Coupling of Histone and DNA Synthesis in the Somatic Cell Cycle

Abstract. *The coupling of histone and DNA synthesis was examined in the temperature-sensitive hamster fibroblast cell line K12. By monitoring total cellular histone synthesis at various times after quiescent cells were stimulated to proliferate at permissive and nonpermissive temperatures, a direct correlation was found between the rates of DNA and histone synthesis. Furthermore, when DNA synthesis was blocked by the K12 mutation, histone synthesis was reduced to the basal rate.*

The idea that histone and DNA synthesis are tightly coupled during the cell cycle (*1–3*) was challenged recently by Groppi and Coffino (*4*). They found that histones synthesized during the G_1 period of interphase remain in the cytoplasm and do not become associated with chromatin until the onset of DNA synthesis in the S period; hence, previous studies failed to detect histone synthesis during G_1 because the histones were extracted from isolated nuclei or chromatin before analysis. Furthermore, they contended that mouse lymphoma (S49) and Chinese hamster ovary cells synthesize histones at equivalent rates during the G_1 and S periods.

We investigated the coupling of histone and DNA synthesis in the hamster fibroblast cell line K12, a mutant that is unable to progress through G_1 at a nonpermissive temperature. [The mutation is expressed 4 hours before initiation of DNA synthesis (*5, 6*).] We measured the rate of total cellular histone synthesis at various times after quiescent cells were stimulated to proliferate at both permissive (35°C) and nonpermissive (40.5°C) temperatures. By analyzing histones extracted from total cell lysate instead of isolated nuclei or chromatin, we included in our measurement histones that may remain in the cytoplasm after their synthesis. Contrary to the findings of Groppi and Coffino, our results indicate a direct correlation between the rates of DNA and histone synthesis in these cells.

We first determined whether the rate of histone synthesis is related to the onset of DNA synthesis during a normal cell cycle. K12 cells were isolated from Chinese hamster Wg1A fibroblasts (*7*). Cells that were able to grow at 35°C but not at 40.5°C were selected for study and synchronized by serum deprivation. Upon addition of fresh medium to stimulate proliferation, the cells were incubated at 35°C and briefly exposed at different times to [*methyl*-^{3}H]thymidine in order to measure the rate of DNA synthesis. As shown in Fig. 1a, when the cells were incubated at 35°C they progressed through a G_1 phase. There followed a wave of DNA synthesis, which ended about 24 hours after growth stimulation was initiated. To simultaneously monitor the rate of histone synthesis during the cell cycle, we labeled an identical set of cells with [^{3}H]lysine at the same time points. At the end of the labeling period, total cell lysates of these cells were prepared by the histone extraction procedure, which enables histones to dissociate from the DNA and become soluble (*4*). These lysates were applied to polyacrylamide gels (*8*). There was a marked increase in the labeling of the histone

