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Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: Evidence for a transcriptional effect

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ABSTRACT **Evidence from epidemiological studies, clinical trials, and animal experiments indicates that inhibitors of prostaglandin synthesis lower the risk of colon cancer. We tested the hypothesis that abnormal expression of prostaglandin H synthase 2 (*PHS-2*), which can be induced by oncogenes and tumor promoters, occurs during colon carcinogenesis by examining its level in colon tumors. Human colon cancers were found to have an increased expression of PHS-2 mRNA compared with normal colon specimens from the same patient ($n = 5$). *In situ* hybridization showed that the neoplastic colonocytes had increased expression of *PHS-2* ($n = 4$). Additionally, five colon cancer cell lines were shown to express high levels of PHS-2 mRNA even in the absence of a known inducer of *PHS-2*. To study the basis for this increased gene expression, we transfected a colon cancer cell line, HCT-116, with a reporter gene containing 2.0 kb of the 5′ regulatory sequence of the *PHS-2* gene. Constitutive transcription of the reporter gene was observed, whereas normal control cell lines transcribed the reporter only in response to an exogenous agonist. We conclude that *PHS-2* is transcribed abnormally in human colon cancers and that this may be one mechanism by which prostaglandins or related compounds that support carcinogenesis are generated.**

The study of the genetic events leading to colon carcinoma has shown that early mutations generate premalignant changes in the colonic epithelium. It is also likely that additional mutations and epigenetic progression factors are required for the development of a malignancy (1). Many studies [reviewed by Marnett (2)] have suggested that prostaglandins may be one of these progression factors. For example, colon cancers secrete more prostaglandins than normal colon tissue (2, 3). Additionally, reactive byproducts of prostaglandin synthesis might be relevant mutagens (2). The regular use of medications that inhibit prostaglandin synthesis (e.g., aspirin and indomethacin) is associated with a decreased risk of death from colon cancer (4), decreased incidence of colon cancer (5–8), and decreased incidence of colon adenomas (6, 7, 9, 10). Inhibitors of prostaglandin synthesis protect against chemically induced colon cancer in animal models (2). These inhibitors appear to act early in the process of colon carcinogenesis, as the use of an inhibitor in patients with adenomatous polyposis coli reduced the number and size of polyps by ≈60% (11, 12).

The key step in the conversion of arachidonic acid to prostaglandins is catalyzed by prostaglandin H synthase (PHS, also known as cyclooxygenase), and inhibition of this reaction by aspirin, indomethacin, and other anti-inflammatory drugs accounts for all or most of their therapeutic effects. PHS converts free arachidonic acid into prostaglandin H_2, which is a pivotal metabolite that serves as the precursor for terminal prostaglandins. Specific synthases catalyze the conversion of prostaglandin H_2, and the products of a given tissue depend on which of these enzymes is expressed. Recently, it was discovered that there is a second PHS (PHS-2) that is not present constitutively in most tissues but is induced by growth factors, cytokines, and tumor promoters (13–16). The clinical and animal studies of the effects of prostaglandin synthesis inhibitors on colon carcinogenesis would not have distinguished between the two types of PHS. Interestingly, *PHS-2* was discovered by investigators searching for genes induced by a tumorigenic stimulus, such as phorbol ester in mouse 3T3 cells (13) and transformation of chicken fibroblasts with the Rous sarcoma virus (14). Overexpression of *PHS-2* in a rat intestinal cell line resulted in resistance to apoptosis, which provides another potential link in the pathway to carcinogenesis (17). Thus, it seems likely that *PHS-2* might be the relevant target for the antitumor actions of prostaglandin synthesis inhibitors.

In this study, we tested the hypothesis that the overexpression of the *PHS-2* gene is associated with colon tumor formation. Colon cancers were examined for *PHS-2* expression by Northern blot analysis, quantitative PCR, and *in situ* hybridization to histological sections of neoplastic and normal human colon. Lastly, the promoter region of *PHS-2* was tested in a colon cancer cell line for its ability to express a reporter gene. Expression of *PHS-2* was upregulated in all tumor tissues examined. In addition, the *PHS-2* promoter was constitutively active in a colon cancer cell line, suggesting that the upregulation of *PHS-2* expression occurs at the transcriptional level and is associated with the neoplastic transformation of colonic epithelial cells.

