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Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma

(double minute chromosomes/chromosome aberrations/retroviruses/tumorigenesis)

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ABSTRACT **Two human neuroendocrine tumor cell lines derived from a colon carcinoma contain either numerous double minute chromosomes (COLO 320 DM) or a homogeneously staining marker chromosome (COLO 320 HSR). We found amplification and enhanced expression of the cellular oncogene c-*myc* in both COLO 320 DM and HSR cells, and we were able to show that the homogeneously staining regions of the COLO 320 HSR marker chromosome contain amplified c-*myc*. From previous and present karyotypes, it appears that the homogeneously staining regions reside on a distorted X chromosome. Therefore, amplification of c-*myc* has been accompanied by translocation of the gene from its normal position on chromosome 8 (8q24). Because double minute chromosomes were features of primary cultures from the original tumor, it seems reasonable to suspect that amplification of c-*myc* may have contributed to tumorigenesis.**

The oncogenes of retroviruses arose by transduction of genetic loci (known as "cellular oncogenes") from normal cells (1). Because cellular oncogenes can resemble very closely their viral progeny, it has been postulated that cellular oncogenes might themselves contribute to neoplastic growth (reviewed in ref. 1). The postulate has gained some credence from recent discoveries that implicate both the enhanced expression (2, 3) and mutation (4, 5) of cellular oncogenes in specific forms of tumorigenesis.

The expression of cellular oncogenes could be augmented by at least two kinds of genetic change. Damage to DNA or insertion of foreign controlling elements (such as those contained in the genomes of retroviruses) could stimulate transcription from the genes, or amplification of the genes could increase the number of templates available for transcription. In cells selected for resistance to certain metabolic inhibitors, amplified genes have been found in two previously mysterious structures: (*i*) double minute (DM) chromosomes that lack centromeres and segregate unpredictably at cell division (6–8) and (*ii*) homogeneously staining regions (HSRs) of centromeric chromosomes (6, 9). DM chromosomes apparently embody unstable forms of amplified DNA and may be the predecessors to HSRs, which are generally stable (6, 10).

DM chromosomes and HSRs are recurrent features in malignant human tumors (11). Recent findings indicate that—like their counterparts in drug-resistant cells—both of these cytogenetic abnormalities may represent amplified DNA (12). We report here the amplification and enhanced expression of a cellular oncogene (c-*myc*) (13, 14) in human neuroendocrine tumor cells that contain either DM chromosomes or a marker chromosome with extensive HSRs.

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MATERIALS AND METHODS

Tumor cells were obtained from American Type Culture Collection. The recombinant plasmid clones containing chicken viral oncogene inserts have been described (15–18). Other oncogene DNA fragments were prepared from recombinant plasmids supplied by the following investigators: *fps* (C. Hammond), *yes* (M. Yoshida), *rel* (H. Temin), *mos* (C. van Beveren and I. Verma), *abl* (D. Baltimore), Ha-*ras* and Ki-*ras* (R. Ellis and E. Scolnick), and *fes* (C. Sherr). Radioactive DNAs were prepared with reverse transcriptase and either [α-^{32}P]dCTP or [^{3}H]dCTP to achieve specific activities of 2–5 $\times$ 10^7 cpm/μg and 1–2 $\times$ 10^7 cpm/μg of template, respectively. Procedures for transfer of RNA and DNA to nitrocellulose, hybridizations, and washings were those reported earlier (15), except that 40% instead of 50% formamide was used. The details of our assay for RNAs transcribed from c-*onc*s will be reported elsewhere.

Chromosomal spreads, trypsin-Giemsa staining (G-banding), and quinacrine hydrochloride staining (Q-banding) followed published procedures (19, 20). *In situ* hybridizations were performed as described by Harper and Saunders (21) except that, for v-*myc* probes, 40% instead of 50% formamide was used during hybridizations.

