nal cortex in the regio superior of the hippocampus proper. For this reason, cells were also found in layer III (the site of the cells of origin of the normal entorhinal afferents to the regio superior). Therefore, the possibility that some of the reinnervating fibers might also originate from this group of cells in layer III (which give rise to the normal crossed projections to the regio superior) cannot be excluded.

If the reinnervating contralateral entorhinal afferents did originate exclusively from cells in layer II, it would still be clear that some fibers which reinnervate the granule cells also originate from other sources. For example, acetylcholinesterase-containing fibers from the septal nucleus (*3*) and dentate commissural fibers from the contralateral hippocampus (*3*) also appear to proliferate in response to the lesion, reoccupying synaptic space which is normally the territory of ipsilateral entorhinal afferents. Thus, input is restored to denervated granule cells by one system which appears to be homologous with the normal circuitry (except with regard to laterality), and by other fiber systems which bear no obvious relation to the circuitry that was destroyed by the lesion. The functional consequences of this preservation of some aspects of normal specificity of neuronal interconnection, with concomitant loss of other specific features, must still be ascertained.

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Activation of Histone Gene Transcription by Nonhistone Chromosomal Phosphoproteins

Abstract. *Hybridization analysis of RNA transcripts from HeLa S3 cell chromatin to histone complementary DNA indicates that a chromosomal phosphoprotein fraction activates transcription of histone messenger RNA sequences in vitro with chromatin from a phase in the cell cycle when histone genes are normally silent.*

Although the factors involved in genetic regulation are beginning to be understood in prokaryotes, the mechanisms and specific macromolecules involved in the control of gene readout in eukaryotes remain elusive. However, the discovery of the enzyme reverse transcriptase (*1*) has enabled researchers to synthesize sensitive DNA probes for specific messenger RNA's (*2, 3*), and has led to the development of experimental systems that offer promise for elucidating the kinds of macromolecules involved in eukaryotic gene regulation. One system under study is that of histone gene transcription during the growth cycle of HeLa cells, where, by the technique of hybridization with a complementary DNA (cDNA) probe in which reverse transcriptase and polyadenylated histone messenger RNA (mRNA) are used (*4*), it has been shown that histone gene transcription from chromatin takes place only during the S phase of the cell cycle (*5*). Such phase-specific transcription of histone genes is consistent (i) with the restriction of histone synthesis to the S phase of the cell cycle (*6, 7*) and (ii) with in vitro translation (*7, 8*) and hybridization studies (*9*) which indicate that histone mRNA's are associated with polyribosomes only during S phase. Thus, synchronized populations of HeLa cells offer a viable model system for the study of factors affecting regulation of the transcription of a specific set of genes by allowing the fairly easy attainment of two populations in which it is known that histone genes are transcribed in one (S phase cells) and not in the other population (G1 cells) (*5*). By transcribing isolated chromatin from S and G1 cells in vitro and analyzing the RNA products for their abilities to form hybrids with histone cDNA, it is apparent that the factors responsible for the differences in transcriptional capabilities of S phase and G1 cells are endogenous to the isolated chromatin material (*5*). By dissociating chromatin from G1 and S phase HeLa cells in a mixture of NaCl (3*M*) and urea (5*M*) and reconstituting various components of the two chromatins, it has also been possible to determine that a component of the S phase nonhistone chromosomal proteins is responsible for the transient activation of histone gene transcription during the period of the cell cycle when DNA replication occurs (*5*).

Nuclear phosphoproteins have been implicated in the control of gene expression (*10–12*), and recent studies have shown that HeLa cell chromatin reconstituted with nonhistone chromosomal proteins which were partially dephosphorylated enzymatically has a decreased ability to serve as a template for histone gene transcription (*11*). We report experiments directed toward determining whether or not the component of S phase chromatin responsible for activation of histone gene transcription can be found in the phosphoprotein fraction of the nonhistone chromosomal proteins.

