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Biochemistry

Identification of a sequence responsible for periodic synthesis of yeast histone 2A mRNA

(*H2A–lacZ* fusions/yeast transformation/transcriptional control/*ars*)

MARY ANN OSLEY* AND LYNNA HEREFORD*

Rosenstiel Center and Department of Biology, Brandeis University, Waltham, Massachusetts 02254

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ABSTRACT Sequences required for the regulated expression of a yeast histone 2A (*H2A*) gene have been investigated by using fusions between this gene and the *Escherichia coli lacZ* gene. Fusions containing the entire spacer region in which divergent transcription of the *H2A* and *H2B* genes is initiated result in low-level constitutive synthesis of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) in yeast. Regulated expression (which is characterized by periodic synthesis during the S phase of the cell cycle) is restored when a 1.3-kilobase *Hin*dIII fragment containing a small region of the 3′ end of the *H2B* gene is present in either orientation. The regulatory activity in this region appears to be coincident with a sequence that supports autonomous replication in yeast.

Yeast histone mRNA levels are tightly controlled during the cell division cycle such that they are maximally accumulated during the S phase (1). The major regulatory mechanism responsible for this pattern of accumulation acts at the level of transcription. We have recently demonstrated that the histone genes are periodically transcribed during cell division and, moreover, that activation and termination of transcription can be distinguished from one another by virtue of their dependence on temporally separated cell cycle events (2). Activation of transcription occurs late in G1 at or before the last known step required for initiation of chromosome replication, whereas termination of transcription occurs early in S (2). The close relationship between the transcription of histone genes and the state of chromosome replication has suggested a simple model in which the periodic synthesis of histone mRNA is regulated by the DNA replication cycle. Thus, we have proposed that changes in chromatin structure preceding the initiation of DNA synthesis are sufficient to activate histone gene transcription; termination of transcription results from the initiation of DNA synthesis or replication through the region containing the histone genes, which restores the chromatin in this region to its prereplicative state (2).

In light of our suggestion that transcriptional regulation may be determined by the periodic activation of DNA replication sites, it was with considerable interest that we noted the presence of a sequence capable of supporting autonomous replication in yeast at the 3′ end of the gene for histone H2B in each of the two genetically unlinked *H2A–H2B* gene copies (3). To determine the possible involvement of this sequence in the periodic transcription of the histone genes, we have asked whether the region of DNA that contains it is required for the regulated expression of a *H2A* gene fused to the bacterial gene encoding β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23). Regulated expression of the fusion gene in yeast occurs only when the DNA segment containing the replication activity is present. Deletion mapping of this region suggests that the replication and regulatory activities are coincident. We have therefore identified a DNA segment responsible for regulating cell-cycle-specific gene expression that may also have a role in chromosome replication.

MATERIALS AND METHODS

Strains, Plasmids, and Media. *Escherichia coli* strains LG90 (F^- *Δlac pro*) (4) and C600 (*leuB thr pro thi*) (5) were obtained from L. Guarente and J. Yarger, respectively. The *Saccharomyces cerevisiae* strain DBY747 (*a his3Δ leu2-3 leu2-112 ura3-52*) was obtained from D. Botstein. Plasmid pLG200 was obtained from L. Guarente (6). Yeast histone gene plasmids have been described by Hereford *et al.* (3). Standard methods were used to transform *E. coli* (7) and yeast (8) with plasmid DNA. In some yeast transformations, plasmids were targeted (9) to *leu2* or *Ty* locations by linearizing plasmids with *Sal* I or *Xho* I restriction endonuclease, respectively.

E. coli cells were grown in LB (complete) or M9-minimal medium (10). Where appropriate, ampicillin was added to a final concentration of 50 μg/ml. Yeast were grown in YNB-minimal medium (1) supplemented with 100 μg of the appropriate amino acids per ml and 0.1% yeast extract and Bactopeptone. *S. cerevisiae* cells were synchronized with α-factor as described by Hereford *et al.* (1, 2).

