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Rearrangement at the 5′ End of Amplified c-*myc* in Human COLO 320 Cells Is Associated with Abnormal Transcription

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The proto-oncogene c-*myc* is amplified in sublines of human COLO 320 cells carrying either homogeneously staining chromsomal regions or double minutes. COLO 320 cells carrying homogeneously staining chromosomal regions have 15 to 20 copies of an apparently normal c-*myc* allele and 1 to 2 copies of an abnormal c-*myc* allele lacking exon 1 and express high levels of a normal c-*myc* mRNA 2.5 kilobases in size. COLO 320 cells carrying double minutes have about 25 copies each of the normal allele and the abnormal allele but express preferentially an abnormal c-*myc* mRNA 2.2 kilobases in size. Nucleotide sequence analyses revealed that the break point of rearrangement resulting in the loss of exon 1 in the abnormal allele lies within a region frequently rearranged in human and murine B-cell tumors.

Amplification of cellular oncogenes is a correlate to malignant progression of certain types of human tumors (for a review, see K. Alitalo and M. Schwab, Adv. Cancer Res. in press). Structural analyses have revealed that the topography of the amplified oncogene, when compared with that of the single-copy counterpart in normal cells, is unaltered in the majority of tumors. Exceptions to this rule include intragenic rearrangements of c-*abl* in human chronic myeloid leukemia cell line K-562 (4) and of c-*erbB* in human epidermoid carcinoma cell line A431 (17) and several human glioblastomas (9).

The human cell line COLO 320, which is derived from a colonic carcinoma composed of neuroendocrine cells (APUDoma; 12), carries amplified c-*myc* that is localized in double minutes (DMs) and homogeneously staining chromosomal regions (HSRs; 2, 10). Sublines have been established carrying either one of these chromosomal abnormalities. Preliminary analyses indicated that COLO 320 contains an abnormal c-*myc* allele, in addition to the apparently normal c-*myc* allele (2).

For definition of the abnormal allele in greater detail, the DNA isolated from COLO 320 sublines carrying either DMs (COLO 320-DM cells) or HSRs (COLO 320-HSR cells) and, for comparison, from human cell line HL-60 and skin fibroblasts was digested with restriction endonucleases, fractionated through agarose gels, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled v-*myc* probe (18). DNA from all cell lines yielded upon digestion with restriction endonucleases a set of common fragments detectable with the v-*myc* probe, including a 13.5-kilobase-pair (kbp) *Eco*RI fragment, a 6.2-kbp *Eco*RI/*Xho*I fragment, 2.7- and 1.4-kbp *Sst*I fragments, and a 3.6-kbp *Eco*RI-*Eco*RV fragment (Fig. 1). These fragments conform to previously published descriptions of human c-*myc* (3). In addition, COLO 320 cells yielded abnormal restriction endonuclease fragments not seen in the DNA from HL-60 cells or fibroblasts, including a 9.5-kbp *Eco*RI fragment, a 3.8-kbp *Eco*RI-*Xho*I fragment, and a 3.3-kbp *Sst*I fragment. (The faint signals in Fig. 1, lane b, of the *Eco*RI-*Xho*I digest above the 6.2-kbp fragment and of the *Eco*RI-*Eco*RV digest above the 3.6-kbp fragment are not reproducible and presumably result from partial DNA cleavage.) The signal obtained for the abnormal allele was much lower in COLO 320-HSR cells than in COLO 320-DM cells. Serial dilutions of DNA suggested that the abnormal allele is present in COLO 320-DM cells in approximately 25 copies, roughly the same number at which the normal allele is present, and in COLO 320-HSR cells in approximately 1 to 2 copies, in addition to the roughly 15 to 20 normal c-*myc* copies (2; data not shown).

For further studies, the normal and abnormal c-*myc* alleles were molecularly cloned. DNA was partially digested with

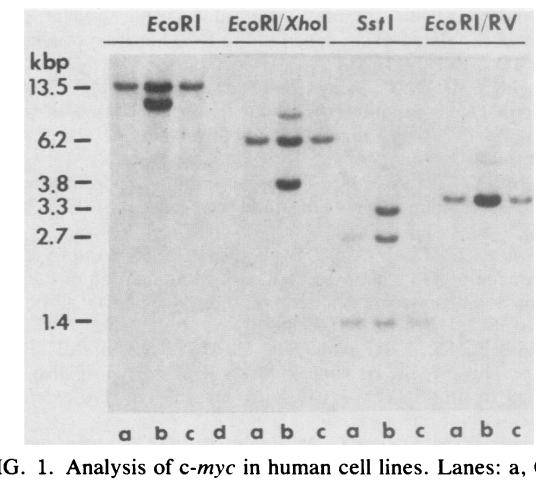

FIG. 1. Analysis of c-*myc* in human cell lines. Lanes: a, COLO 320-HSR cells; b, COLO 320-DM cells; c, HL-60 cells; d, skin fibroblasts. DNA was digested with restriction endonucleases, and 10 μg per lane was fractionated through an agarose gel. The DNA was transferred to nitrocellulose filters, to which ^{32}P-labeled v-*myc* (18) was then hybridized under stringent conditions (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–50% formamide, 42°C). The filters were washed at 50°C in 0.1× SSC and autoradiographed.

