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S Phase Activation of the Histone H2B Promoter by OCA-S, a Coactivator Complex that Contains GAPDH as a Key Component

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Summary

We have isolated and functionally characterized a multicomponent Oct-1 coactivator, OCA-S which is essential for S phase-dependent histone H2B transcription. The p38 component of OCA-S binds directly to Oct-1, exhibits potent transactivation potential, is selectively recruited to the H2B promoter in S phase, and is essential for S phase-specific H2B transcription in vivo and in vitro. Surprisingly, p38 represents a nuclear form of glyceraldehyde-3-phosphate dehydrogenase, and binding to Oct-1, as well as OCA-S function, is stimulated by NAD^+ but inhibited by NADH. OCA-S also interacts with NPAT, a cyclin E/cdk2 substrate that is broadly involved in histone gene transcription. These studies thus link the H2B transcriptional machinery to cell cycle regulators, and possibly to cellular metabolic state (redox status), and set the stage for studies of the underlying mechanisms and the basis for coordinated histone gene expression and coupling to DNA replication.

Introduction

Histones are essential components of eukaryotic chromosomes and play key roles in their maintenance, replication, and function. The genes that encode the various histone subtypes (H1, H2A, H2B, H3, and H4) are present in multiple copies, regulated at both transcriptional and posttranscriptional levels, and expressed predominantly in an S phase- and DNA replication-dependent fashion. Earlier studies on transcriptional mechanisms identified essential subtype-specific regulatory elements (SSREs) in the promoters of the S phase-inducible histone genes, as well as transcription factors that bind directly to these elements (reviewed in Osley, 1991). The cognate SSRE binding factors, however, seem not to account for the S phase regulation of histone gene transcription. For example, the SSRE of the human H2B gene contains a conserved octamer sequence, 5′-ATG CAAAT-3′, that, along with the TATA box, is necessary and sufficient for S phase-dependent H2B transcription (LaBella et al., 1988). However, the ubiquitous octamer binding protein (Oct-1) that acts through this element (Fletcher et al., 1987) cannot account for the S phase regulation since it shows no variation in expression level, DNA binding activity, or modification during the G1 to S and S to G2 phases of the cell cycle (Segil et al., 1991). These studies suggested the possible involvement of an S phase-specific cofactor(s) or modification of the general transcription machinery in conferring S phase-specific H2B transcription.

Transcriptional cofactors facilitate functional communication between sequence-specific transcriptional activators bound to distal regulatory elements and the basal transcription machinery assembled on proximal core promoter elements (reviewed in Malik and Roeder, 2000). While most cofactors are ubiquitous and mediate the function of diverse activators on a variety of genes, a subset play more specialized roles in gene activation and thus provide a paradigm for cell- and gene-specific transcriptional regulation. This was exemplified by the biochemical identification of the B cell-specific coactivator OCA-B (*O*ct *C*o-*A*ctivator from *B* cells; Luo et al., 1992), which mediates B cell-specific immunoglobulin (Ig) promoter activation in conjunction with either the ubiquitously expressed Oct-1 or the B-cell-enriched Oct-2. OCA-B binds to the POU domains of the Oct factors and is the major determinant of B cell-restricted, octamer-dependent Ig promoter activity (reviewed in Luo and Roeder, 1999). Purified Oct-1 and OCA-B were shown to fully stimulate Ig promoters in a system reconstituted with highly purified general initiation factors and a general coactivator fraction termed USA; however, the failure of Oct-1 to stimulate transcription from an octamer-containing H2B promoter in this assay system led us to postulate the existence of an *O*ct-1 *Co*Activator in *S* phase (OCA-S) that confers high-level H2B promoter activation (Luo and Roeder, 1995).

More recent studies of cyclin E/cdk2, a kinase known to regulate the G1/S transition and multiple S phase events including histone synthesis (reviewed in Ewen, 2000), have identified a substrate (NPAT) that has been implicated in histone gene regulation. Thus, overexpression of NPAT stimulates H2B, H3, and H4 promoter activities in a manner that is dependent on the SSREs within each promoter and that requires phosphorylation of NPAT by cyclin E/cdk2 (Ma et al., 2000; Zhao et al., 2000). Chromatin immunoprecipitation (ChIP) assays further indicated an association of NPAT, as well as cyclin E, with the H2B, H3, and H4 promoters (Zhao et al., 2000). These studies demonstrated an apparently global role for NPAT in the regulation of histone transcription, although its mechanism of action on individual histone genes, and whether it acts directly or indirectly, is not yet known.

Here, we describe the functional identification and purification of the OCA-S activity. Further characterization of this multicomponent cofactor has established a key role(s) for the p38 subunit, identical to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in mediating S phase-inducible H2B promoter activation, as well as a link between OCA-S and NPAT.

Results

OCA-S Copurifies with Seven Polypeptides

Although high level octamer-dependent transcription of the H2B promoter was observed in nuclear extracts from

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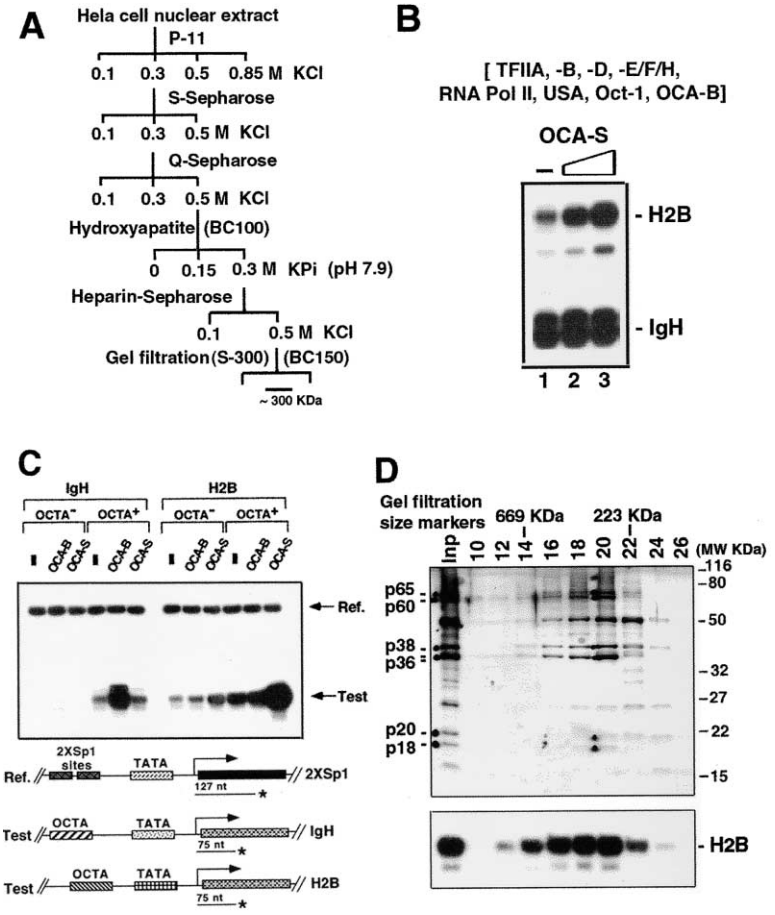

Figure 1. Identification and Purification of the OCA-S Activity

(A) Purification scheme.

(B) Identification of the OCA-S activity. Reactions contained the purified components indicated at the top of the image and 0 (lane 1), 6 (lane 2), or 15 (lane 3) μg of P11 0.3 M KCl OCA-S fraction. Transcription levels were measured by primer extension with 75 nt (IgH) and 110 nt (H2B) signals.

(C) Differential functions of OCA-B and OCA-S on IgH and H2B promoters. Templates containing promoter sequences with an intact ($OCTA^+$) or mutated ($OCTA^-$) octamer element were assayed in the presence of a reference Sp1-dependent template. The system contained the same components as in (B) except for the addition of Sp1 and omission of OCA-B. Coactivators OCA-B (recombinant) and OCA-S (hydroxyapatite fraction) were added as indicated.

