Evidence that the familial adenomatous polyposis gene is involved in a subset of colon cancers with a complementable defect in c-myc regulation

(cell fusion/oncogene expression/restriction fragment length polymorphism/chromosome 5/tumor-suppressor gene)

MICHAEL D. ERISMAN*, JOAN K. SCOTT†, AND SUSAN M. ASTRIN‡
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

Communicated by Alfred G. Knudson, Jr., February 23, 1989 (received for review September 8, 1988)

ABSTRACT Human colorectal carcinomas frequently express elevated levels of c-myc mRNA in the absence of a gross genetic change at the c-myc locus. To test the hypothesis that these tumors are defective in a gene function necessary for the regulation of c-myc expression, we fused an osteosarcoma cell line that exhibits normal c-myc regulation with two colon carcinoma cell lines that express deregulated levels of c-myc mRNA. The levels of c-myc transcripts in all of the hybrid clones examined were normal and were induced normally by a mitogenic stimulus. Since rates of c-myc mRNA turnover in the colon carcinoma cells were found to be comparable to those in normal cells, increased message stability cannot account for the increased steady-state levels of transcripts. Our findings suggest that loss of function of a trans-acting regulator is responsible for the deregulation of c-myc expression in a major fraction of colorectal carcinomas. Analysis of restriction fragment length polymorphisms in tumor/normal tissue pairs from patients with primary colorectal lesions indicated that deregulation of c-myc expression in the tumors is correlated with frequent loss of alleles of syntenic markers on chromosome 5q; allele loss on 5q could be detected in 9 of 19 tumors expressing deregulated levels of c-myc mRNA, but not in any of 8 tumors expressing normal levels of c-myc RNA. Chromosome 5q is the region known to contain the gene for familial adenomatous polyposis, an inherited predisposition to colon cancer. These findings, together with the earlier finding that the colonic distribution of tumors exhibiting deregulated c-myc expression is similar to that reported for familial polyposis, provide evidence that loss of function of the familial adenomatous polyposis gene is involved in a subset of colorectal cancers in which c-myc expression is deregulated.

The deregulated expression of the c-myc oncogene has been recognized as an initiating or contributing factor in the development of chicken bursal lymphomas (1), murine plasmacytomas (2), and human Burkitt lymphomas (3). In each case, a cis-acting genetic change has been identified that affects the transcriptional activity of the c-myc locus; these changes are the result of insertional mutagenesis by viruses (chicken) or reciprocal chromosomal translocations involving the immunoglobulin loci (mouse and man). Increased expression of the c-myc locus also occurs in some human lung (4) and breast (5) carcinomas as a result of gene amplification that appears to occur during tumor progression.

A majority of human colorectal carcinomas also have significantly elevated levels of c-myc mRNA and protein (6–13). Twenty percent of the primary adenocarcinomas of the colon examined by Erisman et al. had levels of c-myc mRNA that were 5- to 40-fold higher than those observed in normal colonic mucosa (6), and the levels of the encoded protein (p64c-myc) observed in tumor-derived cell lines were correspondingly high (8). Gross change at the c-myc locus is rarely seen in these tumors and is limited, in such cases, to moderate levels of gene amplification (6, 8, 11). As a result, it was suggested (6) that colorectal carcinomas have a defect in another gene that normally acts in trans to regulate expression of the c-myc locus. This hypothesis predicts that c-myc regulation might be restored in hybrids between colon carcinoma cells that express deregulated c-myc and cells that regulate the gene normally. We have tested this prediction in the present work.

We have also explored the possibility of a relationship between c-myc regulation and the gene for familial adenomatous polyposis (FAP). FAP is characterized by the development of large numbers of polyps during the first 20–30 years of life and the subsequent appearance of frank colorectal adenocarcinoma as early as age 40. The “two-hit” mechanism, originally proposed by Knudson (14) to explain the sporadic and inherited forms of retinoblastoma (RB), can be applied to oncogenesis involving the FAP locus. That is, if the FAP gene acts phenotypically as a suppressor of neoplastic disease (15), or “anti-oncogene” (16), the inheritance of a defect in one copy would result in a predisposition to cancer (17), since loss or mutation of the other allele would lead to oncogenesis. The possibility that this gene is involved in a subset of tumors that express deregulated c-myc arose from the earlier finding that, like primary tumors in individuals with FAP, a disproportionate number of colorectal tumors with elevated levels of c-myc mRNA arise in the left (descending) colon (7, 9).

