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Advances in Brief

Histone H3 Messenger RNA *in Situ* Hybridization Correlates with *in Vivo* Bromodeoxyuridine Labeling of S-Phase Cells in Rat Colonic Epithelium[1]

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Abstract

Measurements of cell cycle phase fractions, particularly S-phase, are useful for studies of cell biology and carcinogenesis. Up-regulation of histone gene expression is tightly coupled to the G_1-S-phase transition of the cell cycle, and mRNA levels rise 30-100-fold during S-phase. Labeling of histone H3 mRNA using *in situ* hybridization (ISH) was assessed as a measure of S-phase cells and compared with that found using *in vivo* 5-bromodeoxyuridine (BrdUrd) labeling in formalin-fixed rat colonic crypts under baseline, modified 72-h starvation, and 24-h refeeding conditions. The labeling index scored in single-labeled sections by histone H3 ISH tightly correlated with that found by *in vivo* BrdUrd labeling ($r = 0.99$, $P < 0.0001$) and clearly discriminated between the control, starved, and refed states ($P < 0.001$). In 180 crypt sections double labeled using histone H3 ISH and BrdUrd, 92% of 1572 labeled cells exhibited both nuclear BrdUrd and cytoplasmic histone H3 label. It is concluded that histone H3 ISH is an accurate measure of the S-phase fraction and provides an alternative to *in vivo* BrdUrd labeling in rat colon. This finding warrants validation in human studies.

Introduction

Disregulation of the cell cycle and increased proliferation in tissues have been associated with carcinogenic propensity. Thus, several chemoprevention strategies have targeted disregulated proliferation. In the colon, measurements of the proliferative fraction of crypt cells and the spatial distribution of proliferating cells have served as surrogate biomarkers of colon cancer risk and response to intervention in animal and human studies, including chemoprevention trials (1, 2). Such studies, however, are often limited by the lack of widely applicable, accurate, and reproducible measures of specific cell cycle phases, including S-phase (2). *In vivo* labeling of DNA by nucleotide analogues such as [^{3}H]thymidine or BrdUrd[3] has proven useful in identifying S-phase cells in animal experiments, but this "gold standard" is not generally suitable for human studies, especially when repeated samplings are obtained from the same individual. The alternative procedure of *in vitro* labeling of DNA with nucleotide analogues can introduce artifact and variability related to the *ex vivo* incubation procedure (3), and, in addition, is not applicable to the study of archival specimens. Recent studies have resorted, therefore, to measurements of proliferation-related antigens, such as PCNA (2, 4, 5) and Ki-67 (2, 6, 7). Although these markers can be readily studied by immunohistochemistry or flow cytometry of fresh or archival specimens, they lack S-phase specificity, and their expression can be dissociated with proliferation in pathological conditions.

The measurement of cell cycle-regulated mRNA and protein species may provide cell cycle phase specificity as well as relevant biological correlation. Histones are key structural proteins important in the organization of eukaryotic DNA into chromatin. Although posttranslational modification of histones can occur throughout the cell cycle, histone gene expression is tightly associated with the timing of DNA replication (8). Transcription of histone mRNA begins at late G_1 and peaks in S-phase, when mRNA levels rise 20-100-fold over that in other phases of the cell cycle (8-10). Because histone mRNA lacks the polyadenylate tail, it is quickly degraded upon completion of S-phase, at which time the mRNA half-life is only 8-14 min (10, 11). Detection of histone H3 mRNA expression by ISH is therefore a potentially useful method for identifying S-phase cells on histological tissue sections (12-16). The present study was designed to assess the utility of histone H3 ISH as a marker of S-phase cells in colonic crypt sections. A modified rat starvation-refeeding model expected to induce three discrete levels of proliferative activity in colonic crypts was used to determine the correlation between histone H3 ISH and *in vivo* BrdUrd labeling. The results demonstrate a high degree of correlation between the two assays and suggest that H3 ISH is a useful proliferation biomarker which could be used as a surrogate end point in chemoprevention trials.