Assay for β-Galactosidase. (*i*) Plate assays. Bacteria were spotted onto MacConkey–lactose plates (10). 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal; Sigma) was added to yeast minimal-medium plates as described by Rose *et al.* (11) at a concentration of 100–200 μg/ml.

(*ii*) Culture assays. Exponentially growing cultures of yeast cells were grown to a density of $\approx 3 \times 10^6$ cells per ml in YNB-minimal medium minus leucine. Three- to 6-ml aliquots were harvested by centrifugation and the pellets were resuspended in 0.5 ml of Z buffer (10) plus 0.5 g of glass beads. The suspensions were swirled in a Vortex mixer at highest speed for 10–20 sec and β-galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl β-D-galactoside (10). After termination of the reaction, the liquid phase was removed from the glass beads and clarified by centrifugation. β-Galactosidase activity in synchronous cultures was assayed by the same procedure, using 3-ml aliquots removed at intervals throughout the division period.

RNA Gels. Total RNA was extracted from exponential cultures of yeast, fractionated on 1.5% agarose/methylmercury hydroxide gels, and transferred to diazobenzyloxymethyl-paper as described (1). Hybridization probes were labeled with ^{32}P by nick-translation (12).

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Abbreviations: *ars*, autonomously replicating sequence; kb, kilobase(s).

* Present address: Sidney Farber Cancer Institute and Dept. of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

RESULTS

Construction of *H2A–lacZ* Fusions. Several features of the organization of the *H2A–H2B* gene pair found at the *TRT1* (3) locus and a partial restriction map of this region are summarized in Fig. 1A. Transcription of the histone genes is divergent and initiates within the ≈800 nucleotides of spacer DNA that separates them (3). A 1.1-kilobase (kb) *Hin*dIII fragment encompasses the entire spacer region as well as a portion of the 5′ coding sequences of both the *H2A* and the *H2B* genes. A second *Hin*dIII fragment of 1.3 kb is immediately adjacent to the 1.1-kb region and includes the 3′ end of the *H2B* gene and its flanking sequences. Within this fragment is a 1-kb region bounded by one of the *Hin*dIII sites and an internal *Bam*HI site that contains a sequence behaving like a yeast *ars* (for autonomously replicating sequence) (14, 15). This *ars* was identified by two criteria. First, when present on an integrative plasmid, YIp5 (14), it promotes high-frequency yeast transformation (≈200–500 colonies per μg of DNA). Second, it allows autonomous replication of this plasmid in yeast, as assayed by both the mitotic instability of plasmid sequences and the ability to detect closed circular plasmid DNA in transformant cells (data not shown).

A histone–β-galactosidase hybrid gene was constructed *in vitro* by fusing the 1.1-kb *Hin*dIII fragment to *lacZ* carried on plasmid pLG200 (6; Fig. 1B). The *lacZ* fragment on this plasmid contains the gene for β-galactosidase fused to a portion of the *lacI* gene, but it lacks both a bacterial promoter and the first several amino acids of the hybrid enzyme and is therefore unable to direct synthesis of β-galactosidase in either bacteria or yeast (6). When this plasmid is cut with *Hin*dIII, the 5′ end of the *lacZ* fragment is exposed in a unique translational reading frame (6). Insertion of the 1.1-kb histone fragment in either orientation produces a fusion of *H2A* or *H2B* to *lacZ* in the correct reading frame (16, 17). Although both hybrid genes are expressed in yeast, our analysis has been restricted to the *H2A–lacZ* fusion (pfusA1-1, Fig. 1B) because the levels of β-galactosidase produced by this fusion are much higher than those produced by the *H2B* fusion (data not shown). Two additional plasmids were constructed by inserting the *ars*-containing 1.3-kb *Hin*dIII fragment from *TRT1* (Fig. 1A) into the distal *Hin*dIII site on the pfusA1-1 fusion plasmid. Insertion of this fragment in the correct orientation (pfusA1-2; Fig. 1C) reconstitutes an intact *H2B* gene, whereas insertion in the opposite orientation (pfusA1-3; Fig. 1C) results in the separation of the 5′ and 3′ *H2B* coding regions by the *ars*.