We have therefore sought further independent evidence for a correlation between loss of FAP gene function and c-myc deregulation. Linkage analyses in polyposis probands (18, 19) have localized the FAP locus (APC, for adenomatous polyposis coli) to chromosome 5 near bands 5q21–q22. In addition, allele loss on 5q has also been documented in >20% of sporadic (nonfamilial) colorectal carcinomas (20). In the experiments reported here, we tested whether allele loss on chromosome 5q is correlated with c-myc gene deregulation.

MATERIALS AND METHODS

Cell Culture and Isolation of Intraspacific Hybrids. The colon carcinoma (American Type Culture Collection) and osteosarcoma (Human Genetic Mutant Repository) cell lines were propagated in RPMI 1640 as described (8). A stable, G418-resistant transformant of the osteosarcoma cell line was presumptive of FAP, familial adenomatous polyposis; RB, retinoblastoma; HAT, hypoxanthine/aminopterin/thymidine.

*Present address: Clinical Research and Development, Smith Kline & French Laboratories, Swedeland, PA 19479.
†Present address: Biotechnology and Microbiology Division, Wyeth-Ayerst Laboratories, Radnor, PA 19087.
‡To whom reprint requests should be addressed.
prepared by electroporation (21, 22) of linearized pSV2neo plasmid DNA (23). Polyethylene glycol-mediated cell fusions (24) were carried out using trypsin-treated, late-logarithmic-phase cells that were incubated in suspension with phytohemagglutinin (40 μg/ml) for 30 min at 37°C prior to fusion (25) or by direct polyethylene glycol-mediated fusion of monolayer cocultures. Selection with hypoxanthine/aminopterin/thymidine (HAT) medium containing G418 sulfate (400–800 μg/ml) was imposed and continuously maintained beginning 24–36 hr after fusion. Individual clones were isolated and expanded for molecular analyses. Approximate population doubling times were determined from cell counts during logarithmic growth.

Measurement of mRNA Half-life. Early-log-phase cell cultures were treated with actinomycin D (5 μg/ml) 36–48 hr after their last feeding. Total cellular RNA was recovered from treated cells at specific times after addition of the drug, and dot blots were prepared as described below. The relative abundance of c-myc mRNA was determined by scanning densitometry of dot blots, and the rate of turnover was estimated by regression analysis of the relative densities obtained.

RNA and DNA Analysis. Total cellular RNA was recovered from late-log-phase cell cultures and primary tumor tissue lysed in guanidinium isothiocyanate and fractionated on cesium chloride cushions as described (8). Dot blot and Northern blot (26) analyses were carried out with glyoxalated total cellular RNA that was hybridized with a 32P-labeled probe for exon 3 of the human c-myc gene (6, 8). A 32P-labeled β2-microglobulin probe (27) was used as a control for RNA integrity (6). Methylene blue staining of rRNA and RNA size standards (Bethesda Research Laboratories) on Northern blots was carried out by published methods (28). Total cellular DNA was recovered from primary tumor tissue as described (6). Southern blot (29) analysis of HinfI-digested tumor DNAs was carried out exactly as described previously (6) with a 32P-labeled human minisatellite probe called λMS8 (D5S43) (30), which hybridizes to sequences on chromosome 5 (5q34–qter). The relative densities of the bands representing polymorphic alleles observed in Southern blots hybridized to this probe were determined by a computerized scanning densitometer. Loss of heterozygosity in informative cases was defined as ≥50% loss of a given band intensity relative to that of the companion allele when normalized to the ratio of band intensities in the matched normal control.

RESULTS

Characterization of Colon Carcinoma–Osteosarcoma Cell Hybrids. The human colorectal adenocarcinoma cell lines SW1116 and DLD-1 are characteristic of many primary colorectal tumors (6, 11, 12) and tumor-derived cell lines (8) in that (i) the steady-state levels of c-myc mRNA are 8–10-fold higher than those observed in tissue and cells from normal colonic mucosa and (ii) these levels are not significantly modulated in response to mitogenic stimuli. Neither cell line exhibits rearrangement or amplification of the c-myc locus. To determine whether the defect in c-myc regulation can be complemented in trans, we fused each of these cell lines with a human cell line that regulates the gene normally. The fusion partner used was a murine sarcoma virus-transformed, thymidine kinase-deficient, human osteosarcoma cell line (GM5887) resistant to the cytotoxic effects of G418. The GM5887 (HAT+, G418+) parent exhibits the low-level, steady-state expression of the c-myc gene typically observed in asynchronously growing populations of normal cells that have not been treated with mitogens (31). Fusion of this cell line with either of the colorectal cell lines (HAT−, G418−), followed by stringent selection in drug-containing medium, provided hybrid clones (HAT+, G418+) that were subsequently evaluated for steady-state levels of c-myc RNA and for the ability to modulate c-myc expression in response to a mitogenic stimulus. Drug-insensitive revertants of the parental lines have not been observed; thus, it seems likely that the stringently selected clones are colon carcinoma–osteosarcoma hybrids. The possibility of selective loss of a cis-activated allele(s) from the colon donor could not be addressed directly, since sufficient restriction fragment length polymorphisms appropriate for this analysis were not available. However, the genetic stability of inraspecific hybrids in general and the fact that identical results were obtained for c-myc expression in 12 clones from two different fusions make the fortuitous loss of c-myc alleles from the colon parent seem an unlikely explanation for the results. Moreover, the chromosomal complements of these two hybrids were found to approximate the sum of the parental karyotypes (data not shown).

