Coupled Transcription and Translation Within Nuclei of Mammalian Cells

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It is widely assumed that the vital processes of transcription and translation are spatially separated in eukaryotes and that no translation occurs in nuclei. We localized translation sites by incubating permeabilized mammalian cells with [^{3}H]lysine or lysyl–transfer RNA tagged with biotin or BODIPY; although most nascent polypeptides were cytoplasmic, some were found in discrete nuclear sites known as transcription "factories." Some of this nuclear translation also depends on concurrent transcription by RNA polymerase II. This coupling is simply explained if nuclear ribosomes translate nascent transcripts as those transcripts emerge from still-engaged RNA polymerases, much as they do in bacteria.

The nuclear membrane is the defining feature of eukaryotes. It divides the cell into two compartments, and it is widely assumed that translation is restricted to only one—the cytoplasm. Indeed, it is often suggested that this membrane evolved to segregate splicing and translation so that one process would not interfere with the other. However, three types of evidence are consistent with some translation occurring within nuclei. First, nuclei contain the necessary components (*1*–*4*), but these might not be active there. Second, isolated nuclei can aminoacylate tRNAs and incorporate radiolabeled amino acids into protein (*2*, *3*, *5*), but contaminating cytoplasmic machinery on the outer nuclear membrane could be responsible for such synthesis. The phenomenon of nonsense-mediated decay (NMD)—the degradation of transcripts bearing termination codons close to their 5′ ends—provides a third type of evidence. NMD is a quality-control mechanism that checks newly made messages; any carrying premature stop codons introduced by faulty splicing encode truncated proteins and are degraded quickly (*6*, *7*). Although most NMD is cytoplasmic, a fraction seems to be nuclear, and this fraction poses a challenge to the current consensus. How might the only machinery able to recognize a termination codon—an active cytoplasmic ribosome—trigger degradation of a transcript while it is still in the nucleus? This phenomenon could be explained if some translation occurred within nuclei, with premature termination by a nuclear ribosome destabilizing the message or altering the splicing.

Translation sites are usually localized by autoradiography after growth in a protein precursor such as [^{3}H]leucine. However, this approach requires long incubation times to allow the tracer to equilibrate with internal pools and be converted to the aminoacyl tRNA, and this gives time for completed proteins to leave synthetic sites. Therefore, we established an in vitro translation system that used a low temperature and suboptimal precursor concentrations so few proteins, which contain an average of ~350 amino acid residues, are completed during the reaction and able to escape from synthetic sites. Cells (*8*) are permeabilized in a "physiologi-

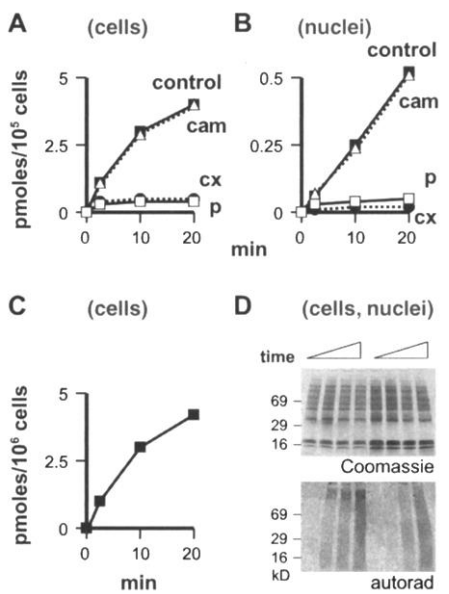

Fig. 1. Protein synthesis in permeabilized HeLa cells and isolated nuclei. (**A** and **B**) The effects of inhibitors on [^{3}H]lysine incorporation. cam, cx, p: + 0.3, 1, or 0.3 mg/ml chloramphenicol, cycloheximide, or puromycin. (**C**) [^{3}H]Leu incorporation in the presence of biotin-lysine-tRNA. (**D**) Sizes of nascent proteins. Proteins made (after 0, 2, 5, and 20 min) by permeabilized cells or nuclei in [^{35}S]Met were run on a gel (four lanes on left or right, respectively), stained with Coomassie blue, and photographed, and an autoradiogram was prepared.

