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Cell cycle-regulated binding of nuclear proteins to elements within a mouse H3.2 histone gene

(intragenic elements)

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ABSTRACT **The histone gene family in mammals consists of 15–20 genes for each class of nucleosomal histone protein. These genes are classified as either replication-dependent or -independent in regard to their expression in the cell cycle. The expression of the replication-dependent histone genes increases dramatically as the cell prepares to enter S phase. Using mouse histone genes, we previously identified a coding region activating sequence (CRAS) involved in the up-regulation of at least two (H2a and H3) and possibly all nucleosomal replication-dependent histone genes. Mutation of two seven-nucleotide elements, α and Ω, within the H3 CRAS causes a decrease in expression in stably transfected Chinese hamster ovary cells comparable with the effect seen upon deletion of the entire CRAS. Further, nuclear proteins interact in a highly specific manner with nucleotides within these sequences. Mutation of these elements abolishes DNA/protein interactions *in vitro*. Here we report that the interactions of nuclear factors with these elements are differentially regulated in the cell cycle and that protein interactions with these elements are dependent on the phosphorylation/dephosphorylation state of the nuclear factors.**

The histone gene family is highly conserved among higher eukaryotes (1). The expression of the replication-independent histone genes in vertebrates is low and constitutive throughout the eukaryotic cell cycle. These genes encode the so-called replacement-variant histones (1, 2). Their products accumulate only in nondividing cells that have ceased to produce the replication-dependent histones (3). In contrast, the expression of the replication-dependent histone genes is tightly linked to the onset of DNA synthesis (2, 4). These histone genes are activated in late G_1 phase and accumulation of mRNA transcripts peaks during S phase (5–7). The amount of histone mRNA in the cell is regulated at both the transcriptional and posttranscriptional levels (7, 8). Posttranscriptional regulation of histone genes occurs at the level of 3′ processing of pre-mRNA and mRNA degradation (8). Transcriptional regulation of certain classes of histone genes is mediated by promoter proximal class-specific elements interacting with their cognate trans-acting factors (9–17). Posttranslational modification by phosphorylation of these factors has been implicated in cell cycle regulation of human H2b and H4 histone gene expression (10, 18–20). However, these factors and their target promoter sequences are not shared among the different classes of histone genes.

Our identification of a sequence present in the coding region of two replication-dependent mouse histone genes (H2a and H3), responsible for a 20-fold effect on the level of expression of these genes (21, 22), was the first report of an element common to more than a single class of histone genes. We demonstrated by *in vivo* transfection experiments of a chimeric H2a.2 gene containing the H3.2 coding region activating sequence (CRAS) that the H3.2 CRAS is capable of functionally replacing the H2a.2 CRAS by restoring high expression (22). Expression of site-directed H3.2 CRAS mutants in stably transfected Chinese hamster ovary (CHO) cells has functionally verified that two 7-bp subsequences common to H2a, H3, H2b, and H4 mouse histone genes are responsible for the loss of expression observed upon deletion of the 110-bp H3.2 CRAS (23, 24). These elements are designated the CRAS α and Ω. Deletion or mutation of the H3.2 α or Ω element alone results in a 4-fold drop in expression in stable transfectants when compared with the expression of the intact gene. However, a much greater decrease in expression is observed when both the α and Ω sequences are mutated, suggesting cooperativity between these two elements (23).

The α and Ω elements act as binding sites for mouse nuclear proteins (23–25). These DNA/protein interactions have been studied extensively via electrophoretic mobility-shift assay (EMSA), DNase I footprinting, and methylation interference and are similar if not identical among the different classes of replication-dependent histone genes (refs. 23–25 and 38). In contrast, a replacement variant H3 gene, H3.3, contains mutations in both α and Ω elements in the H3 coding sequence and does not interact with mouse nuclear proteins (23, 24). In fact, the naturally mutated H3.3 "CRAS α" element (five out of seven nucleotide changes) encodes the diagnostic amino acids (codons 89 and 90) characteristic to every vertebrate H3.3 known (1, 26, 27). The presence of the CRAS α and Ω elements in the replication-dependent histone genes and the absence of these sequences in a replication-independent H3.3 gene suggest that the CRAS elements are involved in the correct regulation of the replication-dependent histone genes in the cell cycle. Here we report that the CRAS α and Ω binding activities are present only in G_1 phase extracts, that these activities are regulated in an opposite fashion by phosphorylation/dephosphorylation, and that a cyclin D_1-dependent factor is capable of phosphorylating the CRAS α binding protein(s).

MATERIALS AND METHODS

Preparation of Nuclear Extracts from Logarithmically Growing and Synchronous Populations of Cells. Mouse myeloma cells were grown in spinner cultures and logarithmic phase nuclear extracts were prepared as described (22, 28). To prepare cell cycle phase-specific extracts, logarithmically growing CHO cells were plated ($6–8 \times 10^6$ per 75-cm^2 flask) in McCoy's 5a medium supplemented with 10% calf serum and

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Abbreviations: CRAS, coding region activating sequence; CHO, Chinese hamster ovary; EMSA, electrophoretic mobility-shift assay; CIAP, calf intestinal alkaline phosphatase; PP2A, protein phosphatase 2A; PTP-1B, protein tyrosine phosphatase 1B.

