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Expression of c-*myb* Protooncogene and Other Cell Cycle-related Genes in Normal and Neoplastic Human Colonic Mucosa[1]

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ABSTRACT

The expression of c-*myb*, c-*myc*, histone H3, and ornithine decarboxylase genes was examined by Northern blot analysis in the normal and neoplastic mucosa of ten subjects affected by colon cancer. The mRNA levels of c-*myb* protooncogene were detected at low levels in all normal samples but were increased in the neoplastic mucosa of six cases in comparison to the normal counterpart. In five of these six cases the mRNA levels of c-*myc*, histone H3, and ornithine decarboxylase mRNAs were also increased, suggesting that there is a relation between the high expression of c-*myb* and the fraction of cycling neoplastic cells.

INTRODUCTION

The normal physiological function of the protooncogene c-*myb* (1), the cellular homologue of v-*myb*, the avian myeloblastosis virus-transforming gene (2), is still largely unknown.

Studies of its expression in the RNAs derived from different tissues in chickens and mice (3, 4) show that a mRNA species hybridizable with c-*myb* gene partial sequences is present in fairly detectable amounts only in cells of the hemopoietic lineages at early stages of differentiation. Similar results were obtained studying the c-*myb* expression in cellular lines derived from human tumors (5, 6) as well as in fresh tissues of different human malignancies (7, 8). On the basis of these observations the hypothesis of a functional relation between c-*myb* and the early stages of hemopoietic differentiation was suggested. Further observations made in murine leukemic myeloid cells induced to differentiate (9) as well as in human normal lymphocytes stimulated with mitogens (10) permit the hypothesis of a possible relation of c-*myb* expression with cell proliferation rather than with the differentiation pathways.

Studies of the variations of c-*myb* expression in chicken fresh and cultured cells (11) show that the expression of this protooncogene is in fact cell-cycle dependent in several, not only hemopoietic, cell types; however, in the immature thymocytes the observed high levels of c-*myb* mRNA are not linked to proliferation.

On the other hand, a high c-*myb* expression was found in two cell lines derived from a single human colon adenocarcinoma (12) and later in several cell lines derived from human small cell lung cancers (13). These latter results confirm the possibility that c-*myb* has a function not limited to hemopoietic differentiation, although these observations were done in cell lines, so that the results cannot be simply transferred to fresh tissues. This caution holds particularly in the case of colon carcinoma cell lines, in which the c-*myb* overexpression is associated with a gene amplification related to the presence of giant marker chromosomes not normally found in primary colon carcinoma (12).

To ascertain the presence of c-*myb* expression in primary human colon cancers we examined the total RNAs derived from fresh tissues of different cases together with samples of the normal colonic mucosa of the same subjects.

In the same RNAs, we examined also the level of expression of c-*myc*, histone H3, and ornithine decarboxylase genes, in an attempt to establish possible relations between c-*myb* expression and the expression of these cell cycle-related genes. The experimental material allowed us to investigate the level of c-*myb* expression in a normal nonhemopoietic fresh tissue as well as in a tumor considered the result of a multistep carcinogenic process (14).

In this tumor the activation and overexpression of two other oncogenes, K-*ras* (15) and c-*myc* (16, 17), have been reported, at least in the majority of the cases.

MATERIALS AND METHODS

Tissue Samples. Ten colon specimens located in all sections of the colon were obtained directly after surgical resection and processed on ice. They were opened, and the contents were rinsed off with cold phosphate-buffered saline. Tissue samples were taken from the tumor as well as from the normal areas. Ulcerated and necrotic tissue was dissected of the tumor tissue, and the submucosa and muscularis were removed from normal tissue. Thus, only the intact tumor and normal mucosa were used for RNA extraction. Samples were taken from 10 adenocarcinomas and 10 normal colons from the same subjects. They were immediately stored in liquid nitrogen until use in about 1 wk.