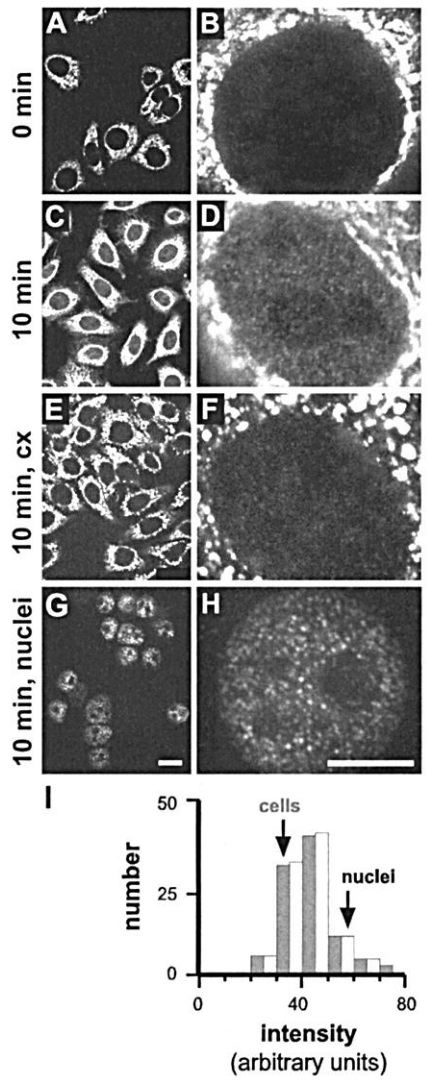

Fig. 2. Biotin peptides: light microscopy. Permeabilized HeLa cells (**A** to **F**) and isolated nuclei (**G** and **H**) were allowed to extend nascent proteins in biotin-lysine-tRNA, sites containing biotin were indirectly immunolabeled, and single equatorial sections through nuclei were collected by "confocal" microscopy. Scale bars, 10 (left) and 5 (right) μm. (A and B) Zero time control; mitochondria contain high levels of endogenous (covalently bound) biotin, whereas nuclei contain background levels. (C and D) Extension for 10 min; nuclei and cytoplasm contain more biotin, and nuclear labeling is concentrated in discrete sites. (E and F) Extension for 10 min in cycloheximide (1 mg/ml); most nuclear labeling is abolished. (G and H) Extension for 10 min, isolated nuclei; most cytoplasm has been lost, and nuclear foci appear sharper because they aggregate during isolation. (**I**) Intensities of nucleoplasmic labeling in >100 cells like those in (C) or (G) were measured, and numbers of cells or nuclei with intensities (arbitrary units) of 0 to 10, 10 to 20, etc. are plotted. Gray boxes, permeabilized cells; open boxes, isolated nuclei.

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cal" buffer (PB) (*9*) and allowed to extend nascent polypeptides by ~15 residues (*10*) in the presence of a tagged precursor, the other 19 amino acids, aminoacyl-tRNA synthetases, guanosine triphosphate (GTP), and an energy-regenerating system (*11*). Before localizing tagged peptides, we established the biochemical characteristics of the system using [^{3}H]lysine; incorporation was sensitive to inhibitors of eukaryotic protein synthesis (cycloheximide and puromycin), but not bacterial synthesis (chloramphenicol; Fig. 1A). Nuclei freed of >95% cytoplasmic ribosomes (*8*) also incorporated [^{3}H]lysine at ~9% the rate of permeabilized cells (Fig. 1B), consistent with previous results (*5*). After extension by ~four residues in [^{35}S]methionine, autoradiography showed that labeled products had the sizes expected of a complex population of nascent polypeptides (Fig. 1D).

