22. We thank S. Burstein, E. Beutler, and R. McMillan for bone marrow samples; H. Abrams for help with manuscript preparation; and I. Verma and D. Miller for advice and for providing the helper plasmid pSAM. Supported in part by the Clayton Foundation for Research, California Division and by Public Health Service grants AM 13622, GM 28223, HD20034, GM 17702, RR-00827, the Weingart Foundation, the Gould Family Foundation, the Kroc Foundation, the Keck Foundation, the Stallone Foundation for Autism Research, and the Academic Senate Committee on Research of the University of California, San Diego.

28 May 1985; accepted 6 September 1985

Isolation of a Mammalian Sequence Capable of Conferring Cell Cycle Regulation to a Heterologous Gene

Abstract. *A hybrid gene containing the 5′ sequence of a hamster histone H3 gene and the coding sequence of the bacterial neomycin-resistance gene* (neo) *was constructed. Upon transfection into the hamster fibroblast cell line K12, the hybrid gene exhibited cell cycle–dependent regulation, as evidenced by the maximal accumulation of the* neo *transcripts during synthesis of DNA in the cell cycle. In addition, cells arrested in the prereplicative phase, as a consequence of the K12 temperature-sensitive mutation, produced significantly less* neo *messenger RNA.*

ALEXANDER ARTISHEVSKY
ALBERT GRAFSKY
AMY SHIU LEE*
Department of Biochemistry, University of Southern California School of Medicine, Los Angeles 90033

* To whom reprint requests should be addressed.

A fundamental problem in the study of cellular proliferation is the control mechanisms by which cells regulate temporal events during the cell cycle. The problem is compounded in mammalian cells because of the complexity of the genome and the general lack of well-defined cell-cycle mutants. Our approach has been to identify the elements of the mammalian genome controlling the cell cycle expression of specific sets of genes through the use of the well-studied histone gene system (*1*). Mammalian cells contain multiple copies of each of the histone genes and their variants, some of which are regulated temporally during the cell cycle at both transcriptional and post-transcriptional levels (*2, 3*). For example, the histone H3.2 gene is transcribed during the early S phase when DNA synthesis is initiated, and histone H3.2 messenger RNA (mRNA) accumulates measurably during the peak of DNA synthesis (*3, 4*). As the cells progress into late S and postreplicative (G2) phases, H3 mRNA declines correspondingly. This strict interdependence of the increased rate of histone mRNA transcription and the onset of DNA synthesis suggests that sequences in or around this cell cycle–regulated histone gene may represent specific control elements necessary for the temporal regulation of its transcription.

To test this hypothesis, we constructed a hybrid gene in which the 5′ sequence of a hamster H3 gene was fused to the bacterial neomycin-resistance gene (*neo*) (Fig. 1). We have described the subcloning of a 3.7-kilobase (kb) hamster genomic sequence containing a hamster H3 gene into the plasmid pUC8 (*3*). Partial DNA sequencing of this recombinant, pAAD3.7, revealed that the hamster H3 gene was similar to the mouse H3.2 gene (*5*). Further restriction mapping of pAAD3.7 showed that a 1.1-kb Pvu II fragment contained about 1 kb of 5′ flanking sequence plus the nucleotides encoding the first 20 amino acid residues of histone H3. This fragment was therefore isolated and tested for the presence of cell cycle–regulatory sequences. For a marker gene, we used the bacterial neomycin resistance gene (*neo*) (*6*). The use of this marker gene allowed us to discriminate expression of the exogenous chimeric gene from that of the endogenous histone H3 genes and to select stable transformants that had integrated the marker gene into their genomes at various chromosomal sites. We removed the herpes simplex virus thymidine kinase (HSV-tk) promoter from plasmid pNEO3 (*7*) and in its place inserted the hamster 1.1-kb Pvu II fragment containing the 5′ sequence of the hamster H3 gene. This hybrid gene, unlike other *neo* transfection vectors commonly used (*8*), did not contain any SV40 enhancer or promoter sequences. The only eukaryotic sequences in the hybrid gene were the hamster DNA and a short stretch of HSV-tk DNA containing the polyadenylate [poly(A)] addition site in the original pNEO3 construct. One hybrid gene, pHN/7, contained the hamster and the *neo* sequences fused in the same transcriptional orientation. This plasmid was used in all subsequent