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5% CO_2 at 37°C 24 hr before mitotic selection. CHO cells traverse the cell cycle in approximately 12 hr under these growth conditions (8). Selection of cells was achieved by use of a semiautomatic mitotic shaker apparatus, using the Terasima and Tolmach (29) method as modified by Schneiderman *et al.* (30). Mitotic cells were pooled on ice for no more than 4 hr, then plated at time 0. After plating, the cells were allowed to progress through the cell cycle under the conditions described above for harvest at different time points. For the 0-hr extract, cells were immediately harvested and nuclear extracts prepared. Cells were plated in parallel for each time point on glass slides and labeled with BrdUrd for 15 min before the end of the time point. The cells from the 0-hr sample were allowed to attach to the glass slide for 15 min and were labeled with BrdUrd for additional 15 min. Detection of labeled cells used immunofluorescence reagents from Boehringer Mannheim. The number of cells in S phase was determined by counting several hundred cells on photomicrographs for each time point and calculating the percentage showing nuclear fluorescence (due to BrdUrd incorporation) as an indicator of entry in S phase. Nuclear extracts for the various time points were prepared from 5–10 $\times$ 10^6 cells as described by Stauber and Schumperli (31).

EMSA. The mouse H3.2 CRAS was excised from the appropriate subclone and 3′ end-labeled with [α-^{32}P]-dATP and -dCTP using the Klenow fragment of DNA polymerase I (21, 22). The nucleotide sequence of the H3.2 gene may be retrieved from the GenBank data base under the EMBL accession number X16148. The labeled probe fragment was excised from a 6% native gel and used for detection of the CRAS α and Ω interactions (22, 24). Synthetic oligonucleotides for detection of only the CRAS Ω interactions were radioactively labeled as above and used in EMSA (23). The oligonucleotide sequences are: H3.2 CRAS Ω duplex:

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GTGCGCGAGATCGCGCAGGACT
    CGCTCTAGCGCGTCCTGAcacg
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Lowercase letters indicate nucleotides that are not found in the H3 sequence but are complementary to the 5′ overhang. Binding conditions and electrophoretic separation of DNA/protein complexes were as described (22). Human H2b octamer oligonucleotides were obtained from Pharmacia.

Partial Purification of CRAS-Binding Proteins. Mouse myeloma cell nuclear extract (20, 26) was dialyzed against 20 vol of buffer (20 mM Hepes, pH 7.9/0.2 mM EGTA/0.2 mM EDTA/100 mM KCl/20% glycerol/2 mM dithiothreitol) and was then applied to DNA-cellulose resin (40-ml bed volume; Pharmacia) equilibrated with this buffer. Proteins were eluted with the same buffer containing 200 mM, 400 mM, or 1 M KCl. Fractions were collected and aliquots analyzed by EMSA.

Protein Phosphatase Assays. Calf intestinal alkaline phosphatase (CIAP, Boehringer Mannheim), protein phosphatase 2A [PP-2A, Upstate Biotechnology (Lake Placid, NY)], or protein tyrosine phosphatase 1B (PTP-1B, Upstate Biotechnology) were incubated with nuclear extract for the specified time at 37°C. PP-2A is a serine/threonine phosphatase, PTP-1B is a tyrosine phosphatase, and CIAP is nonspecific in its action. After preincubation with the indicated phosphatase, an aliquot (4–6 μg of total protein) was added to the binding reaction with radioactive DNA probe and incubated for 20 min at 4°C, followed by EMSA. ATP (Sigma) was added to a final concentration of 0.1 mM in some reactions (as indicated in the figure legends) during the pretreatment at 37°C.

Cyclin D_1 Immunoaffinity Column Fractionation of Nuclear Extracts. Mouse myeloma cell nuclear extract was applied to a cyclin D_1 immunoaffinity column (Santa Cruz Biotechnology), and eluted from the column as described by the manufacturer. The flow-through, wash, and eluate fractions were analyzed for CRAS-binding activity by EMSA.

