified. Additionally, the cell lines employed in each study were very different and, interestingly, Dahler *et al.* (3) noted that the repressor activity does not appear to be active in immortalized cells of an epithelial lineage. Because HeLa cells are immortalized and epithelioid in origin, it is quite possible that the repressor activity is low in these cells and, in fact, we detected only low levels of such activity.

The CDP (CCAAT displacement protein) transcription factor was originally identified in *Drosophila* as the Cut-homeodomain protein and has been shown to regulate terminal differentiation, phenotype and exit from the cell cycle (52-56). CDP is a DNA binding protein (57,58) and has been shown to possess tumor suppressor activity acting in opposition to activated oncogenes, such as c-*myc*, largely through repression of transcription (59). CDP can also function in the activation of gene expression through interaction with other multi-protein transcription complexes (60). CDP can associate with the cyclin A/CDK1/retinoblastoma protein complex composing the HiNF-D transcription complex, an important component in repression of histone H4 and p21/*waf1* expression and as a substrate for the CDK1 kinase (58,60-62). We have termed the CDP-like transcription factor binding site the CDK1 transcription activation sequence number one (CTAS-1) enhancer of the human CDK1($p34^{cdc2}$) promoter. CTAS-1 appears responsible for some, though not all, of the CDK1 transcription activation activity observed as cells re-enter G1-phase. The evidence presented suggests strongly that the transcription factor(s) bound to the OARE enhancer includes a protein with affinity for the CDP binding site or perhaps authentic CDP. Because CDP is usually associated with repression of gene expression, it is unclear whether the CDP-like protein is activating the CDK1 promoter or part of a CDK1 derepression system. If this factor proves to be CDP or a related transcription factor that can also be phosphorylated by CDK1, it would provide a mechanism by which CDK1 could regulate its own expression. This would make the OARE region encoding the CDP-like CTAS-1 binding site a potential regulator of the transition from G0 to G1-phase during cell cycle re-entry.

The complex structure of the CDK1 promoter probably reflects the complexity and number of different signals that must be successfully integrated to correctly regulate CDK1 expression. Our data suggests existence of both transcription enhancers and repressors localized to the Site I region of the CDK1 promoter (3,29). This is consistent with the complexity of inductive and suppressive proliferation signals integrated through cyclin/CDK complexes. A similar strategy has been suggested for the histone H4 gene of which CDP is also a regulatory component (60,63). In this model, multiple transcription factor complexes respond to signaling pathways as cells pass through sequential phases of the cell cycle. We would propose a similar strategy could be operative for the CDK1 promoter. This proposed model would include regulation at the CTAS-1 enhancer, especially during the G0/G1-phase transition. Thus, the CTAS-1 enhancer and other sequences within Site I may play a role in regulating how CDK1 expression responds to many different signals in a context that is appropriate for cell cycle phase.

Acknowledgements

The authors thank Drs. V. van Santen and C. Dykstra for valuable consultations during the course of this investigation and Dr. B. Smith and Dr. L. Wolfe for critical reading of the manuscript. This work was supported by the Food Animal and Disease Research Program at Auburn University, College of Veterinary Medicine, U.S.A.

References

1 Stein JL, van Wijnen AJ, Lian JB and Stein GS: Control of cell cycle regulated histone genes during proliferation and differentiation. Int J Obes Relat Metab Disord *20 Suppl 3*: S84-S90, 1996.

2 Bird RC: The cyclin/cyclin-dependent kinase (cdk) complex: regulation of cell cycle progression and nuclear disassembly: *In*: Nuclear Structure and Gene Expression (Bird RC, Stein GS, Lian JB and Stein JL, eds). San Diego, Academic Press, 1997, pp 145-174.

3 Dahler AL, Jones SJ, Dicker AJ and Saunders NA: Keratinocyte growth arrest is associated with activation of a transcriptional repressor element in the human cdk1 promoter. J Cell Physiol *177*: 474-482, 1998.

4 Ekholm SV and Reed SI:Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol *12*: 676-684, 2000.

5 Itzhaki JE, Gilbert CS and Porter AC: Construction by gene targeting in human cells of a "conditional" CDC2 mutant that rereplicates its DNA. Nat Genet *15*: 258-265, 1997.