RESULTS

c-*myc* Expression Is Enhanced in COLO 320 Cells. COLO 320 cells were established from a carcinoma of the colon, but they have proven to be representative of neuroendocrine tumors known as APUDomas (containing amine precursor uptake and decarboxylase activities), which secrete serotonin, catecholamines, and parathyroid hormone (22). DM chromosomes were present in the initial subcultures of COLO 320 DM and have persisted in cells of the DM line for several years, but they are absent from the COLO 320 HSR cell line, which arose and became dominant in some subcultures of COLO 320 DM cells during growth *in vitro* (22). The HSR cells have acquired instead a marker chromosome, most of which stains homogeneously in G-banding (see Fig. 4 below).

We first studied the expression of 12 cellular oncogenes in the COLO 320 cells. Polyadenylylated RNA was isolated from the cells and transcribed into radioactive cDNA, which was then hybridized to DNA fragments of viral oncogenes bound to nitrocellulose filters. Of the oncogenes tested, only c-*myc* was

Abbreviations: APUDoma, a tumor containing amine precursor uptake and decarboxylase activities; DM, double minute; HSR, homogeneously staining region; kb, kilobases; kbp, kilobase pairs; G-banding, trypsin–Giemsa banding; Q-banding, quinacrine staining.

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abundantly expressed in COLO 320 HSR cells (Fig. 1*A*). By contrast, none of the oncogenes displayed enhanced expression in the colon carcinoma cells WiDr (23). Quantitative analyses showed that 320 HSR RNA is ≈25-fold enriched in *myc*-related sequences as compared to the RNA of WiDr cells. Similar experiments with RNA from the COLO 320 DM cells showed an increase of about 30-fold (data not shown).

c-*myc* RNA Is Transcribed from the Complete c-*myc* Gene. The size of polyadenylylated c-*myc* RNA in COLO 320 and other tumor cell lines was determined by using ^{32}P-labeled v-*myc Pst* I fragment as a hybridization probe (Fig. 1*B*). The 2.7-kilobase (kb) size and the signal intensity were similar to those of c-*myc* mRNA in HL-60 cells that also contain increased levels of c-*myc* RNA and DNA (26–29). Longer exposures of the autoradiogram detected a c-*myc* RNA of 2.7 kb in other tumor cells and in normal human fibroblasts (Fig. 1*B*). A similar size has been reported for c-*myc* RNA in normal tissues from several diverse species (30, 31).

The human genome contains one apparently complete c-*myc* gene and several putative c-*myc* pseudogenes, which retain homology only to part of a 5′ exon represented in v-*myc* (13, 14). The experiment shown in Fig. 2 demonstrates that the radioactive cDNA has homology also to a 3′ exon, which is missing from the truncated, putative c-*myc* pseudogenes. We conclude that the high levels of c-*myc* RNA in COLO 320 cells are transcribed from the complete c-*myc* locus.

c-*myc* Is Amplified in COLO 320 Genomes. We next asked whether amplification of the c-*myc* gene in the COLO 320 cells might account for its increased expression. The v-*myc* probe was used to compare c-*myc* sequences in genomic DNA from the COLO 320 DM and HSR cells and from other cells. Only faint bands corresponding to restriction fragments of c-*myc* loci were seen in DNA samples from control human cells (Fig. 3*A*, HSF lanes). The corresponding bands in COLO 320 were intensely labeled. In addition, bands were found with mobilities distinct from normal c-*myc* fragments (asterisks in Fig. 3 *A* and *B*). These variant fragments were only weakly labeled in blots of COLO 320 HSR DNA (Fig. 3 *A* and *B*) and were not seen at all in the analysis of DNA from HL-60 cells, in which the c-*myc* gene is amplified also (28, 29).

When the v-*myc*/c-*myc* hybrids were melted and the washed filter was probed similarly with the molecularly cloned Ki-*ras* oncogene fragment from Kirsten sarcoma virus, all lanes containing DNA derived from the above samples showed radioactive bands of similar size and intensity (Fig. 3*A*, Ki-*ras* lanes). We conclude that c-*myc* is amplified in COLO 320 cells.