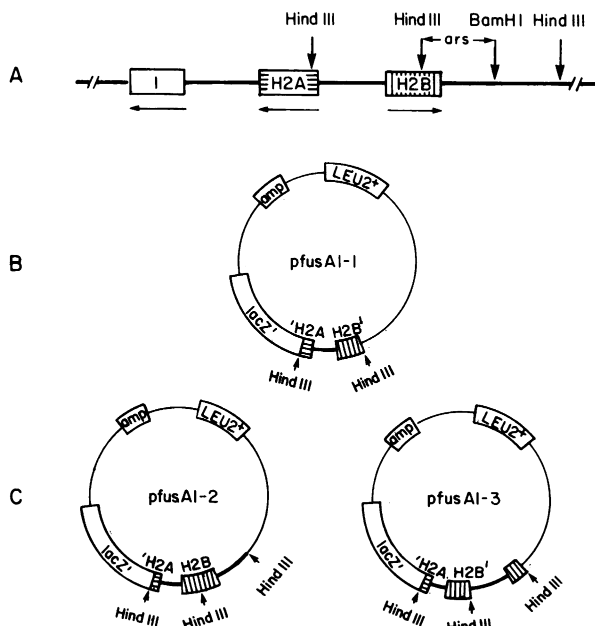

FIG. 1. Construction of *H2A* fusion plasmids. (*A*) Organization of genes at the *TRT1* locus, showing *H2A* and *H2B* genes and nonhistone protein 1 gene (3). The arrows represent the direction of transcription. The two *Hin*dIII fragments used to construct the fusion plasmids are indicated. The 1.1-kb *Hin*dIII fragment includes 40 nucleotides of *H2A* coding sequence, 236 nucleotides of *H2B* coding sequence, and ≈800 nucleotides of spacer DNA separating the two genes. The 1.3-kb *Hin*dIII fragment contains the 3′ end of the *H2B* gene and its flanking DNA and also includes a sequence supporting autonomous replication in yeast (*ars*), which has been localized to a 1-kb *Hin*dIII/*Bam*HI fragment. (*B*) pfusA1-1 was created by inserting the 1.1-kb *Hin*dIII fragment shown in *A* into the unique *Hin*dIII site at the 5′ end of the *lacI–lacZ* fragment carried on plasmid pLG200 (6). This plasmid also contains a *Pst* I fragment containing the yeast *LEU2*$^+$ gene (13) to provide a selectable marker for maintenance of the plasmid in yeast. *amp*, Ampicillin resistance. (*C*) pfusA1-2 results from the insertion of the 1.3-kb *Hin*dIII fragment shown in *A* into pfusA1-1 in the correct orientation. pfusA1-3 has this same fragment in the reverse orientation.

Expression of *H2A* Fusions in Yeast. To determine the roles of the 1.1-kb and 1.3-kb regions in the expression or regulation of the *H2A* gene, we took advantage of the fact that, in yeast, integration of plasmid sequences introduced by transformation occurs by homologous recombination (8). The plasmids that we constructed will therefore integrate either at the *TRT1* locus or at loci homologous to the selectable marker placed on the plasmid for maintenance in yeast. The selectable marker that we used is the yeast *LEU2*$^+$ gene (Fig. 1 *B* and *C*) from plasmid CV9 (13). Because the DNA fragment containing this gene also contains a portion of a repetitive *Ty* sequence (13), integration will occur either at the *leu2* locus or at random *Ty* sites within the genome.