6 Frey RS, Li J and Singletary KW: Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: involvement of dc2, p21waf/cip1, p27kip1, and Cdc25C expression. Biochem Pharmacol *61*: 979–989, 2001.
7 Veronique A, Smits J, Klompmaker R, Vallenius T, Rijksen G, Makela TP and Medema RH: p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. J Biol Chem *275*: 30638–30643, 2000.
8 Chellappan SP, Giordano A and Fisher PB: Role of cyclin-dependent kinases and their inhibitors in cellular differentiation and development. Curr Top Microbiol Immunol *227*: 57-103, 1998.
9 You J and Bird RC: Selective induction of cell cycle regulatory genes cdk1 (p34cdc2), cyclins A/B, and the tumor suppressor gene Rb in transformed cells by okadaic acid. J Cell Physiol *164*: 424-433, 1995.
10 Onishi T, Zhang W, Cao X and Hruska K: The mitogenic effect of parathyroid hormone is associated with E2F-dependent activation of cyclin-dependent kinase 1 (cdc2) in osteoblast precursors. J Bone Miner Res *12*: 1596-1605, 1997.
11 Liu H and Bird RC: Characterization of the enhancer-like okadaic acid response element region of the cyclin-dependent kinase 1 (p34cdc2) promoter. Biochem Biophys Res Commun *246*: 696-702, 1998.
12 Ishii T, Shimizu M, Kanayama Y, Nakada S, Nojima H and Oda K: Differential activation of cyclin and cyclin-dependent kinase genes by adenovirus E1A12S cDNA product. Exp Cell Res *208*: 407-414, 1993.
13 Dalton S: Cell cycle regulation of the human cdc2 gene. EMBO J *11*: 1797-1804, 1992.
14 Yamamoto M, Yoshida M, Ono K, Fujita T, Ohtani-Fujita N, Sakai T and Nikaido T: Effect of tumor suppressors on cell cycle-regulatory genes: RB suppresses p34cdc2 expression and normal p53 suppresses cyclin A expression. Exp Cell Res *210*: 94-101, 1994
15 Furukawa Y, Terui Y, Sakoe K, Ohta M and Saito M: The role of cellular transcription factor E2F in the regulation of cdc2 mRNA expression and cell cycle control of human hematopoietic cells. J Biol Chem *269*: 26249-26258, 1994.
16 Morishita R, Gibbons GH, Horiuchi M, Ellison KE, Nakama M, Zhang L, Kaneda Y, Ogihara T and Dzau VJ: Gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. Proc Natl Acad Sci USA *92*: 5855-5859, 1995.
17 Tsubota Y, Takeuchi A, Shimizu M, Nakajima T, Nojima H and Oda K: Negative regulation of the rat cdc2 promoter in G1 by the silencer element. Cell Growth Differ *9*: 257-265, 1998.
18 North S, Espanel X, Tavitian B, Brun G and Gillet G: Two distinct regulatory elements control quail cdc2 transcription: possible involvement in the control of retinoblast differentiation. Cell Growth Differ *7*: 339-349, 1996.
19 North S, Espanel X, Bantignies F, Viollet B, Vallet V, Jalinot P, Brun G and Gillet G: Regulation of cdc2 gene expression by the upstream stimulatory factors (USFs). Oncogene *18*: 1945-1955, 1999.
20 Taylor WR, DePrimo SE, Agarwal A, Agarwal ML, Schonthal AH, Katula KS and Stark GR: Mechanisms of G2 arrest in response to overexpression of p53. Mol Biol Cell *10*: 3607-3622, 1999.
21 Tommasi S and Pfeifer GP: *In vivo* structure of the human cdc2 promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of cdc2 expression. Mol Cell Biol *15*: 6901-6913, 1995.
22 Taylor WR, Schonthal AH, Galante J and Stark GR: p130/E2F4 binds to and represses the cdc2 promoter in response to p53. J Biol Chem *276*: 1998-2006, 2001.
23 Sugarman JL, Schonthal AH and Glass CK: Identification of a cell-type-specific and E2F-independent mechanism for repression of cdc2 transcription. Mol Cell Biol *15*: 3282-3290, 1995.
24 Zwicker J, Lucibello FC, Wolfraim LA, Gross C, Truss M, Engeland K and Muller R: Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. EMBO J *14*: 4514-4522, 1995.
25 Badie C, Itzhaki JE, Sullivan MJ, Carpenter AJ and Porter AC: Repression of CDK1 and other genes with CDE and CHR promoter elements during DNA damage-induced G(2)/M arrest in human cells. Mol Cell Biol *20*: 2358-2366, 2000.
26 Calabretta B and Nicolaides NC: c-myb and growth control. Crit Rev Eukaryot Gene Expr *2*: 225-235, 1992.
27 Ku DH, Wen SC, Engelhard A, Nicolaides NC, Lipson KE, Marino TA and Calabretta B: c-myb transactivates cdc2 expression *via* Myb binding sites in the 5'-flanking region of the human cdc2 gene. J Biol Chem *268*: 2255-2259, 1993.
28 Born TL, Frost JA, Schonthal A, Prendergast GC and Feramisco JR: c-Myc cooperates with activated Ras to induce the cdc2 promoter. Mol Cell Biol *14*: 5710-5718, 1994.
29 Wen SC, Ku DH, De Luca A, Claudio PP, Giordano A and Calabretta B: ets-2 regulates cdc2 kinase activity in mammalian cells: coordinated expression of cdc2 and cyclin A. Exp Cell Res *217*: 8-14, 1995.
30 Chen H, Campisi J and Padmanabhan R: SV40 large T antigen transactivates the human cdc2 promoter by inducing a CCAAT box binding factor. J Biol Chem *271*: 13959-13967, 1996.
31 Tanimoto A, Chen H, Kao CY, Moran E, Sasaguri Y and Padmanabhan R: Transactivation of the human cdc2 promoter by adenovirus E1A in cycling cells is mediated by induction of a 110-kDa CCAAT-box-binding factor. Oncogene *17*: 3103-3114, 1998.
32 Tanimoto A, Kao CY, Chang CC, Sasaguri Y and Padmanabhan R: Deregulation of cdc2 gene expression correlates with overexpression of a 110 kDa CCAAT box binding factor in transformed cells. Carcinogenesis *19*: 1735-1741, 1998.
33 Kao CY, Tanimoto A, Arima N, Sasaguri Y and Padmanabhan R: Transactivation of the human cdc2 promoter by adenovirus E1A. E1A induces the expression and assembly of a heteromeric complex consisting of the CCAAT box binding factor, CBF/NF-Y, and a 110-kDa DNA-binding protein. J Biol Chem *274*: 23043-23051, 1999.
34 Yun J, Chae HD, Choy HE, Chung J, Yoo HS, Han MH and Shin DY: p53 negatively regulates cdc2 transcription *via* the CCAAT-binding NF-Y transcription factor. J Biol Chem *274*: 29677-29682, 1999.
35 Liu N, Lucibello FC, Korner K, Wolfraim LA, Zwicker J and Muller R: CDF-1, a novel E2F-unrelated factor, interacts with cell cycle-regulated repressor elements in multiple promoters. Nucleic Acids Res *25*: 4915-4920, 1997.