Biotin-lysine-tRNA [N^{ε}-(*N*-biotinyl-6-aminohexanoyl)-lysyl-tRNA^{Lys}] and BODIPY-lysine-tRNA^{Lys} [4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propanoyl-lysyl-tRNA^{Lys}] are used to label newly made proteins in cell-free translation reactions; we extended their use to localize translation sites using conditions like those in Fig. 1C where polypeptides are extended by ~15 residues (range 12 to 19; $n = 4$) in 10 min (*10*). We were concerned that the tags might label only some nascent polypeptides, so we compared the incorporation of biotin-lysine-tRNA with that of [^{3}H]leucine and [^{35}S]methionine and found that the biotin was incorporated into protease-sensitive material much like the two radiolabels (*12*).

The immunolocalization of newly made biotin polypeptides is complicated by endogenous biotin; some is easily extracted, and some is covalently attached to the three biotin-dependent carboxylases in the mitochondrial matrix (*13*). After adding the charged tRNA tagged with biotin, the reaction was terminated immediately and soluble biotin extracted; then any covalently bound biotin was immunolabeled (*14*). Biotin in mitochondria was readily detected, but nuclei contained almost none (Fig. 2, A and B). After incubation with biotin-lysine-tRNA, the cytoplasm became progressively more labeled and discrete sites containing biotin appeared in both nucleoli and nucleoplasm (Fig. 2, C and D). Cycloheximide reduced this labeling (Fig. 2, E and F).

It remained possible that some biotin peptides were made in the cytoplasm and imported into nuclei, but similar labeling was obtained with isolated nuclei (*8*) lacking >95% cytoplasmic ribosomes (Fig. 2, G and H). No mitochondrial signal was seen outside nuclei, confirming that most cytoplasm had been removed; cycloheximide also reduced the nuclear signal to 4% (*12*). The nuclear periphery was unlabeled, so protein synthesis at the outer nuclear membrane followed by import was not responsible for internal labeling. Moreover, the same nucleoplasmic intensity was obtained with samples of permeabilized cells and isolated nuclei treated in parallel (Fig. 2I); removing >95% cytoplasmic ribosomes did not affect nuclear intensity. These nuclei also remained intact so cytoplasmic ribosomes could not enter during isolation to initiate on nuclear transcripts; nucleoplasmic incorporation (measured as in Fig. 2I) was unaffected when nuclear import was blocked by growth for 30 min in thapsigargin (0.5 μg/ml) (*15*) followed by lysis in the drug, a treatment that ensured a 40-kD fluorescein-dextran could no longer enter (*16*). Even if cytoplasmic ribosomes were to enter, they could not initiate on nuclear mRNAs; adding 50 μM aurintricarboxylic acid (ATA) to inhibit translational initiation but not elongation (*17*) did not affect nucleoplasmic labeling, although 0.1 and 1 mM ATA, which inhibit elongation (*17*), reduced labeling by 15 and 91%, respectively (*16*). All these controls confirm that cytoplasmic translation cannot account for the nuclear labeling.

Essentially similar results were obtained after localizing translation sites more directly (*11*, *14*) with BODIPY-Lys-tRNA (Fig. 3, A