As a consequence of integration of pfusA1-1 at these different genomic locations, the sequences flanking it vary greatly. When pfusA1-1 is integrated at the *TRT1* locus, it displaces the resident *H2A* gene such that the fusion gene is properly positioned with respect to the adjacent *ars* (cf. Figs. 1A and 2A). At *leu2* (Fig. 2B) or any other chromosomal location, however, the only histone gene sequences present are those 5′ sequences in which transcription is initiated. If the latter sequences are all that are required for expression and regulation, we would expect the levels of β-galactosidase to be independent of the chromosomal location of the fusion gene.

Leucine-independent transformants of pfusA1-1 were initially screened on 5-bromo-4-chloro-3-indolyl β-D-galactoside

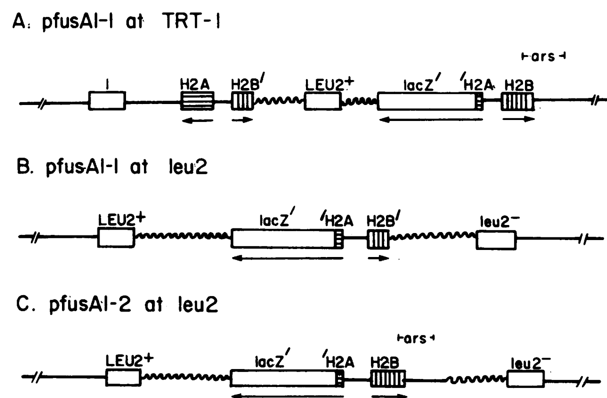

FIG. 2. Orientation of *H2A* fusion genes after integration. Organization of genes at either the *TRT1* or the *leu2* locus after integration of two of the fusion plasmids shown in Fig. 1. Wavy lines indicate plasmid sequences. The positions of *ars* are shown when they are present. Arrows represent the transcriptional orientation of integrated and resident genes.

indicator plates. Two phenotypic classes, which differed markedly in their degree of β-galactosidase expression, were obtained. In all cases, transformants producing high levels of enzyme contained the hybrid gene integrated at the *TRT1* locus, as determined by Southern blot analysis (data not shown). This difference was confirmed by enzyme assays in culture, which showed 4-fold differences between β-galactosidase levels (Table 1).

To determine whether the increase in β-galactosidase activity when pfusA1-1 is integrated at the *TRT1* locus is due to the proximity of the fusion gene to the *ars*, we next transformed yeast with pfusA1-2 and pfusA1-3 (Fig. 1*C*). If the 1.3-kb region containing this sequence is involved in the expression of β-galactosidase, we would expect that the position effect observed with pfusA1-1 would disappear, because now a fusion gene integrated at any chromosomal location would be positioned next to this region (Fig. 2*C*). Because of the *ars* in the 1.3-kb fragment, both pfusA1-2 and pfusA1-3 replicate autonomously. Although many of the plasmids eventually integrated at the *TRT1* locus, it was necessary to target these plasmids to *leu2* or *Ty* locations by linearization within the appropriate sequences (ref. 9; *Materials and Methods*).

The β-galactosidase levels of the pfusA1-2 and pfusA1-3 transformants were remarkably similar and, moreover, were now independent of the genomic location of the fusion gene. The enzyme levels produced by fusions integrated at *leu2* or *Ty* locations are 70–95% of those produced by pfusA1-1 integrated at *TRT1* (Table 1). Interestingly, enzyme levels are somewhat higher when the 1.3-kb *ars* fragment is in the reverse orientation (Fig. 1*C*; Table 1). Although it is not known whether this difference is related to the presence of an additional functional *H2B* gene when the fragment is in the correct orientation (Fig. 1*C*), these results demonstrate that the enhancing effect of the 1.3-kb fragment is not dependent on an intact, adjacent *H2B* gene.

Regulation of *H2A* Fusions. To determine whether the presence of the *ars* fragment has either a general enhancing effect on *H2A* gene transcription or a more specific role in cell-cycle regulation, β-galactosidase activity was followed in synchronous cells that carried the fusion gene, in the absence or the presence of this fragment, integrated at either the *TRT1* or *leu2* locus.