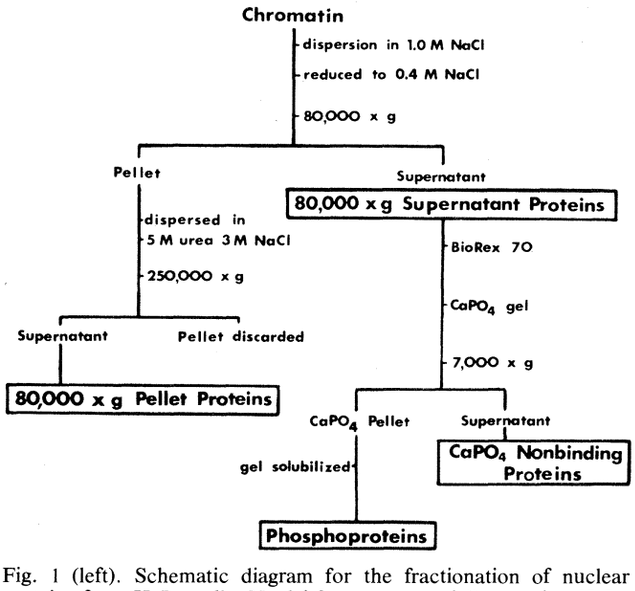
Phosphoproteins were isolated from chromatin obtained from HeLa cells (*5*)

Table 1. Characteristics of chromosomal protein fractions. The percentage of nuclear proteins and specific activities of the various protein fractions isolated from HeLa cell chromatin. Exponentially growing HeLa cells (1500 ml) were centrifuged at 1500 rev/min for 5 minutes and resuspended in 50 ml of phosphate-free Eagle's minimum essential medium containing 2 percent fetal calf serum and 10 mc of ^{32}P (obtained from ICN in water) for 1 hour. The protein fractions were isolated as described in the legend to Fig. 1. Protein concentrations were determined by the spectrophotometric method (*17*), and radioactivity was measured by counting 10-μl portions of the protein fractions in 10 ml of Triton-toluene scintillation fluid (*18*).

	Percent of total chromosomal protein	Recovery (mg)	Specific activity (count/min per mg)
Total nuclear protein	100.0	25.5	8.25×10^5
80,000*g* supernatant	33.0	8.4	7.36×10^5
80,000*g* pellet proteins	66.7	17.0	9.80×10^5
$CaPO_4$ nonbinding proteins	16.2	4.13	2.30×10^5
Phosphoproteins	16.8	4.28	3.20×10^6

during the log phase of growth (*12*). This method (Fig. 1) involves dispersing chromatin in 1*M* NaCl, decreasing the NaCl concentration to 0.4*M*, and precipitating the nucleohistone by centrifugation at 80,000*g*. The pellet was solubilized in a mixture of 5*M* urea and 3*M* NaCl, and the supernatant obtained after sedimenting the DNA by centrifugation is referred to as the "80,000*g* pellet protein" (Fig. 1). This protein fraction contained more than 90 percent of the histones and approximately 40 percent of the nonhistone chromosomal proteins. The 80,000*g* supernatant, containing approximately 60 percent of the nonhistone chromosomal proteins, was treated briefly with BioRex 70 to remove small traces of histones and was then exposed to calcium phosphate gel in a ratio of 0.46 mg of gel per milligram of protein. This gel was removed by low-speed centrifugation; we call the proteins that bind to $CaPO_4$ gel under these conditions phosphoproteins. It has been known for some time that proteins rich in phosphorus bind to $CaPO_4$ gel, although the mechanism is not well understood. The phosphoproteins are subsequently recovered by solubilizing the gel in 0.3*M* EDTA, *p*H 7.5, 0.33*M* $(NH_4)_2SO_4$, and then dialyzed against 5*M* urea and 3*M* NaCl. The proteins remaining in the supernatant are referred to as the "$CaPO_4$ nonbinding proteins," although these proteins will bind to $CaPO_4$ gel when exposed to the gel in the absence of the phosphoproteins. Simultaneously, protein fractions were isolated in an identical manner from cells labeled with ^{32}P for 1 hour. The ability of each fraction to activate the histone gene was correlated with the degree of phosphorylation, and the fractions were also examined by polyacrylamide gel electrophoresis. The phosphoprotein fraction scheme subdivides the nuclear proteins into three electrophoretically distinguishable fractions (Fig. 2). These fractions also differ as to their specific activity with respect to ^{32}P, with the proteins bound to the $CaPO_4$ gel exhibiting a tenfold greater phosphorylation (3.2×10^6 count/min per milligram) than the $CaPO_4$ nonbinding proteins (2.3×10^5 count/min per milligram) (Table 1).