MATERIALS AND METHODS

Analysis of Colon Samples by Reverse Transcriptase (RT)–PCR. Specimens of adenocarcinoma of the colon were paired with specimens of normal mucosa from the same person. All samples were obtained from the tumor bank of the Huntsman Cancer Institute at the University of Utah. Each sample had been rapidly frozen in liquid nitrogen and stored at −80°C. Approximately 350 mg of each sample was minced with a razor on ice and passed through a Dounce glass homogenizer. Nonhomogenized particles were removed by centrifugation. Total RNA was isolated by the guanidinium isothiocyanate method (18). cDNA synthesis and RT-PCR using primers for *PHS-2* and *GAPDH* were performed as described (16). Products were analyzed on 1.3% agarose gels stained with ethidium

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Abbreviations: PHS, prostaglandin H synthase; RT, reverse transcriptase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U44805).

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bromide. The predicted sizes of the PCR products for *PHS-2* and *GAPDH* were 890 and 550 bp, respectively.

Northern Blot Analysis. HCT-116, DLD-1, and human umbilical vein endothelial cells were grown to near confluence and then serum-starved for 48 h. Endothelial cells were stimulated with interleukin 1α (10 units/ml) for 2 h as a positive control for induction of *PHS-2* mRNA or vehicle control before total RNA was isolated as before. Northern blot analysis was performed using ^{32}P-random-prime-labeled cDNA probes to *PHS-2* as described (16).

***In Situ* Hybridization.** An additional four tumor specimens containing adjacent normal mucosa were obtained as described above. Specimens were fixed in 10% paraformaldehyde for 24 h before embedding in paraffin and sectioning into 5-μm slices. A 2.9-kb fragment of *PHS-2* cDNA (nt 325–3270) and the full-length cDNA of *PHS-1* were cloned into pBS/(KS-) (16). Plasmids were linearized by restriction digests and purified. Full-length sense and antisense ^{33}P-labeled RNA probes were synthesized using SP6 and T3 RNA polymerases (Stratagene). Probe size was adjusted to an average of ≈150 nt by incubation in 0.2 M carbonate buffer (pH 10.2) at 60°C (19). Slide preparation, hybridization, and detection were performed as described (19).

Genomic Cloning, Mapping, and Construction of Reporter Genes. We isolated yeast artificial chromosomes (YAC) containing the genomic sequence by screening a library (20) using a PCR-based row/column method in 96-well plates. A λ European Molecular Biology Laboratory sublibrary was constructed from the DNA of the YAC containing the *PHS-2* sequence and was screened with a cDNA encoding human *PHS-2*. Positive clones were isolated, and their inserts were subcloned into the pUC plasmid vector for further analysis. Restriction mapping and hybridization studies showed that the human *PHS-2* spans 12 kb (*Eco*RI fragments of 4 and 8 kb). The intron–exon boundaries were mapped by PCR using primers based on the cDNA sequence. To construct reporter genes, a 2.0-kb fragment of the promoter region of human *PHS-2*, *Eco*RI(−1840) to *Msp*A1I(+123) (5 bp upstream of ATG) was cloned into the *Hin*dIII site upstream of the firefly luciferase gene in the pGL2-Basic vector (Promega) after linker-ligation. The pSV-β-galactosidase control vector (Promega) was used to control for transfection efficiency.

Cell Culture. The human colon cancer cell lines HCT-116, DLD-1, Caco-2, LS 174T, and SK-CO-1 were obtained from American Type Culture Collection. The last three lines were grown in Eagle's minimal essential medium/10% fetal bovine serum (FBS). HCT-116 and DLD-1 cell lines were grown in McCoy's 5A/10% FBS and RPMI 1640 medium/10% FBS, respectively. BALB/c 3T3 cells were grown in Dulbecco's modified Eagle's medium/10% FBS. Human endothelial cells (21) and HCT-116 cells that overexpress the *APC* gene were cultured as described (22). The media for all of the above cells lines contained penicillin (100 units/ml) and streptomycin (100 μg/ml). The human breast epithelial cell line 184A1 (obtained from M. R. Stampfer, Lawrence Berkeley Laboratory), which is immortalized but not tumorigenic, was grown in serum-free MCDB 170 supplemented as described (23). Before RNA isolation or transfection, cells were placed in their respective media containing 0.5% FBS (defined as low-growth medium) for 48 h to avoid serum-induction of *PHS-2*. For 184A1 cells, low-growth medium consisted of MCDB 170 lacking epidermal growth factor (EGF), bovine pituitary extract, and hydrocortisone and containing a monoclonal antibody to the EGF receptor (10 μg/ml) (23). MCDB 170 was made as described (23); FBS was from HyClone and all other media were obtained from GIBCO/BRL.

