MOLECULAR AND CELLULAR BIOLOGY, Feb. 1989, p. 869–873
0270-7306/89/020869-05$02.00/0

Vol. 9, No. 2

Cell Cycle Regulation of H2b Histone Octamer DNA-Binding Activity in Chinese Hamster Lung Fibroblasts

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Received 15 August 1988/Accepted 16 November 1988

The promoter regions of H2b histone genes contain a 14-base-pair element which includes the octamer ATTTGCAT. Mutational analysis has implicated the octamer element in the cell cycle-dependent expression of H2b histone genes. In this report, we address the question of whether the DNA-binding activity of the octamer transcription factor is itself cell cycle regulated. By using a gel mobility shift assay, we measured the relative amounts of octamer-binding activity during various phases of the cell cycle in serum-synchronized Chinese hamster fibroblasts. We found that the activity increased approximately fivefold between late G1 phase and early S phase and then decreased threefold between late S phase and G2 phase. These cell cycle-dependent changes in octamer DNA-binding activity may in part account for the selective transcription of H2b histone genes in late G1 and S phases.

Eucaryotic chromatin contains approximately equal masses of DNA and histones. In a typical somatic cell, this ratio is maintained by a tight coupling of histone and DNA synthesis (for a review, see reference 19; D. Schlumperli, Trends in Genetics, in press). Thus, the majority of histone genes are activated in late G1 phase and are transcribed through most of S phase; at the end of S phase, histone mRNAs are rapidly degraded. Any maneuver that interferes with DNA synthesis also results in the immediate cessation of histone gene transcription and the rapid degradation of histone mRNA. The mechanisms underlying cell cycle-dependent gene expression have been investigated intensively. S phase-dependent expression of histone genes is achieved through regulation of transcriptional and posttranscriptional processes (19; Schlumperli, in press). Mutational analyses of mammalian histone promoters have identified several DNA sequence elements that are responsible for cell cycle-dependent transcriptional regulation (3, 7, 14) including the H2b subtype-specific promoter element CCTTATT TGCATAAG, a highly conserved sequence found between the CCAAT and TATA elements of H2b histone genes from a variety of phylogenetically diverse organisms (13, 31). This element includes the octamer sequence ATTTGCAT also found in promoters and enhancers of immunoglobulin and several snRNA genes (4, 5, 10, 17, 18, 20, 22) and in the origin of replication of adenovirus (26). Two different transcription factors that bind to the octamer consensus sequence have been purified. One of these, OTF-1, is ubiquitous (11); the second, OTF-2, is found exclusively in lymphoid cells (27). It has been proposed that OTF-1 mediates the cell cycle-dependent expression of histone genes (11, 14). OTF-2 is, at least in part, responsible for the lineage-specific expression of immunoglobulin genes (27, 32). Recently, O'Neill et al. demonstrated that OTF-1 is functionally identical to the DNA replication factor NF-III, suggesting a dual role for this octamer-binding factor in histone gene transcription and DNA replication (21).

If the binding of a factor such as OTF-1 to the octamer sequence of H2b histone genes were important for cell cycle regulation, as is suggested by the site-directed mutagenesis experiments, then the activity of OTF-1 would be expected to increase in late G1 phase, remain high during S phase, and subsequently decline. Consistent with this view, Fletcher and colleagues (11) showed that an octamer-binding factor present in extracts derived from S-phase HeLa cells enhanced transcription of a human H2b gene in vitro, while extracts prepared from cells in G2 phase did not. In contrast with these findings, Dalton and Wells reported that the H2b octamer binding activity is not cell cycle regulated in avian cells (8). However, a thorough study of the activity of the H2b octamer binding activity throughout the mammalian cell cycle is still needed. As a first step toward this goal, we have monitored the DNA-binding activity of the H2b octamer-binding factor through the mammalian cell cycle. We prepared whole cell extracts from serum-synchronized Chinese hamster lung (K12) fibroblasts at various stages of the cell cycle and assayed these extracts for octamer-binding activity by using a gel mobility shift assay.