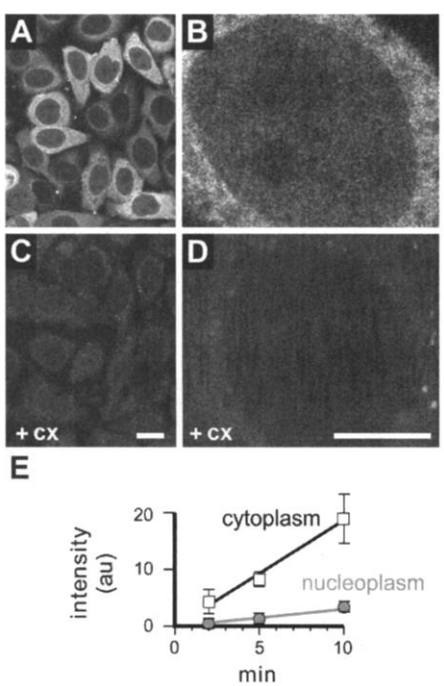


Fig. 3. BODIPY peptides: light microscopy. (**A** to **D**) HeLa cells were permeabilized, incubated (10 min) ± BODIPY-Lys-tRNA ± cycloheximide (1 mg/ml), fixed, and imaged. Scale bars, 10 (left) and 5 (right) μm. (**E**) Intensities (arbitrary units) of nuclear and cytoplasmic regions of >100 cells at different times. Autofluorescent background was ~10% of the nucleoplasmic signal at 2 min, and treatment with RNase A (100 μg/ml; 30 min; 37°C) or proteinase K (500 μg/ml; 30 min; 30°C) reduced intensities by 5 or 98%, respectively.

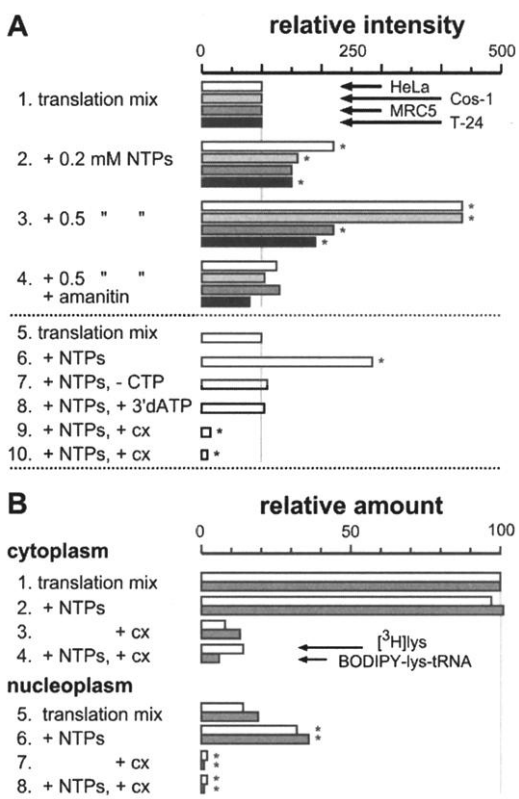
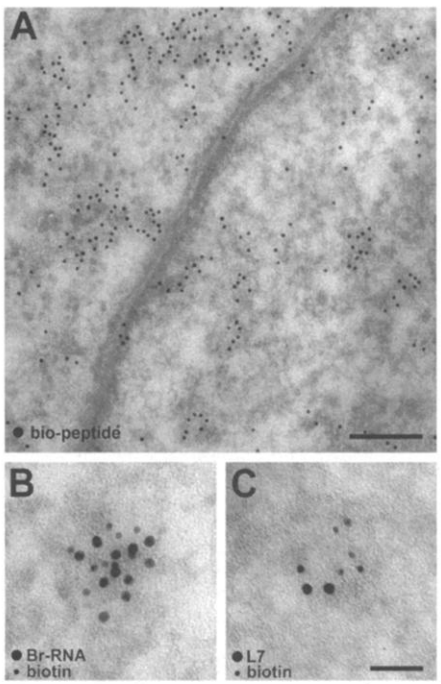