Materials and Methods

Animals and Study Design. Inbred 10-week-old male F344 rats (Charles River Breeding Laboratories, Wilmington, MA) were housed three per cage and fed a semipurified diet (AIN 76A) *ad libitum* for 3 weeks. The rats were then subjected to a starvation-refeeding schedule modified to allow the rats access to feces. This experimental model results in small changes in proliferation status of the colonic epithelium, suitable for testing the sensitivity of a new methodology. Three groups of six rats each were studied: one at the baseline fed state, the second at 72 h of starvation, and the third after 72 h of starvation followed by 24 h of refeeding. All rats had free access to water. The study was approved by the Institutional Animal Care Committee.

One h before sacrifice, at 7:00 a.m., the rats were given an i.p. injection of BrdUrd (Sigma Chemical Co., St. Louis, MO) at a dose of 6 mg/100 g body weight. Following sacrifice, a segment of distal colon, 3 cm from the anus, was resected and fixed in 10% buffered formalin for 6 h and then embedded in paraffin. Serial 4-μm sections were cut and mounted on 3-aminopropyltriethoxysilane-coated slides. Diethyl pyrocarbonate water (0.04%) was used in slide preparation and in all of the subsequent steps to prevent RNase activity.

BrdUrd Immunohistochemistry. Mouse anti-BrdUrd IgG (clone IU-4; CALTAG Labs, South San Francisco, CA) served as the primary antibody utilizing the avidin-biotin complex immunoperoxidase technique (ABC kit; Vector Labs, Burlingame, CA). In brief, deparaffinized, rehydrated sections were incubated with 0.1% protease (Sigma) at room temperature for 30 min, followed by incubation with 3% H_2O_2 in methanol for 20 min, then with 0.2 N HCl for 20 min and then with 0.1 M boric acid buffer. The sections were covered with normal goat serum and incubated with the primary antibody (diluted 1:500 in goat serum) for 3 h at room temperature, followed by washing

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[3] The abbreviations used are: BrdUrd, 5-bromo-2'-deoxyuridine; ISH, *in situ* hybridization; LI, labeling index; PCNA, proliferating cell nuclear antigen.

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in PBS containing 0.1% Triton X-100 and the addition of biotinylated goat antimouse IgG for 1 h at 37°C. The rinsed sections were then covered with avidin-biotin complex for 30 min at room temperature. The sections were developed in 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and weakly counterstained with Mayer's hematoxylin.

Histone H3 ISH. The fluorescein-labeled histone H3 mRNA DNA probe and ISH detection system were obtained from DAKO Corporation (Carpinteria, CA). The histone H3 probe is a 550-base pair, single-stranded, antisense fluorescein-labeled DNA fragment that hybridizes to the entire coding region of the human histone H3 mRNA, including the 5′ and 3′ untranslated regions. In brief, the deparaffinized slides were digested with 0.8% pepsin in 0.2 N HCl for 5 to 10 min at 37°C, washed in distilled water, and incubated with the histone H3 probe, in the hybridization buffer supplied by the manufacturer, for 2 h at 55°C. The slides were then washed in prewarmed stringent wash buffer (DAKO) for 15 min at 55°C, rinsed in trizma-buffered saline, and incubated with alkaline phosphatase-conjugated antifluorescein antibody (DAKO) for 20 min at room temperature. After washing in trizma buffered saline, the slides were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (DAKO) for 1 to 2 h at room temperature in the dark and weakly counterstained with 0.1% Nuclear Fast Red (Sigma) in 5% aluminum sulfate.

Double Labeling. Slides processed for BrdUrd immunohistochemistry according to the above procedure were washed in distilled water after treatment with diaminobenzidine, and then incubated with the histone H3 probe as indicated above. As a control for signal loss in the double-labeling experiments, serial single-labeled sections were compared.

LI Calculation and Statistical Analysis. Coded slides were examined at a magnification of ×1000. To overcome the potential bias in scoring slides which were double labeled with BrdUrd and histone H3 ISH, single-labeled slides by each of the two methods were separately scored to determine the LI (the percentage of total epithelial cells which are labeled) and the distribution of the labeled cells along the crypt axis. For each label, 20 U-shaped longitudinally cut crypt sections were scored per rat by established scoring criteria (17). In addition, 10 double-labeled crypt sections per rat were scored to determine the per cell and per crypt correlation of the two labels.