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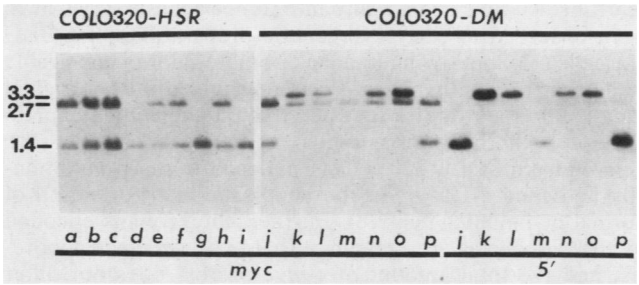

FIG. 2. Charon 30–c-*myc* recombinants. Lanes a through i, recombinants from COLO 320-HSR cells; j through p, recombinants from COLO 320-DM cells. DNA was isolated from the recombinants, digested with restriction endonuclease *Sst*I, and analyzed as described in the legend to Fig. 1 with ^{32}P-labeled v-*myc*. As the probe specific for the 5′ region of c-*myc* we used a 500-base-pair *Pst*I fragment containing a portion of exon 2 (3). The numbers at the left are in kilobase pairs.

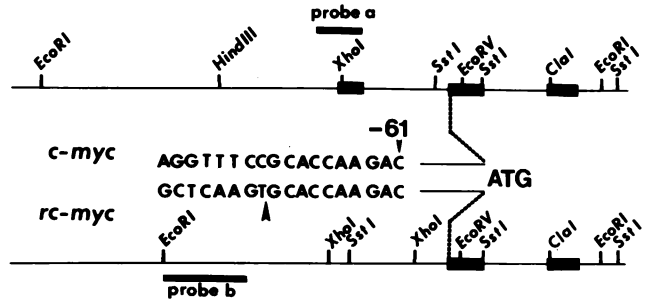

FIG. 3. Structure of normal c-*myc* and abnormal c-*myc* (c-*myc* and rc-*myc*, respectively) in COLO 320 cells. Solid black boxes indicate exons 1, 2, and 3. For application of probes a and b, see Fig. 4. The sequence compares the normal and abnormal c-*myc* alleles in the region of the recombination breakpoint. Nucleotide −1 is the first nucleotide 5′ flanking the ATG start codon.

restriction endonuclease *Mbo*I and cloned into bacteriophage Charon 30. DNA of 16 recombinants containing c-*myc* of the HSR and DM sublines was isolated and digested with restriction endonuclease *Sst*I. All recombinants from the HSR subline contained only the normal 2.7- and 1.4-kbp restriction fragments also predominantly seen in total genomic DNA (data not shown). In contrast, about 50% of the recombinants from the DM subline yielded the normal 2.7- and 1.4-kbp fragments, and the other 50% yielded the normal 2.7-kbp fragment and the abnormal 3.3-kbp fragment.

The c-*myc* gene is composed of three exons, of which only exons 2 and 3 encode the c-*myc* protein and are homologous to v-*myc* (3). Exon 2 at the 5′ end of the coding region is contained within the 1.4-kbp *Sst*I fragment which, however, does not contain exon 1; exon 3 is localized within the 2.7-kbp fragment. When nitrocellulose filters containing DNA from recombinants of both the HSR and DM sublines were hybridized with the v-*myc* probe, both the 1.4- and 2.7-kbp as well as the abnormal 3.3-kbp fragments were detected (Fig. 2, lanes a through p). In contrast, when a filter carrying DNA from recombinants of the DM subline was hybridized with an exon 2 probe derived from c-*myc*, either the 1.4-kbp fragment of the normal allele or the 3.3-kbp fragment of the abnormal allele yielded a signal. These results showed that the molecular alteration resulting in the 3.3-kbp fragment is localized in the 5′ region of c-*myc* or its 5′-flanking region.

We used nucleotide sequencing to analyze the molecular alteration resulting in the abnormal c-*myc*. The 3.3-kbp *Sst*I fragment of the abnormal c-*myc* was digested with restriction endonuclease *Taq*I or *Sac*II and cloned into bacteriophages M13. Nucleotide sequencing was performed by the dideoxynucleotide method (15). Our analyses revealed that the abnormal c-*myc* does contain the normal translational start codon and that the DNAs upstream of the ATG codon of normal c-*myc* and abnormal c-*myc* are identical over 70 base pairs (Fig. 3). Starting at nucleotide −71, the sequences of normal c-*myc* and abnormal c-*myc* diverge.