Levels of c-myc Gene Expression in the Hybrids. To determine steady-state levels of c-myc mRNA for the parental cell lines and their hybrids, dot blots of total cellular RNA were hybridized to an exon 3 probe from the human c-myc gene (Fig. 1a). Levels of expression in SW1116 (lane B') and DLD-1 (lane B) cells from late-log-phase cultures were 8–10-fold higher than those observed in cell lines or tissue from normal colonic mucosa (8). Low, basal levels of expression

![Fig. 1. Levels of c-myc mRNA in the colorectal cell lines and hybrids.](image-url)
characteristic of normal, asynchronously growing cells were observed in the GM5887 parent (lane A). Twelve hybrid cell clones generated by the fusion of GM5887 with either SW1116 (A–B') or DLD-1 (A–B) were analyzed. The hybrids consistently expressed significantly reduced levels of c-myc mRNA, comparable to those in the GM5887 parent. These data provide evidence that the mechanisms controlling c-myc expression in GM5887 can act in trans to restore regulation of the c-myc loci from the colon carcinoma parents.

Northern blots of 10 μg of total cellular RNA (Fig. 1b) confirmed this result. The significantly higher levels of c-myc mRNA observed in the parental colon carcinoma cell lines were not observed in the hybrids. Basal levels of normal-sized, 2.4-kb c-myc transcripts were observed in the GM5887 and hybrid cell lines. Hybridization with a β2-microglobulin probe (27) showed that the levels of β2-microglobulin mRNA in GM5887, SW1116, and two of their hybrids were similar (Fig. 1c), indicating that the observed reduction in c-myc mRNA in the hybrids was not the result of a generalized diminution of mRNA levels. Expression of the β2-microglobulin gene in the DLD-1 cell line was reproducibly lower than in the other parental lines and hybrids examined. The integrity of RNA in each sample was confirmed by methylene blue staining of rRNA (Fig. 1d). The staining also indicated that the relative amount of RNA in each lane was the same.

**Regulation of c-myc Gene Expression in the Hybrids.** The reduced expression of the c-myc gene in the hybrid cell lines is apparently the result of restoration of normal regulation of the gene, rather than complete suppression of expression. Cultured fibroblasts and epithelial cells with properly regulated levels of c-myc mRNA undergo a transient increase in expression after mitogenic stimulus (31). As shown in Fig. 2, the osteosarcoma cell line, GM5887, exhibited such a transient increase (≥10-fold) within 2 hr of being treated with trypsin and replated at reduced density in fresh, serum-containing medium. Within one population doubling time, expression of the c-myc locus in the growing culture returned to basal levels. In contrast, the colorectal cell lines, DLD-1 and SW1116, expressed elevated levels of c-myc mRNA constitutively and underwent a minor (∼2-fold) response to mitogenic stimulus. Hybrid 31h33mC6, which was generated by the fusion of GM5887 and SW1116, expressed c-myc mRNA at basal levels in late-log-phase cultures (Fig. 1) and exhibited a transient increase in c-myc expression in response to mitogenic stimulus, similar to that observed with the GM5887 parent (Fig. 2). Thus, fusion resulted in the reestablishment of the control of c-myc expression that is characteristic of normal cells in culture.

**Measurement of mRNA Stability in Parental Cell Lines.** We have begun to investigate the molecular mechanism of deregulation of c-myc expression by examining the rate of c-myc mRNA turnover in the parental colon cell lines. The half-life of c-myc mRNA in actinomycin D-treated, log-phase cultures of SW1116 and DLD-1 was measured in two separate experiments. Both cell lines have average c-myc mRNA half-lives of ∼26 min (data not shown). These values are comparable to those reported for other normal and neoplastic human cells (10–20 min; ref. 33) but considerably shorter than the very extended half-lives (60–90 min) of truncated myc transcripts in murine plasmacytomas (34). The half-life determined for the GM5887 parent under identical conditions is ∼38 min (data not shown). Given that the half-life of c-myc mRNA in the colon carcinoma cells is shorter than that measured for the GM5887 parent, changes in posttranscriptional stability of the message seem unlikely to account for the increased steady-state mRNA levels. These findings suggest that the “re-regulation” of expression observed in the hybrids must occur at the level of synthesis or processing, rather than degradation of transcripts.