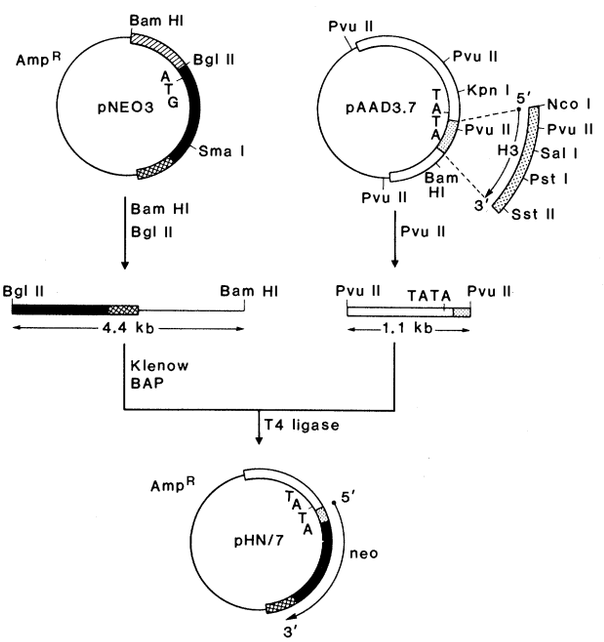

Fig. 1. Construction of the hamster histone H3-*neo* hybrid gene. The preparation of the 4.4-kb Bam HI–Bgl II fragment from the vector plasmid pNEO3 has been described (*7*). After extraction from low-melting agarose, the fragment was treated with the Klenow fragment of DNA polymerase to fill in the 5′ protruding ends generated by Bam HI and Bgl II. The fragment was subsequently treated with the bacterial alkaline phosphatase to prevent vector self-polymerization. Digestion of pAAD3.7 with Pvu II yielded four fragments with sizes of 2.4 (pUC8 vector), 1.5, 1.4, and 1.1 kb. The 1.1-kb fragment contained the 5′ flanking sequence of the histone H3 gene as well as sequences encoding for the first 20 amino acids of H3. This fragment was extracted from low-melting agarose and ligated to the *neo* vector with T4 DNA ligase. The ligated mixture was transfected into HB101, and ampicillin-resistant colonies were selected. Plasmids were prepared from individual colonies and the orientation of the hamster 1.1-kb insert with respect to the *neo* gene was determined by restriction mapping. In the plasmid, designated pHN/7, the orientation of transcription of the hamster histone fragment was the same as that of the *neo* gene, which is 1.2 kb long. If the RNA initiates at the histone promoter (TATA sequence, as indicated) and terminates at the poly(A) addition site downstream from the *neo* gene, the size of the RNA transcribed from the hybrid gene is expected to be around 1.5 kb.

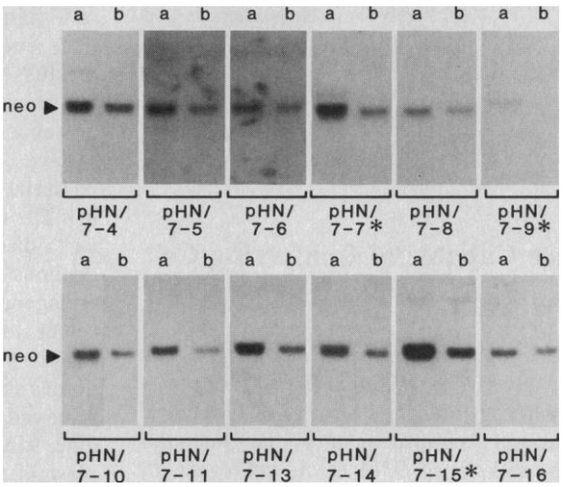

Fig. 2. Effect of G_1 arrest on the production of pHN/7 hybrid gene transcripts in the individual stable transformants. K12 cells were transfected with pHN/7 (*11*). A total of 12 individual G418 resistant clones were selected and expanded into mass cultures. For each clone, two sets of cells were prepared. Cells were seeded in 150-mm culture dishes and grown to about 50 percent confluency in Dulbecco's minimal essential medium. At this time, one set (lane a) was allowed to continue to grow at 35°C for an additional 24 hours. The other set (lane b) was incubated at the nonpermissive temperature of 40.5°C for 24 hours. Total cytoplasmic RNA was extracted from both sets of cells (*3*); 15 μg of each RNA sample was applied to formamide-formaldehyde agarose gels and, after electrophoresis, was blotted onto nitrocellulose filters. The RNA gel blots were hybridized with a nick-translated 1-kb Bgl II–Sma I *neo* fragment of pNEO3 (Fig. 1). The specific activity of this probe was 3×10^7 count/min per microgram of DNA. The blots were exposed for 30 hours to Kodak XAR-5 film at −70°C, through the use of a Lightning-plus intensifying screen. The size of the *neo* transcripts was about 1.5 kb. Asterisks indicate three individual transformants chosen for the more detailed cell cycle analysis shown in Fig. 3.

transformation experiments. If this Pvu II fragment contained the control elements for cell cycle regulation, the expression of the marker gene would be temporally regulated during the cell cycle.