RESULTS

The DNA-Binding Activities of CRAS α and Ω Factors Are Cell Cycle Regulated. We have previously shown that the H3.2 CRAS α and Ω elements are required for normal expression *in vivo* and interact with nuclear proteins *in vitro* (23, 24). To examine the possibility that the interactions of the α and Ω proteins with their target elements are regulated in the cell cycle, we prepared nuclear extracts from synchronous populations of CHO cells in specific phases of the cell cycle by mitotic shake-off (see *Materials and Methods*). Cells were plated and extracts prepared for the following time points: 0 hr

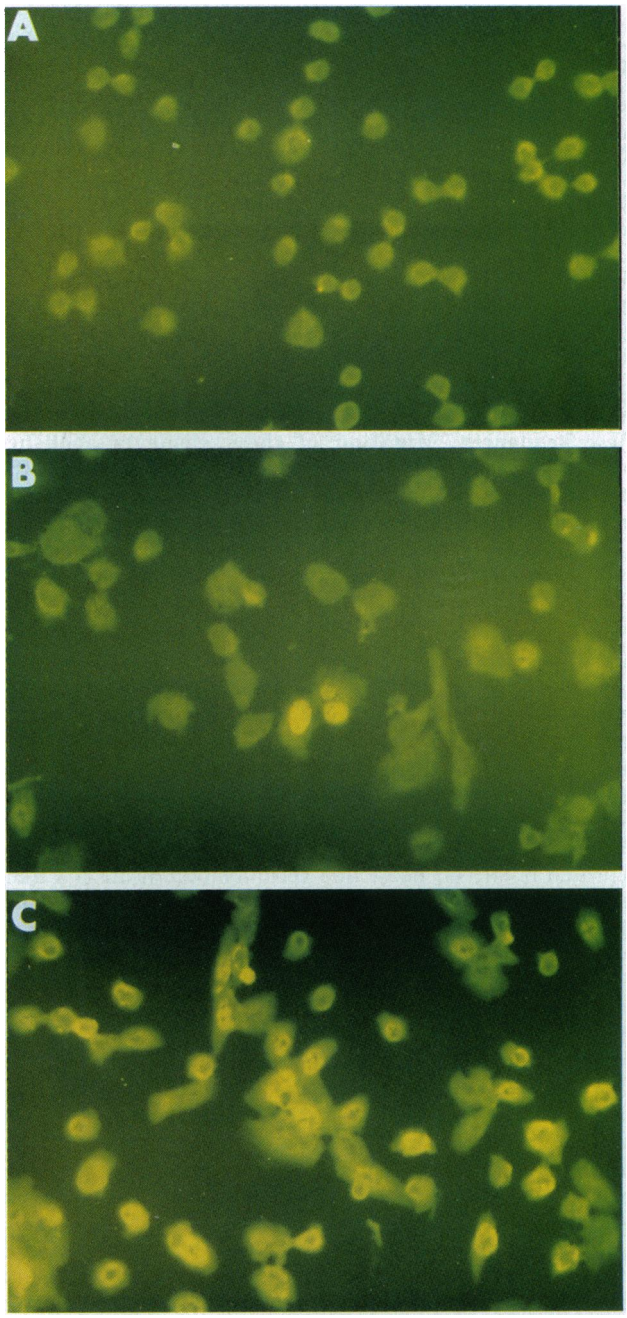

FIG. 1. Synchronous CHO cell populations are the source of phase-specific nuclear extracts. CHO cells were synchronized by mitotic shake-off and then plated for 0, 2, or 5 hr. The cell populations were labeled with BrdUrd and stained for immunofluorescent detection of cells incorporating BrdUrd. (*A*) CHO cells 30 min after plating (M phase); *B*, 2 hr after plating (G_1 phase); *C*, 5 hr after plating (S phase). (×40.)

(M phase), 2 hr (G_1 phase), and 5 hr (S phase). Synchrony of these populations of cells was determined via incorporation of BrdUrd as an indicator for DNA synthesis (S phase). Fig. 1 shows fluorescent photomicrographs of CHO cells labeled with BrdUrd. Mitotic cells (Fig. 1*A*) show only background fluorescence. Two percent of the cells from the 2-hr sample (Fig. 1*B*) show nuclear fluorescence. However, the majority of cells (>80%) from the 5-hr time point (Fig. 1*C*) show nuclear fluorescence. These results confirm that the extracts prepared from cells plated in parallel with those shown in Fig. 1 are representative of synchronous populations of cells harvested at a specific point in M, G_1, or S phase.

We tested these nuclear extracts for the presence of the α and Ω binding activities. Results of EMSA using labeled CRAS fragments and M-, G_1-, and S-phase extracts are shown in Fig. 2. A reaction containing the probe and extract prepared from logarithmically growing CHO cells is shown in lane 1 in Fig. 2*A*. The CRAS α binding activity was barely detectable in M phase (lane 2), increased dramatically by 2 hr after mitosis (G_1 phase, lane 3), and was significantly lower than in G_1 phase 5 hr after mitosis (S phase, lane 4). The shake-off experiment with preparations of new extracts was repeated three times with identical results. Experiments using phase-specific extracts with synthetic duplex oligonucleotides containing only the Ω element are shown in Fig. 2*B*. The pattern of the Ω binding activity in the cell cycle is identical to that of the α binding activity. Ω binding activity was missing from M-phase extracts (lane 2), present in extracts prepared from cells in G_1 phase (lane 3), and very low in S-phase extracts (lane 4). Additionally, we tested these phase-specific extracts for Oct-1 binding activity. The octamer-dependent DNA binding activity and *in vitro* transcriptional effect of Oct-1 upon transcription from the H2b promoter have been extensively studied (9, 15, 18, 19). Previous studies performed with synchronized cells have shown that Oct-1 DNA-binding activity is missing in mitotic extracts and is not significantly different in G_1- and S-phase extracts (18–20). An experiment using our phase-specific extracts reproduced the results of these previous studies (Fig. 2*C*). The Oct-1/octamer DNA complex in the figure is designated by O. Oct-1 binding activity was absent in M-phase extracts (lane 2) and present in both G_1 and S phases without significant variability between the two extracts (lanes 3 and 4). These results show that the dramatic increase in the CRAS binding activities of the α and Ω factors observed in G_1-phase extracts is genuine and is not due to extract variability.