Fig. 4. Coupled transcription and translation. (**A**) Cells were permeabilized and incubated (10 min) with biotin-lysine-tRNA ± supplements shown, and sites containing biotinylated polypeptides were imaged (as in Fig. 2D); then the average fluorescence intensity over the nucleoplasm in >100 cells was measured and expressed relative to that found without supplements. In the first experiment (rows 1 to 4) with HeLa (subtetraploid human carcinoma), Cos-1 (SV40-transformed monkey cell), MRC5 (diploid human fibroblast), and T-24 (human bladder carcinoma) cells, increasing NTPs increases nucleoplasmic labeling, but not if α-amanitin (10 μg/ml) is present. In the second experiment with HeLa (rows 5 to 10), adding 0.5 mM NTPs increases nucleoplasmic labeling, but not if CTP is omitted or 1 mM 3′dATP (cordycepin triphosphate) is present. Adding cycloheximide (cx; 1 mg/ml) with (row 9)—or 3 min before (row 10)—biotin-lysine-tRNA also decreases labeling. *, difference significant at 99.9% level (Student's *t* test). (**B**) HeLa cells were permeabilized and incubated with [^{3}H]Lys or BODIPY-Lys-tRNA ± supplements, and the tagged peptides were detected by autoradiography or direct imaging. Average numbers of grains (open boxes) or fluorescence intensities (gray boxes) of >50 cells are expressed relative to values found in row 1. *, difference compared with value in row 5 significant at 99.99% level (Student's *t* test).

to D); the nucleoplasmic signal constituted 14% of the total (Fig. 3E) and was again reduced by cycloheximide (Fig. 4B, row 7).



D

fraction colocalized (biotin with marker)

marker	observed	theoretical
Br-RNA	0.52	0.07*
SR	0.68	0.12*
L7	0.48	0.15*
eIF4E	0.59	0.18*
NAC	0.40	0.04*
20S (β)	0.25	0.06*
FALDH	0.04	0.06

Fig. 5. Biotin peptides: electron microscopy. (**A**) Permeabilized HeLa cells were incubated (10 min) in biotin-lysine-tRNA, and sites containing biotin were indirectly immunolabeled with 10-nm gold particles; most particles are found over ribosome-rich regions of the cytoplasm, but some are in clusters over the nucleoplasm. Incubation with cycloheximide (1 mg/ml) reduced nucleoplasmic labeling by 92%. Scale bar, 200 nm. (**B**) Permeabilized cells were incubated (10 min) in biotin-lysine-tRNA and Br-UTP, and sites containing biotin-polypeptides and Br-RNA were indirectly immunolabeled with 5- and 10-nm gold particles, respectively; the two labels lie close to each other. (**C**) L7 marked by 10-nm particles and biotin polypeptides by 5-nm particles. Scale bar, 50 nm. (**D**) Observed versus random colocalization; the significance of the fraction of biotin peptides colocalizing with various markers was determined by superimposing a grid of 40-nm squares on images like those in (B) and (C), counting the fraction of squares containing both types of particles, and comparing the results with those expected of a Poisson distribution. *, probability that association arose by chance < 0.001 (P^2 test).

Cytoplasmic labeling increased fivefold faster than nuclear labeling (Fig. 3E); this suggests that a nuclear transcript might typically associate with only one ribosome as each cytoplasmic message associates with ~five. Autoradiography after incorporation of [^{3}H]-lysine (*11*) confirmed that nuclei were responsible for ~15% cellular incorporation (Fig. 4B). Therefore, [^{3}H]lysine, biotin-Lys-tRNA, and BODIPY-Lys-tRNA, which are detected with different methods, all gave similar results.

Immunogold labeling (*18*) of permeabilized cells incubated with biotin-Lys-tRNA (*11*) confirmed that the nuclear interior contained ~10% nascent peptides in the cell (Fig. 5A). Immunofluorescence also showed that all components of the translation machinery tested were present in nuclei (*14*), including respectively 25, 16, 21, 38, and 68% of initiation factors eIF2α, eIF3, eIF4γ, eIF4E (detected with a polyclonal antibody), and eIF4E (detected with a monoclonal antibody), and 25 and 9% of the ribosomal proteins, L7 and QM [see also (*1*, *4*)]. As QM may mark active ribosomes (*19*), this is consistent with the nucleoplasm containing ~25% of the machinery but 10 to 15% activity.