Nested analyses of variance methods were applied to the data, assuming that each crypt section was nested within each rat and each rat was nested within its treatment group. Differences in LI among the three treatment groups were compared, and all three pairwise comparisons were also performed after Bonferroni adjustment for multiple comparisons. A two-sided $P \leq 0.05$ was considered significant. Correlation analysis was then performed to assess the degree of association between the proliferation indexes obtained using BrdUrd and histone H3 ISH. Pearson's and Spearman's correlation coefficients were calculated for continuous and discrete data, respectively. The variance components for random effects were calculated to estimate variability both between and within rats and variability between crypt sections.

Results

***In Situ* Expression of Histone H3 mRNA in Normal Rat Colon Mucosa.** To assess the utility of histone H3 ISH as a marker of S-phase cells, histone H3 ISH was compared to *in vivo* BrdUrd labeling in single- and double-labeled colonic crypt sections. In single-labeled sections histone H3 ISH produced distinct cytoplasmic labeling with no significant background (Fig. 1*A*). The clarity of the ISH label was generally maintained in double-labeled slides (Fig. 1*B*), and cytoplasmic histone H3 was readily distinguished from nuclear BrdUrd. In many instances, however, the colors were less distinct in double-labeled than in single-labeled slides. In 180 double-labeled rat crypt sections, 92% of 1572 H3 ISH or BrdUrd labeled cells were

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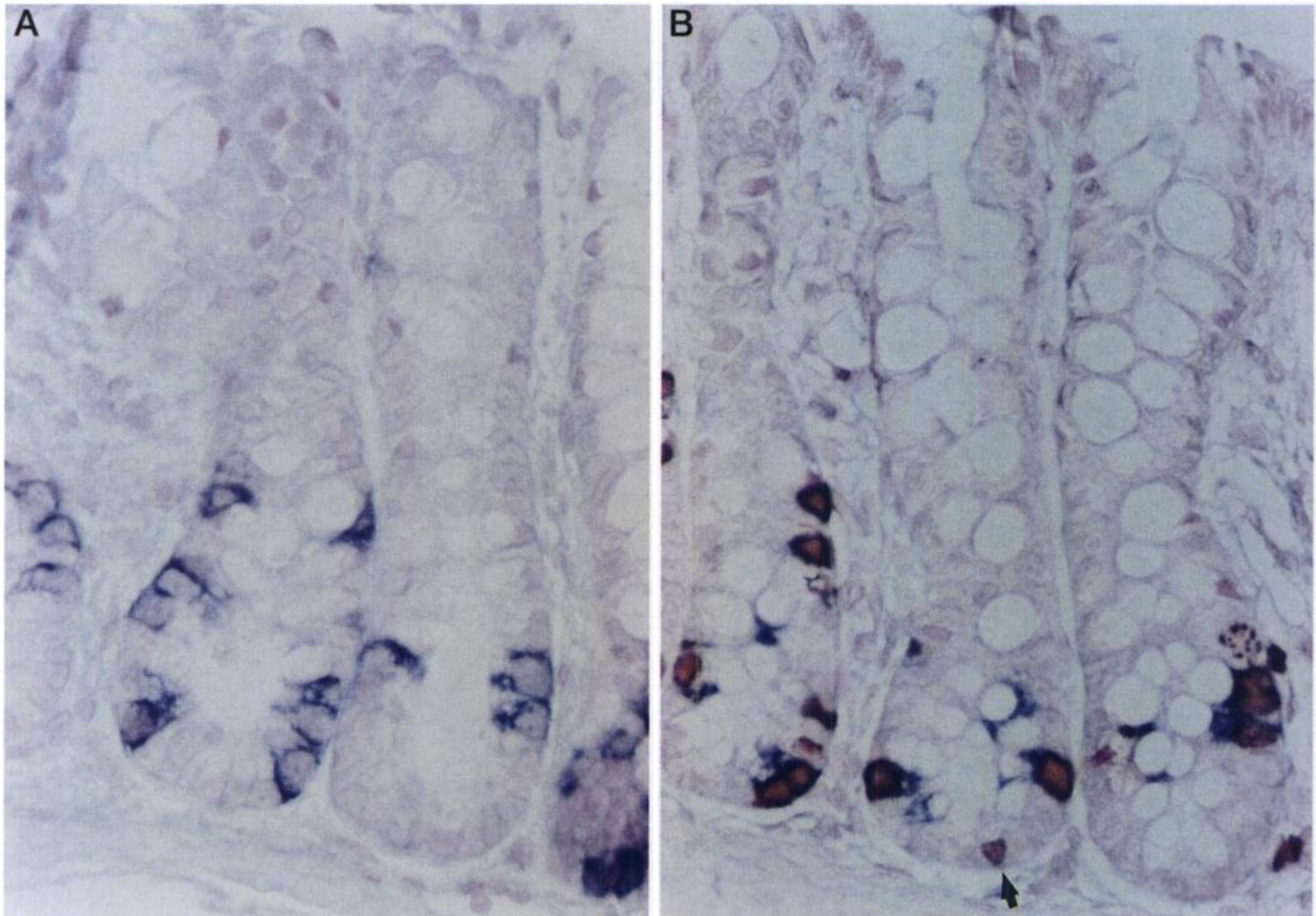