CIAP Treatment of Nuclear Extract Affects α and Ω Binding Activities, but in an Opposite Fashion. Lane 1 in Fig. 3*A* shows the control reaction products formed upon incubation of nuclear extract with labeled CRAS fragment at 4°C. Lane 2 shows that preincubation of nuclear extract at 37°C for 30 min, before addition to the binding reaction, abolished the CRAS α complex. In the same reaction, however, the CRAS Ω complex was enhanced. Preincubation periods of 15 min to 1 hr at 37°C produced the same results (data not shown). Addition of CIAP (0.5 or 1.0 unit) to nuclear extract for 30 min at 37°C produced quite a different result (lanes 3 and 4). After limited CIAP treatment, the α complex was detected, while the Ω complex was no longer observed. Higher CIAP concentrations (>2.0 units) caused the disappearance of the α complex (lane 5).

Evidence that a kinase might be involved in the loss of the α binding activity at 37°C was obtained after fractionation of nuclear extract (see *Materials and Methods*). EMSA of crude mouse myeloma nuclear extract is shown in lanes 1–3 in Fig. 3*B*. Lanes 1 and 2 of Fig. 3 *A* and *B* are directly comparable. The extract in lanes 1 and 2 was incubated at 4°C and for 30 min at 37°C, respectively, before addition to the binding reactions. Unlike the result observed in lane 2 of Fig. 3*A*, the crude nuclear extract in lane 3 of Fig. 3*B* showed stable α binding activity at 37°C. The expected loss of the α binding activity at 37°C was observed only after addition of exogenous ATP to the 37°C reaction (Fig. 3*B*, lane 3). These results were due to depletion of small molecules (e.g., ATP) from the crude nuclear extract (column load) by dialysis. More direct evidence for the involvement of a kinase was obtained with the 0.4 M KCl DNA-cellulose column fraction, containing the CRAS α and Ω binding activities. As in lanes 1 and 2, the 0.4 M fraction

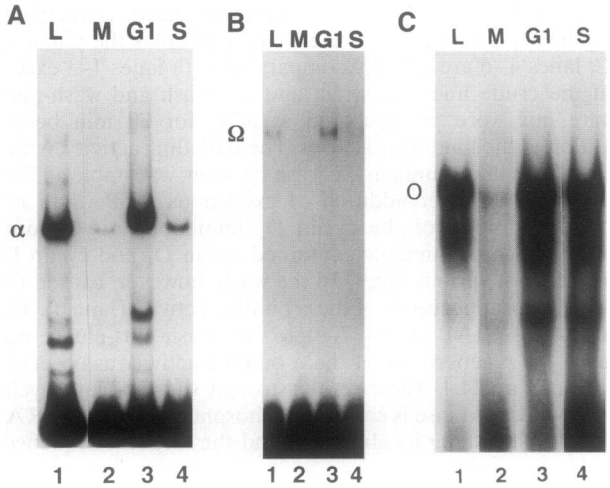

FIG. 2. Binding of the α and Ω factors to the H3 CRAS is cell cycle regulated. Nuclear extracts prepared from synchronous cell populations shown in Fig. 1 were used in all parts of this figure. Lanes: 1, asynchronous, logarithmically growing cells (L); 2, M-phase cells; 3, G_1-phase cells; and 4, S-phase cells. The amount of protein used was the same for all lanes within each panel. (*A*) Probe, 3′ end-labeled full-length H3.2 CRAS fragment. The CRAS α complex is designated by α. (*B*) The probe, 3′ end-labeled H3.2 synthetic oligonucleotides containing the CRAS Ω binding site (see *Materials and Methods*). The Ω complex is designated by Ω. (*C*) Probe, labeled human H2b octamer oligonucleotides. The human H2b octamer complex is designated by O.