Gel electrophoretic mobility shift assay for H2b-specific octamer-binding protein. Figure 1 shows that the core octamer sequence of the H2b-specific element is highly conserved across large phylogenetic distances (13, 31). Indeed, the sea urchin octamer element matches its human counterpart in all eight positions and shows only two mismatches in the bordering nucleotides of the 14-base-pair H2b-specific consensus element. This high degree of conservation enabled us to use a sea urchin H2b histone gene promoter fragment to assay the mammalian octamer-binding protein. This promoter fragment, spanning the region −41 (*Bam*HI) to −98 (*Dde*I) of the sea urchin α-H2b gene (6), contains one copy of the H2b subtype-specific element and core octamer sequence, as well as a CCAAT element.

We tested the ability of factors in whole cell extracts of K12 cells to bind specifically to the sea urchin H2b promoter fragment. The fragment shown in Fig. 2A was labeled at its 3′ termini using T4 DNA polymerase and [α-^{32}P]dATP (3,000 Ci/mmol). It was incubated (10,000 cpm; approximately 1 ng) with whole cell protein extract prepared from S-phase cells (3, 16) (2 μg) and poly(dI-dC) (3 μg), and resultant DNA-protein complexes were electrophoresed on a low-salt polyacrylamide gel as previously described (12). Three protein complexes were formed (Fig. 2B, lane 2, I, II, and III). To

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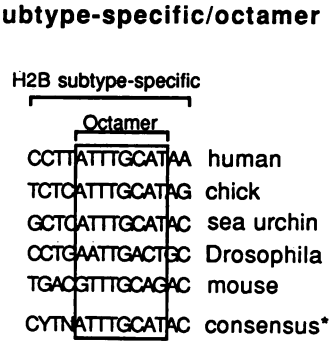

FIG. 1. Nucleotide sequences of H2b subtype-specific elements from a variety of animal groups (31). The box indicates the central, highly conserved octamer element.

determine whether any of these complexes resulted from the selective binding of a factor(s) to the octamer element, we performed a competition experiment for which we used an oligonucleotide bearing the sea urchin H2b-specific sequence (including the octamer) as a specific competitor. We tested the competitive ability of two other oligonucleotides as controls, one containing the sea urchin CCAAT element and the other containing the TATA element. The nucleotide sequences of these oligonucleotides are given in Fig. 2A. All three oligonucleotides were annealed with their opposite strands and self-ligated to an average chain length of several hundred nucleotides prior to their use in competition assays.

Complex I was greatly reduced by the addition of a 30-fold molar excess of the H2b octamer oligonucleotide to the binding reaction but not by the addition of CCAAT or TATA oligonucleotides (Fig. 2B, lanes 3 through 5), indicating that