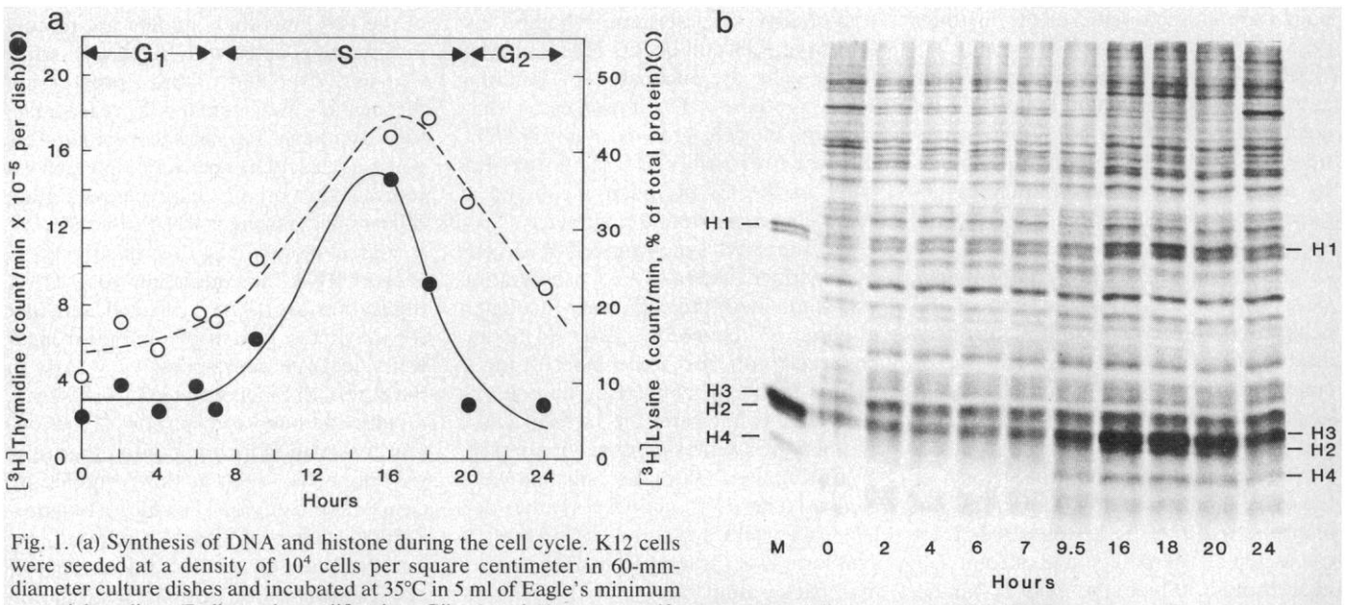

Fig. 1. (a) Synthesis of DNA and histone during the cell cycle. K12 cells were seeded at a density of 10^4 cells per square centimeter in 60-mm-diameter culture dishes and incubated at 35°C in 5 ml of Eagle's minimum essential medium (Dulbecco's modification; Gibco) and 10 percent calf serum. Most cells were synchronized after 4 days. All cultures but the controls then received fresh medium and were incubated at 35°C for various lengths of time. DNA and histone synthesis were assayed at each time point. The rate of DNA synthesis was monitored by labeling cultures with [*methyl*-^{3}H]thymidine (0.25 μCi/ml) for 30 minutes, precipitating the material with trichloro-acetic acid, and measuring its activity in counts per minute (*6*). The rate of histone synthesis was monitored by labeling cultures for 30 minutes with lysine-deficient Eagle's minimum essential medium (Dulbecco's modification) containing [^{3}H]lysine (40 uCi/ml) and 10 percent dialyzed calf serum. The cells were washed twice with cold phosphate-buffered saline and resuspended in 0.5 ml of histone lysis buffer (*4*) containing 8*M* urea, 2 percent NP40, 1.6 mg protamine sulfate per milliliter, 0.3*M* NaCl, and 0.5 percent 2-mercaptoethanol. The amount of [^{3}H]lysine incorporated in each sample was then determined. (b) Autoradiograms of gels to which the lysates were applied. The samples were adjusted to 10 percent glycerol, 5 percent 2-mercaptoethanol, 2 percent sodium dodecyl sulfate (SDS), and 0.0625*M* tris (*p*H 6.8); boiled in a water bath for 1 minute; and a given amount (about 1×10^5 count/min) was loaded onto each lane of 12.5 percent polyacrylamide SDS slab gel. Bromophenol blue was used as a marker. To identify the positions of the histones, we prepared purified histones from cell nuclei (*4*). An amount of this preparation equivalent to about 4×10^4 count/min was applied to the marker (*M*) lane. Conditions for gel electrophoresis and fluorography were as described by Lee (*11*). The gels were dried and exposed to Kodak XAR-5 film at −70°C for 2 days. To measure the amount of [^{3}H]lysine incorporated into histones (a), the autoradiogram was scanned and the area under the peaks was measured by an electronic digitizer (Numonics Corp).

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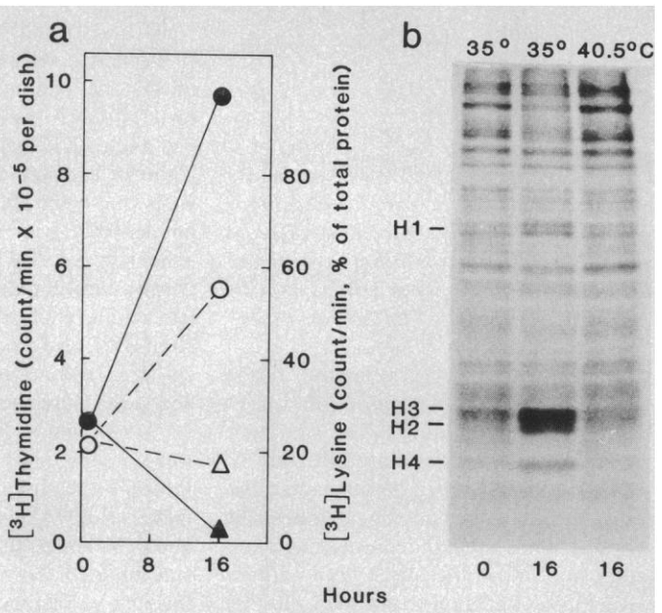