Each of the four protein fractions was analyzed in the following manner for its ability to activate the transcription, in vitro, of histone mRNA sequences in G1



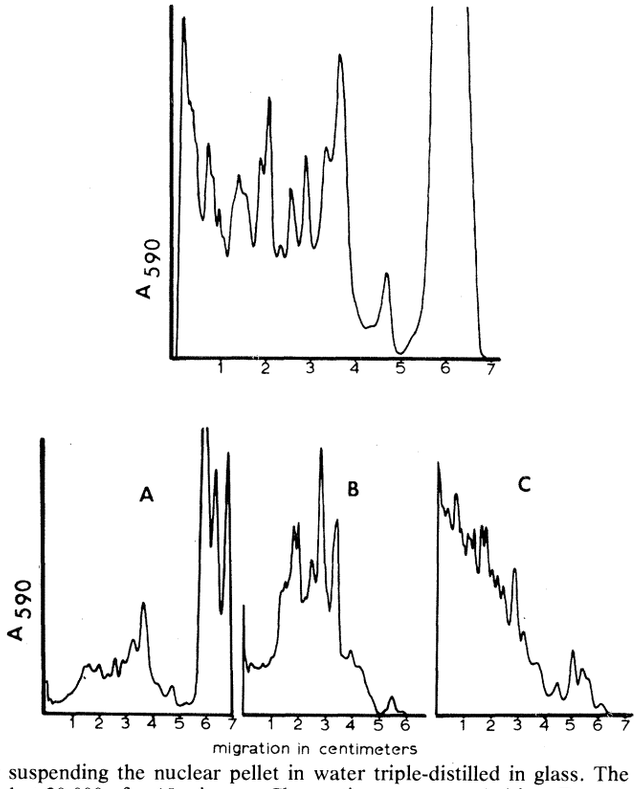


Fig. 1 (left). Schematic diagram for the fractionation of nuclear proteins from HeLa cells. Nuclei from exponentially growing HeLa cells were obtained by washing cells four times in 80 volumes of Earle's balanced salt solution and lysing the cells in 80 volumes of a solution of 80 m*M* NaCl, 20 m*M* EDTA, and 1 percent Triton X-100 (*p*H 7.2). The nuclei were washed three times with the lysing medium and then twice with 0.15*M* NaCl, 0.01*M* tris (*p*H 8.0). Nuclei prepared by this method are free of cytoplasm when examined by phase contrast and electron microscopy (*15*). Lysis of nuclei was achieved by suspending the nuclear pellet in water triple-distilled in glass. The chromatin was allowed to swell at 4°C for 20 minutes and then centrifuged at 20,000*g* for 15 minutes. Chromatin was suspended in a Dounce homogenizer in 1.0*M* NaCl and 0.05*M* tris, *p*H 7.5, at a concentration of 2 mg/ml. Then 1.5 volumes of 0.02*M* tris, *p*H 7.5, was added dropwise, and the mixture was briefly homogenized and centrifuged at 80,000*g* for 1 hour. The pellet from this centrifugation was dispersed in a solution of 5*M* urea, 3*M* NaCl, and 0.01*M* tris, *p*H 8.3; the mixture was then centrifuged at 250,000*g* for 24 hours; the proteins in this supernatant are those that sedimented at the 80,000*g* (here termed 80,000*g* pellet proteins). The proteins in the supernatant after centrifugation at 80,000*g* (here termed the 80,000*g* supernatant proteins) were treated with BioRex 70 [previously equilibrated with 0.4*M* NaCl and 0.02*M* tris-HCl (*p*H 7.5)] at a ratio of 20 mg of BioRex per milligram of protein. The suspension was stirred for 5 to 10 minutes and then centrifuged at 6000*g*. Calcium phosphate gel was added to the resulting supernatant in a ratio of 0.46 mg of gel per milligram of protein; the mixture was stirred for 5 to 10 minutes and then centrifuged at 6000*g*. The proteins remaining in the supernatant are referred to as $CaPO_4$ nonbinding proteins. The pellet of $CaPO_4$ gel was washed in 40 ml of 1.0*M* $(NH_4)_2SO_4$ and 0.05*M* tris (*p*H 7.5) and solubilized in a solution of 0.3*M* EDTA (*p*H 7.5) and 0.33*M* $(NH_4)_2SO_4$ in a ratio of 0.2 ml of solution per milligram of gel. The insoluble residue was removed by centrifugation for 15 minutes at 33,000*g*; the supernatant consisted of the protein fraction referred to as the phosphoproteins. Fig. 2 (right). Electrophoretic profiles of the total nuclear proteins (top), the 80,000*g* pellet proteins (A), the $CaPO_4$ nonbinding proteins (B), and the phosphoproteins (C) isolated as described (Fig. 1) from exponentially growing HeLa cells. Portions of each sample were dialyzed against 0.1 percent sodium dodecyl sulfate (SDS), 0.01*M* sodium phosphate, *p*H 7.0, 0.1 percent β-mercaptoethanol, and subsequently fractionated electrophoretically according to molecular weight on 7.5 percent acrylamide, 0.28 percent bisacrylamide gels according to the procedure reported (*16*). Electrophoresis was for 6½ hours at 8 ma per gel. The gels were fixed in a mixture of 12.5 percent trichloroacetic acid, 7 percent acetic acid, and 40 percent ethanol overnight and stained in 0.25 percent Coomassie brilliant blue in the above solution for 5 hours. After the gels were destained in a solution of 7 percent acetic acid and 10 percent ethanol, they were scanned at 590 nm.