The copy number of c-*myc* loci in DM and HSR cells was estimated by comparing the intensity of hybridization signals from serially diluted *Sst* I-digested cellular DNAs (Fig. 3*B*). The intensity of the signal from the 2.7-kb c-*myc* 3′ fragment was approximately equal with 1 μg of COLO 320 DM DNA, 1–2 μg of HSR DNA, 2 μg of HL-60 DNA, and 32 μg of human skin fibroblast DNA. We conclude that the haploid number for c-*myc* is 16 in HL-60 cells (in accord with previous estimates; see refs. 28 and 29), 32 in COLO 320 DM cells, and 16–32 in COLO 320 HSR cells.

Karyologic Examination of COLO 320 Cells. The chromosomes of G-banded COLO 320 HSR and DM were very similar to those of the original cell lines (22), including the modal chromosome number, the number of intact chromosomes, and the majority of marker chromosomes. However, a structural change was found in the large HSR marker chromosome, which apparently is derived from the X chromosome (22). Instead of one large homogeneously staining region in its long arm, the HSR chromosome now appeared to contain the Xp11 to Xp22 region with one remaining G band (Xq21) (Fig. 4 *A* and *C Insets*), which

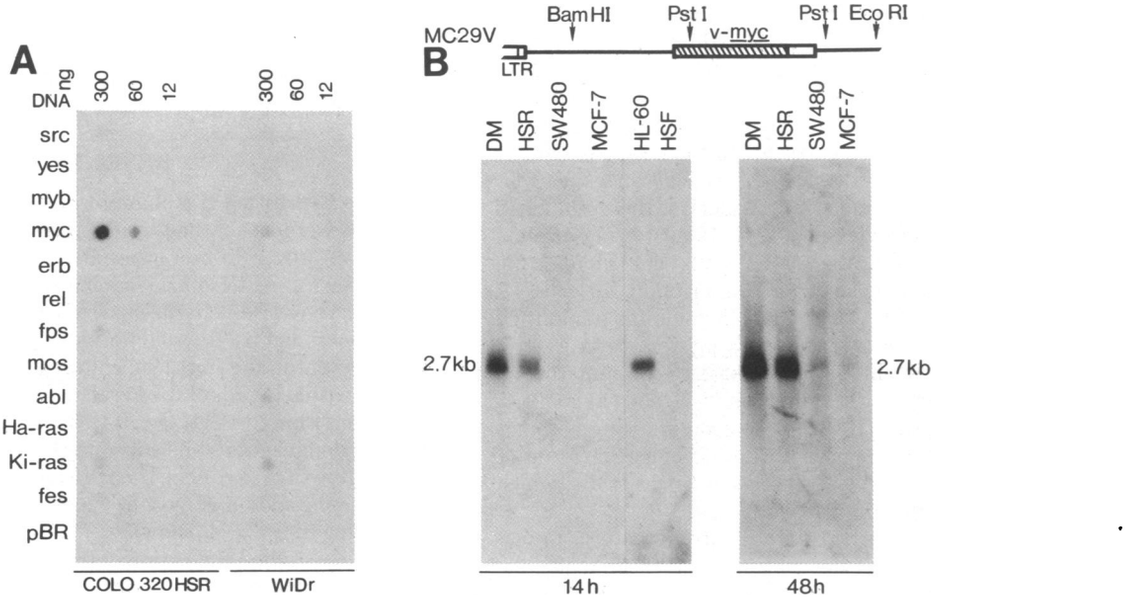

FIG. 1. (*A*) Expression of cellular oncogenes in COLO 320 HSR cells and WiDr colon carcinoma cells. Five microliters of different concentrations (2.4–60 ng/μl) of oncogene-specific recombinant plasmid DNA fragments (*src*, *yes*, *myb*, etc.) and control DNA (pBR322) were bound to nitrocellulose filters and hybridized to radioactive cDNA (3×10^6 cpm) transcribed from polyadenylylated RNA isolated from the cells. (*B*) c-*myc* RNAs in tumor cells. (*Lower*) Five micrograms of polyadenylylated RNAs from the COLO 320 DM and HSR cells, SW 480 colon carcinoma cells that also contain DMs (24), MCF-7 breast carcinoma cells, HL-60 leukemia cells, and human skin fibroblasts (HSF) were electrophoresed in a 0.8% agarose gel run in the presence of formamide, transferred to nitrocellulose, and hybridized with ^{32}P-labeled 3.5-kb *Bam*HI–*Eco*RI fragment of v-*myc* (15). The gel was exposed for 14 hr to compare the intensities of DM, HSR, and HL-60 bands and for 48 hr to see the size of *myc* RNA (about 2.7 kb) in other cells. (*Upper*) Diagram of the MC 29 provirus (MC29V) shows the topography of the restriction fragments *Bam*HI–*Eco*RI and *Pst* I (v-*myc* nucleotides 158–1676) used in this study. The protein coding region deduced from the nucleotide sequence (25) is hatched. LTR, long terminal repeat of the provirus.