Fig. 2. Effect of the K12 mutation on DNA and histone synthesis. Synchronized K12 cells were stimulated to proliferate at 35° or 40.5°C. After 14 hours the cells were labeled with [*methyl*-^{3}H]thymidine or [^{3}H]lysine for 2 hours and then processed as described in the legend to Fig. 1. (a) The rate of DNA and histone synthesis: [*methyl*-^{3}H]thymidine incorporation at 35°C (●) and 40.5°C (▲); percentage of [^{3}H]lysine incorporated into histones at 35°C (○) and 40.5°C (△). (b) Autoradiogram of labeled proteins.

proteins at the peak of DNA synthesis (Fig. 1b). With the exception of one or two protein bands at the high molecular weight range, most other cellular proteins were synthesized in relatively constant amounts during the different labeling periods. Further analysis of the lysates by two-dimensional gel electrophoresis revealed that the *p*H values of the labeled proteins at their isoelectric points are characteristic of the histones (*9*).

Since histone proteins are resolvable as major bands by one-dimensional gel electrophoresis, it was possible to quantitate the labeling in the different samples by analyzing densitometer tracings obtained after scanning the autoradiograms of the gels presented in Fig. 1b. The autoradiograms used for the tracings were calibrated so that the area of the peaks obtained from the tracings was directly proportional to the amount of label incorporated into each protein band. From the densitometer tracings, we measured the areas of the peaks corresponding to histone proteins and to the total cellular proteins. The [^{3}H]lysine incorporated into the histones was then calculated as the percentage of total cellular protein. Since the rate of amino acid uptake and incorporation into cellular protein is relatively constant during the cell cycle (*4*, *10*), the rates of histone synthesis in cells at different stages of the cell cycle can be compared. When K12 cells were incubated at 35°C there was no detectable DNA synthesis above background levels up to 7 hours after stimulation of proliferation (Fig. 1, a and b). At approximately 9 hours, DNA synthesis had started. A fivefold increase in [*methyl*-^{3}H]thymidine incorporation was observed at 16 hours. Concomitantly, there was a threefold to fourfold increase in the rate of histone synthesis. Thus the rate of histone synthesis is proportional to the rate of DNA synthesis when the cells are progressing through the G_1 and S periods. Similarly, at G_2, when the rate of DNA synthesis declines, the rate of histone synthesis also declines.

The K12 cell line provides a unique opportunity to determine whether histone synthesis still occurs independently if the cells are incapable of initiating DNA synthesis. The rationale is that, although the cells grow normally at 35°C, they are irreversibly blocked at the midpoint of the G_1 period if incubated at 40.5°C for more than 6 hours (*7*).

We prepared synchronized K12 cells as described above. Once proliferation was stimulated, the cells were incubated at either 35° or 40.5°C. After 14 hours, both cell cultures were labeled for 2 hours with [*methyl*-^{3}H]thymidine or [^{3}H]lysine to measure the rates of DNA and histone synthesis, respectively (Fig. 2). During the 2 hours the cells that were incubated at 35°C actively synthesized DNA as well as histones. The rate of synthesis was three to four times higher than background rates. For the cells incubated at 40.5°C, DNA synthesis was not initiated because of the mutation. At the same time, histone synthesis remained at about the background level. The increased synthesis of three high molecular weight proteins at 40.5°C is a unique characteristic of this mutant cell line (*11*).

We conclude that in these hamster fibroblast cells histone synthesis is coordinately expressed with DNA synthesis; if DNA synthesis is blocked by a mutation, histone synthesis is reduced to the basal level. Since the cells maintain normal levels of RNA metabolism (*12*) and continue to synthesize normal amounts of most cellular proteins for at least 24 hours at 40.5°C (*11*), it is unlikely that the mutation affects histone synthesis in any specific way.

In earlier studies of the relation between DNA and histone synthesis in mammalian cells (*13–17*), the methods for synchronization were either selective detachment of mitotic cells or the use of excess thymidine or hydroxyurea, which artificially uncoupled DNA and histone synthesis. The cells synchronized by the latter method were arrested at the borderline between the G_1 and S periods. The cells we used were blocked at the midpoint of G_1. Furthermore, previous conclusions were deduced from data obtained with histones extracted from nuclei or chromatin, with the assumption that histones become associated with the chromatin immediately after their synthesis.