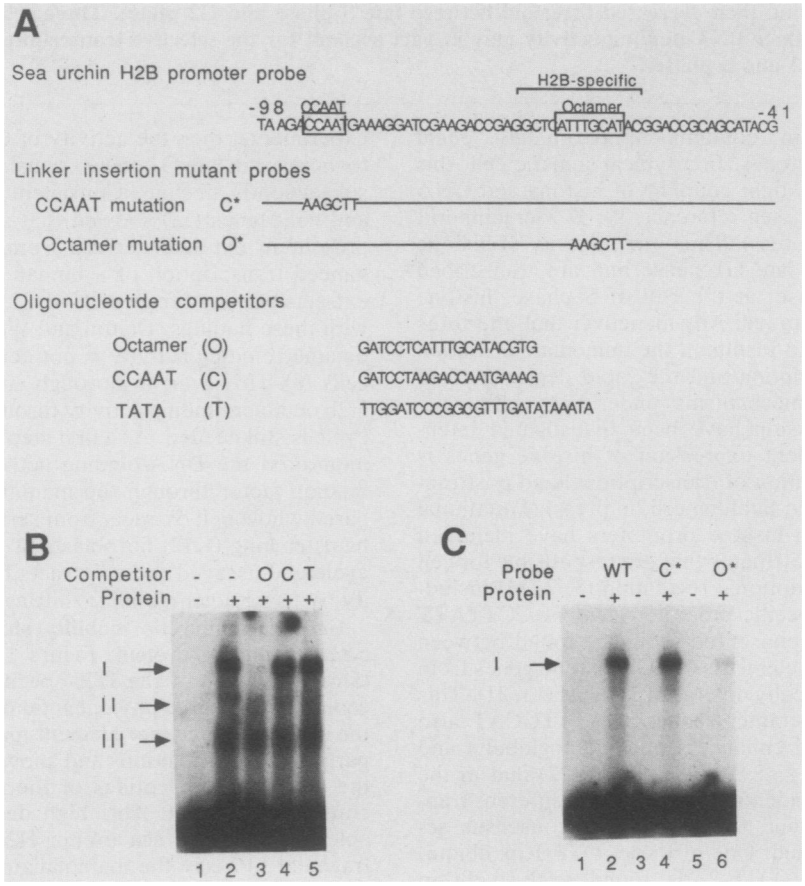

FIG. 2. Gel shift assay for H2b octamer-binding proteins. (A) Schematic map of sea urchin (*Strongylocentrotus purpuratus*) α-H2b gene promoter region showing linker insertion mutations and competitor oligonucleotides. The boxed DNA sequences are CCAAT and octamer consensus elements. The H2b-specific element comprises the octamer and the indicated surrounding bases. For linker insertion mutations C* and O*, the indicated bases (a *Hin*dIII restriction site) were substituted for the wild-type sequence by oligonucleotide-directed mutagenesis. (B) Effect of oligonucleotide competitors on the binding of factors to the sea urchin H2b promoter fragment. Radiolabeled H2b probe (1 ng; 0.025 pmol) was incubated with S-phase (11 h after serum addition) whole cell protein extract, poly(dI-dC) (3 μg), and specific oligonucleotide competitors (0.75 pmol). DNA-protein complexes were resolved on low-salt gels and visualized by autoradiography (12). Lanes: 1, probe incubated without protein extract; 2 through 5, probe incubated with 2 μg of S-phase extract; 3 through 5, probe incubated with S-phase extract and octamer (O), CCAAT (C), and TATA (T) oligonucleotides, respectively. Arrows indicate the major DNA protein complexes. Complex I was highly reproducible; complexes II and III varied in intensity on different gels. (C) Effect of linker insertion mutations on the binding of factors to the sea urchin H2b promoter fragment. DNA fragments bearing the mutations indicated in 2A were radiolabeled, incubated with S-phase whole cell extracts, and analyzed on a low-salt gel as in 2B. Lanes: 1, 3, and 5, wild-type (WT), CCAAT (C*), and octamer (O*) probes, respectively, incubated without protein extract; 2, 4, and 6, WT, C*, and O* probes, respectively, incubated with protein extract. Arrow points to a DNA-protein complex that migrated to the same relative position as complex I in 2B. Complexes II and III were not visible in this autoradiogram.