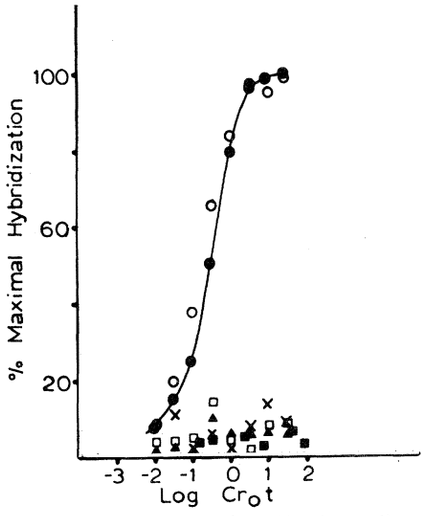


Fig. 3. Kinetics of annealing of histone cDNA to in vitro transcripts of chromatin from G1 HeLa cells dissociated and reconstituted (■), or dissociated and reconstituted in the presence of 80,000*g* supernatant proteins (●), 80,000*g* pellet proteins (▲), $CaPO_4$ nonbinding proteins (□), phosphoproteins (○), or phosphoprotein where no RNA polymerase was added to the transcription assay (x). Expotentially growing HeLa S3 cells were maintained in suspension culture in Joklik-modified Eagle's minimum essential medium supplemented with calf serum (3.5 percent) and fetal calf serum (3.5 percent). Cells were synchronized as described, and G1 cells were obtained by selective detachment of mitotic cells from semiconfluent monolayers. Chromatin was prepared from nuclei treated with detergent (*5*). The G1 chromatin was dissociated by dispersement in a solution of 5*M* urea, 3*M* NaCl, and 0.01*M* tris, *p*H 8.3; and 1-mg portions were reconstituted by the gradient dialysis procedure of Bekhor *et al.* (*19*) in the presence of 1 mg of 80,000*g* supernatant proteins, 80,000*g* pellet proteins, $CaPO_4$ nonbinding proteins, or phosphoproteins. The details of these methods and evidence for fidelity of chromatin reconstitution have been reported (*3, 5, 14, 19, 20*). Transcription of the reconstituted chromatins was allowed to proceed for 70 minutes at 37°C. The incubation mixture in a final volume of 2 ml contained 0.04*M* tris, *p*H 8.0; 4 m*M* $MgCl_2$; 1 m*M* $MnCl_2$; 0.02 m*M* EDTA; 0.008 percent β-mercaptoethanol; 0.4 m*M* each of adenosine triphosphate, cytidine triphosphate, uridine triphosphate, and guanosine triphosphate; 1 mg of DNA as chromatin; and 200 units of RNA polymerase isolated from *E. coli* (*21*). RNA transcripts were isolated and prepared for nucleic acid hybridization analysis (*5*); 1.5 μg of RNA was annealed with ^{3}H-labeled cDNA (500 count/min; 27,000 dpm/ng) at 52°C in sealed glass capillary tubes in 15 μl of solution containing 50 percent formamide, 0.5*M* NaCl, 25 m*M* Hepes buffer, *p*H 7.0, and 1 m*M* EDTA in the presence of 3.75 μg of *E. coli* RNA as carrier. The reaction mixtures were assayed for hybrid formation with the use of single-strand–specific S1 nuclease isolated from *Aspergillus oryzae* (*22*). Each sample was incubated for 20 minutes in 2.0 ml of a solution containing 30 m*M* sodium acetate, 0.3*M* NaCl, 1 m*M* $ZnSO_4$, 5 percent glycerol, *p*H 4.6, and S1 nuclease at a concentration sufficient to degrade at least 95 percent of the single-stranded nucleic acids present. The amount of radioactive DNA resistant to digestion was determined by precipitation with trichloroacetic acid. Cr_0t, moles of ribonucleotides per liter times seconds.