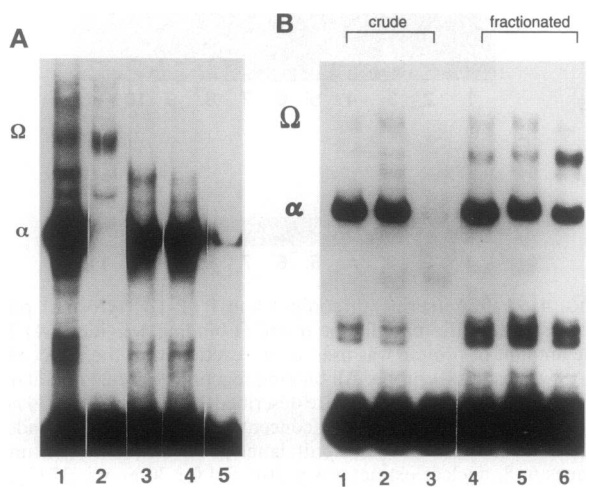

FIG. 3. The state of phosphorylation differentially affects α and Ω binding activities of the H3.2 CRAS. (*A*) Crude mouse nuclear extract was preincubated before addition to the binding reaction (4°C, 20 min) with or without CIAP. Probe, the intact H3.2 CRAS fragment. Lanes: 1, 4°C for 30 min; 2, 30 min at 37°C; 3, 0.5 unit of CIAP for 30 min at 37°C; 4, 1.0 unit of CIAP, 30 min at 37°C; 5, 2.0 unit of CIAP, 30 min at 37°C. (*B*) Large-scale mouse crude nuclear extract prepared for column fractionation (lanes 1–3) or DNA-cellulose column 0.4 M KCl fraction (lanes 4–6) (see *Materials and Methods*) was used in this panel. Lanes: 1, 4°C for 30 min; 2, 37°C for 1 hr; 3, 37°C for 1 hr, 0.1 mM ATP final concentration. The same conditions were duplicated for the 0.4 M KCl DNA cellulose fraction (lanes 4–6).

was incubated at 4°C (lane 4) or at 37°C (lane 5). Addition of ATP to the preincubation at 37°C with the partially purified column fraction had little effect on the α complex formation as is seen in lane 6 of Fig. 3*B*. We conclude from these results that the kinase(s) responsible for the loss of α binding activity in lane 3 of this panel does not elute with the CRAS-binding proteins in the 0.4 M fraction. The column fractionation experiment provides evidence that an ATP-dependent activity, a kinase, present in crude nuclear extracts prepared from logarithmically growing mouse cells, is responsible for the loss of α binding activity at 37°C. This loss, mediated by ATP-dependent phosphorylation, is freely reversible by removal of the phosphate group with CIAP, restoring the DNA-binding activity (Fig. 3*A*, lanes 3 and 4).

Next we examined the effects of two specific protein phosphatases, PP2A (a serine/threonine phosphatase) and PTP-1B on the α and Ω binding activities. The results are presented in Fig. 4. In Fig. 4*A*, lanes 1 and 2 are control reactions containing crude extract, preincubated at 4°C and 37°C, before addition to the binding reactions. In lanes 3–11, the extract was preincubated for 30 min at 37°C with increasing concentrations of PP-2A (lanes 3–5), CIAP (lanes 6–8), and PTP-1B (lanes 9–11), before addition to the binding reaction. As Fig. 4*A* shows, incubation for 30 min at 37°C abolished α complex formation, while the Ω complex was unaffected (lane 2). However, addition of PP-2A and PTP-1B at the appropriate concentrations (lanes 4 and 10, respectively) to the extracts for

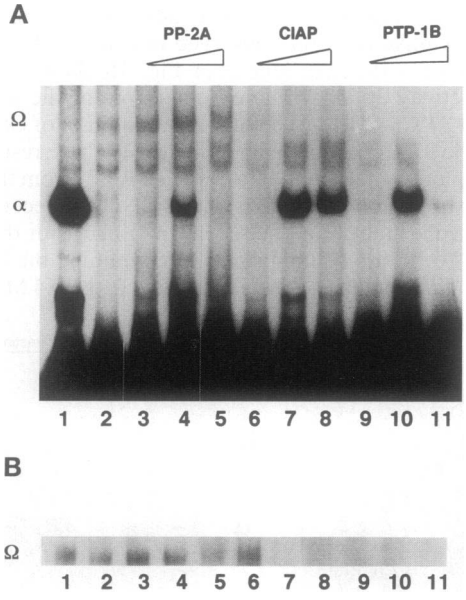

FIG. 4. PP-2A (serine/threonine) and PTP-1B (tyrosine) phosphatases affect differentially the α and Ω binding activities. (*A*) The same amount of protein was used in all reactions and the intact H3.2 CRAS was used as a probe. All binding reactions were 20 min at 4°C. Preincubation times ± enzyme are described. Lanes: 1, 4°C for 30 min; 2, 37°C for 30 min; 3–5, increasing concentrations of PP-2A were added for 30 min at 37°C (lane 3, 0.01 unit; lane 4, 0.06 unit; lane 5, 0.1 unit). In lanes 6–8, nuclear extract was pretreated for 30 min at 37°C with increasing concentration of CIAP (lane 6, 0.5 unit; lane 7, 1.0 unit; lane 8, 2.0 units). In lanes 9–11, crude nuclear extract was incubated with increasing concentrations of PTP-1B (lane 9, 5 μl; lane 10, 8 μl; lane 11, 10 μl). (*B*) Binding reactions contained the same amount of protein, and a labeled *Sal* I–*Ava* II CRAS fragment, containing only the Ω binding site, was used as probe. Incubation times and temperatures are the same as in *A*; lanes 1 and 2 are control reactions, nuclear extract incubated at 4°C and 37°C; lanes 3–5, nuclear extract was incubated with increasing concentrations of PP-2A (lane 3, 0.02 unit; lane 4, 0.04 unit; lane 5, 0.08 unit). In lanes 6–8, nuclear extract was treated with 0.5, 1.0, and 2.0 unit(s) of CIAP, respectively. In lanes 9–11, nuclear extract was treated with increasing concentrations of PTP-1B (lane 9, 1 μl; lane 10, 5 μl; lane 11, 10 μl).