For the recipient cells, we used the hamster mutant cell line K12. When K12 cells are shifted to the nonpermissive temperature (40.5°C), the cells would be blocked at late G_1 because of the temperature-sensitive (ts) mutation (*9*). We have used this ts mutant to demonstrate the coupling of histone and DNA synthesis at both the translational and transcriptional levels (*3*, *10*). In addition, K12 cells are highly competent for calcium phosphate–mediated DNA uptake (*11*), and the general RNA metabolism is intact when incubated at 40.5°C (*12*). Therefore, this well-characterized mammalian cell cycle mutant provides us with a rapid and simple way to prepare synchronized populations of G_1 cells.

Because of these advantages of the K12 cell line, we used it for transfection with the histone-*neo* hybrid gene, pHN/7. Stable transformants were selected on the basis of their resistance to G418. The transformation efficiency of pHN/7 was about 100 resistant colonies

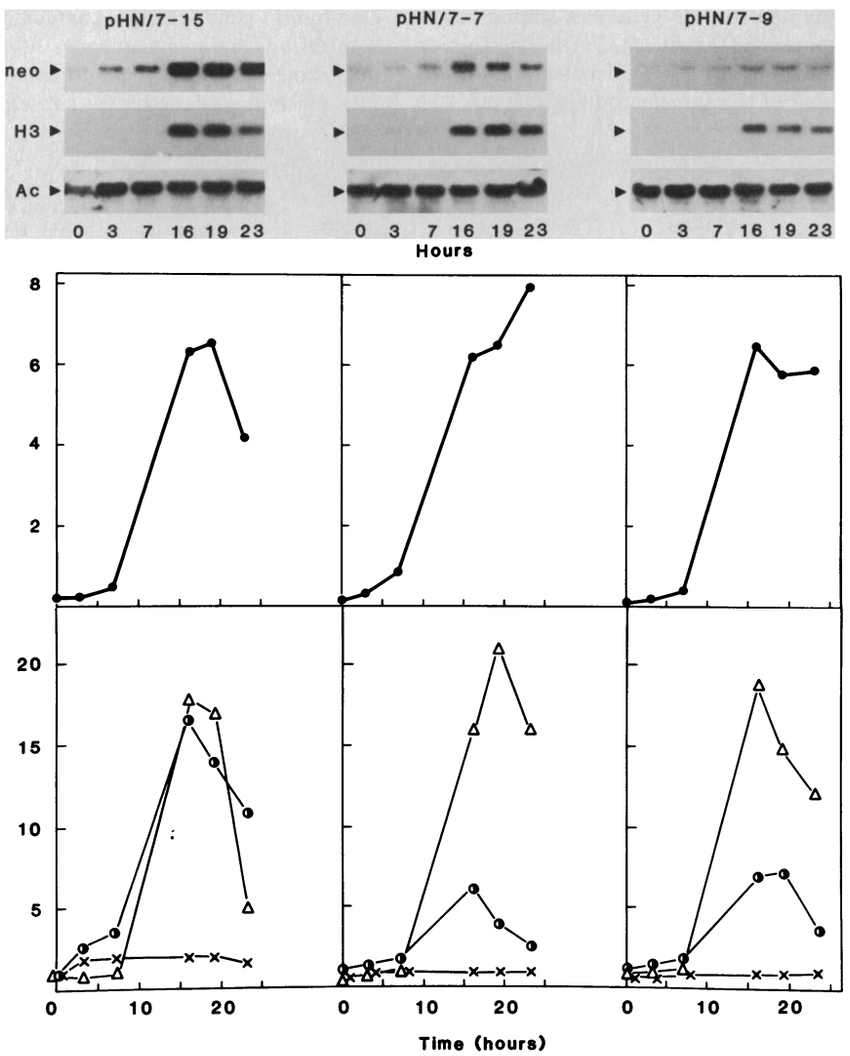

Fig. 3. Cell cycle analysis of the levels of *neo*, histone, and actin mRNA in three individual transformants. Culturing and synchronization of the K12 transformants by serum deprivation occurred as described (*3*). Before synchronization, the cells were taken out of the G418 selection. At time 0, fresh medium was added to the G_1-arrested cells to stimulate their synchronous progression through the cell cycle. The rate of DNA synthesis was monitored by pulse-labeling cells with 0.25 μCi of [*methyl*-^{3}H]thymidine (50 Ci/mmol) per milliliter of medium for 30 minutes. The radioactivity recovered in the trichloroacetic acid–precipitable material indicated the amount of incorporated thymidine (*10*). In parallel, total cycloplasmic RNA was extracted and processed for hybridization (Fig. 2). Three nick-translated probes were used for RNA blot hybridization. The *neo* probe was the one described for Fig. 2. The histone probe was obtained as a 1.1-kb Kpn I–Bam HI fragment of the plasmid pAAD3.7 containing the hamster H3 gene (Fig. 1) and was labeled to a specific activity of 5×10^7 count/min per microgram of DNA. The mouse actin complementary DNA plasmid (*3*) was digested with Pst I to yield a 0.7-kb fragment representing a portion of the actin coding sequence. This was nick-translated to a specific activity of 1×10^8 count/min per microgram of DNA. The autoradiograms for the histone H3 and actin probes were obtained after 2 hours of exposure, and that for the *neo* probe after 24 hours. The sizes of RNA hybridized to these probes were 1.5 (*neo*), 0.9 (histone), and 2.1 kb (actin). The autoradiograms were quantitated by densitometry to obtain the relative levels of *neo* (◑), histone (△), and actin (x) mRNA during the cell cycle.