For determining whether exon 1 is present at the 5′- end of the abnormal c-*myc* allele in COLO 320 cells, DNA of recombinant bacteriophages containing normal c-*myc* and abnormal c-*myc*, including approximately 5 kbp of flanking sequences upstream of the translational start codon, was digested with restriction endonuclease *Eco*RI, fractionated through agarose gels, and transferred to nitrocellulose filters. The filters were then hybridized with ^{32}P-labeled DNA containing exon 1 of the normal c-*myc* (Fig. 4, probe a). A signal was only seen in the lane containing the normal c-*myc* allele (lane c). Conversely, when a probe derived from the flanking DNA of abnormal c-*myc* was used (Fig. 4, probe b), only the abnormal c-*myc* allele was detected (lane r). (The faint signal in lane c is due to the incomplete removal of probe a from a previous experiment, as confirmed by independent analyses.) Additional studies (data not shown) established that the DNAs flanking the 5′ end of normal c-*myc* and abnormal c-*myc* are unrelated beyond the *Eco*RI restriction site. In conclusion, DNA encompassing exon 1 has been replaced by DNA of unidentified provenance.

Our previous analyses showed that c-*myc* mRNA is present in COLO 320-HSR cells and COLO 320-DM cells at high levels (2). Studies of other tumor cells carrying amplified cellular oncogenes have established that abnormal mRNA is present whenever the oncogene is structurally rearranged (4, 9, 17). We asked whether the molecular rearrangement of c-*myc* in COLO 320 cells results in the synthesis of an abnormal c-*myc* transcript. Polyadenylated RNA of COLO

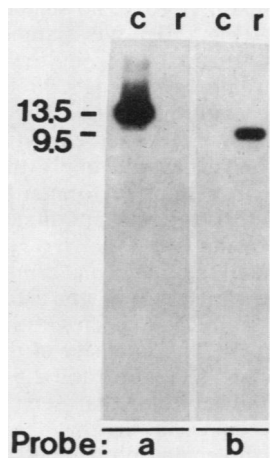

FIG. 4. Absence of noncoding exon 1 in abnormal c-*myc*. DNA of two recombinants containing normal c-*myc* (lanes c) and abnormal c-*myc* (lanes r) was digested with restriction endonuclease *Eco*RI, fractionated through agarose gels, and transferred to nitrocellulose filters. The filters were then analyzed with ^{32}P-labeled probes containing either exon 1 of normal c-*myc* (probe a) or DNA from the region flanking abnormal c-*myc* (probe b). The numbers at the left are in kilobase pairs.

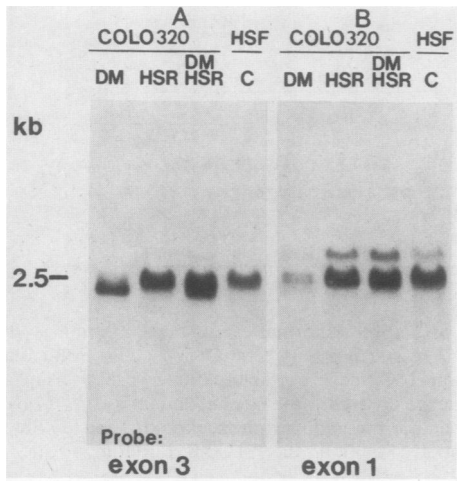

FIG. 5. Analysis of c-*myc* mRNA. Total RNA was isolated from cells lysed in sodium dodecyl sulfate as previously described (16), and polyadenylated RNA was further purified by oligo(dT)-cellulose chromatography. Polyadenylated RNA from COLO 320-DM cells and COLO 320-HSR cells (5 μg per lane) and from skin fibroblasts (HSF; C, comparison; 30 μg per lane) was fractionated through agarose gels in the presence of 2.2 M formaldehyde and transferred to nitrocellulose paper, to which ^{32}P-labeled probes containing exon 3 (A) or exon 1 (B) were then hybridized. In lanes DM/HSR, the RNA from the two cells lines was coelectrophoresed (5 μg each).