If β-galactosidase is not regulated during the cell cycle, we would expect a continuous (exponential or linear) increase in enzyme activity. If the hybrid enzyme shows the same pattern of regulation as histone mRNA (1, 2), then we would expect a peak or step in enzyme activity, depending on the stability of the enzyme. The results of this experiment are shown in Fig. 3. In the absence of the *ars* fragment, the pattern of β-galactosidase activity is very different and dependent on the chromosomal location of the fusion gene. When pfusA1-1 is integrated at *leu2* (Fig. 3*B*), the activity of β-galactosidase increases continuously throughout cell division. Integration at *TRT1* (Fig. 3*C*), however, results in a discrete step in enzyme activity, exactly at the point of maximal histone mRNA accumulation (Fig. 3*A*). This position effect is abolished in *leu2* integrants of either pfusA1-3 (Fig. 3*D*) or pfusA1-2 (data not shown): the presence of the *ars* in either orientation results in a pattern of β-galactosidase activity identical to that observed in *TRT1* integrants of pfusA1-1. These results strongly suggest that the differences in enzyme levels measured in exponentially growing populations of cells reflect differences in cell cycle regulation of the hybrid gene. The *H2A–lacZ* fusion is apparently responsive to normal cell cycle regulation only when it is adjacent to the *ars* fragment, either as a result of integration at the *TRT1* locus (Fig. 2*A*) or after addition of this fragment to the fusion gene carried on plasmids integrated at other locations (Figs. 1*C* and 2*C*).

To confirm that the differences in enzyme levels are an accurate representation of the transcription patterns of the fusion gene, the concentration of mRNA corresponding to this gene was determined. Cells containing pfusA1-1 integrated at *TRT1* or at *leu2* were grown exponentially or arrested in G1 with α-

Table 1. β-Galactosidase levels in cells transformed with *H2A* fusion plasmids

H2A fusion* plasmid	Chromosomal location			β-Galactosidase activity†		Activity relative to *TRT1*‡
	TRT1	*leu2*	Other	Fusion	Average	
pfusA1-1						
1	+			6.8	7.5	(1.0)
2	+			7.1		
3	+			8.5		
4			+	1.6	2.0	0.26
5			+	2.2		
6		+		2.1		
pfusA1-2						
7	+			5.5		0.73
8			+	5.2		0.70
9		+		5.2		0.70
pfusA1-3						
10		+		7.1		0.95
11			+	6.2		0.83
12			+	5.9		0.79

* Cells transformed with plasmids shown in Fig. 1.

† μmol of *o*-nitrophenyl β-D-galactoside hydrolyzed per min per 10^7 cells.

‡ β-Galactosidase activity relative to activity when pfusA1-1 is at *TRT1* locus.