30 min at 37°C restored the α binding activity. The Ω binding activity, however, was unaffected by PP-2A treatment (lanes 3–5), while addition of PTP-1B (lanes 9–11) caused the complete disappearance of the Ω complex at all concentrations, reproducing the effect of CIAP on the Ω complex shown in Figs. 3*A* and 4*A* (lanes 6–8). These results indicate that phosphorylation on both serine/threonine and tyrosine residues are capable of inhibiting the binding of the α factor to its target sequence since dephosphorylation events of both types activate the CRAS α binding activity. Only tyrosine phosphorylation, however, is involved in the activation of the Ω binding activity, since a tyrosine-specific phosphatase abolished its ability to bind its target (lanes 9–11).

To examine the effects of the specific phosphatases upon the Ω activity alone, a probe fragment containing only the Ω sequence was used. In Fig. 4*B*, lanes 1 and 2 are control reactions, containing mouse myeloma nuclear extract incubated at 4°C or at 37°C for 30 min, respectively, before addition to the binding reaction. Lanes 3–5 contain extracts incubated with increasing concentrations of PP-2A, CIAP (lanes 6–8), or PTP-1B (lanes 9–11). Incubation of the extracts with PP-2A had little effect on the Ω activity (lanes 3–5), except at the highest concentration. At higher concentrations, PP-2A addition diminished the Ω activity but we assume this may be due to nonspecific dephosphorylation, or alternatively, to a contaminating phosphatase activity. PTP-1B abolished the Ω complex, even in the lowest concentration (lanes 9–11). The data shown in Fig. 4*B* confirm that tyrosine-specific phosphorylation and dephosphorylation play important roles in the interaction of the Ω factor(s) with the CRAS Ω element.

A Cyclin D_1-Dependent Factor Can Play a Role in the Phosphorylation Events That Regulate the Binding Activity of the CRAS α Factor. Because the CRAS α binding activity dramatically increases in G_1 phase and phosphorylation/dephosphorylation regulates its binding to the CRAS sequence, we looked for possible involvement of the cyclin-dependent kinases. Nuclear extract was applied to a cyclin D_1 immunoaffinity column and the column wash and eluate were assayed for CRAS α binding activity by EMSA. The results are shown in Fig. 5 with the CRAS fragment used as probe in all lanes: lane 1, a control reaction with crude nuclear extract; lane 2, cyclin D_1 column wash; lane 3, the wash plus the eluate at 4°C; lanes 4–6 are directly comparable with lanes 1–3 except that the crude nuclear extract and the wash and wash-plus-eluate mix were preincubated at 37°C for 30 min before addition to the binding reactions. The α binding activity, which eluted from the column in the wash fraction, was stable at 37°C (lane 5), even after addition of exogenous ATP (data not shown). Addition of the cyclin D_1 immunoaffinity column eluate (which presumably contained cyclin D_1 and cyclin D_1 complexes of various types) to the wash, however, caused the complete disappearance of the α binding activity (lane 6). The addition of CIAP (lanes 7–9) to the wash-plus-eluate mix caused the regeneration of the α binding activity as observed in Figs. 3*A* and 4*A*. These results strongly suggest that a cyclin D_1-dependent kinase is capable of phosphorylating the CRAS α factor, abolishing its ability to bind the target α sequence.

DISCUSSION

We have examined the temporal regulation in the cell cycle of the binding of the α and Ω factors to their target sequences in the histone CRAS. The α and Ω binding activities are not present in M phase, increase dramatically in G_1 phase, and return to low levels by S phase (Fig. 2 *A* and *B*). Oct-1 binding activity in these same phase-specific extracts does not vary dramatically in G_1- and S-phase extracts and is missing in M-phase extracts (Fig. 2*C*). No extracts prepared from M- and S-phase cells had any significant α or Ω binding activities, while both activities were present in all G_1-phase extracts examined.