36 Lucibello FC, Liu N, Zwicker J, Gross C and Muller R: The differential binding of E2F and CDF repressor complexes contributes to the timing of cell cycle-regulated transcription. Nucleic Acids Res *25*: 4921-4925, 1997.

37 Zwicker J, Lucibello FC, Jerome V, Brusselbach S and Muller R: CDF-1-mediated repression of cell cycle genes targets a specific subset of transactivators. Nucleic Acids Res *25*: 4926-4932, 1997.

38 Nettelbeck DM, Jerome V and Muller R: A dual specificity promoter system combining cell cycle-regulated and tissue-specific transcriptional control. Gene Ther *6*: 1276-1281, 1999.

39 Yamashita K, Yasuda H, Pines J, Yasumoto K, Nishitani H, Ohtsubo M, Hunter T, Sugimura T and Nishimoto T: Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. EMBO J *9*: 4331-4338, 1990.

40 Schonthal A: Okadaic acid--a valuable new tool for the study of signal transduction and cell cycle regulation? New Biol *4*: 16-21, 1992.

41 Sambrook J, Fritsch EF and Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor, NY Cold Spring Harbor Press, 1989.

42 Lee MG and Nurse P: Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature *327*: 31-35, 1987.

43 Pines J and Hunter T: Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell *58*: 833-846, 1989.

44 Pines J and Hunter T: Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature *346*: 760-763, 1990.

45 Goldberg GS and Lau AF: Transfection of mammalian cells with PEG-purified plasmid DNA. Biotechniques *14*: 548-550, 1993.

46 Liu Q, Yan H, Dawes NJ, Lu Y and Zhu H: Transcriptional activation of the p34cdc2 gene by cdc2 promoter binding factor/nuclear factor-Y in fetal rat ventricular myocytes. Circ Res *82*: 251-260, 1998.

47 Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG and Struhl K: Current Protocols in Molecular Biology, New York, Greene Publishing Assoc. & Wiley, Interscience, 1989.