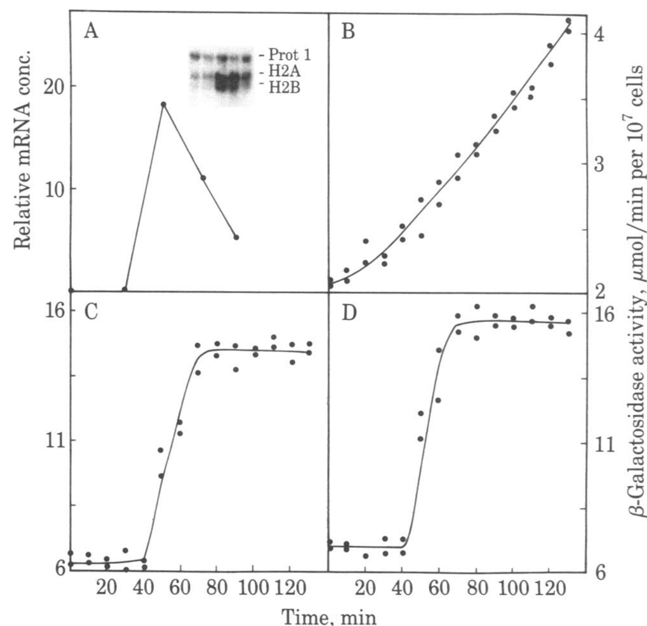

FIG. 3. β-Galactosidase activity during synchronous cell division. Cells were arrested in G1 with α-factor as previously described (1, 2) and, after removal of the pheromone, the activity of β-galactosidase was measured in aliquots removed at the times indicated. The concentration of *TRT1 H2B* mRNA was determined in parallel aliquots by gel analysis (1). (*A*) Relative steady-state levels of *TRT1 H2B* mRNA. Prot 1, protein 1. (*Inset*) Autoradiograph of mRNAs. (*B*) β-Galactosidase activity when pfusA1-1 is integrated at *leu2* (Fig. 2*B*). (*C*) β-Galactosidase activity when pfusA1-1 is integrated at *TRT1* (Fig. 2*A*). (*D*) β-Galactosidase activity when pfusA1-3 is integrated at *leu2*.

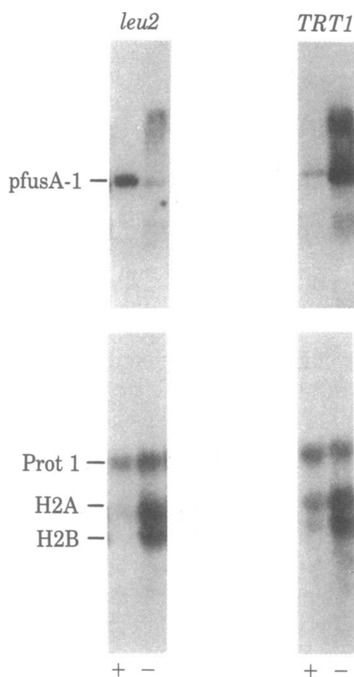

FIG. 4. RNA levels in cells transformed with *H2A* fusion plasmids. Exponentially growing cultures of strain DB747 containing pfusA1-1 integrated at either the *TRT1* or the *leu2* locus were grown to a density of ≈1 × 10^6 cells per ml, and α-factor was added to an aliquot of cells for about 1 cell generation to arrest cells uniformly in G1. RNA was extracted, separated on a methylmercury hydroxide/agarose gel, and transferred to diazobenzyloxymethyl-paper. Transcripts were identified by hybridization to radiolabeled plasmid *TRT1*. Transcripts corresponding to *H2A*, *H2B*, and protein 1 genes as well as those coded by the *H2A* fusion gene are indicated. −, No α-factor; +, addition of α-factor.

factor, and total RNA was isolated and separated on a methylmercury hydroxide/agarose gel. Both histone and fusion gene transcripts were identified by hybridization to plasmid *TRT1* (Fig. 4). In exponential cells the levels of the three *TRT1*-encoded transcripts—H2A, H2B, and protein 1 [a nonhistone gene that is not regulated during the cell cycle (1)]—are comparable in each type of integrant. The fusion gene transcript is reduced in concentration in the *leu2* integrant, however, consistent with the observed decrease in β-galactosidase activity in this strain (Table 1). In G1-arrested cells, the H2A and H2B transcripts decrease in concentration (1), as does the fusion transcript in the *TRT1* integrant. The *leu2* integrant does not show a similar decrease in fusion RNA levels; instead, the concentration of this RNA increases. These results suggest that the observed differences in enzyme levels reflect differences in transcriptional activity.