In prokaryotes, ribosomes copy messages while those messages are made; transcription is coupled to translation (*20*). We tested whether this was so in four different types of eukaryotic cells, initially using biotin-Lys-tRNA. Our standard translation mixture lacks two nucleotide triphosphates (NTPs) required for transcription. When these were added, nucleoplasmic fluorescence increased in a concentration-dependent fashion (Fig. 4A; rows 1 to 3), and α-amanitin sufficient to inhibit RNA polymerase II prevented this increase (row 4). In a second experiment, adding all four NTPs also increased nucleoplasmic fluorescence (rows 5 and 6), whereas adding all but CTP (row 7), or adding the chain terminator, 3'deoxyadenosine triphosphate (3'dATP) (row 8), inhibited the stimulation. This stimulation was also seen with ATP, cytidine triphosphate (CTP), GTP, and Br-uridine triphosphate (UTP) (*16*); as the resulting Br-RNA cannot leave transcription sites (*21*), the increased signal cannot result from cytoplasmic translation of nuclear RNA made in vitro. NTPs also doubled incorporation of [^{3}H]lysine and BODIPY-Lys-tRNA into the nucleoplasm (Fig. 4B, rows 5 and 6), but not the cytoplasm (Fig. 4B, rows 1 and 2). Therefore, nucleoplasmic incorporation of some of all three labels depends on concurrent transcription.

If all translation occurred in the cytoplasm, newly made proteins should enter and spread throughout the nuclei; if some translation were coupled to transcription, some nascent peptides should be found with nascent transcripts. Nascent polypeptides and RNA were extended in the presence of biotin-lysine-tRNA and Br-UTP (*11*), and biotin-peptides and Br-RNA were marked with 5- and 10-nm gold particles (*18*); the two were often found together (Fig. 5B). Newly made biotin peptides in nuclei also colocalized (*18*) with (Fig. 5, C and D) (i) phosphorylated SR proteins that associate with newly made transcripts (*21*), (ii) two components of the translation machinery (L7 and IF4E), (iii) the α subunit of a complex involved in directing nascent polypeptides to the appropriate cellular compartment (i.e., NAC), and (iv) the β subunit of the proteasomal complex that degrades misfolded proteins. They did not colocalize with formaldehyde dehydrogenase (FALDH), which plays no role in translation or transcription. These results are consistent with translation occurring solely in the cytoplasm only if newly made proteins are targeted to these particular nuclear sites, and this seems unlikely.

These results show that lysine coupled to three different tags—^{3}H, biotin, and BODIPY—is incorporated into discrete sites within nuclei and that some of this nuclear translation depends on concurrent transcription by RNA polymerase II. Our results can be combined with those obtained on NMD to give the following model. Ribosomes are assembled within nucleoli and exported to both nucleoplasm and cytoplasm, where they associate with transcripts and become active. Export need not be selective; perhaps 10 times more ribosomes end up in the cytoplasm, as the nucleoplasm is so packed with chromatin. Some nuclear ribosomes are incorporated into nucleoplasmic transcription factories that contain many active polymerases and transcription units (*22*). These ribosomes "proofread" newly made transcripts as they emerge from polymerases. Any engaged ribosomes detecting incorrectly positioned stop codons would trigger degradation of the useless transcripts; simultaneously, truncated and unwanted polypeptides would be degraded by nearby proteasomes. If no premature stop codons are found, the transcript would be exported to the cytoplasm where it could support multiple initiations. Then, the processes of transcription and translation are coupled, much as they are in bacteria.