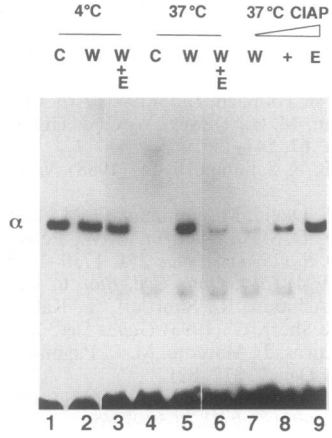

FIG. 5. EMSA of cyclin D_1 immunoaffinity column fractions. Crude mouse nuclear extract, prepared from synchronously growing cells, was applied to a cyclin D_1 immunoaffinity column and eluted as described in *Materials and Methods*. The collected fractions were assayed for α binding activity by EMSA with the full-length H3.2 CRAS as probe. All reactions contained the same amount of protein and reactions 1–3 were held at 4°C for 30 min, before addition to the binding reactions; lane 1, crude nuclear extract (C); lane 2, wash (W); lane 3, wash plus eluate mix (W+E); lanes 4–6, C, W, and W+E incubated for 30 min at 37°C, before addition to the binding reactions. Lanes 7–9, increasing concentrations of CIAP were added to the W+E mix for 30 min at 37°C prior to addition to the binding reaction (lane 7, 0.1 unit; lane 8, 0.5 unit; lane 9, 1.0 unit).

Our nuclear extracts represent a precise point in the progression of the cells through the cell cycle due to the mitotic shake-off technique. For example, our G_1 extract is prepared from cells at a single point in G_1 phase and not the entire G_1 phase. Therefore, all the events occurring in G_1 are not represented in our G_1 extracts. We can conclude only that at 2 hr after mitosis the binding activity of the α and Ω factors are activated, and that by 5 hr, when most cells have entered S phase (see Fig. 1*C*), very little binding activity is detected.

The peak in CRAS binding activities appears to coincide in time with the activation of the replication-dependent histone genes (5–8). These observations support our hypothesis that the α and Ω proteins are involved in regulating the transcription of replication-dependent histone genes in response to cellular signals in the cell cycle. CIAP treatment of the phase-specific extracts did not have an effect on the pattern of binding activities observed in Fig. 2 (data not shown). Therefore, the peak in binding activity in G_1 phase of the α binding protein itself or another factor required for α complex formation might be due to de novo protein synthesis. A more attractive explanation for these results is that in M and S phase, the α binding activity is in a complex with other factors and thus is unavailable for interaction with DNA or action by phosphatases.

Our results support the hypothesis that the phosphorylation state of the α and Ω proteins regulates their binding to the CRAS elements (Fig. 3). Treatment of heated extracts with CIAP restores the DNA binding activity of the α factor, but in the same reaction CIAP treatment causes the disappearance of the Ω–DNA complexes. This result leads us to propose that the α factor binds to the CRAS α element when it is dephosphorylated at a site (or sites) critical for binding activity. Conversely, the Ω protein must be phosphorylated at site(s) critical for binding to its target element, since CIAP treatment abolishes Ω/CRAS interactions. Using specific phosphatases, we showed that both serine/threonine and tyrosine dephosphorylation events are capable of recovering the α binding activity in heated extracts, whereas only tyrosine dephosphorylation affects the Ω binding activity (Fig. 4). Regulation of transcription factors via tyrosine phosphorylation appears to be a general mechanism by which growth factors can modulate gene expression (32, 33) but the involvement of such signal pathways remains to be determined.

Finally, a cyclin D_1-dependent factor is capable of phosphorylating the CRAS α binding activity (Fig. 5). The D cyclin family of proteins has been shown to play key roles in the regulatory events occurring in G_1 phase of the cell cycle (34–37). Our finding provides an indirect link between replication-dependent histone gene expression and an important G_1-phase regulatory protein. It should be remembered that the extract for the immunoaffinity experiment was prepared from an asynchronous cell population. This extract likely contained all possible cyclin D_1 complexes, while the G_1 phase extract (used in Fig. 2) contained only the cyclin complexes present at a precise point in G_1 phase of the cell cycle.

In summary, the data presented here show that phosphorylation and dephosphorylation of the CRAS α and Ω factors play central roles in modulating the binding of the proteins to their target sequences. Evidence obtained from the use of phase-specific nuclear extracts implicate these CRAS elements in the cell cycle regulation of histone gene expression. Further, we have demonstrated indirectly that a G_1 cyclin-dependent factor is capable of phosphorylating of the CRAS α factor *in vitro*. We have not directly shown that the CRAS is required for correct regulation of replication-dependent histone genes in the cell cycle, but three indirect lines of evidence support this hypothesis: (*i*) the critical role of the state of phosphorylation in the interaction of the CRAS-binding proteins with their target elements, (*ii*) the peak of CRAS α and Ω binding activities in G_1 phase of the cell cycle, and (*iii*) phosphorylation of the CRAS α factor by a cyclin D_1-dependent complex. Together, the evidence presented here strongly supports our hypothesis that the CRAS and its cognate factors play important roles in replication-dependent histone gene expression.