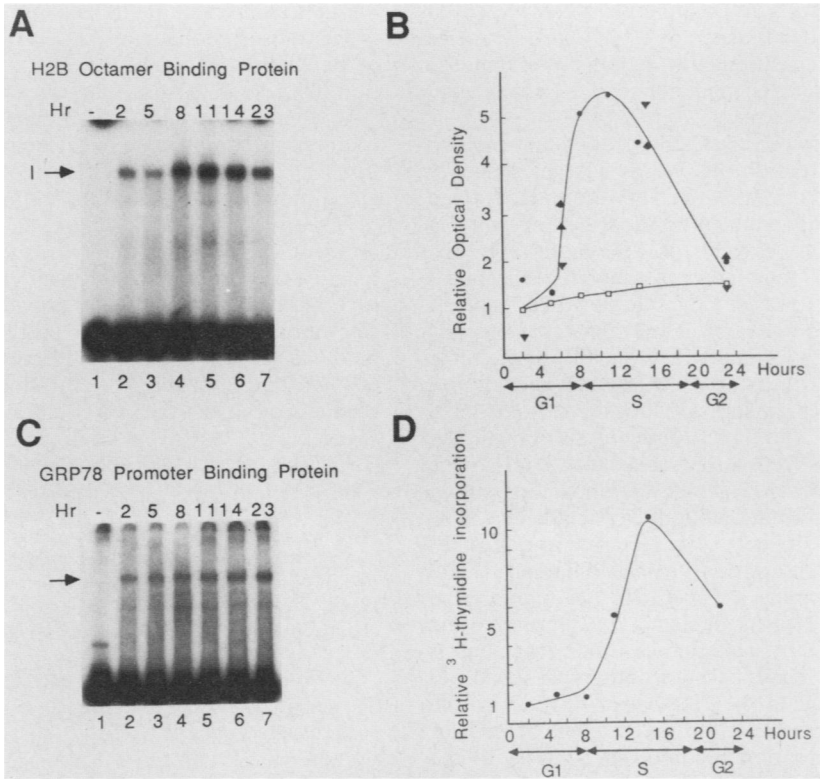

FIG. 3. Binding of factors from extracts of synchronized Chinese hamster lung fibroblasts to H2b and GRP78 promoters. (A) Autoradiogram of gel showing H2b octamer-binding activity during the cell cycle. Whole cell extracts prepared from serum-synchronized cells were incubated with radiolabeled wild-type sea urchin H2b promoter probe (Fig. 2A) and analyzed for octamer-binding activity with the gel shift assay as in Fig. 2A. Lanes: 1, probe incubated without protein extract; 2 through 7, probe incubated with extract prepared from cells at 2, 5, 8, 11, 14, and 23 h after release from serum block, respectively. Arrow points to the octamer complex. (B) Graphic representation of data from 3A and 3C. Autoradiograms shown in 3A and 3C were scanned in a densitometer, and the results were plotted as a function of time after serum addition. For the octamer-binding protein (upper curve), we show data from three separate experiments on three different sets of extracts (closed symbols). □, Values obtained from a scan of the major GRP78 promoter-protein complex on the autoradiogram shown in 3C. (C) Autoradiogram of gel showing GRP78 promoter-binding activity during the cell cycle. The same cell extracts that were analyzed with the H2b probe in 3A were analyzed with a 223-nucleotide fragment of the GRP78 promoter. The gel shift assay was carried out as in 3A. Lanes: 1, probe incubated without protein extract; 2 through 7, probe incubated with extract prepared from cells at 2, 5, 8, 11, 14, and 23 h after release from serum block, respectively. Arrow points to the major complex. (D) Incorporation of [^{3}H]thymidine into cells used for extract preparation for analysis shown in 3A and 3C. The rate of DNA synthesis was monitored by pulse-labeling the synchronized cells with [*methyl*-^{3}H]thymidine (0.7 μCi/ml) for 30 min as described elsewhere (1). The radioactivity in trichloroacetic acid-precipitable material was measured and plotted as relative [^{3}H]thymidine incorporation.

complex I was the result of a specific interaction between an H2b octamer-binding protein and the octamer sequences on the sea urchin H2b promoter fragment. Complexes II and III were unaffected by any of the three oligonucleotide competitors. These complexes were not consistently observed, while complex I was highly reproducible (see Fig. 2C and 3A). To further confirm that complex I was caused by the binding of a protein to the octamer sequence, we prepared a linker insertion mutation in which the central portion of the octamer sequence was substituted with a *Hin*dIII enzyme cleavage site. When we used this mutant DNA as a radiolabeled probe in a gel retardation analysis, we found that complex I was not formed (Fig. 2C [compare lane 2, wild-type probe, with lane 6, octamer mutation; lanes 1, 3, and 5 show the wild-type, CCAAT mutation, and octamer mutation probes with no protein]), while a mutation in the CCAAT element had no effect on complex I (Fig. 2C; compare lane 2 with lane 4 [CCAAT mutation]). These data demonstrate that the formation of complex I requires an intact octamer sequence. We conclude that a gel mobility shift assay, in which the sea urchin H2b promoter fragment is used as the probe, accurately detects an H2b octamer-binding protein, likely OTF-1, in K12 cells. We were unable to detect binding of protein factors to the CCAAT sequence on the sea urchin H2b promoter probe, perhaps because this sequence is located near the end of the probe DNA fragment and therefore does not provide a suitable binding site.

Cell cycle-dependent binding of a K12 protein to the H2b octamer element. We used the gel retardation assay to monitor levels of octamer-binding activity through the mammalian cell cycle. We demonstrated previously that histone gene transcription is regulated during the cell cycle in serum-synchronized Chinese hamster K12 cells. Transcription rate measurements indicated that histone mRNA synthesis began in late G1 phase (7 h after serum release) and reached its maximal rate in early S phase (10 h after serum release) (1). K12 cells were synchronized by serum starvation (3, 9), and whole cell extracts were prepared at various times after release from the serum block (16). To monitor cell synchrony, we measured DNA synthesis rates by pulse-labeling the cells at various times after their release from the serum block (Fig. 3D). The rate of [^{3}H]thymidine incorpo-

ration into trichloroacetic acid-precipitable DNA increased nearly 11-fold by 15 h after release; by 23 h, DNA synthesis had subsided. These measurements demonstrate that the cells were well synchronized and define the approximate boundaries of G1 and S phases.