per 2 million cells transfected with 10 μg of plasmid DNA, a value similar to that obtained with pNEO3, a plasmid in which *neo* transcription is driven by the HSV-tk promoter. Twelve clones were randomly selected and cultured for the analysis of *neo* mRNA in exponentially growing cells (asynchronous culture at 35°C) and in cells arrested in G_1 (cultures shifted to 40.5°C) (Fig. 2). When the *neo* mRNA level of exponentially growing cells was compared with that of cells blocked in G_1, increased amounts of *neo* transcripts were seen in asynchronously cycling cells. This observation for all the transformant clones tested indicates that position of the hybrid gene in the host genome was not a major factor in determining the differential *neo* expression between growing and G_1-arrested cells. Nonetheless, the sites of integration and copy number may have some effect on the transcription of the *neo* RNA, since some transformants had more *neo* RNA than others. Therefore, the sequence elements present on a 1.1-kb Pvu II fragment of the hamster H3 gene may be important for its cell cycle regulation.

To further investigate whether the *neo* mRNA expression was regulated during the cell cycle as it is in the endogenous histone H3 genes, we studied three stable transformants that produced different amounts of *neo* mRNA for further analysis. The transformants were synchronized by serum deprivation, and upon addition of fresh medium, were stimulated to progress through the cell cycle (*3, 10*). The rate of DNA synthesis in these synchronized cells was monitored by [^{3}H]thymidine incorporation. *Neo* transcripts were detectable at low levels during the G_1 period (Fig. 3). As cells entered the S phase, the *neo* RNA began to increase in parallel to the increase in histone H3 mRNA accumulation. In all three cases, at the peak, *neo* mRNA increased 6- to 17-fold. In contrast, actin mRNA remained relatively constant throughout the cell cycle. We conclude that the 1.1-kb Pvu II fragment derived from this particular hamster H3 gene contains some elements necessary for regulated temporal expression of the gene during the cell cycle. It is not yet known whether the sequences encoding for the first 20 amino acid residues of the H3 gene play a role in this regulation.

In studying regulated gene expression during the cell cycle, it is important to show that the gene is regulated temporally. (In contrast, growth might be induced by the addition of fresh medium to starving synchronized cells.) We used two independent methods to address this issue. (i) When DNA synthesis was inhibited by the K12 *ts* mutation, the *neo* mRNA levels decreased substantially (Fig. 2). Since the cells were not starved of serum, the result argued against the effect of nutrient on *neo* mRNA. In addition, when DNA synthesis was inhibited by hydroxyurea, similar results were observed. Together, these results reaffirm the coupling between DNA synthesis and the amount of *neo* mRNA. (ii) Where serum-synchronized cells were used, *neo* mRNA increased concurrently with the increase in host cell DNA synthesis and, as with histone mRNA, decreased as host cell DNA synthesis declined (Fig. 3). This result shows that the hybrid gene is regulated in a cell cycle–dependent manner, and that changes were not a result of overall growth stimulation. However, it is also clear that the regulation of the *neo* RNA is not as stringent as that of histone mRNA (Fig. 3), probably because of both transcriptional and post-transcriptional control in histone mRNA regulation; perhaps only transcriptional control is conferred by the sequences used here.