Our conclusion may be different from that of Groppi and Coffino (*4*) because the phenomenon is dependent on the physiological states of the cells being studied (*15*). Another possible explanation lies in their method for selecting synchronized populations of cells. Both of the cell lines they studied are rapidly proliferating cells with relatively short G_1 periods, and rapid progression through G_1 may demand increased histone synthesis late in the period. This point is particularly pertinent in view of a recent report on the cell-cycle regulation of histone messenger RNA in yeast (*18*). It was demonstrated that histone messenger RNA accumulation and DNA replication are tightly coupled and that the maximum rate of histone messenger RNA accumulation occurs very early in the S period. Since yeast cells proliferate rapidly and have a very short G_1 period, this observation may apply to other fast-growing cells—such as those studied by Groppi and Coffino. The cell sorting procedures they used were not able to separate the late G_1 cells from earlier G_1 cells. Furthermore, without quantitation of the data, labeled histones may appear with similar intensities in gel autoradiograms in such a mixture of cells.

In conclusion, histone synthesis is coordinately expressed with DNA synthesis in the hamster fibroblast cells we studied. Our findings are consistent with published reports that in HeLa cells histone messenger RNA's are found on polyribosomes only during the S period, but are undetectable on polyribosomes of G_1- or S-period cells treated with

inhibitors of DNA synthesis (*19–23*). It is important to determine whether this conclusion has general validity and whether the regulation of histone gene expression resides at the transcriptional or posttranscriptional level (*24*).

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References and Notes

1. T. W. Borun, *Results Probl. Cell Differ.* **7**, 249 (1975).
2. S. C. R. Elgin and H. Weintraub, *Annu. Rev. Biochem.* **44**, 725 (1975).
3. T. Pederson, *Protein Synth.* **2**, 69 (1976).
4. V. E. Groppi and P. Coffino, *Cell* **21**, 195 (1980).
5. B. J. Smith and N. W. Wigglesworth, *J. Cell. Physiol.* **82**, 339 (1973).
6. J. A. Melero and V. Fincham, *ibid.* **95**, 295 (1978).
7. D. H. Roscoe, M. Read, H. Robinson, *ibid.* **82**, 325 (1973).
8. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
9. A. S. Lee, unpublished data.
10. D. M. Prescott, *J. Cell Biol.* **31**, 1 (1966).
11. A. S. Lee, *J. Cell. Physiol.* **106**, 119 (1981).
12. S. Kit and G. N. Jorgensen, *ibid.* **88**, 57 (1976).
13. J. Spalding, K. Kajiwara, G. C. Mueller, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1535 (1966).
14. E. Robbins and T. W. Borun, *ibid.* **57**, 409 (1967).
15. A. Sadgopal and J. Bonner, *Biochim. Biophys. Acta* **186**, 349 (1969).
16. L. R. Gurley and J. M. Hardin, *Arch. Biochem. Biophys.* **130**, 1 (1969).
17. G. Russev, L. Vassilev, R. Tsanev, *Nature (London)* **285**, 584 (1980).
18. L. M. Hereford, M. A. Osley, J. R. Ludwig II, C. S. McLaughin, *Cell* **24**, 367 (1981).
19. T. W. Borun, M. D. Scharff, E. Robbins, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1977 (1967).
20. D. Gallwitz and G. C. Mueller, *Science* **163**, 1351 (1969).
21. W. B. Butler and G. C. Mueller, *Biochim. Biophys. Acta* **294**, 481 (1973).
22. M. Melli, G. Spinelli, E. Arnold, *Cell* **12**, 167 (1977).
23. G. S. Stein, J. L. Stein, W. D. Park, S. Detke, A. C. Lichtler, E. A. Shepard, R. L. Jansing, I. R. Phillips, *Cold Spring Harbor Symp. Quant. Biol.* **42**, 1107 (1978).
24. L. H. Kedes, *Annu. Rev. Biochem.* **48**, 837 (1979).
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β,γ-Peroxy Analogs of Adenosine and Guanosine Triphosphate: Synthesis and Biological Activity

Abstract. *Extended analogs of adenosine triphosphate (ATP) and guanosine triphosphate (GTP), in which a peroxide bridge replaces the terminal bridge-oxygen of the triphosphate chain, have been synthesized. The ability of* β,γ*-peroxy-ATP to inhibit or substitute for ATP in representative enzyme systems and that of* β,γ*-peroxy-GTP, for GTP in protein synthesis was tested.*

We have introduced the concept of testing the geometrical restrictions of nucleotide binding sites by examining the biological properties of synthetic purine analogs with defined dimensional alterations within their heterocyclic nuclei (*1–3*). Compounds in the first series of these dimensional probes have a purine-like ring system which is laterally extended by 2.4 Å as a result of the introduction of a benzene ring between the terminal imidazole and pyrimidine rings.