chromatin, which is ineffective as a template for histone gene transcription (*5*). The G1 chromatin was dissociated in the mixture of 5*M* urea and 3*M* NaCl and then reconstituted in the presence of one of the four chromosomal protein fractions. The reconstituted chromatins were transcribed with (fraction 4) *Escherichia coli* RNA polymerase, and the RNA transcripts were assayed for their abilities to form hybrids with a ^{3}H-labeled single-stranded DNA complementary to histone mRNA's that are resistant to S1 nuclease and precipitable by trichloroacetic acid. Conditions used for chromatin reconstitution (*13, 14*) and transcription (*5*), for nucleic acid hybridization (*5*), and for the preparation and characteristics of the histone cDNA probe (*4, 5, 9*) have been described.

Dissociated G1 chromatin reconstituted alone or in the presence of 80,000*g* pellet proteins or $CaPO_4$ nonbinding proteins, does not serve as a template for the in vitro transcription of RNA sequences which hybridize with histone cDNA (Fig. 1)—even at Cr_0t (concentration of ribonucleotides in moles per liter times the time in seconds) values of 30. However, dissociated G1 chromatin reconstituted in the presence of 80,000*g* supernatant proteins or the phosphoproteins is capable of transcribing RNA that hybridizes to histone cDNA. The kinetics of the hybridization reaction between histone cDNA and RNA transcripts from native S phase chromatin ($Cr_0t_{\frac{1}{2}} = 2 \times 10^{-1}$) are similar to those of the hybridization reaction between histone cDNA and RNA transcripts from G1 chromatin reconstituted with 80,000*g* supernatant proteins or the phosphoproteins ($Cr_0t_{\frac{1}{2}} = 2.5 \times 10^{-1}$). These results suggest that the ability to activate histone mRNA sequence transcription resides in a component of the nohistone chromosomal proteins, which is soluble in 0.4*M* NaCl and has a high affinity for $CaPO_4$ gel. When RNA polymerase is omitted from the transcription reaction, and RNA is isolated (with an amount of carrier *E. coli* RNA equivalent to the amount of RNA transcribed in the presence of polymerase) from G1 chromatin reconstituted with the phosphoproteins, the isolated RNA does not show any significant extent of hybridization with histone cDNA up to a Cr_0t value of 30. This experiment indicates that endogenous histone-specific sequences associated with the phosphoprotein fraction do not contribute significantly to the hybridization observed between histone cDNA and transcripts of G1 chromatin reconstituted in the presence of phosphoprotein.

The observation that a protein or group of proteins in the phosphoprotein fraction can activate transcription of histone mRNA sequences coupled with previous results demonstrating the importance of phosphorylation to histone gene transcription (*11*), points to the possibility that activation of histone gene readout in HeLa cells is attributable to a phosphoprotein. The phosphoproteins are a complex and heterogeneous class of chromosomal polypeptides—the specificity and selectivity of the component or components that activate transcription of histone genes remains to be defined. Furthermore, the mechanism by which these proteins interact with other elements of the genome to render histone genes transcribable is yet to be resolved.

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5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole Inhibits Initiation of Nuclear Heterogeneous RNA Chains in HeLa Cells

Abstract. *The nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at 75 to 150 micromolar concentrations inhibits the synthesis of nuclear heterogeneous RNA (hnRNA) in HeLa cells by 60 to 70 percent. The sedimentation profile of hnRNA labeled with [³H]uridine for 45 seconds after brief treatment (45, 90, or 180 seconds) with DRB showed a progressive decrease in the labeling of shorter hnRNA molecules relative to longer molecules. Prior exposure of the cells to actinomycin D, an inhibitor of RNA chain elongation, did not alter the sedimentation profile of hnRNA. These results suggest that DRB preferentially inhibits the initiation of hnRNA chains so that after exposure to DRB for a brief period the longer nascent chains still remain to be finished and thus incorporate a greater share of the pulse label. By progressively increasing the time of exposure to DRB, and measuring the rate of increase in the average size of the labeled, nascent RNA, it was estimated that the chains were growing at rates between 50 and 100 nucleotides per second.*

The halobenzimidazole riboside 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is a selective and reversible inhibitor of nuclear heterogeneous RNA (hnRNA) synthesis in human (HeLa), murine (L), avian (chicken), and insect (chironomid) cells (*1*, *2*). We have attempted to determine the mechanism by which DRB inhibits the synthesis of hnRNA molecules in mammalian cells.