Transfection Studies. HCT-116, 184A1, and 3T3 cells were grown in their respective complete media in six-well polystyrene tissue culture plates (Falcon) to ≈60% confluence and then placed in low-growth medium for 48 h before transfection to avoid growth factor induction of *PHS-2*. The *PHS-2* promoter construct (2 μg), the pGL-Basic vector (2 μg), or water (2 μg) was cotransfected with 2 μg of the pSV-β-gal vector using LipofectAMINE (GIBCO/BRL) following the manufacturer's directions. After 6 h, transfection medium was replaced with low-growth medium, and the cells were incubated for 48 h before lysing with reporter lysis buffer (Promega). Aliquots were assayed for luciferase activity by scintillation counting and for β-galactosidase by spectrophotometric detection. Six experiments were performed for each condition in HCT-116 and 184A1 cells, and three experiments were performed in 3T3 cells. The results are reported as mean ± SE of luciferase activity divided by milliunits of β-galactosidase activity.

RESULTS

***PHS-2* Is Expressed Abnormally in Colon Carcinoma.** To determine whether *PHS-2* is expressed in human colon cancer, we carried out RT-PCR on total RNA extracted from five colon tumors. Expression of *PHS-2* was increased in the tumor samples compared with normal colon tissue from each of the same patients (Fig. 1). To assess which cells overexpressed *PHS-2*, we performed *in situ* hybridization of *PHS-2* mRNA in samples of colon tumors (n = 4) that contained normal epithelium and carcinoma. *PHS-2* was expressed in the carcinomatous epithelial cells but not in the normal epithelial cells (Fig. 2), and there was little or no *PHS-2* observed in other cells except for Peyer's patches, which showed expression. In the same experiments, expression of *PHS-1* was assessed by *in situ* hybridization and low levels of mRNA were observed in both normal and cancerous regions. These results confirmed that there is constitutive, dysregulated expression of *PHS-2* in neoplastic colon epithelium.

To pursue the mechanism for the increased expression, we tested whether colon cancer cell lines also exhibited overexpression of *PHS-2*. When examined by RT-PCR, five different lines each were found to express *PHS-2* mRNA (Fig. 3*A*). RNAs from two of the lines were analyzed by Northern blot analysis as well (Fig. 3*B*); the same result was observed. In contrast, human endothelial cells in their basal state showed little or no expression of *PHS-2* but, after activation with interleukin 1, had levels equivalent to the colon cancer lines.

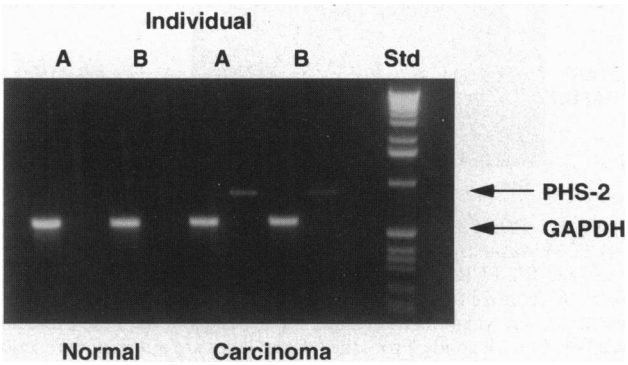

FIG. 1. Human colon carcinomas express *PHS-2*. RNA was extracted from either the carcinomatous area (carcinoma) or an equivalent weight of tissue from an unaffected area (normal) in the colon from five patients; results from two patients, A and B, are shown. RT-PCR was performed as described in *Materials and Methods*. The lanes are alternating reaction products with *GAPDH* (as a control) in the paired left, and *PHS-2* in the right, lane for each of the samples. The extracts of normal tissue are in the first four lanes from the left (Normal) and those from carcinomatous areas are in the next four lanes. The products for *PHS-2* and *GAPDH* were the predicted sizes of 890 and 550 bp, respectively. In both cases, note the increased expression of *PHS-2* in the carcinoma. The results shown are representative of those obtained with samples from all five patients.