Deletion Mapping of *ars* and Regulatory Activities. While it is clear that the 1.3-kb region has an important role in transcriptional regulation, the previous experiments did not distinguish whether the *ars* and regulatory activities are coincident. To determine whether these two activities can be separated, *in vitro* deletion mapping of the 1.3-kb fragment was undertaken. The results of this analysis are shown in Table 2. The data show that, within the limits of 50–100 base pairs, the deletions simultaneously abolish the *ars* and regulatory activities: high transformation frequencies are associated with high β-galactosidase levels, and low transformation frequencies correspond to low levels of enzyme. Thus, to a first approximation, the *ars* region and the regulatory region appear to be the same.

Table 2. Deletion mapping of *ars* and regulatory activities in 1.3-kb fragment

Deletion*	*ars* activity†	β-Galactosidase relative activity‡
pfusA1-2	1.0	0.7
Δ1 (−25)	0.9	0.7
Δ2 (−100)	0.7	0.53
Δ29 (−150)	1.1	0.82
Δ30 (−250)	0.005–0.01	0.28
Δ31 (−300)	0.005–0.01	0.27
Δ4 (−400)	0.005–0.01	ND
pfusA1-1	0.005–0.01	0.26

* pfusA1-2 was linearized with *Bam*HI (see Fig. 1) and treated with 0.2 unit of *Bal* 31 (Bethesda Research Laboratories). The plasmids were then digested with *Sma* I (which cuts in nonessential vector sequences approximately 1.3 kb from the *Bam*HI site), ligated, and used to transform *E. coli* strain LG90. Individual transformant clones were analyzed for the extent of deletion from the *Bam*HI site and appropriate deletion plasmids were used to transform yeast. The numbers in parentheses represent the approximate number of base pairs deleted.

† Transformation frequencies relative to the frequency obtained with pfusA1-2 (≈200 colonies per μg of DNA).

‡ β-Galactosidase levels of plasmids integrated at *leu2* or *Ty* chromosomal locations relative to those produced by pfusA1-1 at *TRT1*. ND, not determined.

DISCUSSION

Using fusions of one of the yeast *H2A* genes to the bacterial gene encoding β-galactosidase, we have identified a DNA segment required for periodic transcription of the *H2A* gene during the cell division cycle. This segment is distinct from other sequences lying in the 5′ region immediately adjacent to the *H2A* gene in which transcription is initiated, which may also have a role in *H2A* gene expression. This is shown by the observation that a fusion gene containing only these immediate 5′ sequences synthesizes low levels of β-galactosidase throughout the cell cycle (Fig. 3*B*) in an apparently unregulated manner. The fusion gene is subject to the pattern of cell cycle control seen for the intact *H2A* gene (Fig. 3 *A* and *C*) only when it is positioned next to the 1.3-kb *Hin*dIII fragment, which includes the 3′ end of the *H2B* gene and its flanking sequences (Fig. 1*A*).

The most interesting aspect of the 1.3-kb *Hin*dIII fragment is that it contains an *ars*. Deletion mapping (Table 2) shows that the *ars* activity apparently coincides with the regulatory activity identified in the 1.3-kb fragment, suggesting that the *ars* has an important role in the regulated expression of histone genes. It is not yet known whether the histone-gene-associated *ars*, or indeed any other *ars*, actually represents a specialized replication sequence. Support for the view that these sequences may be chromosomal replication origins comes from the observation that they occur, as assayed by yeast transformation (15), with approximately the same frequency as the spacing of DNA initiation sites seen by electron microscopy (18). Because transcription of the histone genes is linked to steps required to initiate chromosome replication (2), it is tempting to speculate that the *ars* near these genes may indeed be a replication origin. If this is the case, the data presented here support our notion that the activation and firing of this origin once every cell cycle could be responsible for the periodic synthesis of histone mRNA.

Although it remains to be shown, first, that the *ars* and regulatory activities are truly inseparable and, second, that the histone gene *ars* is used to direct chromosome replication, we suggest that we have identified a potentially novel regulatory mechanism. We also suggest that this mechanism may be used more generally to regulate the expression of genes whose prod-

ucts have either structural or enzymatic roles during the S phase.

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