320-HSR cells and COLO 320-DM cells and, for comparison, from skin fibroblasts was fractionated through denaturing agarose gels and blotted onto nitrocellulose, to which a ^{32}P-labeled 1.2-kbp *Eco*RI-*Cla* fragment containing exon 3 of human c-*myc* was then hybridized (Fig. 5A). Abundant c-*myc* mRNA was detected in COLO 320 cells (Fig. 5). The size of the c-*myc* mRNA in COLO 320-HSR cells was not detectably different from that in skin fibroblasts. In contrast, in COLO 320-DM cells, we found predominantly a smaller transcript approximately 2.2 kilobases (kb) in size. Coelectrophoresis of RNA from HSR and DM cells confirmed the size difference (Fig. 5, lanes DM/HSR). Further, when we used the exon 1 probe, no signal was obtained for the 2.2-kb mRNA (Fig. 5B). We assume that the 2.2-kb mRNA species is transcribed from the abnormal allele, although we do not know the signals directing the initiation of synthesis of the abnormal RNA. The mature abnormal mRNA is substantially larger than expected as the result of the loss of exon 1. This would imply that transcriptional initiation signals map within the foreign DNA flanking the abnormal c-*myc*, but further structural studies are required to resolve this problem.

In addition to the 2.2-kb abnormal c-*myc* mRNA and the 2.5-kb normal c-*myc* mRNA, we consistently observed an approximately 3.0-kb mRNA species with both exon 1 and exon 3 probes (Fig. 5). The intensity of the signal obtained with the exon 1 probe relative to the 2.5-kb c-*myc* mRNA was considerably higher than that seen with the exon 3 probe. Preliminary data showed that the 3.0-kb mRNA is detectable with a molecular probe flanking the 5′ end of c-*myc* excluding exon 1 (M. Schwab and N. Hay, unpublished data). We are now analyzing the molecular basis for the presence of the 3.0-kb mRNA detectable both with the c-*myc* probe and with a probe from the region flanking the 5′ end of c-*myc*.

It is difficult at this point to assess how the combination of amplification, rearrangement, and preferential expression of rearranged c-*myc* could figure into tumorigenesis. DMs have been observed in early cytogenetic preparations of COLO 320 cells (12), but owing to a lack of material it is impossible to determine whether c-*myc* was rearranged in the original tumor. Previous studies revealed that on augmented expression of both the normal c-*myc* and the abnormal c-*myc* derived from COLO 320 cells is sufficient to assist mutationally activated c-Ha-*ras* in the neoplastic transformation of normal mammalian cells (8). Further, the proteins encoded by the two alleles are indistinguishable by gel electrophoresis, and the total amount of c-*myc* protein does not differ detectably between the COLO 320-HSR and COLO 320-DM cell lines (14). Nevertheless, the rearrangement of c-*myc* with a loss of the noncoding exon 1 seen in COLO 320 cells is interesting because the breakpoint occupies a position within the same region of c-*myc* involved in chromosomal translocations in many Burkitt lymphomas and murine plasmacytomas (for reviews, see references 7 and 11).

We were particularly intrigued to find that the major portion of c-*myc* mRNA in COLO 320-DM cells is abnormal despite the roughly equal copy numbers of the normal and abnormal alleles. The absence of normal c-*myc* transcripts in cells containing both a normal c-*myc* allele and a translocated c-*myc* allele has been observed in murine plasmacytomas and human Burkitt lymphomas (for a review, see S. Cory, Adv. Cancer Res., in press), although c-*myc* is not amplified in those tumors. Similarly, cells of lymphomas in transgenic mice produced by injection of c-*myc* genes linked to immunoglobulin enhancers express the enhancer-linked c-*myc* gene but not the endogenous c-*myc* gene (1). Three possible mechanisms have been proposed for the apparent preferential expression of the translocated allele in plasmacytomas and Burkitt lymphomas and that of the enhancer-linked c-*myc* in lymphomas of transgenic mice. One mechanism could be that a mature lymphocyte or plasma cell normally does not express c-*myc* and that this nonpermissiveness can be overcome by c-*myc* translocation (for a review, see Cory, in press). From this it would follow that the expression of c-*myc* in these cells is abnormal and might be related to the neoplastic phenotype. Another proposed mechanism is that c-*myc* expression activates a repressor that regulates transcription of the normal c-*myc* allele but not of the translocated c-*myc* allele (for a review, see reference 7). Another mechanism emerged when it was discovered that posttranscriptional processes regulating the stability of the mRNA can also contribute to the steady-state level of intracellular c-*myc* mRNA (5, 6). It has recently been reported that the abnormal c-*myc* mRNA in COLO 320-DM cells has a longer in vivo half-life than the normal c-*myc* mRNA (13). It is not clear, however, whether the lack of exon 1 alone is responsible for the increased stability of the abnormal c-*myc* mRNA in COLO 320-DM cells. It is also possible that sequences derived from the new DNA that are most likely situated at the 5′ end of the abnormal allele contribute to the increased stability of the abnormal c-*myc* mRNA.

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