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8. Cells were grown, permeabilized, and treated with buffers [PB*, PB*–bovine serum albumin (BSA), and

phosphate-buffered saline+ (PBS+)] described in (*9*). Nuclei were prepared from 4 to 20 × 10^7 HeLa by washing in PBS, resuspension in 10 ml of PB* diluted by 3 volumes of distilled water (PB*-diluted), incubation (2 min; 37°C), addition of 40 ml of PB*-diluted, incubation (15 min), Dounce homogenization (×10 to 15) to release 95 to 99% nuclei (assayed by phase-contrast microscopy), and adding 0.25% Triton X-100; after 5 min, nuclei were spun (250*g*; 5 min) through PB* with 10% glycerol, and the pellet was gently resuspended in PB*-BSA. Standard stereological procedures (*21*) showed that isolated nuclei were contaminated with <5% extranuclear ribosomes (detected by electron microscopy of Epon sections) (*18*) seen in whole cells. Greater than 95% of the nuclei excluded a 500-kD dextran conjugated with fluorescein isothiocyanate (FITC) (Sigma), so larger cytoplasmic ribosomes were unlikely to enter on isolation. Nuclei prepared as above, but without washing with Triton, incorporated the same amount of biotin (measured as in Fig. 2I); 94% also excluded a 70-kD dextran conjugated with FITC (Sigma) but not fluorescein-12-ATP.

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10. Calculations assume that a typical protein contains 5.8, 2, and 9.2% lysine, methionine, and leucine, respectively, and that ~2 × 10^6 ribosomes remain active on ~0.4 × 10^6 transcripts per cell (*23*). The following confirms that few peptides were completed in vitro. HeLa cells were labeled with [^{3}H]Leu in vivo (*12*) or in vitro (*11*), swollen (10 min; 4°C) in one-half dilution PB*-BSA, and broken (passage 20 times through 23 gauge needle), and nascent [^{3}H]peptides were separated from released (completed) ones by pelleting (20,000*g*; 45 min; 4°C). After in vivo labeling for 10 and 30 min, 22 and 48% ^{3}H were recovered in the supernatant, showing that many [^{3}H]peptides were released. Corresponding values after extension in vitro were only 4 and 8%, respectively. Even if all released peptides entered nuclei, they could not account for the nuclear signals seen.
11. Translation "mix" contained PB*-BSA, creatine phosphokinase (20 units/ml), 2.5 mM phosphocreatine, 0.25 mM GTP, tRNA (0.5 mg/ml) (Sigma; bovine liver), aminoacyl-tRNA synthetase (200 units/ml) (Sigma; bovine liver), protease inhibitors for mammalian cells (Sigma), and various supplements. $MgCl_2$ was also added in equimolar amounts to any NTPs. [Synthetases can be omitted when biotin-Lys-tRNA is used; then nucleoplasmic biotin incorporation (measured as in Fig. 2I) falls by 22%.] For Fig. 1, 2x concentrates of permeabilized cells (8 to 50 × 10^6/ml) or nuclei (4 to 20 × 10^7/ml) in suspension and the "mix" ± inhibitors were preincubated separately (3 min; 27.5°C), mixed, and incubated (27.5°C), and 100 μl was removed at various times and mixed with 350 μl of 2% SDS plus 50 μl of 5 M NaOH. After 30 min at 37°C, 100 μl of this were extracted with 10% trichloroacetic acid (TCA) and counted (*9*). For Fig. 1, A to C, supplements were 5 μM L-[4,5-^{3}H]Lys (86 Ci/mmol) + 50 μM amino acids minus Lys, 5 μM L-[4,5-^{3}H]Leu (147 Ci/mmol) + 50 μM amino acids minus Leu, and 1 μM biotin-lysine-tRNA from brewer's yeast (Boehringer) + 5 μM L-[4,5-^{3}H]Leu (147 Ci/mmol) + 50 μM amino acids minus Lys, respectively. For Fig. 1D, supplements were 200 μCi/ml of L-[^{35}S]Met (0.5 Ci/mmol) + 50 μM amino acids minus Met. After incubation, 250 μl was added to 10 ml of PB*, pelleted, rewashed in 10 ml of PB*, and resuspended in 150 μl of PB* diluted plus 1 mM $MgCl_2$, 1 mM dithiothreitol, human placental ribonuclease inhibitor (25 units/ml; Amersham), ribonuclease (RNase)–free deoxyribonuclease (100 units/ml), and protease inhibitors (as above); after 10 min at 37°C and addition of 100 μl of sample buffer, [^{35}S]proteins from 10^5 cells or 3 × 10^5 nuclei were run on 10 to 20% gradient acrylamide gels before autoradiography (*9*). For Figs. 2, 4A, and 5A, supplements were 1 μM biotin-lysine-tRNA from brewer's yeast (Boehringer) + 50 μM amino acids minus Lys; for Fig. 5B, 100 μM CTP and Br-UTP were also added. For Fig. 3, supplements were a 1/10 dilution BODIPY-Lys-tRNA (FluoroTect Green$_{Lys}$; Promega) + 50 μM amino acids minus Lys. For Fig. 4B, supplements were [^{3}H]Lys (100 μCi/ml; 86 Ci/mmol) + 50 μM amino acids minus Lys. After fixation in methanol, cells were washed in 5% TCA, rewashed in water, dried, and covered with dipping film (Ilford K.5) for 3 days; after developing, grain numbers were counted. Cycloheximide (1 mg/ml) was generally used to inhibit protein synthesis as it only reduced incorporation of [^{3}H]uridine by 25% (*12*).
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14. Cells on cover slips were fixed (20 min; 4°C) in 4% paraformaldehyde in 250 mM Hepes (pH 7.4), antigens were indirectly immunolabeled with various antibodies (*12*, *24–26*), nucleic acids were counterstained with 20 μM TOTO-3 (Molecular Probes), images were collected with a confocal microscope (*9*), intensities over nucleoplasm and equivalent areas of the slide were measured (EasiVision software; Soft Imaging Systems), and data were exported to Excel (Microsoft) for background subtraction and analysis. Average intensities (confocal sections) of nucleoplasm and cytoplasm abutting the nucleus were determined and multiplied by the volume fraction of the two compartments (i.e., 400 and 673 μm^3) (*21*) to obtain relative contents. Incorporated BODIPY-Lys was directly detected after paraformaldehyde fixation.
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18. For Fig. 5A, samples in suspension were fixed, treated with osmium, and embedded in Epon (*21*). For Fig. 5, B and C, they were fixed and embedded in LR White, indirectly immunolabeled with various antibodies (*12*, *27–29*) on ultrathin sections, and contrasted with uranyl acetate, and digital images were collected (*21*).
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Snf1—a Histone Kinase That Works in Concert with the Histone Acetyltransferase Gcn5 to Regulate Transcription