Tissue Samples for Microscopy. Whole thickness tissue slices were taken from the tumor as well as from normal mucosa for microscopy. They were fixed in buffered formalin, embedded in paraffin, and routinely stained with hematoxylin-eosin for histological diagnosis. All tumor samples were typed histologically as moderately differentiated adenocarcinomas. No signals of neoplastic invasion were detected in normal samples by routine examination, including carcinoembryonic antigen and high-iron diamine researches.

Nucleic Acids Extraction. Total cellular RNA was extracted from colon tissue according to the method of Frazier *et al.* (18). The DNA extraction was performed essentially as described by Gross-Bellard *et al.* (19).

Molecular Probes. To analyze c-*myb* expression, the plasmid F8, containing an insert corresponding with the 3′-terminal of the human c-*myb* protooncogene (20), was used. The plasmid pMC 41.3, containing an insert corresponding to the 3′-terminal of the human c-*myc* protooncogene (21), was used for detection of c-*myc* expression.

The histone gene expression was examined using the plasmid pST 519, containing a H3 human histone gene sequence (22). The ODC[4] gene expression was studied using the pODC 934 plasmid, containing an insert corresponding to a mouse ODC complementary DNA (23). The β-actin gene expression was studied with a human complementary DNA clone (24). Recombinant plasmid DNAs were isolated and purified by CsCl centrifugation following standard procedures (25).

Blotting and Hybridization Procedures. Total cellular RNA was de-

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[4] The abbreviation used is: ODC, ornithine decarboxylase.

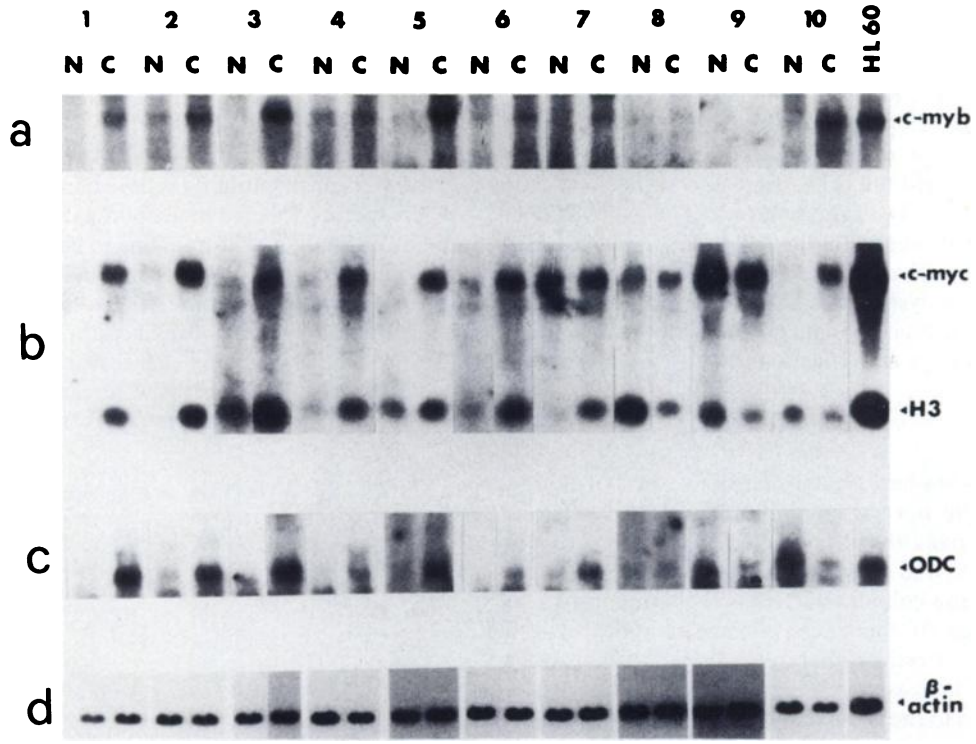

Fig. 1. Northern blot analysis of the RNAs extracted from normal (*N*) and neoplastic (*C*) colonic mucosa of ten subjects with moderately differentiated human colon adenocarcinoma. Fifteen μg of total cellular RNA were hybridized, as described in "Materials and Methods," with cloned probes, labeled by nick-translation (29), representing portions of the human cellular genes c-*myb* (*a*, plasmid pF8), c-*myc* (*b*, plasmid pMC 41.3), histone H3 (*b*, pST 519), the mouse ODC gene (*c*, pODC 934), and β-actin (*d*, plasmid pM1). The hybridizations with the β-actin probe were performed as controls of the amount of RNA present and reacting on each *lane*.