**c-myc Deregulation and Allele Loss on Chromosome 5.** It has been reported (20) that 23% of sporadic colorectal carcinomas show allele loss on chromosome 5q, the chromosome to which the FAP gene maps. This finding has led to the speculation that loss of FAP gene function may be a crucial step in the development of a major fraction of colorectal tumors. What is the relationship between loss of FAP gene function and c-myc deregulation?

We used a locus-specific, minisatellite probe (AMS8) that maps distal (5q34–qter) to the FAP gene (30) to assay for allele loss on 5q in a panel of colorectal tumors; DNA samples from tumor/normal tissue pairs were available for this analysis. Levels of c-myc mRNA in each sample were determined as previously reported (6, 7, 9). Of the 39 cases examined, 26 (67%) had levels of c-myc mRNA ≥5-fold higher than in normal mucosa from the same patient; 13 (33%) had levels of mRNA ≥2-fold higher than corresponding controls.

Tumor and normal DNAs from these 39 cases were digested with the restriction endonuclease Hinfl, and Southern blots of these digests were hybridized to 32P-labeled AMS8 probe. The region detected by this probe is extremely polymorphic; two alleles can be distinguished in ∼90% of individuals due to differences in location of restriction enzyme sites (20). Twenty-seven of the 39 cases we examined (69%) were informative, i.e., exhibited two distinguishable alleles in digests of DNA from normal tissue. The difference between 69% and 90% could be statistical or could reflect real population differences (United States versus United Kingdom). Loss of heterozygosity in the tumors from these 27 cases was judged on the basis of allele loss exceeding 50% in the tumor sample when normalized to the corresponding normal control. Computerized scanning densitometry was used for the quantitation (data not shown). Although allele loss is presumed to be complete in the tumor cells, contamination of the tumor sample with normal cells causes a diminution rather than absolute loss of signal for one allele, as reported by others (20).

Tumors from eight of the informative cases had levels of c-myc mRNA that were not elevated (≥2-fold higher than normal mucosa). None of these tumors exhibited loss of heterozygosity by the above criterion (Fig. 3, top row). The remaining 19 tumors from informative cases had levels of...
c-myc mRNA that were elevated (≥5-fold higher than normal mucosa). Ten of these tumors exhibited no loss of heterozygosity (Fig. 3, middle row); however, 9 of the 19 tumors with high c-myc mRNA (47%) showed loss of one allele (Fig. 3, bottom row). Thus, all 9 tumors exhibiting allele loss of 5q expressed elevated levels of c-myc mRNA; no tumor expressing normal levels of c-myc mRNA showed allele loss.

Two FAP cases were also available for examination (Fig. 3). One of these (FAP 1, middle row) exhibited no loss of heterozygosity in primary tumor tissue resected from either the left or right colon. The tumors exhibited a 5- to 10-fold increase in c-myc mRNA. The second (FAP 2, bottom row) showed allele loss in one of two primary tumors recovered from the same resection of large bowel; both tumors exhibited a 3- to 4-fold elevation of c-myc gene expression.

DISCUSSION

Previous work showed that >80% of primary carcinomas of the left colon exhibit constitutively high levels of c-myc expression (6, 7, 9). We propose that this defect in regulation has an important oncogenic role in this tissue, as is already known to be the case in cancers of the immune system (1–3). Since the deregulation is not accompanied by amplification or rearrangement of the c-myc locus or by a significant change in stability of the message, we have suggested the involvement of a trans-acting regulator of c-myc transcription. In support of this view, we now show that the defect can be corrected by fusion with a cell in which c-myc expression is regulated. These findings provide evidence that lesions in trans-acting regulators can result in deregulation of c-myc.

The identification of these trans-acting genes and elucidation of their mechanisms of action are subjects of major interest.