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Modification of histones is an important element in the regulation of gene expression. Previous work suggested a link between acetylation and phosphorylation, but questioned its mechanistic basis. We have purified a histone H3 serine-10 kinase complex from *Saccharomyces cerevisiae* and have identified its catalytic subunit as Snf1. The Snf1/AMPK family of kinases function in conserved signal transduction pathways. Our results show that Snf1 and the acetyltransferase Gcn5 function in an obligate sequence to enhance *INO1* transcription by modifying histone H3 serine-10 and lysine-14. Thus, phosphorylation and acetylation are targeted to the same histone by promoter-specific regulation by a kinase/acetyltransferase pair, supporting models of gene regulation wherein transcription is controlled by coordinated patterns of histone modification.

Posttranslational modifications of the NH_2-terminal tails within core histones are important determinants of transcriptional regulation. In recent years, specific covalent modifications on histone tails have been characterized, including acetylation, phosphorylation, ubiquitination, and methylation (*1*, *2*). Several transcriptional coactivators, such as the Gcn5 family, possess intrinsic histone acetyltransferase (HAT) activity (*3*), which correlates with gene activation (*4*, *5*). HATs are typically components of high molecular weight protein complexes that are recruited to specific promoters by interaction with DNA-bound transcriptional activators (*6*).

Histone phosphorylation is not as well understood as acetylation. Mitotic chromosome condensation is accompanied by histone H3