(D) Polypeptide composition of the purified OCA-S activity. Upper image, protein profiles revealed by SDS-PAGE analysis. The Input (Inp) lane corresponds to the OCA-S fraction prior to S300 chromatography and the remaining lanes correspond to S300 column fractions (indicated by numbers). Lower image, OCA-S activity examined for the above-described fractions in the assay system described in (B). The upper image also highlights (by dots) the activity peak (#20) along with polypeptides that correlate with the OCA-S activity, with their molecular weights in kDa indicated on the left. The positions of molecular weight (MW) markers for the gel are shown on the right.

dividing HeLa cells, only basal transcription was observed in an assay system that contained purified general transcription factors, the USA coactivator fraction, purified Oct-1, and the B-cell coactivator OCA-B (Luo and Roeder, 1995). However, transcription of the H2B promoter in this assay system was markedly and selectively stimulated by a HeLa nuclear extract-derived 0.3 M KCl phosphocellulose (P11) fraction (Figure 1A) that contained most of this OCA-S activity (Figure 1B). The OCA-S activity in the P11 fraction was further enriched by S-Sepharose, Q-Sepharose, and hydroxyapatite steps, resulting in an overall purification of ~16,000-fold (Figure 1A).

To investigate the promoter specificity of OCA-B versus OCA-S, we compared the functions of recombinant OCA-B and highly purified OCA-S (hydroxyapatite fraction) on wild-type or octamer mutant H2B and immunoglobulin heavy chain (IgH) promoters cloned upstream of the same reporter gene (Figure 1C). OCA-S stimulated octamer-/Oct-1-dependent transcription from the H2B promoter, but not from the IgH promoter, whereas the reverse was true for OCA-B. A weak stimulatory effect of OCA-S on the octamer-independent transcription from the H2B promoter was also observed (Figure 1C), suggesting a potential role of OCA-S in a core promoter interaction.

The OCA-S enriched hydroxyapatite fraction was further purified by chromatography on heparin-Sepharose and then subjected to gel filtration on Sephacryl 300 (S300). Fractions were analyzed by SDS-PAGE (Figure 1D, upper image) and for OCA-S activity (lower image). This step resulted in an overall purification of ~30,000-fold and an estimated native size of ~300 kDa for OCA-S. The OCA-S activity coeluted with seven polypeptides, whose identities were revealed by peptide sequencing. p20 and p18 were identified as nm23-H1 and nm23-H2, respectively, and are thought to possess nucleoside diphosphate (NDP) kinase activity (reviewed in Freije et al., 1998). One polypeptide in the p36 doublet band was identified as uracil-DNA glycosylase (UNG), whereas the other was identified as LDH (lactate dehydrogenase). The 38 kDa protein was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although well-characterized glycolytic enzymes are localized predominantly in the cytoplasm, nuclear localization and DNA binding activities of GAPDH and LDH have been documented (reviewed in Ronai, 1993). The p65 and p60 proteins were identified as Hsp70 and Sti1, which are components of molecular chaperones (reviewed in Frydman and Hohfeld, 1997).

p38/GAPDH Interacts Directly with Oct-1

In view of the interaction of OCA-B with Oct-1/-2 POU domains (Luo and Roeder, 1995), we examined the seven OCA-S-associated polypeptides for similar interaction(s). When in vitro translated proteins were tested in GST pull-down assays, only p38/GAPDH bound to GST-fused POU-1 and POU-2 (Figure 2A). Moreover,

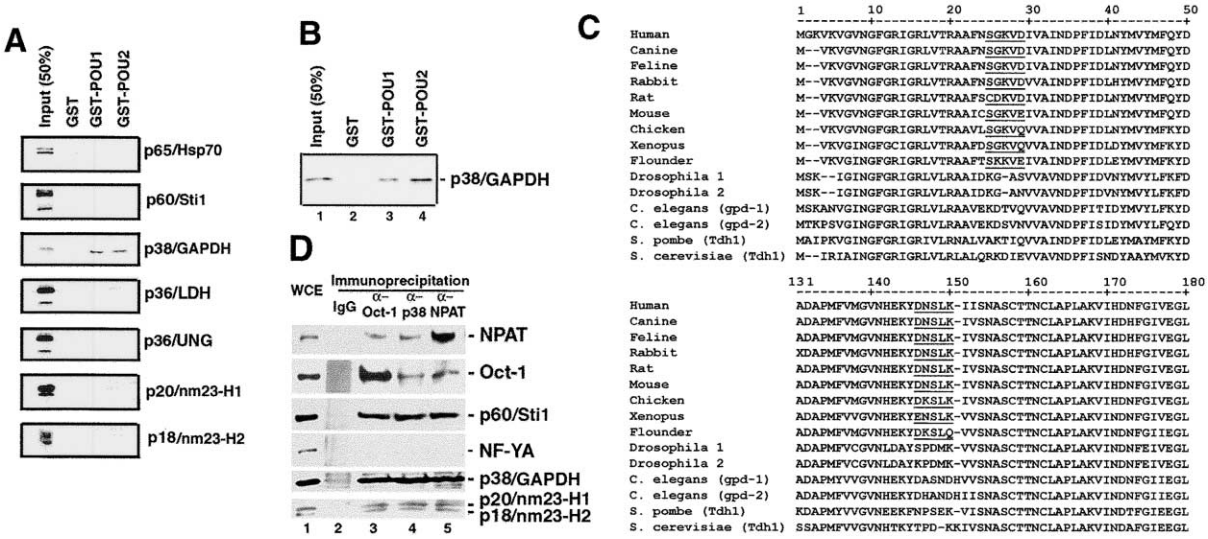

Figure 2. In Vitro and In Vivo Interactions between OCA-S Components and Oct-1/-2 POU Domains and between Oct-1, OCA-S, and NPAT

(A) Interactions of in vitro translated OCA-S components with Oct-1/-2 POU domains.

(B) Interaction of homogeneously purified p38/GAPDH with Oct-1/-2 POU domains.

(C) Alignment of relevant vertebrate and nonvertebrate GAPDH protein sequences. The residues in the two small regions that display significant divergence from vertebrate GAPDHs to nonvertebrate ones are underlined. Numbers indicate amino acid positions. The GenBank accession numbers for these GAPDH sequences from human to *S. cerevisiae* on the list are CAA25833, BAA90817, BAA90818, P51469, P46406, CAA26150, NP_032110, P00356, BAA88638, NP_525108 and NP_542445, CAA888170 and AAB53874, P78958, and NP_012483, respectively.

(D) Coimmunoprecipitation of Oct-1, OCA-S, and NPAT. A T24 whole-cell extract (WCE) was immunoprecipitated with antibodies against Oct-1 (lane 3), p38/GAPDH (lane 4), NPAT (lane 5), or control IgG (lane 2) under salt conditions equivalent to 150 mM NaCl. The intact WCE (lane 1) and immunoprecipitates were subjected to immunoblot with antibodies against NPAT, Oct-1, p60/Sti1, p38/GAPDH, p20/nm23-H1, p18/nm23-H2, or NF-YA.

homogeneous purified recombinant p38/GAPDH also bound to the POU domains (Figure 2B), indicating a direct interaction. p38/GAPDH thus becomes a new member of the Oct-1 POU domain interacting protein family, which includes OCA-B (Luo and Roeder, 1995), VP16 (reviewed in Babb et al., 1997), and SNAP190 (reviewed in Ford et al., 1998).

An alignment of p38/GAPDH protein sequences from a number of vertebrate and nonvertebrate species revealed protein sequence conservation throughout the entire length, except for two small regions (underlined in Figure 2C) where the conservation is restricted to vertebrates. These vertebrate-specific sequences might provide the basis for Oct-1 interactions and transcriptional activation functions (see below) on H2B genes, although the involvement of p38/GAPDH and Oct-1-related POU domain proteins in the regulation of nonvertebrate H2B transcription, via the octamer element, is not yet excluded.