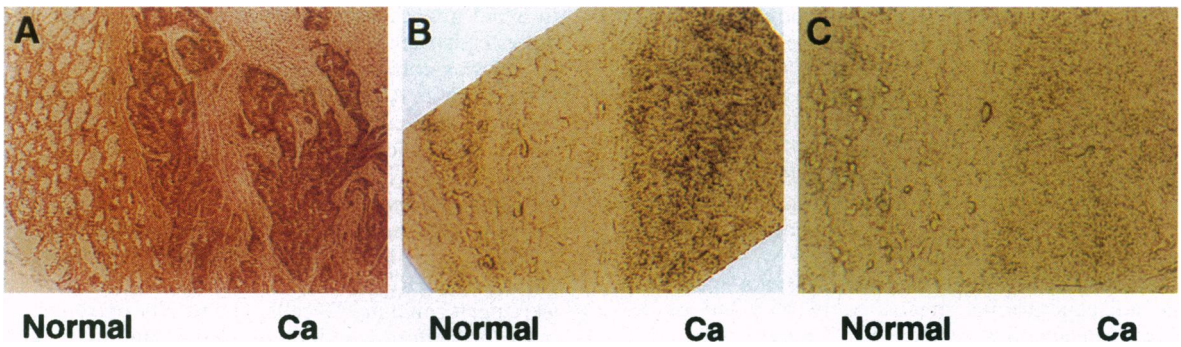

FIG. 2. The carcinomatous epithelial cells in human colon cancers have high-level expression of PHS-2. Samples of tissue from colon cancers were quickly frozen and then fixed and sliced. The RNA probes were generated and the hybridization was performed as described in *Materials and Methods*. (*A*) Section stained with hematoxylin and eosin; the carcinoma is on the right and is labeled Ca and normal epithelium is on the left (Normal). (*B*) A section of similar composition from the same tumor that has been hybridized with an antisense probe to *PHS-2*. Note the intense staining over the carcinomatous cells and absence of staining elsewhere (the sections are not identical, but the tumor occupies the right-hand portion of the tissue). (*C*) Same as *B* but hybridized with a sense probe; there is no staining, indicating that the staining observed in *B* was specific. The results shown are from a single tumor, but high-level expression of *PHS-2* was observed in the carcinomatous colonocytes of four separate tumors, each of which was examined in two independent experiments with equivalent results each time.

Isolation and Characterization of a Genomic Clone of Human *PHS-2*. To examine its transcription, we first isolated a genomic clone containing *PHS-2*. Its organization was determined, and a 4-kb fragment that contained almost 2 kb of upstream sequence (as well as exons 1 through 4) was sequenced. This sequence (GenBank accession no. U44805) did not differ substantially from that reported by Kosaka *et al.* (24). We previously had used PCR screening of human–hamster hybrids to localize *PHS-2* to human chromosome 1 (16). With the genomic clone as a probe, fluorescence *in situ* hybridization confirmed the location on chromosome 1 and placed the *PHS-2* gene on the q arm, also consistent with Kosaka's results (24).

Colon Cancer Cells Exhibit Constitutive Transcription of *PHS-2*. The finding that colon cancers express high levels of *PHS-2* suggested that there may be abnormal transcription of the gene. However, the mRNA contains multiple repeats of AUUUA, which have been implicated as conferring instability (25). Thus, it was possible that the increased mRNA level in colon cancer cells was the result of posttranscriptional events. Therefore, to examine whether there was increased transcription, a reporter gene that consisted of a 2.0-kb fragment of the promoter region of human *PHS-2* upstream of firefly luciferase was constructed. Treatment of reporter-transfected 3T3 cells with serum induced a 4- to 10-fold increase in luciferase activity (data not shown); 3T3 cells containing a control construct (luciferase without 5′ region of *PHS-2*) did not show a response. Next, we transfected the colon cancer line HCT-116, 3T3 cells, and a control epithelial cell line (184A1 breast epithelial cells) with the reporter gene. The data presented in Fig. 4 show that cells transfected with the Basic vector lacking the promoter region of *PHS-2*, or mock-transfectants, showed little or no luciferase activity. In contrast, the cancer cells transfected with the 2.0-kb fragment of the human *PHS-2* promoter demonstrate marked production of luciferase despite being serum-starved. The control epithelial cells and 3T3 cells had a much lower level of transcription in the basal state; there was substantial activity only when they were exposed to serum. These results indicate that HCT-116 colon cancer cells