48 van Santen VL: Characterization of a bovine herpesvirus 4 immediate-early RNA encoding a homolog of the Epstein-Barr virus R transactivator. J Virol *67*: 773-784, 1993.

49 Goodwin GH: The analysis of sequence-specific DNA-binding proteins in cell extracts. *In*: Gel Electrophoresis of Nucleic Acids. A Practical Approach (Rickwood D and Hames BD, eds). Oxford, IRL press, 1990, pp 225-247.

50 Akiyama Y: TFSEARCH: Searching Transcription Factor Binding Sites, 1995. http://www.cbrc.jp/research/db/TFSEARCH.html.

51 Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL and Kolchanov NA: Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. Nucleic Acids Res *26*: 364-370, 1998.

52 Blochlinger K, Bodmer R, Jack J, Jan LY and Jan YN: Primary structure and expression of a product from cut, a locus involved in specifying sensory organ identity in *Drosophila*. Nature *333*: 629-635, 1988.

53 Blochlinger K, Bodmer R, Jan LY and Jan YN: Patterns of expression of cut, a protein required for external sensory organ development in wild-type and cut mutant *Drosophila* embryos. Genes Dev *4*: 1322-1331, 1990.

54 Blochlinger K, Jan LY and Jan YN: Transformation of sensory organ identity by ectopic expression of cut in *Drosophila*. Genes Dev *5*: 1124-1135, 1991.

55 Jack J, Dorsett D, Delotto Y and Liu S: Expression of the cut locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distant enhancer. Development *113*: 735-747, 1991.

56 Liu S, McLeod E and Jack J: Four distinct regulatory regions of the cut locus and their effect on cell type specification in *Drosophila*. Genetics *127*: 151-159, 1991.

57 Harada R, Dufort D, Denis-Larose C and Nepveu A: Conserved cut repeats in the human cut homeodomain protein function as DNA binding domains. J Biol Chem *269*: 2062-2067, 1994.

58 van Wijnen AJ, van Gurp MF, de Ridder MC, Tufarelli C, Last TJ, Birnbaum M, Vaughan PS, Giordano A, Krek, W, Neufeld EJ, Stein JL and Stein GS: CDP/cut is the DNA-binding subunit of histone gene transcription factor HiNF-D: a mechanism for gene regulation at the G1/S phase cell cycle transition point independent of transcription factor E2F. Proc Natl Acad Sci USA *93*: 11516-11521, 1996.

59 Dufort D and Nepveu A: The human cut homeodomain protein represses transcription from the c-myc promoter. Mol Cell Biol *14*: 4251-4257, 1994.

60 Aziz F, van Wijnen AJ, Stein JL and Stein GS: HiNF-D (CDP-cut/CDC2/cyclin A/pRB-complex) influences the timing of IRF-2-dependent cell cycle activation of human histone H4 gene transcription at the G1/S phase transition. J Cell Physiol *177*: 453-464, 1998.

61 van Wijnen AJ, Cooper C, Odgren P, Aziz F, De Luca A, Shakoori RA, Giordano A, Quesenberry PJ, Lian JB, Stein GS and Stein JL: Cell cycle-dependent modifications in activities of pRb-related tumor suppressors and proliferation-specific CDP/cut homeodomain factors in murine hematopoietic progenitor cells. J Cell Biochem *66*: 512-523, 1997.

62 Santaguida M, Ding Q, Berube G, Truscott M, Whyte P and Nepveu A: Phosphorylation of the CCAAT displacement protein (CDP)/Cux transcription factor by cyclin A-Cdk1 modulates its DNA binding activity in G(2). J Biol Chem *276*: 45780-45790, 2001.

63 Aziz F, van Wijnen AJ, Vaughan PS, Wu S, Shakoori AR, Lian JB, Soprano KJ, Stein JL and Stein GS: The integrated activities of IRF-2 (HiNF-M), CDP/cut (HiNF-D) and H4TF-2 (HiNF-P) regulate transcription of a cell cycle controlled human histone H4 gene: mechanistic differences between distinct H4 genes. Mol Biol Rep *25*: 1-12, 1998.

64 Su S and Bird RC: Cell cycle, differentiation and tissue-independent expression of ribosomal protein L37. Eur J Biochem *232*: 789-97, 1995.

65 Seed B and Sheen Y: A simple phase-extraction assay for chloramphericol acetyltransferase activity. Gene *67*: 271-277, 1988.

Received February 3, 2004
Accepted April 5, 2004