Our results show in vivo that the 5′ sequence of a mammalian histone gene is able to confer cell cycle regulation on expression of a heterologous gene to which it is fused in the same transcriptional orientation. This observation is in agreement with a recent report that in in vitro systems, promoter elements in the 5′-flanking sequence of a human histone H4 gene are specifically recognized by cellular factors produced in S-phase nuclear extracts (*13*). A combination of both approaches should yield information on the *trans*-acting regulatory factors and the *cis*-acting elements involved in the regulation. A further investigation into the cell cycle regulation of the histone genes can be achieved by more detailed analysis of the sequence and function of the 1.1-kb fragment. Furthermore, with the isolation of this regulatory-promoter sequence, it may now be possible to direct the expression of any compatible heterologous gene in a cell cycle–regulated manner in mammalian cells.

References and Notes

1. C. C. Hentschel and M. L. Birnstiel, *Cell* **25**, 301 (1981); R. Maxson, R. Cohn, L. Kedes, *Annu. Rev. Genet.* **17**, 239 (1983).
2. R. S. Wu and W. M. Bonner, *Cell* **27**, 321 (1981); D. B. Sittman, R. A. Graves, W. F. Marzluff, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1849 (1983); M. Plumb, J. Stein, G. Stein, *Nucleic Acids Res.* **11**, 2391 (1983); N. Heintz, H. L. Sive, R. G. Roeder, *Mol. Cell. Biol.* **3**, 539 (1983).
3. A. Artishevsky, A. M. Delegeane, A. S. Lee, *Mol. Cell. Biol.* **4**, 2364 (1984).
4. A. J. DeLisle, R. A. Graves, W. F. Marzluff, L. F. Johnson, *ibid.* **3**, 1920 (1983).
5. D. B. Sittman, R. A. Graves, W. F. Marzluff, *Nucleic Acids Res.* **11**, 6679 (1983).
6. J. Davies and D. I. Smith, *Annu. Rev. Microbiol.* **32**, 469 (1978).
7. J. W. Attenello and A. S. Lee, *Science* **226**, 187 (1984).
8. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).
9. T. Ashihara, S. D. Chang, R. Baserga, *J. Cell Physiol.* **96**, 15 (1978); J. A. Melero, *ibid.* **98**, 17 (1979).
10. A. M. Delegeane and A. S. Lee, *Science* **215**, 79 (1982).
11. M.-L. J. Wang and A. S. Lee, *Biochem. Biophys. Res. Commun.* **110**, 593 (1983).
12. A. S. Lee, A. M. Delegeane, V. Baker, P. C. Chow, *J. Biol. Chem.* **258**, 597 (1983).
13. S. M. Hanly, G. C. Bleecker, N. Heintz, *Mol. Cell. Biol.* **5**, 380 (1985).

14. We thank A. M. Delegeane and L. Kung for initial characterizations of the hamster histone H3 subclone, pAAD3.7 and R. E. K. Fournier for critical review of the manuscript. Supported by PHS grant GM 31138. A.S.L. is a recipient of an American Cancer Society Faculty Research Award.

21 March 1985; accepted 12 September 1985

Antiprotozoal Activity of Tricyclic Compounds

The observations by Zilberstein and Dwyer (*1*) on the antileishmanial effects of tricyclic antidepressants are intriguing in the context of earlier studies of the antiprotozoal activities of phenothiazines and other closely related compounds. In 1891, Guttman and Ehrlich (*2*) first noted that methylene-blue dye (Fig. 1a) was effective in the treatment of *vivax* malaria and presumably, therefore, lethal to *Plasmodium vivax*. In several studies, phenothiazines have been found to have lethal effects on protozoa. We demonstrated that chlorpromazine (figure 1b) and several of its derivatives killed *Leishmania donovani* promastigotes, extracellular amastigotes, and amastigotes within human macrophages in vitro (*3*). Furthermore, chlorpromazine reduced the parasite burden of hamsters infected with *L. donovani* (*4*). Similar in vitro effects of these compounds on *Trypanosoma brucei brucei* have been described by Seebeck and Gehr (*5*).

The data presented by Zilberstein and Dwyer are fascinating in relation to the prior observations (*3, 4*). Clomipramine (3-chloroimipramine, figure 1c), the prototype compound demonstrated in their study to have antileishmanial activity, is identical to chlorpromazine (figure 1b) with the exception of two carbons substituted for the sulfur at position 5. The concentration of clomipramine required to kill 50 percent (LD_{50}) of promastigotes of *L. donovani* was 24 μ*M* (1), while the LD_{50} for chlorpromazine was 30 μ*M* (*4*). Similarly, imipramine (clomipramine