We then employed coimmunoprecipitation assays to examine possible in vivo interactions among OCA-S-associated proteins, as well as interactions between these proteins and Oct-1. Since NPAT has been implicated in the transcription of multiple histone subtypes (Ma et al., 2000; Zhao et al., 2000), and thus likely acts upstream of OCA-S during H2B promoter activation, we also explored possible interactions of OCA-S proteins with NPAT. To this end, a whole-cell extract of human T24 bladder carcinoma cells was subjected to immunoprecipitation with anti-Oct-1, anti-p38/GAPDH, anti-NPAT, or control IgG antibodies, and the immunoprecipitates were analyzed by immunoblotting. Anti-Oct-1, anti-p38/GAPDH, and anti-NPAT antibodies all coimmunoprecipitated Oct-1, p18/nm23-H2, p20/nm23-H1, p38/GAPDH, p60/Sti1, and NPAT (Figure 2D), as well as p36/LDH, p36/UNG, and p65/Hsp70 (data not shown). The native H2B promoter also contains a CCAAT box that does not contribute to its cell cycle regulation (Labella et al., 1988). NF-YA, the factor that binds to this element (Fan et al., 2002), served as a control here and was not immunoprecipitated by any of the antibodies (Figure 2D). These results, together with the observed copurification of seven OCA-S-associated proteins, strongly support the notion that these seven proteins form a complex, hereafter designated OCA-S, and that this complex interacts with Oct-1 in vivo, most likely via p38/GAPDH. The observed NPAT-OCA-S interaction further suggests a physical link between NPAT and the H2B transcriptional regulatory machinery, possibly via a transient mechanism (see Figure 6I, Figure 8E, and Discussion).

p38/GAPDH Has an Intrinsic Transactivation Potential

To test whether one or more subunits of the OCA-S complex might possess an intrinsic transactivation potential (activation domain), p18/nm23-H2, p20/nm23-H1, p38/GAPDH, and p60/Sti1, as well as OCA-B as a positive control (Luo and Roeder, 1999), were individually fused to LexA and cotransfected into 293T human kidney cells with a reporter bearing LexA binding sites. The reporter was activated by both LexA-OCA-B and LexA-p38, but not by LexA alone or LexA fused to other

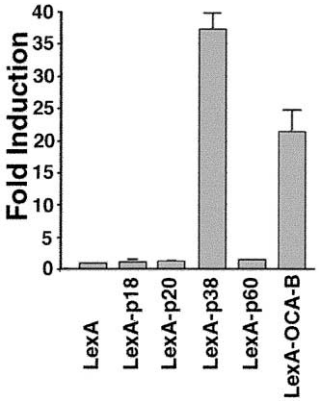

Figure 3. p38/GAPDH Has an Intrinsic Activation Domain

The indicated LexA or LexA fusion proteins were assayed by cotransfection of cognate expression vectors with a luciferase reporter bearing four LexA binding sites in front of an E1B minimal promoter, and values were normalized to activity with LexA alone.

proteins (Figure 3). This result indicates that p38/GAPDH has a transactivation potential that might account for most, if not all, of the OCA-S transcription activity. A prominent role of p38/GAPDH in the OCA-S complex thus prompted focus on p38/GAPDH in further studies of the transcription function of the complex.

p38/GAPDH Is Essential for Octamer-/Oct-1-Dependent H2B Transcription In Vitro

To study the transcription function of the OCA-S complex, we first attempted to immunodeplete OCA-S proteins from HeLa cell nuclear extracts with specific antibodies. Of the available antibodies tested, anti-p38/GAPDH and anti-p60/Sti1 quantitatively removed corresponding proteins under high salt conditions (500 mM KCl; Figure 4A). Depletion of p38/GAPDH, but not p60/Sti1, reduced octamer-dependent H2B transcription by ~4-fold (Figure 4B). Consistent with a role for OCA-S in the core promoter interaction (Figure 1C), depletion of p38/GAPDH and p60/Sti1 also reduced, albeit marginally, transcription from the octamer mutant H2B promoter (Figure 4C). Indicative of promoter specificity, OCA-B-dependent activation of the IgH promoter was intact in either p38/GAPDH- or p60/Sti1-depleted extracts (Figure 4D).

Altogether, these results suggest that p38/GAPDH is an essential nuclear factor for H2B transcription and, further, that it plays the central role in the coactivator function of the OCA-S complex. The apparent dispensability of p60/Sti1 for H2B transcription in vitro (Figure 4B) suggests a functional redundancy for p60/Sti1 in the p60/Sti1-deficient nuclear extract, which contained normal levels of other OCA-S-associated proteins (Figure 4A); however, it is also possible that p60/Sti1 and other untested OCA-S proteins might be required for the transcription of natural (chromatin) templates or for another function(s) of the OCA-S complex (see Discussion).

To verify that the reduction of H2B transcription in p38/GAPDH-depleted nuclear extracts is due directly to a missing p38/GAPDH function, the ability of homogeneous-purified p38/GAPDH to rescue this deficiency was examined. To this end, a HeLa cell line that stably expresses f-p38-HA (p38/GAPDH doubly tagged by Flag and HA epitopes) was established. f-p38-HA protein was purified to homogeneity on anti-Flag agarose followed by anti-HA agarose under high salt (500 mM KCl) conditions, from either nuclear or cytoplasmic (S100) extracts of the cell line (Figures 4E and 4F). The f-p38-HA in these preparations is predominantly monomeric since the endogenous p38/GAPDH did not copurify (compare Figure 4F with 4G). Addition of purified nuclear f-p38-HA to the p38/GAPDH-deficient extracts restored high-level H2B transcription (Figure 4H), thus confirming a transcription coactivation function for nuclear p38/GAPDH. In marked contrast, addition of monomeric cytoplasmic f-p38-HA (Figure 4H) or commercial tetrameric GAPDH from erythrocyte cytoplasm (data not shown), failed to effectively restore the H2B transcription. These results suggest that conformational changes and modifications of p38/GAPDH may be important for its nuclear function in H2B transcription.

Immunodepletion of endogenous p38/GAPDH and affinity purification of f-p38-HA were very inefficient under physiological salt conditions (data not shown). It is conceivable that endogenous p38/GAPDH and tagged f-p38-HA epitopes are blocked by other proteins of the OCA-S complex, thus making p38/GAPDH poorly accessible to antibodies under these conditions. Consistent with this possibility, as well as a high salt-induced dissociation of p38/GAPDH from other OCA-S subunits that remained intact in the p38/GAPDH-depleted extract (Figure 4A), the optimal salt condition either for immunodepletion of endogenous p38/GAPDH or for affinity purification of f-p38-HA on M2 agarose was 500 mM KCl.

p38/GAPDH Is Essential for H2B Transcription In Vivo

To test the role of p38/GAPDH in H2B transcription in vivo, we used the RNA interference (RNAi) method (Elbashir et al., 2001) to block p38/GAPDH expression in human U2OS osteosarcoma cells. As these cells and other cells used throughout this study were routinely passaged and maintained in pyruvate-containing medium, any requirement for p38/GAPDH in the glycolytic pathway could be bypassed. Immunoblot analysis revealed a time-dependent reduction in the nuclear p38/GAPDH protein level, which was already significant at 24 hr and reached ~90% reduction at 60 hr, whereas the levels of Oct-1 and other OCA-S components were unaltered (Figure 5A). RT-PCR analysis showed a reduction, already apparent at 24 hr, in the level of endogenous H2B mRNA in concert with the decline of the nuclear p38/GAPDH level, while the level of β-actin mRNA remained constant (Figure 5C). A second p38/GAPDH-specific short interfering double-strand RNA (siRNA) that targets a different mRNA region was also employed and produced similar results (data not shown). Interestingly, the level of H4 mRNA was also decreased, but this was apparent only at a relatively late time (60 hr) after RNAi treatment (Figure 5D). We reason that inhibition of H4 mRNA expression is secondary to the cell cycle effect caused by inhibition of H2B mRNA expression. This is consistent with previous reports showing that expres-

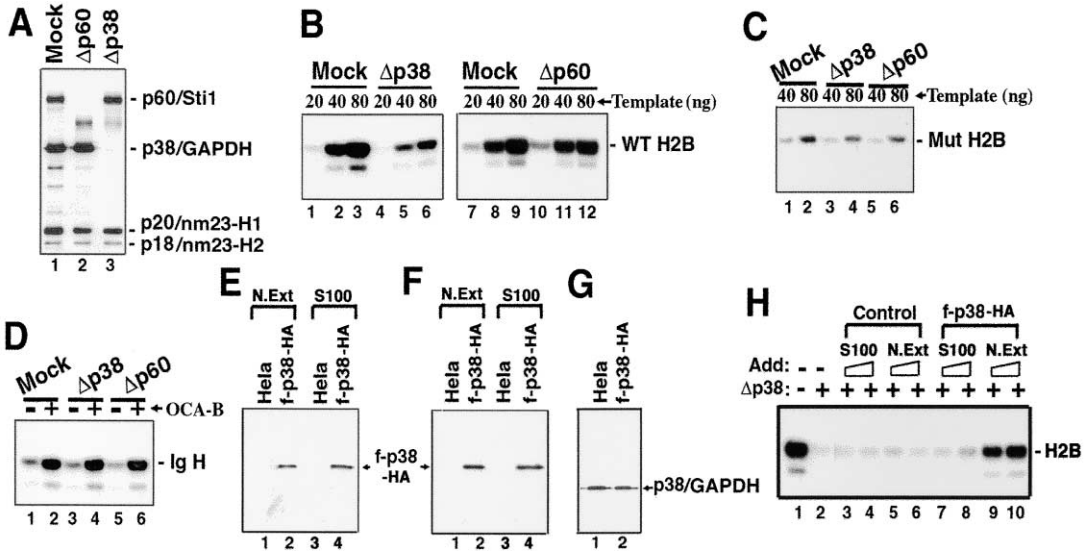

Figure 4. Purified Nuclear f-p38-HA Restores H2B Transcription in a p38/GAPDH-Deficient Nuclear Extract

(A) Immunodepletion of p38/GAPDH or p60/Sti1 from HeLa cell nuclear extracts. Mock- (lane 1), p60/Sti1- (lane 2) or p38/GAPDH- (lane 3) depleted nuclear extracts were examined by immunoblot.