natured with 6.3% formaldehyde and 50% formamide and then fractionated (20 μg per lane) on 1.2% agarose gel in 20 mM MOPS sodium salt, 5 mM sodium acetate, 1 mM sodium EDTA, pH 8.0, and 6.6% formaldehyde. The RNA was transferred to a Gene-Screen membrane (New England Nuclear) by the electroblotting procedure described by Bittner *et al.* (26).

The DNA samples, after digestion with restriction enzymes, were run in 0.8% agarose gel and then transferred to a Gene-Screen membrane with a standard Southern blot procedure (27).

The prehybridization, hybridization, and washing procedures were performed under standard conditions as described by Ferrari *et al.* (28). Radiolabeling of the plasmid DNA at high specific activity was performed by nick-translation essentially as described by Rigby *et al.* (29).

Densitometer scanning of the autoradiograms was performed using a Tesak VDC-501 computerized image analyzer. The accuracy and the linearity of the densitometer readings were tested by analyzing the X-ray films of the same Northern blots developed after different times of exposure.

Table 1 *Comparative analysis of the levels of c-myb, c-myc, histone H3, and ODC genes in neoplastic versus normal colonic mucosa*

The RNAs derived from each neoplastic and corresponding normal colonic mucosa were run on parallel lanes of the same gels. After hybridization with the ^{32}P-labeled probes a densitometer scanning of the autoradiograms was performed. The relative intensity of the bands was expressed as follows: +, the ratio of cancer *versus* normal was >2; −, the ratio was <0.5; and =, the ratio was between <0.5 and 2. Quantitation of the RNA content in the single lanes was performed by hybridizing the same blots to a β-actin probe [clone pM1; Gunning *et al.* (24)].

Case	c-*myb*	c-*myc*	Histone H3	ODC
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	=	+	+	+
7	=	=	+	+
8	=	=	−	=
9	=	=	−	−
10	+	+	−	−

RESULTS

Rather unexpectedly the hybridization of the RNAs derived from normal colonic mucosa with the c-*myb* probe yielded an autoradiographic signal sometimes barely detectable, but in other cases fairly clear (Fig. 1*a*). The same signal, corresponding to a RNA species of about 4.5 kilobases, when compared with the ribosomal subunits in Northern blots, was always clearly evident in the RNAs extracted from neoplastic mucosa (Fig. 1*a*). A comparison of the densitometric scanning of the autoradiograms (Table 1) showed that, in 6 (Nos. 1 to 5 and 10; Fig. 1*a*) of 10 cases, the level of expression of c-*myb* was between 2- and 7-fold increased in neoplastic mucosa with respect to the corresponding normal tissue of the same subject.

In 3 (Nos. 6 to 8) of 10 cases the increase was less than 2-fold, while in 1 case (No. 9) no detectable differences were observed.

The presence of c-*myb* expression in a nonhemopoietic tissue suggested a possible relation with cell proliferation. For this reason we examined, in the same cases, the level of expression of other genes, whose expression in normal cells is thought to be related to the entering of the cell in the cell cycle, as c-*myc* (30) or to the S phase of the cycle, as histone H3 (22, 31), or in a broader sense to the proliferative state of the cell, as ODC gene (32, 33). The analysis of c-*myc* expression (Fig. 1*b*) showed no changes in 3 cases (Nos. 7, 8, 9; Fig. 1*b*) and a more than 2-fold increase in neoplastic *versus* normal colonic mucosa in the remaining 7 cases (Nos. 1 to 6 nad 10; Fig. 1*b*). Six of these 7 cases are those in which an increase of c-*myb* was also observed, although the c-*myc* increase was always slightly higher than that of c-*myb*. In the remaining 3 cases no differ-