showed bright Q-band fluorescence (Fig. 4*A*). The rest of this marker chromosome (Xp11-pter, Xq22-qter) stained homogeneously. The short arm of an X chromosome was involved in a translocation to form a new submetacentric marker. One copy of an intact X chromosome (marked X in Fig. 4*A*) also was present in the cells which were derived from a female patient.

Amplified c-*myc* Copies Map to Homogeneously Staining Chromosomal Regions. The chromosomal location of amplified c-*myc* sequences was studied by *in situ* hybridization. Restriction fragments of v-*myc* and nonrepetitive DNA from molecularly cloned c-*myc* of COLO 320 cells (unpublished data) were labeled with [^{3}H]dCTP by reverse transcription and hybridized to chromosome spreads of COLO 320 cells. The autoradiographic signal was measured as grains in photographic emulsion over individual chromosomes; the identity of the chromosomes was subsequently confirmed by Q-banding.

A representative metaphase spread with the v-*myc* radiographic grains is shown in Fig. 4*B Left*. A majority of all grains were located over the homogeneously staining regions of the large marker chromosome (Fig. 4 *A* and *B*.) This result was confirmed by hybridization in 50% formamide with radioactive human c-*myc* (Fig. 4*C*). The signal-to-noise ratio was improved with the human probe, allowing us to map c-*myc* exclusively to the homogeneously staining region of the HSR marker chromosome (Fig. 4*C Inset*).

In contrast to the abundant specific signal in the COLO 320 HSR chromosomal spreads, we failed to obtain an unequivocal localization of c-*myc* in the COLO 320 DM metaphase spreads (Fig. 4*D*). The negative result would suggest that amplified c-*myc* copies are distributed more randomly among the chromosomes of the COLO 320 DM cells—presumably in the DM chromosomes themselves.

DISCUSSION

Amplified c-*myc* Copies Originate from the Complete Human c-*myc* Gene. The human genome contains one complete c-*myc* gene and several related loci that may be truncated *myc* pseudogenes (14). Amplified c-*myc* copies in COLO 320 cells are derived from the complete c-*myc* gene because (*i*) fragments of amplified c-*myc* conform to the restriction map of cloned nor-