(B–D) H2B or IgH promoter activities in depleted nuclear extracts. Variable doses of H2B promoter templates were used as indicated.

(B) Wild-type H2B promoter activity in p38/GAPDH-depleted nuclear extract (left image, lanes 4–6) or p60/Sti1-depleted nuclear extract (right image, lanes 10–12) in comparison with mock-depleted extract (lane 1–3, 7–9).

(C) Octamer mutant H2B promoter activity in mock- (lanes 1–2), p38/GAPDH- (lanes 3–4), or p60/Sti1- (lanes 5–6) depleted nuclear extracts.

(D) IgH promoter activity in mock- (lanes 1–2), p38/GAPDH- (lanes 3–4), or p60/Sti1- (lanes 5–6) depleted nuclear extracts in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of purified recombinant OCA-B.

(E and F) f-p38-HA purified from either nuclear (N. Ext) or cytoplasmic (S100) extracts of f-p38-HA-expressing Hela cells. M2- and anti-HA-agarose-purified f-p38-HA (lanes 2 and 4) and the eluate from control extracts (lanes 1 and 3) were analyzed by SDS/PAGE-silver staining (E) or by immunoblot with anti-p38/GAPDH antibodies (F).

(G) Position of endogenous p38/GAPDH in the SDS protein gel. Control (lane 1) and f-p38-HA-expressing (lane 2) cell nuclear extracts were analyzed directly by Western blot with anti-p38/GAPDH antibodies. Due to its low expression, f-p38-HA was not observed in the analysis (lane 2), the input (0.2 μl nuclear extract) of which was optimized for detection of endogenous p38/GAPDH. The eluate loaded in (F) corresponds to ~0.2 ml nuclear extract.

(H) H2B promoter activity in normal (lane 1) or p38/GAPDH-depleted nuclear extracts without (lane 2) or with control S100 (lanes 3–4) or nuclear extract (lanes 5–6) eluates, or with f-p38-HA from S100 (lanes 7–8) or nuclear (lanes 9–10) extracts. The two doses of f-p38-HA used were ~10 and 20 ng, respectively.

sion of H3 and H4 was decreased upon deletion of H2B loci in yeast, presumably due to cell cycle arrest in H2B-defective cells (Han et al., 1987).

We thus expected that a block in p38/GAPDH expression would inhibit cell cycle progression into S phase. Indeed, the percentage of S phase cells, monitored by BrdU incorporation, was found to decrease in U2OS cells treated with p38/GAPDH siRNA for 48 hr (Figure 5B). Nevertheless, at an earlier time when the S phase progression was only marginally affected, i.e., at 24 hr after RNAi treatment (Figure 5B), a significant reduction in the level of H2B mRNA was already obvious (Figure 5C). This suggests that this reduction is not due to a nonspecific effect of cell cycle arrest. Conversely, the lag in the reduction of the H4 mRNA synthesis most likely results from a blockage of S phase progression. Therefore, as in yeast, expression of histone genes in mammalian cells is highly coordinated and essential for S phase progression.

To further assess whether the removal of p38/GAPDH has a direct effect on H2B transcription, we examined the effect of p38/GAPDH RNAi on wild-type and octamer-mutated H2B promoter reporters in transfected U2OS cells. The activity of the wild-type promoter was reduced 4- to 5-fold by RNAi treatment, whereas the basal octamer-independent activity of the mutant promoter was unaffected (Figure 5E). These results confirm a p38/GAPDH coactivation function for octamer-/Oct-1-dependent transcription of the H2B promoter.

S Phase-Specific Recruitment of OCA-S/p38/GAPDH to the H2B Promoter

It is expected that a cell cycle-dependent modulation of OCA-S protein abundance and/or activity underlies S phase-specific H2B promoter activation. To investigate possible changes in protein levels, cells were synchronized at various stages by aphidicolin treatment, density arrest (data not shown), or centrifugal elutriation (Figure 6A). Regardless of which approach was used for synchronization, immunoblot analyses of either whole-cell (data not shown) or nuclear (Figure 6B) extracts with available antibodies showed that the protein levels of the OCA-S proteins do not change significantly throughout the cell cycle.

We thus explored another possible modulation, i.e., recruitment of p38/GAPDH and/or other OCA-S proteins to the H2B promoter. As revealed by ChIP assays, the H2B promoter in unsynchronized HeLa cells is occupied

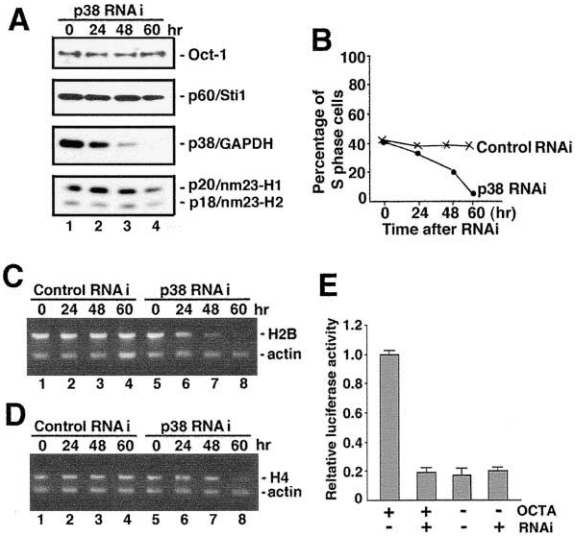

Figure 5. Inhibition of H2B Transcription by p38/GAPDH RNAi

(A) Expression of p38/GAPDH in U2OS cells treated with p38/GAPDH siRNA for 0 to 60 hr was monitored by anti-p38/GAPDH immunoblot analyses of nuclear extracts. Immunoblots with anti-Oct-1, −p60/Sti1, −p18/nm23-H2, and −p20/nm23-H1 antibodies served as controls.

(B) Effect of p38/GAPDH siRNA on S phase progression/DNA synthesis was monitored by BrdU incorporation.

(C and D) RT-PCR analysis of expression levels of H2B (C), H4 (D), and β-actin mRNAs in cells treated with p38/GAPDH-specific siRNA (lanes 5–8) or Ambion's control siRNA (lanes 1–4) for 0 to 60 hr.

(E) Effects of p38/GAPDH siRNA on expression of ectopic wild-type ($OCTA^+$) or mutant ($OCTA^-$) H2B promoters in U2OS cells. Activity was measured at 24 hr posttransfection.

both by Oct-1 and by OCA-S proteins including p18/nm23-H2, p20/nm23-H1, p38/GAPDH, and p60/Sti1 (data not shown). We next focused on the recruitment of Oct-1, p60/Sti1, and p38/GAPDH proteins to the H2B promoter in cells synchronized by centrifugal elutriation. DNA derived from antibody-precipitated chromatin was subjected to PCR using primers for the indicated promoter/DNA regions (Figures 6C-6H). In the assays where 30 cycle PCR was used for amplification, occupancy of the H2B promoter by p38/GAPDH was detected only in S phase, whereas occupancy by Oct-1 was detected in G1, S, and G2 phases (Figure 6C). Importantly, and as strong evidence for its promoter specificity, p38/GAPDH was not detected on the H4 promoter (Figure 6E). By contrast, the Rb family protein Rb2/p130 was selectively recruited to the E2F-recognized H4 promoter in G1, but not S or G2, phase (Figure 6E), as was E2F-4 (data not shown). This is consistent with the suggestion that Rb2/p130 represses E2F-regulated promoter activity at G1 phase via members (mainly E2F-4) of the E2F family (Takahashi et al., 2000). As an additional control, microsatellite DNA was not amplified from any precipitated chromatin DNA (Figures 6G or 6H). These results indicate that p38/GAPDH is recruited specifically to the H2B promoter in an S phase-dependent manner and, in view of a direct p38/GAPDH-Oct-1 interaction (Figure 2), further suggest that this recruitment is central to the S phase regulation of H2B transcription.