ences were observed in c-*myc* expression between neoplastic and normal mucosa. The patterns of expression of histone H3 and ODC genes are closely parallel in our analysis (Fig. 1, *b* and *c*). In 7 (Nos. 1 to 7; Fig. 1, *b* and *c*), of 10 cases there is a clear increase of both H3 and ODC RNAs in neoplastic *versus* normal mucosa, whereas in the remaining 3 cases (Nos. 8 to 10), the expression of histone H3 is decreased in the neoplastic tissue in comparison to the normal mucosa. The expression of ODC mRNA is also decreased in the neoplastic tissue of Cases 9 and 10, while it is unchanged in Patient 8.

In Southern blot analysis of the DNAs derived from normal and neoplastic colonic mucosa (data not shown) we did not find rearrangements nor clear amplifications of the c-*myb* gene.

DISCUSSION

The presence of a low but certainly detectable level of expression of c-*myb* in the normal colonic mucosa of 10 subjects suggests that, in humans, the physiological role of this protooncogene is not restricted to cells of hemopoietic lineages. Moreover, the fact that the colonic mucosa is a continuously self-renewing tissue suggests that there is some relation between c-*myb* expression and cell proliferation. In 6 of 10 cases we found a substantial increase in c-*myb* expression in neoplastic mucosa as compared to the normal one. Since only intact tumor and normal mucosa were used for RNA extraction it seems very unlikely that the detection of c-*myb* transcripts was due to other cell types besides epithelial cells. It should be also noticed that the levels of c-*myb* mRNA are essentially undetectable in peripheral blood mononuclear cells before induction with phytohemagglutinin or interleukin 2 (10, 34). Therefore, even if lymphocytes and macrophages were present in our tissue samples, they could hardly contribute to the detection of c-*myb* transcripts.

Our findings show that a frequent increase in the expression of c-*myb* takes place not only in cultured cells but also in primary tissue of human colon neoplasia in the absence of a clearly detectable amplification of the c-*myb* locus. This phenomenon could be related to the fraction of proliferating cells rather than to the neoplastic process per se.

For this reason, we decided to compare directly the mRNA levels of c-*myb* gene with the expression, in the same RNA populations, of the histone H3 mRNA. Since the expression of the histone genes is restricted to the S phase of the cell cycle, it provides a good estimation of the growth fraction (35). The comparison was extended also to the expression of ODC mRNA, as an indication of the level of polyamines metabolism, which has been related to the proliferation rate of human colon cancer (36). In 5 of 6 cases c-*myb* expression increases in the RNA populations in which both histone H3 and ODC are also clearly increased. This behavior, along with the existence of colon cancers in which no increase of c-*myb* mRNA is detectable, allows us to suggest that the expression of this gene is related to cell proliferation rather than to neoplastic transformation. The frequency of an increased expression of c-*myc* mRNA in our cases (~70%) is in very good agreement with recently reported data in human colon cancers (16). In 6 of 7 of our cases the increase of c-*myc* mRNA expression is accompanied by an increase of histone H3 and ODC mRNAs. Therefore our findings make uncertain the alleged tumor specificity of c-*myc* overexpression which, instead, may simply reflect the increased fraction of cycling cells, as already pointed out (17).

If we assume that the expression of c-*myb* as well as c-*myc* genes plays a physiological, although still undefined, role in normal cell proliferation, we expect to find high expression of these genes in neoplastic cells, when these cells have a proliferative rate higher than the normal ones. Cases 8 and 9 of Table 1 in which a decreased expression of H3 and ODC genes comes with an unchanged expression of c-*myb* and c-*myc* protooncogenes seem to support this view.

However, the hypothesis is based on the assumption that the mRNA species detected in neoplasias are structurally and functionally normal, but this remains to be demonstrated.

In conclusion our findings provide strong evidence that the expression of c-*myb* is not restricted to cells of the hemopoietic tissue but occurs also in normal epithelial cells and suggest that the expression of c-*myb* is in general growth regulated in normal and neoplastic epithelial cells as already shown for normal and leukemic human lymphocytes (10, 37).

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