Whole cell extracts were assayed for octamer-binding activity by the gel retardation method as described above. An example of such a gel is shown in Fig. 3A, and a graphic representation of the data from three separate experiments performed with different extracts is shown in Fig. 3B. Octamer-binding activity remained constant between 2 and 5 h after serum addition, increased fivefold between 5 and 12 h, and declined threefold between 12 and 23 h. These results demonstrate that the DNA-binding activity of a protein (or proteins) that interacts with the H2b subtype-specific octamer element increases in late G1 phase, remains high during much of S phase, and then declines. As a control, the same extracts were assayed for proteins that bind to the promoter of the human GRP78 gene (30), whose expression is regulated by glucose starvation but does not change during the S and G2 phases of the cell cycle (15; A. Lin and A. S. Lee, unpublished results). For this purpose, a human GRP78 promoter fragment spanning a 223-nucleotide region from positions −170 to +53 (24) was labeled at its 3′ termini using [α-^{32}P]dATP and T4 DNA polymerase. This fragment is devoid of the octamer sequence but contains CCAAT and TATA elements (30). The labeled DNA was mixed with the K12 cell extracts and assayed for the binding of cellular factors by the gel retardation method. In contrast to the results with the histone promoter, the binding of cellular factors to this non-cell cycle-regulated promoter showed only a slight increase (less than 1.5-fold) throughout the cell cycle (Fig. 3B and C).

Mutations within the octamer sequence cause a human H2b histone gene to be expressed constitutively during the cell cycle (14), indicating that this sequence plays a major role in cell cycle-dependent expression of H2b genes. With these studies, our demonstration here that the DNA-binding activity of the octamer protein fluctuates during the cell cycle and that the sea urchin α-H2b gene is expressed in a cell cycle-dependent manner after transfection into K12 cells (data not shown) suggest that the binding of the octamer protein to the H2b consensus octamer-binding site may be the key event in the S phase-dependent expression of H2b histone genes. Moreover, binding of this protein may also play a role in H2b DNA replication. Given that OTF-1 and DNA replication factor NF-III are functionally identical (21) and that the octamer-binding activity we detect is probably OTF-1–NF-III, it is possible that the cell cycle-dependent changes in octamer-binding activity govern the timing of H2b DNA replication. We stress that the gel retardation assay detects only octamer protein that is capable of binding to DNA. Thus, the cell cycle-dependent DNA binding of the octamer protein could reflect changes in the fraction of actively binding octamer protein rather than changes in its actual intracellular concentration.

Finally, we note that Dalton and Wells (8) have reported recently that an octamer-binding activity does not fluctuate substantially during the cell cycle of the transformed chicken erythroid cell line AEV ts34. There are at least two probable explanations for the apparent difference between their findings and ours. First, the transformation state of the cells may influence the cell cycle-dependent activity of transcription factors. The K12 cells used in this study were derived from an established Chinese hamster lung fibroblast cell line (25), while the AEV ts34 cells used by Dalton and Wells were virally transformed. Piette et al. have shown that the activity of the transcription factor AP-1 is higher in proliferating NIH 3T3 cells than in quiescent cells (23); however, transformation of these cells to a tumorigenic phenotype with simian virus 40 results in constitutive expression of AP-1 regardless of the proliferative state of the cells. Oncogenic transformation may thus alter the relationship between the activity of a given transcription factor and cell proliferation.

A second explanation is that cells were synchronized by different methods in the two studies. Dalton and Wells blocked cells in the beginning of S phase with aphidicolin, a reversible inhibitor of DNA polymerase α; we used serum starvation to block cells in early G1 phase. Since these methods are likely to act on different cellular processes, it is possible that they differentially affect the pattern of octamer-binding activity in the cell cycle.

We thank Jeff Bell for a critical review of this manuscript. We are grateful to Eva Liu for preparing radiolabeled DNA probe, Yong Kim for performing a [^{3}H]thymidine incorporation assay, and Lynn Williams for synthesizing oligonucleotides.

Ajay Sharma was supported by a Charles Heidelberger Memorial Fellowship. This work was supported by Public Health Service grants HD18582 and GM31108 from the National Institutes of Health to R.M. and A.S.L., respectively.

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