In salivary gland cells of *Chironomus tentans*, DRB appears to inhibit hnRNA synthesis by preventing the initiation of RNA chains without affecting the completion of nascent, or incomplete, chains (*3*). Very brief nucleoside ([³H]uridine) incorporation by HeLa cells (*4*) permits the analysis of nascent RNA molecules. We have used these short-term labeling techniques to determine the effects of DRB on the synthesis of hnRNA in HeLa cells, and have found that DRB selectively inhibits the initiation of at least some of the hnRNA chains.

HeLa S3 cells were grown in suspension culture as described (*2*, *5*). The procedures for labeling hnRNA with [³H]uridine (New England Nuclear, 28.3 c/mmole), and for cell fractionation, RNA extraction, denaturation with 80 percent dimethyl sulfoxide, and sedimentation analysis in sucrose gradients have also been described (*2*, *4*, *5*). Because DRB inhibits the transport of nucleosides (*2*, *6*), it was necessary to make a substantial correction for the effect of DRB on the transport of [³H]uridine (*2*, *6*). In order to evaluate the inhibitory effect of DRB on hnRNA synthesis we determined the effect of DRB on hnRNA synthesis under conditions where the compound would have no effect on the transport of labeled precursor.

Table 1, experiment A, illustrates the inhibition by DRB (150 μ*M*) of the synthesis of hnRNA after the cells were exposed to [³H]uridine for 15 minutes; experiment A also shows the inhibition of [³H]uridine uptake by DRB. Cells treated with a low concentration of actinomycin D (0.05 μg/ml; Merck Sharp & Dohme) under these conditions incorporate more than 90 percent of the ³H into hnRNA (*2*, *7*). Labeling of hnRNA was also assayed (Table 1, experiment B) by [³H]uridine incorporation into DRB-treated and -untreated control cells in the presence of 300 μ*M* uridine. In experiment C, cells were exposed to [³H]-uridine at 4°C for 1 hour and then incubated for 15 minutes at 37°C in the presence or absence of DRB. Both these procedures effectively eliminated the inhibition of nucleoside transport but still demonstrated that 150 μ*M* DRB inhibits close to two-thirds of the labeling of hnRNA. The inhibitory effect of 75 μ*M* DRB on hnRNA synthesis was similar to that of 150 μ*M* DRB (data not shown). A similar inhibition of hnRNA synthesis also occurred when [³²P]phosphate was used as the RNA label (*8*). Thus there is both a DRB-sensitive and a DRB-resistant hnRNA fraction which must be taken

Table 1. Inhibitory effect of DRB (150 μ*M*) on the synthesis of hnRNA in HeLa cells. Triplicate samples of 1 to 2 × 10⁶ cells were used for each determination in spinner medium (*2*, *5*). Treatment with actinomycin D (0.05 μg/ml) was begun 25 minutes before the start of each experiment and continued throughout. In experiments A and B the cells were treated with 150 μ*M* DRB for 15 minutes at 37°C and were then pulsed with [³H]uridine (50 μc/ml) for 15 minutes in the absence (A) or presence (B) of 300 μ*M* uridine (Sigma). In experiment C, [³H]uridine (30 μc/ml) was added to the cells at 4°C for 60 minutes; after washing once with spinner medium the cells were incubated with 150 μ*M* DRB for 5 minutes at room temperature and for 15 minutes at 37°C. At the end of the labeling periods the total cellular and trichloroacetic acid (5 percent TCA)–precipitable radioactivity was determined as described (*2*, *6*).

Condition	[³H]uridine incorporation			
	Total cellular (count/min)	TCA-precipitate		Ratio TCA-prec./total (percent of control)
		Radioactivity (count/min)	Percent of total	
	Experiment A: labeling at 37°C			
Control	5,197,330	190,082	3.7	
DRB	1,010,808	8,803	0.87	24
	Experiment B: labeling at 37°C; 300 μM uridine added			
Control	155,898	6,320	4.1	
DRB	143,411	3,097	2.2	54
	Experiment C: labeling at 4°C			
Control	1,735,525	132,695	7.6	
DRB	1,809,789	48,532	2.7	36