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1. Zweidler, A. (1984) in *Histone Genes: Structure, Organization and Regulation*, eds. Stein, G., Stein, J. & Marzluff, W. (Wiley, New York), pp. 373–395.
2. Wu, R. S. & Bonner, W. M. (1981) *Cell* **27,** 321–330.
3. Groppi, V. E. J. & Coffino, P. (1980) *Cell* **21,** 195–204.
4. Osley, M. A. (1991) *Annu. Rev. Biochem.* **60,** 827–861.
5. Graves, R. A. & Marzluff, W. F. (1984) *Mol. Cell. Biol.* **4,** 351–357.
6. Heintz, N., Sive, H. L. & Roeder, R. G. (1983) *Mol. Cell. Biol.* **2,** 539–550.
7. Sittman, D. B., Graves, R. A. & Marzluff, W. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1849–1853.
8. Harris, M. E., Bohni, R., Schneiderman, M. H., Ramamurthy, L., Schumperli, D. & Marzluff, W. F. (1991) *Mol. Cell. Biol.* **11,** 2416–2424.
9. Heintz, N. (1991) *Biochim. Biophys. Acta* **1088,** 327–339.
10. van Wijnen, A. J., Ramsey-Ewing, A. L., Bortell, R., Owen, T. A., Lian, J. B., Stein, J. L. & Stein, G. (1991) *J. Cell. Biochem.* **46,** 174–189.
11. Dailey, L., Roberts, S. B. & Heintz, N. (1988) *Genes Dev.* **2,** 1700–1712.
12. Gallinari, P., LaBella, F. & Heintz, N. (1989) *Mol. Cell. Biol.* **9,** 1566–1575.
13. LaBella, F. & Heintz, N. (1991) *Mol. Cell. Biol.* **11,** 5825–5831.
14. Artishevsky, A., Wooden, S., Sharma, A., Resendez, E., Jr., & Lee, A. (1987) *Nature (London)* **328,** 823–827.
15. LaBella, F., Sive, H. L., Roeder, R. G. & Heintz, N. (1988) *Genes Dev.* **2,** 32–39.

16. Dalton, S. & Wells, J. R. E. (1988) *EMBO J.* **7,** 49–56.
17. LaBella, F., Gallinari, P., McKinney, J. & Heintz, N. (1989) *Genes Dev.* **3,** 1982–1990.
18. Roberts, S. B., Segil, N. & Heintz, N. (1991) *Science* **253,** 1022–1026.
19. Segil, N., Roberts, S. B. & Heintz, N. (1991) *Science* **254,** 1814–1816.
20. Fletcher, C., Heintz, N. & Roeder, R. G. (1987) *Cell* **51,** 773–781.
21. Hurt, M. M., Pandey, N. B. & Marzluff, W. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4450–4454.
22. Hurt, M. M., Bowman, T. L. & Marzluff, W. F. (1991) *Mol. Cell. Biol.* **11,** 2929–2936.
23. Kaludov, N. K., Pabon-Pena, L. & Hurt, M. M. (1996) *Nucleic Acids Res.* **24,** 523–531.
24. Bowman, T. L. & Hurt, M. M. (1995) *Nucleic Acids Res.* **23,** 3083–3092.
25. Bowman, T. L. (1994) Ph.D. dissertation (Florida State Univ., Tallahassee).
26. Franklin, S. G. & Zweidler, A. (1977) *Nature (London)* **266,** 273–275.
27. Wells, D. & Kedes, L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2834–2838.
28. Shapiro, D., Sharp, P. A., Wahli, W. & Keller, M. (1988) *DNA* **7,** 47–55.
29. Terasima, T. & Tolmach, L. J. (1963) *Exp. Cell Res.* **3,** 344–351.
30. Schneiderman, M. H., Dewey, W. C. & Highfield, D. P. (1971) *Exp. Cell Res.* **67,** 147–155.
31. Stauber, C. B. & Schumperli, D. (1988) *Nucleic Acids Res.* **16,** 9399–9413.
32. Larner, A. C., David, M., Feldman, G. M., Igarashi, K., Hacket, R. H., Webb, D. S. A., Sweitzer, S. M., Petricoin, E. F., III & Finbloom, D. S. (1993) *Science* **261,** 1730–1733.
33. Karin, M. (1994) *Curr. Opin. Cell. Biol.* **6,** 415–424.
34. Quelle, D., Ashmun, R., Shurtleff, S., Kato, J., Bar-Sagi, D., Russel, M. & Sherr, C. (1993) *Genes Dev.* **7,** 1559–1571.
35. Baldin, V., Lucas, J., Marcote, M. J., Pagano, M. & Draetta, G. (1993) *Genes Dev.* **7,** 812–821.
36. Sherr, C. J. & Roberts, J. M. (1995) *Genes Dev.* **9,** 1149–1163.
37. Sherr, C. J. (1993) *Cell* **73,** 1059–1065.
38. Bowman, T. L., Kaludov, N. K., Klein, M. & Hurt, M. M. (1996) *Gene*, in press.