Fig. 1. Rat colonic crypt sections: *A*, single labeled by histone H3 mRNA ISH (blue cytoplasmic staining); *B*, double labeled by addition of *in vivo* BrdUrd immunohistochemistry (brown nuclear staining, *arrow* at a singly labeled cell). Cells in which both labels are identified have a blue cytoplasmic rim around the BrdUrd-positive brown nucleus.

Table 1 *Cell-labeling parameters determined by in vivo BrdUrd labeling and by histone H3 mRNA ISH in distal colon of control, starved, and refed rats*

	BrdUrd IHC[a]			H3 ISH		
	Control	Starved	Refed	Control	Starved	Refed
Total cells (no.)[b]	40.5 ± 3.0	40.4 ± 2.5	40.8 ± 2.1	39.5 ± 2.5	40.3 ± 2.2	40.5 ± 2.3
Labeled cells (no.)	8.5 ± 0.4[c]	5.8 ± 0.1[d]	10.9 ± 0.4[e]	8.7 ± 0.3[c]	5.9 ± 0.1[d]	10.7 ± 0.4[e]
LI (%)	10.4 ± 0.3[c]	7.2 ± 0.1[d]	13.3 ± 0.5[e]	10.9 ± 0.2[c]	7.2 ± 0.2[d]	13.1 ± 0.5[e]
Labeled cell position:						
5th percentile	0.9	1.0	0.8	0.8	1.0	0.9
50th percentile	8.2	7.9	8.3	7.7	7.9	8.4
95th percentile	17.4	15.5	18.3	16.7	16.0	18.0

[a] IHC, immunohistochemistry.
[b] Data are mean ± SD per crypt column.
[c-e] Means in the same line not sharing a common superscript letter are significantly different at $P < 0.001$.

double labeled. The Spearman's correlation coefficient between *in vivo* BrdUrd label and *in vitro* histone H3 ISH label per crypt in double-labeled slides was 0.98 ($P < 0.0001$). Cells singly labeled with either BrdUrd or histone H3 ISH were spatially distributed within the proliferative compartment in a pattern that was indistinguishable from that of double-labeled cells.

Validation Study: Rat Starvation-Refeeding Model. A modified rat starvation-refeeding model was utilized to ascertain whether the LI determination by histone H3 ISH could significantly distinguish three levels of proliferative activity in colonic crypt epithelium. Rats had a mean (± SD) body weight of 240.0 ± 8.0 g at baseline, 207.7 ± 5.4 g after a 72-h modified starvation, and 224.1 ± 8.2 g following 24-h of refeeding. Crypt height (cell number per crypt column) was 40.5 ± 3.0 cells at baseline and did not change during the experiment (Table 1). The mean LI measured by BrdUrd and by histone H3 ISH changed significantly ($P < 0.001$) with starvation and refeeding. As shown in Table 1, the mean (± SD) LI was 10.4 ± 0.3% (BrdUrd) and 10.9 ± 0.2% (H3 ISH) at baseline, 7.2 ± 0.1% (BrdUrd) and 7.2 ± 0.2% (H3 ISH) after 72 h of starvation, and 13.3 ± 0.5% (BrdUrd) and 13.1 ± 0.5% (H3 ISH) after refeeding. The LI measurements by *in vitro* histone H3 ISH correlated closely with measurements by *in vivo* BrdUrd labeling, with a Pearson correlation coefficient of 0.99 ($P < 0.0001$; Fig. 2). The compartmental distribution of labeled cells along the crypt axis was similar with both methods and did not change with starvation or refeeding (Table 1). Labeled cells were distributed almost exclusively within the bottom 60% of the crypt. The 50th and 95th percentiles of labeled cells were located along the crypt axis at cell positions (mean ± SD for all of the rat groups) 8.1 ± 0.2 (BrdUrd), 8.0 ± 0.4 (H3 ISH), 17.2 ± 1.2 (BrdUrd), and 16.9 ± 1.0 (H3 ISH), respectively.

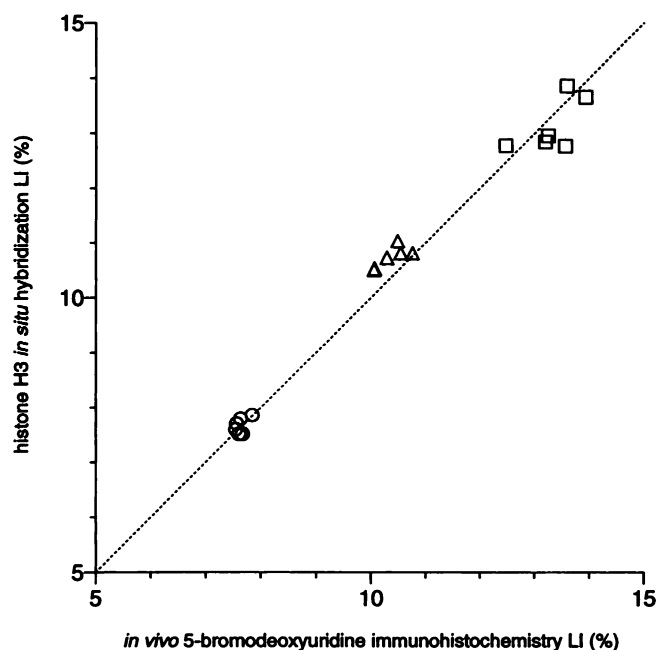

Fig. 2. Correlation between LIs determined by *in vitro* histone H3 ISH and by *in vivo* BrdUrd immunohistochemistry in distal colon of control (△), modified 72-h starved (○), and 24-h refed (□) rats ($r = 0.99$, $P < 0.0001$).

Variance component analysis was used to calculate the variations in LI attributable to the interventions, rats, and crypt sections. With both labeling methods the interventions contributed to 59% of the total variability, and differences between crypt sections contributed to 41% of the total variability. Differences between rats within the same intervention group were insignificant and did not contribute to the observed variability. Crypt-to-crypt variability was lowest in the starved state (SD, 1.9%) and highest in the control and refed states (SD, 2.7%), $P < 0.001$, suggesting that starvation contributed toward uniformity of proliferative activity.

Discussion

Accurate and reproducible cell cycle phase-specific measurements are essential to studies of cellular growth regulation. In particular, a precise measure of S-phase fractions in colonic tissue specimens is needed for studies of colonic carcinogenesis and cancer prevention (2). Recently, histone H3 ISH was found to be specific for S-phase in a number of cell lines as well as in human and animal tissues (12–16). In the present study, histone H3 ISH was found to closely correspond to *in vivo* BrdUrd labeling in sections of diet-perturbed rat colonic crypts. The findings suggest that histone H3 ISH is an accurate tool for determination of S-phase cells in rat colon.

Nucleotide analogues such as [^{3}H]thymidine and BrdUrd, incorporated *in vivo*, have served as standard S-phase-specific markers (2). However, the need for *in vivo* administration of these nucleotides has limited their use in human studies and in fixed or archival specimens. *In vivo* labeling of DNA is considered an accurate measure of S-phase except for minor limitations related to pulse time, cellular distribution and metabolism of nucleotides, and presence of S_0 cells (*i.e.*, cells in S-phase but not replicating DNA; Ref. 3). [^{3}H]thymidine and BrdUrd are also incorporated during unscheduled DNA synthesis but at a lower level. As an alternative to *in vivo* labeling, fresh specimens have been incubated with [^{3}H]thymidine or BrdUrd *in vitro* (3). The *in vitro* method has been useful in small studies in which labor-intensive standardization procedures were observed (1). However, *in vitro* labeling of DNA has been fraught with problems related to irregular incorporation of label as well as to interassay and interlaboratory variations (2).