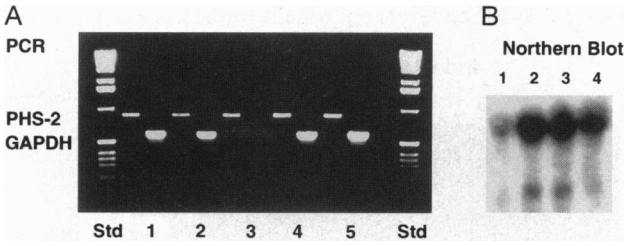

FIG. 3. *PHS-2* is constitutively expressed in colon cancer cell lines. (*A*) RNA was extracted from five different human colon cancer cell lines, and RT-PCR was performed using primers specific for *PHS-2* or *GAPDH* (control for the amount of RNA loaded). The outside lanes are molecular weight markers, and the products were of the predicted size (see Fig. 1 legend). From the left the lanes are alternating reaction products with *PHS-2* in the left lane and *GAPDH* in the paired right for each for the five carcinoma cell lines: lane 1, HCT-116; lane 2, SK-CO-1; lane 3, DLD-1; lane 4, Caco-2; and lane 5, LS 174T. The lane second from the right is a negative (i.e., no template) control for the PCR with *PHS-2* primers. The *GAPDH* product from DLD-1 cells was omitted from this experiment. (*B*) Northern blots from two of the positive lines. Lanes 3 and 4 contain RNA extracted from the HCT-116 and DLD-1 lines, respectively. The negative control was human endothelial cells (16) in the absence of agonist (lane 1), and the positive control was endothelial cells exposed to interleukin 1α (10 units/ml) for 2 h (lane 2). The mRNA for *PHS-2* is 4.3 kb (upper band); there is a small amount of reactivity with 18S RNA (lower band). Note that the mRNA of *PHS-2* in each colon cancer line is almost equal to that in maximally induced endothelial cells.

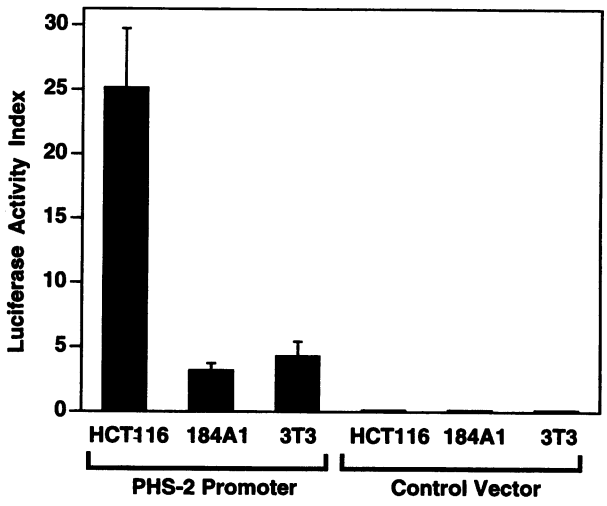

FIG. 4. Constitutive transcription of *PHS-2* in a colon cancer cell line. A reporter gene constructed from 2.0 kb of the 5′ sequence of the *PHS-2* gene and the luciferase gene in the pGL2-Basic vector was cotransfected with the pSV-β-gal control vector into serum-starved HCT-116, 184A1, and 3T3 cell lines. Cells were maintained in a starved state for 48 h before cell lysates were analyzed. Expression was assessed as luciferase activity and was normalized to the β-galactosidase activity, "luciferase activity index" (n = 6 for HCT-116 and 184A1, n = 3 for 3T3 cells). (Error bars = standard error.)

have increased RNA for *PHS-2* as a result of constitutive transcription controlled by the *PHS-2* promoter.

Patients with germline mutations of the *APC* gene have colon polyps that regress in response to inhibitors of PHS (11). Additionally, normal intestinal epithelium does not express *PHS-2*. One hypothesis is that normal APC protein suppresses the expression of *PHS-2* and that its absence leads to increased expression of *PHS-2*. To test this, we assayed HCT-116 cells that overexpress the *APC* gene (22). In three experiments using quantitative PCR, there was no difference in the level of *PHS-2* in wild-type HCT-116 compared with those overexpressing *APC* (data not shown). Thus, we conclude that this tumor suppressor does not directly regulate *PHS-2* expression.