The genetic predisposition to colorectal carcinoma (18, 19, 35), and the development of a major fraction of nonfamilial colorectal carcinomas (20), appears to involve the loss of function of the FAP gene. This gene is presumed to act in a recessive manner in oncogenesis, as is characteristic of the tumor-suppressor genes (15), or anti-oncogenes, as postulated by Knudson (16). Such genes have been shown to be involved in the development of Wilms tumor (36), RB (37–39), and other neoplastic diseases (40). The evidence for involvement of the FAP locus in sporadic colon cancer comes from the observation that >23% of colorectal carcinoma samples show allele loss on chromosome 5q (20), the chromosome to which the FAP gene locus maps. The loss represents a conversion to homozygosity for alleles on 5q, including the FAP locus. Presumably, the function of both copies of the FAP gene must be lost in order for a tumor to develop. It is thus assumed that one allele suffers a small local lesion (point mutation or small deletion) and that the second allele is lost either through a local lesion or by a more global event, such as large deletion or a loss of the entire chromosome (often followed by duplication of the mutant chromosome), or by somatic recombination leading to homozygosity of the defect. The global events result in loss not only of the normal FAP allele but also of heterozygosity for all the genes distal to it, including the locus to which the minisatellite probe used in this study hybridizes. That one of four tumors from FAP patients showed allele loss on 5q is consistent with the idea that loss of FAP gene function can occur both by a gross mechanism and by a local lesion.

It is significant that 9 (47%) of the 19 tumors with elevated c-myc expression showed loss of heterozygosity of 5q markers, whereas none of the 8 tumors with normal levels of c-myc expression showed allele loss. The value of 47% is very close to the 50% allele loss observed for the RB and Wilms tumor genes, where it is presumed that loss of gene function occurs in all tumors but that the type of mechanism that would generate loss of heterozygosity for syntenic markers operates in only about 50% of cases. By analogy, we propose that all cases of c-myc deregulation are accompanied by a mutation.
or loss of both copies of a critical anti-oncogene located on chromosome 5q. Our colon tumor data, in conjunction with the observation that the four tumors from FAP patients we analyzed expressed elevated levels of c-myc mRNA, lend support to this proposal. It is possible that there is a causal relationship between loss of FAP gene function and c-myc deregulation. The FAP gene product may act to regulate c-myc either directly or through another gene or product. Alternatively, there may not be a causal relationship between the two, but both may be required for oncogenesis in a subset of colon cancers.

The possibility that oncogenes and anti-oncogenes might interact has been a topic of considerable interest and speculation. A model has recently been proposed (41) in which the interaction occurs entirely at the protein level—i.e., a protein encoded by an oncogene (the adenovirus EIA gene) binds to a protein encoded by an anti-oncogene (the RB gene). The RB gene codes for a 110-kDa nuclear phosphoprotein (42) whose function appears to be necessary for the normal control of cell proliferation. It has been suggested that binding of the EIA protein to the RB gene product interferes with its normal function and therefore, like loss of the RB gene, leads to uncontrolled growth and tumor formation (41). We suggest that the FAP gene product, like the RB gene product, is necessary for control of cell proliferation and, further, that loss of FAP gene function is correlated with deregulated c-myc expression. The mechanism by which the RB product controls cell proliferation is not known. It does, however, appear to be a DNA-binding protein (42), and may therefore function to regulate directly a gene, like c-myc, whose product is necessary for cell division.

There is a subset of colon carcinomas in which c-myc expression is not deregulated. In addition, not all hereditary colon cancer involves the FAP locus (43); therefore, this locus is probably not involved in all sporadic colon cancer. We surmise that the tumors that express regulated c-myc represent a distinct subgroup that involves somatic mutation at a site other than the FAP locus—i.e., at the site of an anti-oncogene other than FAP, possibly the hereditary nonpolyposis gene.

In conclusion, our data provide evidence that loss of function of a trans-acting regulator of c-myc transcription is a mechanism of c-myc deregulation in human colon carcinoma. In addition, there is a correlation between this deregulation and loss of heterozygosity on chromosome 5q, the chromosomal location of the FAP gene. Whether there is a causal relationship between the FAP gene product and trans regulation of c-myc remains to be determined.

We gratefully acknowledge the cooperation of the Departments of Surgery and Pathology of the Fox Chase Cancer Center; the gift of AM85 DNA from Dr. A. Jeffreys (Northwich Cheshire, U.K.); densitometric analysis of Southern blots by D. Lipton; karyotype analysis by H. Punnett; critical review of the manuscript by J. Taylor, K. Buetow, L. Cohen, N. M. Scalka, E. Rose, and A. Knudson; preparation of the figures by the Special Services group at the Institute for Cancer Research; and preparation of the manuscript by Donna Hnosko, Annmari Shepherd, and Gloria Szymanski. This work was supported by Grant CA40636 from the National Institutes of Health to S.M.A., by Core Grant CA06927 and Biomedical Research Support Grant RR05539 to the Fox Chase Cancer Center, and by an appropriation from the Commonwealth of Pennsylvania.