The lack of an immunohistochemical measure of S-phase in fixed tissue resulted in the use of antibodies to proliferation-associated proteins such as PCNA (4, 5) and Ki-67 (6, 7). PCNA is currently the most widely used proliferation related measure in the colon. PCNA is an auxiliary protein of DNA polymerase δ, which is transcribed and translated throughout the cell cycle but is maximally expressed in late G_1, S-phase, and early G_2 (4, 5). PCNA immunohistochemistry of

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colonic crypts results in a gradient of staining intensity encompassing nonproliferating cells and is subject to numerous factors not specifically linked to the cell cycle. PCNA is under transcriptional and posttranscriptional controls involving mRNA stability, and mRNA levels are influenced by growth factors and oncogene expression (18). The PCNA protein has a long half-life of 20 h and therefore persists throughout the cell cycle in rapidly proliferating cells (18). PCNA also has a role in unscheduled DNA synthesis (19). These factors may account for the poor correlation between *in vivo* labeling of DNA by iododeoxyuridine and *in vitro* PCNA immunohistochemistry observed in human colon specimens (20) in contrast to studies in rodents (21).

Similar to PCNA, the Ki-67 antibody has been widely used to measure the cycling cell fraction, especially in studies of hematopoietic cells and lymphoid tissue (7). The Ki-67 antibody identifies a recently sequenced protein present in the nuclei of cells in G_1, S-phase, G_2, and M but not in cells in G_0 (6). Its application to studies of colonic epithelium has been limited by the heterogeneity of Ki-67 immunostaining, by the large fraction of epithelial cells expressing Ki-67, and by the poorly understood biological correlate of Ki-67 expression (2, 22, 23). The need remains for a more specific and consistent measure of S-phase.

The use of histone H3 ISH as measure of S-phase is supported by the present study as well as by current knowledge of histone gene expression. Histone protein is synthesized in constant proportion to duplicating DNA (8). Its gene expression is not otherwise induced by growth factors or metabolic stimuli and is also not required for DNA repair (8–10). The mRNA has a very short half-life relative to the duration of S-phase (10, 11). Its low baseline levels are not detected by ISH, but the high levels during S-phase are readily detectable (8–10). The histone H3 gene is known to be highly conserved across species and is ubiquitously expressed in eukaryotic cells (12–16). Hence, the human cDNA probe used in the current study is expected to be widely applicable. Potential problems with the use of ISH to detect histone H3 mRNA may relate to tissue integrity and fixation and mRNA degradation by RNase. Thus far, these have not been found to be significant problems in rapidly fixed tissue when formalin or methacarn was used (16).

ISH for histone H3 mRNA was found to correspond closely to *in vivo* BrdUrd incorporation into DNA in rat colon. Minor differences between the two labels are expected. BrdUrd persists in labeled cells that have completed the S-phase during the 1 h between injection of label and sacrifice. Histone H3 ISH, on the other hand, is expected to identify a small number of S-phase cells that escape the sensitivity of BrdUrd labeling as well as some cells in late G_1. Also, due to nuclear truncation in histological sections, some S-phase cells might be detected due to cytoplasmic H3 mRNA even when the nucleus is not apparent. Nonetheless, 92% of labeled cells had dual signals. This is consistent with the expected long S-phase in normal rat colon and possibly with the poor sensitivity of histone H3 ISH for cells in late G_1, when histone H3 mRNA levels are relatively low. Histone H3 ISH and *in vivo* BrdUrd labeling had similar accuracy in discriminating among the three levels of proliferative activity in baseline, starved, and refed rats and the LI determined using the two methods correlated closely ($r = 0.99$).

The present findings support previous reports of the S-phase specificity of histone *H3* gene expression detected by ISH in histological tissue preparations. As a tool for identifying S-phase cells, histone H3 ISH offers a number of advantages. The label has a well-defined biological correlate. It does not require *in vivo* administration, *in vitro* incubation, or use of radioactive isotopes. It can be used in formalin- and methacarn-fixed tissue, and therefore can be applied to routinely acquired and possibly to archival specimens. It also has the unique property of cytoplasmic staining which enables the concomitant study of nuclear antigens in the same sample. Histone H3 ISH is likely to be a useful method for identification of S-phase cells in the colon and offers an alternative to current methods in the study of fixed tissue specimens. Histone H3 ISH would be especially useful when S-phase specificity is important. Additional validation studies in human specimens are warranted.

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