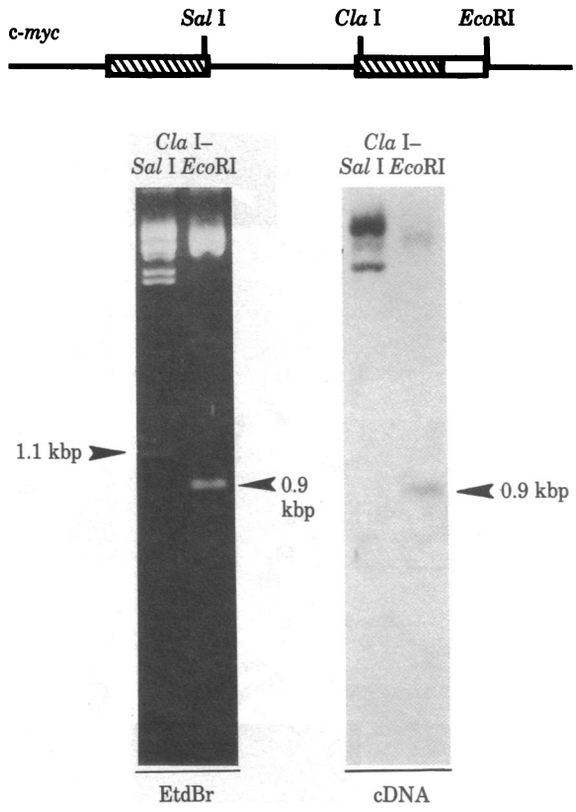

FIG. 2. Identification of the template for c-*myc* RNA in COLO 320 cells. Samples (1 μg) of polyadenylylated RNA from COLO 320 DM and HSR cells were pooled and copied into ^{32}P-labeled cDNA. (*Upper*) The c-*myc* plasmid was cleaved with *Cla* I and *Sal* I to liberate a 1.1-kilobase-pair (kbp) fragment containing an intron of c-*myc* (13) and with *Cla* I and *Eco*RI to obtain a 0.9-kbp fragment bearing an exon located 3′ of the intron (13), as shown diagrammatically at the top of the figure. (*Lower*) The fragments were separated by electrophoresis in a 1.7% agarose gel, transferred to nitrocellulose, and hybridized with radioactive cDNA.

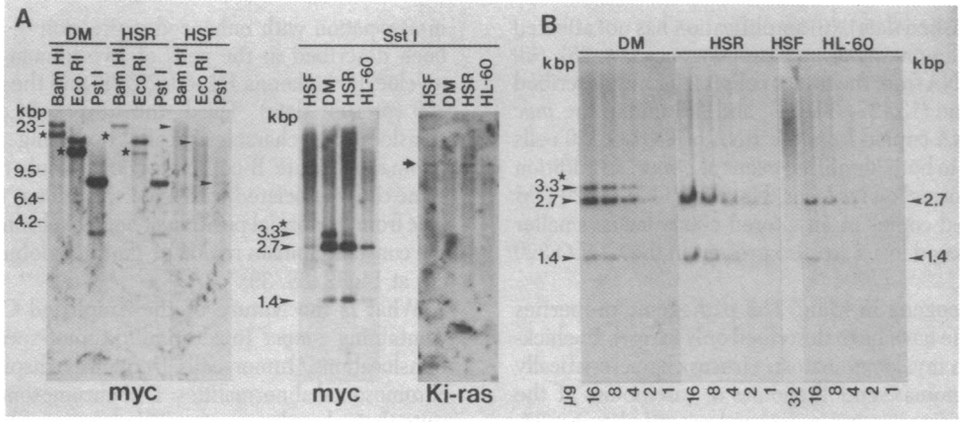

FIG. 3. Analysis of c-*myc* in COLO 320 cells. (*A*) DNA from the DM and HSR cells and from HSF and HL-60 cells was digested with enzymes *Bam*HI, *Eco*RI, *Pst* I, and *Sst* I. Fragments (25 μg) were analyzed by Southern blotting with the radioactive v-*myc Pst* I probe (see Fig. 1). Subsequently, the filter containing the *Sst* I lanes was treated with alkali to remove the hybridized DNA and rehybridized with a 1.0-kbp fragment of Ki-*ras* sequences from HiHi-3 plasmid (ref. 32; a gift from R. Ellis and E. Scolnick). Unlabeled arrowheads point to (single-copy) c-*myc* signals from human skin fibroblasts. Note that DM and HSR lanes also contain major labeled fragments (marked by asterisks) not seen in other cells. Arrow adjacent to the Ki-*ras* panel points to the major fragment (about 10 kbp) of Ki-*ras* cDNA. Longer exposure caused other bands to be seen also, all of which were of equal intensity in the different lanes. (*B*) