Use of Intracellular H3 Messenger RNA as a Marker to Determine the Proliferation Pattern of Normal and 7,12-Dimethylbenz[a]anthracene-transformed Hamster Oral Epithelium

David T. W. Wong,2 Ming Yung Chou,3 Long-Chang Chang, and George T. Gallagher

ABSTRACT

One of the major goals in cancer research and diagnosis is to identify in a tissue the population of actively dividing cells and their pattern of growth and to differentiate the proliferation patterns of normal and transformed tissues. We now describe a method for determining the proliferation pattern of any tissue (normal, diseased, or transformed), applicable in any mammalian species. This method is based on the fact that the transcription of histone H3 gene in mammalian cells is tightly coupled to DNA synthesis during cellular division. Resting cells or cells that just exited the cell cycle will have no detectable H3 mRNA. The presence of H3 mRNA in a cell is thus a good indicator of its proliferation status. We carried out in situ hybridization of H3 mRNA in hamster oral epithelia exhibiting a variety of altered growth patterns as a consequence of exposure to the chemical carcinogen, 7,12-dimethylbenz[a]anthracene to demonstrate the usefulness of this technique. This application does not require in vitro manipulation of tissues nor does it require the prior administration of a tracer. The proliferation pattern at a single moment in time instead of an accumulated pattern over a period of time is produced. Finally, since the technique of in situ hybridization can be applied to archival tissues, retrospective studies can be done. This application should find usefulness in a wide variety of experimental research settings, particularly cancer research.

INTRODUCTION

Cancer is a disease process in which the primary defect is deregulated growth of the affected tissues. The ability to assess the proliferation pattern of any transformed tissue is of great importance toward cancer diagnosis, treatment, and prognosis. This capability is also important in experimental research, since altered proliferation is often a response of biological tissues to different insults and chemical modifiers.

Currently, measurement of the tumor-proliferative index is accomplished by morphological quantitation of the mitotic index, thymidine-labeling index, and flow cytometry (1–3). Recently, Hoffman et al. (4) have demonstrated a technique for the ex vivo determination of the proliferative capacity of normal and transformed human tissues. Each of these methods has its own merits and limitations. Most require in vitro manipulation of the tumor tissue. Examination of mitotic figures is the most common and most physiological. However, the mitotic phase of the cell cycle is a small fraction of the total cell cycle time; most cycling cells not in the mitotic phase will not be detectable. None of the methods, with the exception of examining mitotic figures, allows retrospective studies to be performed.

We now describe a method for determining the proliferation pattern of any normal or transformed tissue in any mammalian species. The application utilizes the advantage of the in situ hybridization technique, which allows the simultaneous examination of histological features and detecting cellular mRNA levels. The expression of the histone H3 gene is known to be tightly coupled to DNA synthesis in all cell types examined (normal and malignant) (5). In cycling cells, the level of H3 mRNA reaches a peak at the S phase and then rapidly disappears toward the end of G1 (5). Cells traversing through M phase will contain little or no H3 mRNA. Histone mRNAs are not polyadenylated and are rapidly degraded when S phase is completed. Nondividing cells will contain no H3 mRNA. While cells in S phase will have high levels of H3 mRNA, the levels in G1 phase are about 5% of S-phase levels. With the exception of the M phase, cycling cells traversing G1, S, or G2 phases of the cell cycle should contain detectable levels of H3 mRNA.

We now demonstrate the successful application of this method to illustrate altered proliferation patterns of hamster oral epithelium undergoing malignant transformation by the carcinogen chemical DMBA (6). The sensitive nature of the in situ hybridization technique, the advantage of no need for a tracer, and the capability to work on archival tissues should make this application a useful method to determine tissue proliferation patterns.

MATERIALS AND METHODS

Cell Culture. HCPC-1 cells are DMBA-transformed Syrian hamster oral epidermoid carcinoma cells isolated and established by Odukoya et al. (7). They are maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (penicillin, 100 units per ml; streptomycin, 100 μg per ml; and amphotericin B, 0.25 μg per ml) (Whittaker MA Bioproducts, Walkersville, MD).

For [3H]thymidine labeling, cells in S phase were labeled with [3H]thymidine (Amersham, 16.0 Ci/mmol), at 1.0 μCi/ml of medium, for 15 min before fixation. For DNA synthesis inhibition experiments, 10 mM hydroxyurea (H-8627; Sigma) was administered to exponentially growing HCPC-1 cells for 1 h.

Animals and Tissues. Twenty 60- to 90-day-old male Syrian golden hamsters were purchased from the Charles River Breeding Laboratories in Wilmington, MA. Hamsters were housed, 4 per cage, in an air-conditioned (76°F) animal room on a 12-h light-dark cycle and fed with a commercial stock diet (Purina formula chow) and tap water ad libitum.

Epidermoid carcinomas were induced in the cheek pouches of Syrian hamsters according to the method of Odukoya et al. (7). This is a 14-wk tumor induction protocol with the DMBA-treated hamster oral epithelium developing defined histopathological lesions (hyperplasia, dysplasia, and carcinoma). The chemical carcinogen was a 0.5% solution of DMBA (0.5 g/100 ml) (D-3254; Sigma Chemical Co., St. Louis, MO) dissolved in mineral oil (U.S.P.). The left buccal pouch of the hamsters was painted 3 times weekly with either 0.5% DMBA in mineral oil or mineral oil only, using a No. 4 soft sable brush. The body weight of the hamsters was recorded every week. Buccal pouches of
hamsters were grossly examined at weekly intervals for epithelial changes and tumor development from Wk 2 to Wk 14. Two hamsters were sacrificed at the end of Wk 0, 2, 4, 6, 8, 10, 12, and 14, and both pouches were excised from each. These tissues were used for in situ hybridization.

**In Situ Hybridization.** DMBA and mineral oil-treated hamster cheek pouch tissues were processed for in situ hybridization according to a modified method of Zeller et al. (8). Excised hamster cheek pouches were immediately fixed in freshly prepared 4% paraformaldehyde in 1× phosphate-buffered saline (4 g/100 ml) for 2 h at 4°C. They were then dehydrated through increasing percentages of ethanol and then xylene and finally embedded into Paraplast wax. Eight-μm sections were cut and mounted onto gelatin-coated glass slides. Prior to hybridization, each tissue section was pretreated and prehybridized. Pretreatment included tissue rehydration, mild acid hydrolysis, postfixation of tissues with 4% paraformaldehyde, and finally treatment in a 0.25% (v/v) concentration of acetic anhydride in 0.1 M triethanolamine (pH 8). Prehybridization of the pretreated tissue section was a 2-h incubation at 50°C of the tissue sections with sufficient quantities (~25 µl/section) of the hybridization solution (containing 50% deionized formamide; 0.3 M NaCl; 10 mM Tris—HCl, pH 8.0; 1 mM EDTA, pH 8.0; 1× Denhardt’s solution; 500 µg/ml of tRNA; 500 µg/ml of polyadenylic acid, 50 µm dithiothreitol; 10% polyethylene glycol) without the probe. Hybridization of the tissue sections was performed immediately following pretreatment and prehybridization. A 35S-labeled antisense (−) single-stranded RNA probe to the rat H3 cDNA was used. The mouse EGF riboprobe was used as a negative control. The single-stranded RNA probes were added directly to the prehybridization solution on the glass slide (2×10^6 cpm per section), mixed well, and then incubated overnight at 50°C under a glass coverslip, sealed with rubber cement. Posthybridization washings followed the overnight hybridization which includes an RNAse digestion step: 15 min twice in 50% formamide, 2× SSC, and 20 mM 2-mercaptoethanol, at 50°C; 15 min twice in 50% formamide, 2× SSC, 20 mM 2-mercaptoethanol, and 0.5% Triton X-100, at 50°C; 5 min twice in 2× SSC and 20 mM 2-mercaptoethanol, at room temperature; RNase digestion for 30 min at 37°C that consisted of 40 µg/ml of RNase A, 2 µg/ml of RNase T1, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 15 µl NaCl; 15 min in 2× SSC and 20 mM 2-mercaptoethanol, at room temperature; 4 times each 30 min in 2× SSC and 20 mM 2-mercaptoethanol, at 50°C with gentle shaking; and once for 10 min in 2× SSC at room temperature. The slides were then dehydrated, dipped in Kodak NTB2 emulsion, and processed for autoradiography. All slides were counterstained with Giemsa (No. SG-28; Fisher).

**Molecular Probes.** The rat histone H3 cDNA probe was kindly provided to us by Dr. William F. Marzluff (9). The fragment of DNA containing the rat histone H3 cDNA was cloned into pGEM3 in an orientation such that transcription from the T7 promoter resulted in an antisense RNA probe. Production of labeled riboprobes was done by using an RNA transcription kit (Promega, Madison, WI), [35S]-UTP (SJ.40383 and SP6/T7 grade; 850 Ci/mol; Amersham, Arlington Heights, IL) and T7 RNA polymerase (Stratagene, La Jolla, CA). Typically, 80 to 90% incorporation was obtained. Each in vitro transcription reaction yields ~230 ng of synthesized RNA with a specific activity of ~3×10^6 cpm/µg of RNA. The final specific activity of the probe used for in situ hybridization is ~2×10^6 cpm per section. A heterologous riboprobe, the mouse EGF, was used as a negative control (a kind gift from Dr. G. Bell, Chiron Corp., Emeryville, CA).

**RESULTS**

**In Situ Demonstration of Histone H3 mRNA Synthesis Coupled to DNA Synthesis in Hamster HCPC-1 Cells.** We have previously demonstrated that, by RNA blot analysis and [3H]-thymidine labeling in the hamster oral carcinoma cells (HCPC-1), the expression of the H3 gene is tightly coupled to the S phase of the cell cycle (10). We now proceeded to determine if such linkage can be demonstrated at the cellular level by examining cellular levels of H3 mRNA by in situ hybridization.

Fig. 1 shows cultures of HCPC-1 cells growing in the absence (A) and presence (B) of the DNA synthesis inhibitor hydroxyurea at 10 mmol, and subjected to in situ hybridization analysis for H3 mRNA content. Autoradiography exposure time was for 10 days to dramatize the difference of H3 mRNA labeling. In the exponentially growing culture (Fig. 1A), cells are labeled in a bimodal manner in which ~50% of cells contained grain densities that were significantly higher than that of background. Consistent with the known profile of histone H3 gene expression during the cell cycle, cells traversing through the mitotic phase of the cell cycle are virtually free of H3 mRNA labeling (arrow). Labeling with the antisense mouse EGF riboprobe did not produce a specific cellular labeling pattern (data not shown). Inhibition of DNA synthesis by hydroxyurea (10 mmol, 1 h) in these HCPC-1 cells greatly reduced the H3 mRNA labeling (Fig. 1B). We have previously determined the half-life of the H3 mRNA in these cells to be ~1.6 h (11). The minimal H3 mRNA labeling in these hydroxyurea-treated cells is likely due to residual H3 mRNA. Parallel cultures of actively growing, non-hydroxyurea-treated HCPC-1 cells labeled with [3H]thymidine followed by autoradiography revealed that ~47% were found to be in S phase. Thus a good agreement was obtained between metabolic labeling by [3H]thymidine and H3 mRNA-positive cells. These results indicate that H3 mRNA labeling of these hamster oral tumor cells by in situ hybridization is a valid procedure to mark the population of dividing cells.

![Detection of H3 mRNA in hamster oral carcinoma cells (HCPC-1)](image)

Fig. 1. Detection of H3 mRNA in hamster oral carcinoma cells (HCPC-1) grown in the absence (A) and presence (B) of the DNA synthesis inhibitor hydroxyurea (10 mmol). The *arrow* in A indicates a cell in the mitotic phase. Exposure is for 10 days at 4°C × 200.
**In Situ** Detection of H3 mRNA in Normal, Hyperplastic, Dysplastic, and Carcinomatous Hamster Oral Epithelium. Hamster cheek pouches treated for different lengths of time with the chemical carcinogen DMBA were examined for a pattern of H3 mRNA labeling by *in situ* hybridization. It is known that DMBA can induce specific epithelial alterations in a time-dependent manner (hyperplasia in 4 to 6 wk; dysplasia in 6 to 8 wk; and carcinomas from 8 wk onward) (6). Representative histological lesions were chosen to demonstrate typical H3 mRNA labeling (Figs. 2 to 4). Fig. 2 shows low- and high-power views of normal cheek pouch epithelium labeled for H3 mRNA. Only an occasional cell in the basal layer of the normal epithelium (obtained at Wk 0) was marked by the H3 riboprobe. This is in agreement with the known biology of normal epithelium in that proliferative cells reside primarily in the basal layer. No cells in the underlying connective tissue were observed to be labeled.

Fig. 3A represents a hyperplastic epithelium (4 wk of DMBA treatment) and labeling for H3 mRNA by *in situ* hybridization. The affected epithelium is thickened to ~7 to 8 cell layers (normal epithelium, ~3 to 4 cell layers). Virtually every other cell in the basal layer was clearly positive for H3 mRNA. However, all H3 mRNA positive cells resided in the basal compartment.

Fig. 3B represents a moderately dysplastic epithelium from an 8-wk DMBA-treated hamster cheek pouch. Like the hyperplastic epithelium (Fig. 3A) H3 mRNA positive cells are more frequent in the basal layer. However, in addition, H3 mRNA positive cells are no longer confined to the basal layer and suprabasilar H3 mRNA labeling can clearly be seen, suggesting a disorderly maturation pattern.

Fig. 4A represents an early invasive, moderately to poorly differentiated epidermoid carcinoma from a tumor-bearing hamster cheek pouch treated with DMBA for 12 wk. Intense H3 mRNA labeling can be seen in the cords and strands of transformed epithelium at the invading front. In the highly proliferative areas such as the invading front, mitotic figures can be frequently seen, but they are devoid of H3 mRNA labeling (data not shown). Fig. 4B is a section of a well-differentiated epidermoid carcinoma from a hamster cheek pouch treated with DMBA for 14 wk. H3 mRNA labeling in the transformed epithelium shows predominantly a basal orientation. In Fig. 4 one can occasionally detect an H3 mRNA positive cell in the dermal portion of the section. These are likely to be proliferating endothelial cells, fibroblasts, or lymphocytes. Hybridization of serial sections of Figs. 2 to 4 with the mouse EGF riboprobe produces no labeling of the epithelium (data not shown).

**In Situ** Detection of H3 mRNA Positive Cells in Archival Hamster Oral Epithelial Tissues. It is known that proper tissue fixation and subsequent processing are of vital importance in order to ensure success with the *in situ* hybridization technique.
H3 mRNA LEVELS AND PROLIFERATION PATTERNS

However, if we can demonstrate the tissue proliferation pattern in a retrospective manner on archival tissues that were routinely fixed in 10% formalin, our proposed method would be of great value in medical research. We hypothesized that the high abundance cellular level of H3 mRNA in dividing cells might permit detection of H3 mRNA in archival tissues. To test this hypothesis, we obtained 10 wax blocks of hamster oral epithelium that were treated with DMBA for different lengths of time and subjected to conventional 10% formalin fixation and subsequent processing. All these blocks are more than 3 yr old. Hybridization of these tissues with the rat H3 antisense riboprobe revealed that all ten specimens contained readily detectable H3 mRNA positive cells in the DMBA-treated epithelium. The intensities of the hybridization signals appear to be similar to tissues optimally prepared for in situ hybridization. Fig. 5A is a section from a normal hamster cheek pouch labeled for H3 mRNA in a similar pattern to the optimally fixed tissues presented in Fig. 2; i.e., only an occasional basal cell is labeled. Fig. 5B is from a hyperplastic hamster cheek pouch treated with DMBA for 5 wk. Similar to Fig. 3A, an increased frequency of H3 mRNA positive cells is seen in the basal layer, demonstrating epithelial hyperplasia. Hybridization of serial sections with the mouse EGF riboprobe produced no labeling of the epithelium (data not shown).

DISCUSSION

The ability to determine the proliferation pattern of normal and transformed tissues is of great importance in cancer research and cancer treatment planning. With the exception of examining mitotic figures, all of the current methods for determining tumor-proliferating capacity require either in vitro manipulation of the tumor tissue or the prior administration of tracer substances.

In this study, using an experimental approach described by Lawrence et al. (12), we demonstrate by in situ hybridization that the expression of the histone H3 gene is a good marker for cellular proliferation in hamster oral tumor cells. The significant reduction of detectable H3 mRNA upon DNA synthesis inhibition by hydroxyurea and the good agreement with metabolic [3H]thymidine-labeling experiments allow us to conclude that the in situ detection of H3 mRNA is a valid marker for identifying dividing cells.

Our results demonstrate that H3 mRNA in situ hybridization can be performed on archival tissues. We have tested ten hamster blocks, each more than 3 yr old. All of these specimens were labeled very prominently for H3 mRNA. The intensities of the in situ hybridization signals are similar to signals detected in tissues prepared optimally for in situ hybridization. This should be of interest to investigators needing to perform retrospective studies on archival tissues.

Our application of the in situ hybridization technique to determine the H3 mRNA content of cells as an indirect means for identification of dividing cells allows an objective method to determine the proliferation pattern of tissues. This method has the following advantages. (a) No in vitro manipulation of tissues is required. (b) No prior administration of tracer sub-
stances is needed. (c) This application allows retrospective studies on archival tissues. (d) This technique can be conveniently coupled to routine biopsy and thus could be considered noninvasive. (e) Normal or diseased tissues can be examined, in any mammalian species, because of the highly conserved nature of the histone genes. (f) Minimal tissue is required. (g) The tissue to be examined is in its native state with regard to tissue architecture. (h) The proliferative status of a tumor at the time of biopsy is determined, instead of a cumulative index over an extended period of time. (i) This application can be coupled to a nonradioactive detection system which should make archival and clinical pathological studies much more widespread, especially in clinical situations where an answer about the proliferating status of the tissue being examined would be known the next day, instead of a wk exposure using autoradiography.

REFERENCES

Use of Intracellular H3 Messenger RNA as a Marker to Determine the Proliferation Pattern of Normal and 7,12-Dimethylbenz[a]anthracene-transformed Hamster Oral Epithelium

David T. W. Wong, Ming Yung Chou, Long-Chang Chang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/16/5107

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.