The presence of p60/Sti1 on the H2B promoter, but not on the H4 promoter or microsatellite DNA, was detected when 40-cycle PCR was used (Figure 6D versus 6F or 6H). This suggests a specific, albeit possibly more indirect, association of p60/Sti1 with the H2B promoter. Apparently, this association is also S phase-dependent (Figure 6D). Similarly, 40-cycle PCR, but not 30-cycle PCR, revealed an association (possibly more indirect) of NPAT with both H2B and H4 promoters (Figures 6D and 6F). This is in line with an upstream and broader role of NPAT in regulating the transcription of all histone subtypes (Zhao et al., 2000; Ma et al., 2000).

The S phase-specific association of NPAT with the H2B promoter (Figure 6D) suggests a cell cycle-dependent NPAT-OCA-S interaction. To test this possibility, we monitored interactions between ectopic Flag- and HA-tagged NPAT (f-NPAT-HA) and endogenous OCA-S components by coimmunoprecipitation of extracts from U2OS cells that were synchronized at G1 or S phase following transfection. Although the expression of endogenous NPAT is induced at the G1/S transition (Zhao et al., 2000), ectopic f-NPAT-HA expression was similar at G1 and S phases (Figure 6I, lanes 4 and 5). Nonetheless, OCA-S proteins (p60/Sti1, p38/GAPDH, p20/nm23-H1, and p18/nm23-H2) were found in f-NPAT-HA coimmunoprecipitates from S phase cells but not those from G1 phase cells (Figure 6I), suggesting an S phase-dependent NPAT-OCA-S interaction in vivo.

Requirement of p38/GAPDH for S Phase-Specific H2B Promoter Activation In Vitro

To provide more direct evidence that p38/GAPDH is involved specifically in S phase H2B transcription, we first attempted to recapitulate cell cycle dependent H2B transcription in vitro using nuclear extracts of HeLa cells separated by elutriation at various stages (Figure 6A). In line with in vivo results (LaBella et al., 1988), an S phase nuclear extract supported high-level H2B transcription, whereas G1 and G2 phase nuclear extracts supported only low-level H2B transcription (Figure 7A, lanes 1–4). In contrast, OCA-B-dependent IgH promoter activity was equally active in random or staged extracts (Figure 7A, lanes 5–8).

This assay system thus offered an opportunity to assess the OCA-S dependency of H2B activities in both S phase and G1 or G2 phase. To this end, we used a direct antibody inhibition assay in which nuclear extracts were incubated with anti-p38/GAPDH or anti-p60/Sti1 antibodies prior to being used for transcription. In an initial test of this approach and consistent with the immunodepletion data (Figure 4), anti-p38/GAPDH, but not anti-p60/Sti1, antibodies inhibited H2B transcription in an extract from unsynchronized cells (Figure 7B). Similarly, anti-p38/GAPDH, but not anti-p60/Sti1, antibodies inhibited H2B transcription in S phase extract (Figure 7C, lane 7 versus lane 3; Figure 7D). In marked contrast, neither of the antibodies had any effect on H2B transcription in either G1 or G2 phase extracts (Figure 7C, lanes 6 and 8 versus lanes 2 and 4; Figure 7D). That the inhibitory effect is specific for the in vitro-recapitulated S phase H2B transcription clearly strengthens the notion that p38/GAPDH, as a key component of a bona fide Oct-1 coactivator, is indeed responsible for S phase-inducible transcription of the H2B gene.

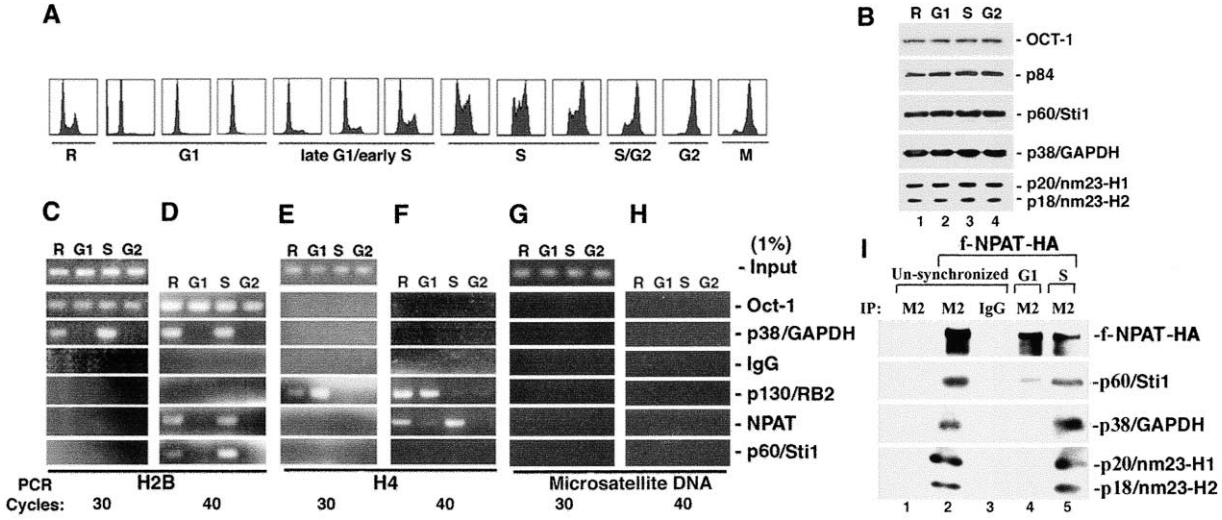

Figure 6. S Phase-Specific Association of OCA-S Components with the H2B Promoter and with NPAT

(A) FACS analyses of HeLa cells synchronized at different stages by elutriation. R, unsynchronized cells.

(B) Immunoblot of p18/nm23-H2, p20/nm23-H1, p38/GAPDH, p60/Sti1, Oct-1, and p84 in nuclear extracts of HeLa cells separated by centrifugal elutriation at different cell cycle stages. The p84 control is a nuclear matrix protein that is not cell cycle regulated.

(C–H) ChIP assays on elutriation-synchronized cells with antibodies against Oct-1, p38/GAPDH, p60/Sti1, NPAT, Rb2/p130, or normal IgG. Precipitated chromatin DNA was amplified by PCR, for either 30 or 40 cycles, with primers specific for H2B (C and D) or H4 (E and F) promoter or microsatellite DNA (G and H).

(I) An S phase-dependent OCA-S-NPAT interaction. Whole-cell extracts were made from cells transfected with empty (lane 1) or f-NPAT-HA-expressing (lanes 2–5) vectors. Cells were either unsynchronized (lanes 1–3) or synchronized at either G1 or S phase (lanes 4 and 5). Immunoprecipitation was carried out with either anti-Flag (M2) (lanes 1, 2, 4, and 5) or control IgG (lane 3) antibodies conjugated to agarose. Immunocomplexes were blotted with anti-p60/Sti1, −p38/GAPDH, −p20/nm23-H1, and −p18/nm23-H2 antibodies and by an anti-HA antibody (to detect tagged NPAT).

Modulation of OCA-S Function by NAD^+/NADH

NAD^+ and NADH are essential coenzymes for the enzymatic activities of cytosolic GAPDH and LDH and, further, have been shown to modulate the functions of several factors involved in transcriptional regulation (see below). Hence, our findings that p38/GAPDH is an essential OCA-S component, and that p36/LDH is also part of the OCA-S complex, make it important to determine whether there is a link between the OCA-S activity and the cellular metabolic state/redox status. To this end, we first employed a GST pull-down assay to test the effects of NAD^+/NADH on the binding of purified recombinant human p38/GAPDH to the Oct-1 POU domain. As shown in Figure 8A, NAD^+ significantly enhanced this interaction in a dose-dependent manner (lower image, compare lane 1 with lanes 2–6). On the other hand, increasing concentrations of NADH inhibited the interaction between POU-1 and p38/GAPDH (lower image, compare lane 1 with lanes 7–11). As a control, the interaction between POU-1 and OCA-B was not affected by either NAD^+ or NADH (upper image). This suggests that both the stimulatory and the inhibitory effects are specific for p38/GAPDH.

We next measured the influence of NAD^+/NADH on the association of p38/GAPDH (via Oct-1) with an H2B promoter (containing both octamer and CAAT box elements) in the context of a nuclear extract fraction. In an initial test of the specificity of such an association, HeLa nuclear extract was mixed with streptavidin agarose-immobilized DNA fragments bearing either wild-type or mutant H2B promoter sequences (Figure 8B), and H2B promoter-associated proteins were analyzed by immunoblotting with corresponding antibodies. As indicated (Figure 8B), p38/GAPDH and Oct-1 bound to promoter fragments in an octamer-dependent manner, whereas NF-YA bound to promoter fragments in a CAAT-box-dependent manner, and little or no cooperative binding was observed. We then explored the effects of NAD^+/NADH on the association of p38/GAPDH with the wild-type H2B promoter. To avoid the influence of possible endogenous (free) NAD^+/NADH, we analyzed such an association in a nuclear extract-derived chromatographic fraction (P11, 0.3 M KCl) that was enriched for OCA-S (Figure 1A) and Oct-1 (data not shown) but depleted of free NAD^+/NADH. We observed stimulatory (NAD^+) and inhibitory (NADH) effects on p38/GAPDH promoter association (Figure 8C, lower image) that were nearly identical to those observed for binding of purified p38/GAPDH to the Oct-1 POU domain (Figure 8A, lower image). By contrast, the direct association of Oct-1 with the H2B promoter was unaffected by either NAD^+ or NADH (Figure 8C, upper image).

To test the effects of NAD^+/NADH in a more physiological setting, we asked whether H2B transcription in a nuclear extract could be modulated by NAD^+/NADH. To reduce the possible influence of endogenous NAD^+/NADH and other redox system(s) in HeLa nuclear extracts, we employed a partially purified reconstituted transcription system consisting of three nuclear extract-derived P11 chromatographic fractions (0.3, 0.5, and 0.85 M KCl; Figure 1A) and highly purified native TFIIA. This system is capable of supporting both H2B and

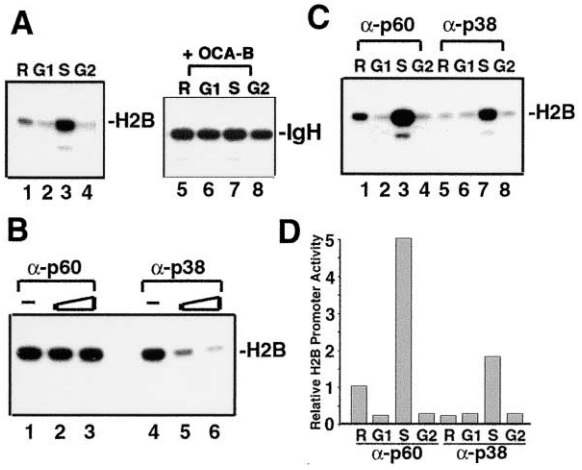

Figure 7. Antibody-Mediated Inhibition of S Phase H2B Transcription

(A) H2B (lanes 1–4) and OCA-B-dependent IgH (lanes 5–8) promoter transcription in nuclear extracts from random (R) or HeLa cells synchronized at G1, S, or G2 phase by elutriation.

(B) Antibody-mediated inhibition of H2B transcription in nuclear extracts of unsynchronized HeLa cells following preincubation with buffer (lanes 1 and 4) or two doses (2 μl and 5 μl) of anti-p60/Sti1 (lanes 2–3) or anti-p38/GAPDH (lanes 5–6) antibodies.

(C) Antibody-mediated inhibition of H2B transcription in nuclear extracts from randomly growing cells (R) or from cells synchronized at G1, S, or G2 phase. Extracts were preincubated with 5 μl of either anti-p60/Sti1 (lanes 1–4) or anti-p38/GAPDH (lanes 5–8) antibodies before being assayed for H2B transcription.

(D) Quantitation of H2B transcription inhibition data from (C). Data in all lanes are normalized to that in lane 1 (as 1).

OCA-B-dependent IgH transcription (Figure 8D). Consistent with the coenzyme effects on the p38/GAPDH-Oct-1 interaction, H2B transcription was stimulated by increasing concentrations of NAD^+ (Figure 8D, upper image, lanes 2–6) and inhibited by increasing concentrations of NADH (Figure 8D, upper image, lanes 7–11). In contrast, the OCA-B-dependent IgH promoter activity was unaffected by either NAD^+ or NADH (Figure 8D, lower image). The concentrations of NAD^+ or NADH required for their stimulatory or inhibitory effects on H2B transcription (Figure 8D) are considerably higher than those that modulate the Oct-1-p38/GAPDH interactions (Figures 8A and 8C). This could be due to persistence of endogenous redox system(s) in the partially purified transcription system and/or other complications associated with the reactions. Nevertheless, the results from all three approaches lead us to conclude that the redox status of NAD^+/NADH can significantly modulate the interaction of Oct-1 with p38/GAPDH, as well as H2B transcription.

Discussion

In an extension of our previous studies that defined the key H2B promoter regulatory element (e.g., LaBella et al., 1988) and the interacting transcriptional activator Oct-1 (Fletcher et al., 1987), we report the biochemical purification of a multicomponent transcriptional coactivator, OCA-S, which appears to be the major determinant for S phase activation of the H2B promoter. We also show that the p38 subunit provides the key Oct-1 recognition and coactivation functions and, remarkably, that it represents a nuclear form of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Furthermore, the transcription function of OCA-S is modulated by NAD^+ and NADH. Along with a demonstrated interaction of the OCA-S complex with NPAT, a cyclin E/cdk2 kinase substrate and global regulator of histone gene transcription (reviewed in Ewen, 2000), these results offer new insights into the regulation of histone gene transcription and set the stage for further analysis of the basis, possibly involving other OCA-S subunits, of coordinate histone gene transcription and linkages to both DNA replication and cellular metabolic state.

Key Role for p38/GAPDH in OCA-S Function

That p38/GAPDH is an essential OCA-S component specific for H2B transcription was clearly established by functional studies involving RNAi-mediated depletion in cells and immunodepletion, followed by complementation with purified p38/GAPDH, in nuclear extracts. Strongly supporting these conclusions, p38/GAPDH showed S phase-specific function in vitro and S phase-specific association with the H2B promoter in vivo. Further indicative of its essential role in OCA-S coactivator function, p38/GAPDH interacts directly with Oct-1, thus providing the main anchor for the OCA-S complex to promoter bound Oct-1, and has an intrinsic activation domain that likely interacts with the general transcription machinery. Finally, while the finding of a nuclear form of GAPDH as a key component of OCA-S was surprising, it is important to note that there are previous reports of diverse cytoplasmic and nuclear GAPDH functions that include apoptosis and DNA replication/repair (reviewed in Sirover, 1999).

Possible Modes for the Modulation of OCA-S Coactivation Function

The S phase-specific recruitment of p38/GAPDH/OCA-S to the H2B promoter is probably central to the regulation of the OCA-S activity. Since we observed no changes in the levels of OCA-S subunits throughout the cell cycle, the most likely scenarios for the modulation of OCA-S activity include posttranslational modifications by, or interactions with, other S phase-specific factors that facilitate intracellular translocations and/or promoter occupancy (including Oct-1 interactions) of one or more OCA-S subunits. In this regard, the selective function of affinity purified nuclear p38/GAPDH, relative to the cytoplasmic form, clearly points to some modification(s).

Also of note is the modulation of p38/GAPDH-Oct-1 interactions and OCA-S transcription activity by NAD^+/NADH redox status. NAD^+ and NADH were earlier shown to influence the functions of several transcription (co)-factors (Imai et al., 2000; Rutter et al., 2001; Zhang et al., 2002), including one (CtBP) that proved to be an NAD^+-dependent dehydrogenase (Kumar et al., 2002). Our study shows that the OCA-S transcriptional coactivator complex contains two classical dehydrogenases, p38/GAPDH and p36/LDH, at least one of which is essential for the activity of the coactivator complex on

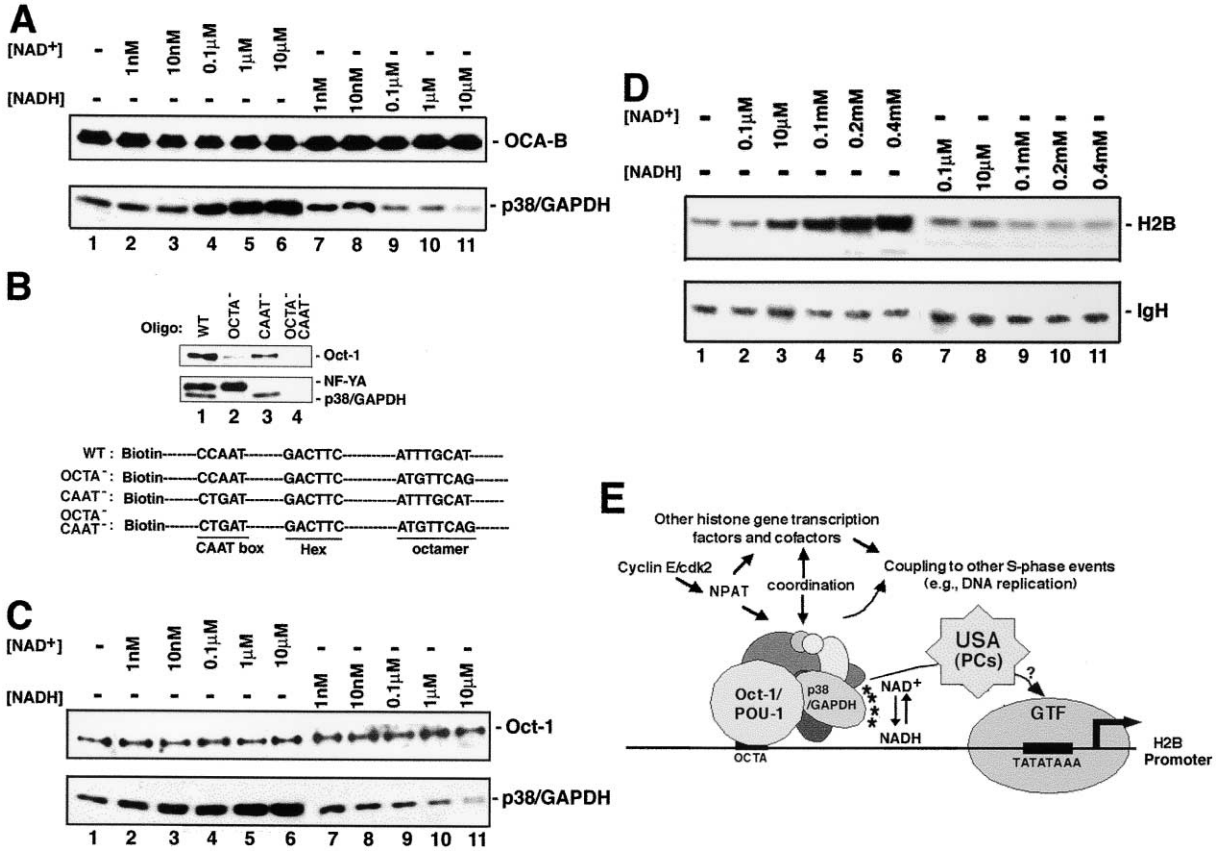

Figure 8. Modulation of POU Domain Binding Activity of p38/GAPDH and H2B Transcription by NAD^+/NADH and a Model for OCA-S-Mediated Coactivation

(A) Effects of NAD^+/NADH on the interaction between purified p38/GAPDH and the Oct-1 POU domain. The interaction between GST-fused POU domain and either recombinant p38/GAPDH or OCA-B was analyzed in a buffer containing either no (lane 1) or increasing concentrations (lanes 2–11) of NAD^+ or NADH, as indicated, by GST pull-down assays as described in Figure 2B. Bound proteins were detected by anti-OCA-B or anti-p38/GAPDH immunoblots.

(B) Octamer-dependent association of p38/GAPDH with an H2B promoter fragment in biotin-streptavidin pull-down assays. HeLa nuclear extract was incubated with biotin-labeled, streptavidin-agarose-immobilized oligonucleotides that contained either the wild-type (lane 1, WT), octamer-mutated (lane 2, $OCTA^-$), CAAT box-mutated (lane 3, $CAAT^-$), or octamer/CAAT box double-mutated (lane 4, $OCTA^-CAAT^-$) H2B promoter sequences. Proteins bound to these oligonucleotides were detected by immunoblotting with anti-Oct-1, −NF-YA, and −p38/GAPDH antibodies. Schematic structures of the oligonucleotides are shown in the lower image.

(C) Effects of NAD^+/NADH on octamer-dependent association of p38/GAPDH with the H2B promoter. Using the same pull-down assays described in (B), the P11 0.3 M KCl fraction of HeLa nuclear extract was incubated with the wild-type oligonucleotides in the absence (lane 1) or presence (lanes 2–11) of the indicated concentrations of NAD^+ or NADH. Bound p38/GAPDH and Oct-1 (as a control) were detected by immunoblots with corresponding antibodies.

(D) Effects of NAD^+/NADH on H2B transcription in vitro. Transcription reactions were carried out in the absence (lane 1) or in the presence (lanes 2–11) of indicated concentrations of NAD^+ or NADH in the system specified in the text. The reactions with the IgH promoter template, as a control, also contained purified OCA-B.

(E) Model for OCA-S-mediated coactivation of the H2B promoter. See text for a detailed explanation.

S phase-specific H2B transcription. Further, our intriguing demonstration of significant NAD^+/NADH-dependent modulations of p38/GAPDH interactions (with free and promoter bound Oct-1) and function (via OCA-S) in H2B transcription prompts speculation regarding two scenarios: (1) that NAD^+/NADH binding and/or dehydrogenase activities associated with p38/GAPDH and/or p36/LDH play important roles in the transcription coactivation function of OCA-S and (2) that histone transcription and cell cycle (S phase) progression may be linked, in part, to overall cellular (or nuclear) metabolic state/redox status. Further studies of the structural basis for the NAD^+/NADH-modulated Oct-1-p38/GAPDH interaction, the mechanisms by which the coenzymes modulate H2B transcription, and the physiological role(s) played by these modulations, promise to be of great interest.

OCA-S: a Multisubunit Transcription Cofactor Complex

That the OCA-S activity resides within a multicomponent complex is evidenced by copurification of the activity with seven stoichiometric polypeptides over a 30,000-fold range, by coelution of the activity with these polypeptides as a high molecular weight entity in gel filtration, and by coimmunoprecipitation of those components (p60/Sti1, p38/GAPDH, p20/nm23-H1, and p18/nm23-

H2) for which antibodies are available. Furthermore, as revealed by ChIP assays, these proteins also occupy the H2B promoter in an S phase-dependent manner, presumably as part of a cognate H2B promoter binding complex. The fact that a single homogeneous f-p38-HA protein can restore H2B transcription to p38/GAPDH-deficient nuclear extracts probably reflects a scenario in which f-p38-HA can reassociate with other OCA-S components in nuclear extracts to acquire transcription competency.

While p38/GAPDH is a key OCA-S component for H2B promoter coactivation, the roles of other subunits and other functions of the complex remain unknown. p20/nm23-H1 and p18/nm23-H2 are members of the nm23 family that have been variably associated with cancer metastasis (reviewed in Freije et al., 1998) and, as revealed by ChIP assays (data not shown), also occupy active H2B promoters, thus suggesting some function(s) related to H2B transcription. Another OCA-S component, p36/UNG, has been shown to play a role in removing dUMP from U:A pairs during DNA replication (Nilsen et al., 2000), raising the possibility of crosstalk, via p36/UNG, between the S phase events of histone transcription and DNA replication/repair. The indication, from its presence in OCA-S, that p36/LDH is associated with transcriptional regulation is consistent with a reported nuclear localization and general DNA binding activity of p36/LDH (Ronai, 1993). As previously suggested, the NAD^+/NADH binding and dehydrogenase activities of p36/LDH, possibly in conjunction with p38/GAPDH, could also participate in the modulation of OCA-S-mediated H2B transcription. The OCA-S components p65/Hsp70 and p60/Sti1/Hop are also subunits of a chaperone complex capable of promoting maturation of certain transcription factors (reviewed in Kimmins and MacRae, 2000). It is thus conceivable that nuclear p65/Hsp70 and p60/Sti1 may help maintain a transcriptionally potent form of the OCA-S complex.

An OCA-S-NPAT Interaction Links Cell Cycle Regulators and H2B Transcription Machinery

Importantly, our results show that Oct-1 occupies the H2B promoter at G1, S, and G2 phases of the cell cycle, whereas OCA-S (notably p38/GAPDH) does so in an S phase-specific manner and is indeed critical for S phase-inducible coactivation. Promoter bound OCA-S likely acts to facilitate recruitment and/or function of general transcription factors and positive cofactors (PCs), including Mediator (Malik and Roeder, 2000), in the USA fraction (Figure 8E). The cyclinE/cdk2 substrate NPAT could also play a role in these processes; however, its demonstrated role in regulating multiple histone subtype genes makes it more likely that NPAT functions at an upstream level, perhaps in the recruitment/activation of individual histone gene-specific (co)factors like OCA-S (Figure 8E). In support of an indirect role of NPAT in H2B transcription, NPAT was not detected in our purified transcriptionally active OCA-S fraction but, instead, was observed to associate in a more dynamic and S phase-dependent manner with OCA-S (Figure 6I). Therefore, it is most likely that OCA-S is a submodule of a larger dynamic complex containing NPAT, OCA-S, and Oct-1.

Consistent with the notion that transcription of histone subtypes is tightly coordinated and coupled to DNA replication in concert with S phase progression (reviewed in Osley, 1991), inhibition of H2B transcription by RNAi-mediated depletion of p38/GAPDH led to blockage of cell cycle progression and an eventual inhibition of transcription of H4 (Figure 5). Although cyclin E/cdk2 is known to be essential for initiation of both DNA replication and histone biosynthesis in concert with S phase entry (reviewed in Ewen, 2000), the mechanisms by which the above-described coordination and coupling throughout the S phase are not yet fully understood. A recent report suggests that the coupling of histone expression to DNA synthesis following S phase entry is not regulated at the level of cyclin E/cdk2, but rather by other (unknown) factors (Nelson et al., 2002). We speculate that OCA-S, a multisubunit and potentially multifunctional complex harboring a transcription cofactor activity, could provide an efficient way to achieve this concerted regulation. Thus, functions that mediate the coordination of multiple histone subtypes, couple histone synthesis to DNA replication, and transmit the S phase cell cycle regulatory signals, could be exerted through additional subunits of OCA-S either directly or via NPAT (Figure 8E). Our biochemical identification of the multisubunit OCA-S complex and cell cycle study of H2B transcription thus provides a paradigm for further exploring the mechanisms underlying this complex regulation.

Experimental Procedures

Plasmids and Antibodies

Mammalian expression vectors for OCA-S-associated proteins are pCIN4-based (Fondell et al., 1996) and involve CMV promoter-driven transcription of in-frame inserted cDNAs encoding f-p38-HA, LexA alone, and LexA fusions with p18/nm23-H2, p20/nm23-H1, p38/GAPDH, and p60/Sti1. The mammalian expression vector for NPAT (pcDNA3.1-f-NPAT-ha) is pcDNA3.1-based (Invitrogen) and encodes an NPAT that is both Flag- and HA-tagged. Antibodies against p38/GAPDH, p20/nm23-H1, p18/nm23-H2, p60/Sti1, and NPAT were raised in rabbits. Rabbit antibodies against Oct-1, Rb2/p130, NF-YA, and E2F-4 were purchased from Santa Cruz Biotechnologies, and mouse antibodies against p84 were from Novus Biologicals.

Protein Purification

The BC buffer system (Luo et al., 1992) was used in all chromatographic steps for OCA-S purification. The nuclear extract-derived P11 OCA-S fraction was enriched on S-Sepharose, Q-Sepharose, and hydroxyapatite. For the latter step, the indicated potassium phosphate salt concentrations in BC100 (100 mM KCl) were used to step-elute proteins. The activity was then concentrated by heparin-Sepharose and by Amicon centricon. This preparation was equilibrated in BC150 (150 mM KCl) and subjected to gel filtration on Sephacryl 300 (S300).

f-p38-HA was affinity-purified from nuclear or cytoplasmic (S100) extracts of a stable f-p38-HA-expressing HeLa cell line, using anti-Flag (mouse MAb M2, Sigma) and anti-HA (rat MAb 3F10, Roche) agarose affinity resins.

Homogeneous recombinant p38/GAPDH was obtained through successive steps of bacterial expression as a GST fusion protein, purification on an affinity column, removal of GST by thrombin digestion, and repurification by Q-Sepharose.

Cell Synchronization

Synchronization of transfected U2OS cells followed a procedure described in Zhao et al. (1998). Separation of suspension HeLa-S cells by centrifugal elutriation was carried out as described in Mendez and Stillman (2000).

GST Pull-Down Assays

OCA-S-associated proteins were in vitro translated and ^{35}S-labeled by the TNT system (Promega). GST, GST-POU1, and GST-POU2 were bacterially expressed (Luo and Roeder, 1995). Each fusion protein (10 μg), immobilized on glutathione-Sepharose beads, was mixed either with 2.5 μl of each in vitro translated OCA-S-associated protein or with 30 ng p38/GAPDH (Figure 2B), 100 ng p38/GAPDH (Figure 8A), or 250 ng OCA-B (Figure 8A) in the binding buffer (50 mM Tris, [pH 7.5], 150 mM NaCl, 4 mM EDTA, 0.5% NP40, 0.5% BSA, proteinase inhibitor cocktail, and, when appropriate, NAD^+/NADH). The binding reactions were incubated for 1 hr at room temperature.

Biotin-Streptavidin Pull-Down Assay

Biotin-streptavidin pull-down assays were performed as described (Fan et al., 2002). In brief, double-stranded oligonucleotides, corresponding to positions −91 to −30 of the human H2B promoter, were end-labeled by biotin and bound to streptavidin-agarose beads. The resultant beads were incubated, in the presence or absence of NAD^+/NADH, with either HeLa nuclear extract or a derived P11 0.3 M KCl column fraction for 20 min at room temperature. The protein-DNA-streptavidin-agarose complexes were washed, and the eluted proteins resolved by SDS-PAGE and detected by immunoblotting.

Chromatin Immunoprecipitation (ChIP) Assays

The ChIP assays were performed as described in Shang et al. (2000), using 1×10^6 HeLa cells synchronized by centrifugal elutriation for each antibody. Primers for histone promoters and microsatellite sequences were described in Zhao et al. (2000).

RNA Interference and RT-PCR Assays

Two duplexes of siRNAs that target two distinct regions on the p38/GAPDH mRNA (AUUCCAUGGCACCGUCAAG and GUCAACGGAU UUGGUCGUA) were designed (Dharmacon). RNAi assays were carried out in U2OS cells as described in Elbashir et al. (2001).

BrdU Incorporation Assays

RNAi-treated cells were grown on glass coverslips in 24-well plates. BrdU (10 μg/ml) was added to the medium for 1 hr before anti-BrdU immunostaining was conducted as described (Ma et al., 2000). One hundred cells were randomly picked and the numbers of BrdU-positive cells were counted for each sample. The percentage of S phase cells was averaged from three counts.

Transfection Assays

To assess the transactivation potential of OCA-S proteins, vectors expressing LexA or LexA fusions to p18/nm23-H2, p20/nm23-H1, p38/GAPDH, p60/Sti1, or OCA-B were each cotransfected into 293T cells with a luciferase reporter bearing LexA binding sites. At 48 hr posttransfection, cells were harvested for luciferase activity measurement. To assess H2B promoter activity in vivo, U2OS cells were cotransfected with wild-type or octamer-mutated reporters (Zhao et al., 2000) and, when appropriate, p38/GAPDH siRNA. At 24 hr posttransfection/RNAi treatment, cells were harvested for analysis of luciferase activity. For all transfections, a Renilla luciferase reporter was cotransfected for the normalization of transfection efficiency.

Immunodepletion and Direct Antibody Inhibition

Antigens were crosslinked to CNBr-activated Sepharose and the resultant resins used to affinity-purify specific antibodies (IgGs). For immunodepletion of p38/GAPDH and p60/Sti1, the corresponding affinity-purified IgGs were crosslinked to protein-A-Sepharose to generate antibody-affinity resins. The depleted nuclear extracts in BC500 (Luo and Roeder, 1995) were dialyzed to BC100 for in vitro transcription assays. For direct antibody inhibition, the affinity-purified anti-p38/GAPDH or anti–p60/Sti1 IgGs in BC500 were incubated with nuclear extracts for 3 hr at 4°C. The treated nuclear extracts were then dialyzed to BC100.

In Vitro Transcription

Conditions for both crude and purified assay systems, as well as DNA templates, were described in Luo and Roeder (1995). For the reconstituted transcription systems described in Figure 1, RNA pol II, general initiation factors, and the USA cofactor fraction were purified as in Ge et al. (1996), Oct-1 and Sp1 as in Luo et al. (1992), recombinant OCA-B as mentioned in Luo and Roeder (1999), and OCA-S as described above.

Peptide Sequencing

Identities of the polypeptides that copurified with OCA-S were revealed by protein sequence analyses following resolution by SDS/PAGE. Derived sequences include: PEELVDYK (p18/nm23-H2); TFI AIKPDGVQR (p20/nm23-H1); SADTLWGIQK (p36/LDH M chain) and SADTLWDIQK (p36/LDH H chain); GSAIDRK (p36/UNG); VVDLMAH MASK (p38/GAPDH); AAALEAMK (p60/Sti1); and LQDFFNGRDLNK (p65/Hsp70).

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