DISCUSSION

Diverse findings suggest that prostaglandins have a role in the pathogenesis of colon cancer. For example, the incidence of colon cancer in humans is reduced in individuals that take nonsteroidal antiinflammatory drugs (2). There is direct support for a role for prostaglandins, or related compounds, in the growth of colonic polyps because they have been shown to regress in patients that take sulindac, an inhibitor of prostaglandin synthesis (11, 12). However, these studies do not provide insight into whether PHS-1, PHS-2, or both are the target(s) of such therapy. Our *in situ* hybridization and RT-PCR experiments demonstrate that PHS-2, but not PHS-1, mRNA is elevated in human colon cancers. We cannot say whether this is a primary or downstream event in the pathogenesis of colon cancer. However, results of other studies, showing that PHS inhibitors slow or prevent progression of disease and can lead to regression of polyps, suggest that products derived from the reactions catalyzed by PHS-2 are important early in the disease process.

In addition to the substantial evidence derived from the use of PHS inhibitors, Eberhart *et al.* (26) showed by Northern blot analysis that colon carcinomas (12 out of 14) had increased mRNA for *PHS-2*, but not *PHS-1*, as compared with normal colon tissue. *PHS-2* expression was observed in 6 of 14 adenomas, which supports the hypothesis that abnormal expression of this enzyme is the basis for the therapeutic effects of nonsteroidal antiinflammatory drugs on colon polyps. Subsequently, Kargman *et al.* (27) confirmed that PHS-2 was present in colon carcinomas, as 19 of 25 protein extracts from carcinomas were positive for PHS-2 by Western blot analysis. In contrast, in the same study, 23 of 25 normal samples were negative for PHS-2. Their results differed from those of Eberhart *et al.* (26) in that they did not detect PHS-2 in four premalignant polyps. These studies strongly suggested that the increased PHS-2 was in the carcinomatous colonocytes, but it remained possible that adjacent tissue was the source, perhaps as a part of a secondary inflammatory reaction. Our studies with *in situ* hybridization addressed this issue, as did the recent report of immunohistochemical studies by Sano *et al.* (28), who found that *PHS-2* expression was markedly increased in the tumor cells but not in normal tissue (even that immediately adjacent to the tumor), although some inflammatory cells were positive (as we found in Peyer's patches).

We found that HCT-116 colon carcinoma cells, in contrast to many other cultured cells, constitutively express *PHS-2* even without an added stimulus. Extensive studies since its discovery have led to a general view that *PHS-2* has low-level or no expression in most unstimulated tissues but is rapidly induced in response to growth factors, tumor promoters, and cytokines (29, 30). The isolation of genomic clones for *PHS-2* from different species and characterization of their promoter regions have yielded insights into the regulation of this gene (reviewed in ref. 30). The 5′ regulatory sequence contains SP-1, NFκB, NF-IL6, AP-1, and AP-2 sites as well as a serum response element (30–32). The cis-acting element that confers responsiveness to gonadotropic hormones and forskolin has been defined (33), and Herschman and colleagues (34) have shown that an ATF/CRE site located between −40 and −80 in the murine promoter is required for induction of *PHS-2* by the oncogene *v-src*. We can conclude that some factor overexpressed by carcinoma cells results in upregulation of the *PHS-2* promoter.

HCT-116 cells stably transfected to overexpress the *APC* gene do not show any change in the increased level of PHS-2 mRNA. This suggests that other genetic alterations that occur early in the adenoma–carcinoma sequence play a role in the upregulation of the *PHS-2* locus. It should be noted that this cell line (HCT-116) may represent a minority of colon carcinomas as its *APC* gene is intact. It will be important to test other lines that lack normal APC to determine if *PHS-2* is upregulated. Alternatively, the increase in *PHS-2* expression may be an epigenetic event. Clinical and animal studies have shown that inhibition of prostaglandin synthesis interrupts the cascade of cellular events initiated by the early mutation(s). This raises the possibility that interventions that block the overexpression of *PHS-2* would have a favorable effect on patients with, or at risk for, colonic neoplasms.

In summary, we have shown that *PHS-2* is abnormally expressed in the epithelial cells of human colon cancer and in cell lines derived from colon carcinomas. Our experiments with transfected cells indicate that the increased expression is, at least in part, due to abnormal, constitutive transcription controlled by the *PHS-2* promoter. These results, coupled with animal studies, epidemiological observations, and clinical trials suggest that abnormal induction of *PHS-2* is an early event in colon carcinogenesis and that compounds derived from its catalytic action play an important role in this process. These may be prostaglandins, products derived from other fatty acids such as linoleate (35), or derivatives of xenobiotics (2), which might function either as progression factors or as mutagens. The recent report of a mouse with targeted deletion of the *PHS-2* gene should help define the role of this enzyme in colon carcinogenesis (36).

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