Another method of stretching out a nucleotide molecule is by inserting an additional oxygen atom between the central (β) and terminal (γ) phosphorus atoms in the triphosphate chain to form a peroxide linkage (Fig. 1). In such a structure, the peroxydiphosphate moiety, comprising the β and γ atoms, has known geometry as determined by x-ray crystallography of lithium peroxydiphosphate hydrate (*4*). The presence of the peroxide bridge results in the lengthening of the triphosphate chain (*5, 6*) by slightly more than one angstrom unit when maximally extended. The actual distance may vary because of internal rotation in the nonlinear chain (*7*).

Both 5′-adenylyl peroxydiphosphate (AMP-PO_2P) and 5′-guanylyl peroxydiphosphate (GMP-PO_2P) were prepared by a general phosphate coupling procedure (*8*). Reaction of peroxydiphosphate with the appropriate nucleoside 5′-phosphorimidazolidate and subsequent purification by ion-exchange chromatography (*9*) provided AMP-PO_2P and GMP-PO_2P in yields of 60 to 70 percent. The identity and purity of the peroxy nucleotide analogs were verified by chromatographic analysis and standard spectroscopic methods. Their ^{31}P nuclear magnetic resonance spectra differed from those of the normal nucleoside triphosphates ATP and GTP both in chemical shifts (from 85 percent phosphoric acid) and in coupling constants (*J*), as expected. For example, the resonances for the phosphorus atoms adjacent to the peroxide linkage in AMP-PO_2P are shifted about 11 ppm downfield from their positions in the ATP spectrum. A smaller *J* (spin-spin coupling constant) value of 13 Hz was measured between the central and terminal phosphorus atoms of the peroxy analog compared with a value of 20 Hz for ATP. This difference reflects three-bond versus two-bond coupling.

The peroxy analogs have chemical properties that make them suitable for enzyme studies. They are stable in neutral or basic solution, as is peroxydiphosphate (*5, 10*), within the *p*H range used in most enzyme assays. The AMP-PO_2P analog is capable of binding divalent metal cations, a strict requirement for all known nucleotide-dependent phosphate displacement reactions, although the AMP-PO_2P-Mg^{2+} association is about 3 percent of that of the ATP-Mg^{2+} association (*11*). The difference is parallel to the relative association of peroxydiphosphate versus pyrophosphate with Mg^{2+} (*5, 12*). It is also consistent with the finding that stabilization due to chelation is less for a seven-membered ring than for a five- or six-membered ring (*13*). At this time, however, we cannot say whether the cause of the lower association of AMP-PO_2P-Mg^{2+} lies (i) in less pyrophosphate ligation with Mg^{2+} than in ATP-Mg^{2+}, (ii) in lower affinity of the seven-membered ring β,γ-peroxydiphosphate-Mg^{2+} pair, or (iii) in altered affinity of Mg^{2+} for the terminal dianion in the β,γ-peroxy analog (*14, 15*).

The potential usefulness of the peroxy nucleotide analogs as probes of the limitations of enzyme binding and mechanism depends upon their ability to inhibit or substitute for the natural cofactors in enzyme systems. To initiate our studies on the biological reactivities of the peroxy derivatives, we selected enzymes that require ATP as donor of adenylyl, pyrophosphoryl, and phosphoryl groups. The conditions used gave consistent results for both ATP and AMP-PO_2P but not necessarily optimal activity (Table 1). Dithiothreitol (*16*) was added to each assay to prevent oxidation of reactive sulfhydryl groups caused by peroxide release.

Adenylyl transfer reactions, represented here by firefly luciferase and NAD^+ pyrophosphorylase, proceed by nucleophilic attack at the α-phosphorus of ATP with cleavage of the α-P-O linkage and displacement of pyrophosphate (*17*). The peroxy analog of ATP is an acceptable substrate for representatives of this class of enzymes, displaying similar enzyme binding and a reduced rate of catalysis compared with ATP. With luciferase in the presence of luciferin, AMP-PO_2P brought about light emission at the same wavelength, 562 nm, as did ATP. In a test of biological activity with tyrosyl-tRNA synthetase, one of the adenylyl transferases responsible for the activation of transfer RNA's (tRNA) for protein synthesis, AMP-PO_2P was found to be about 50 percent as effective as ATP in the formation of the aminoacyl-

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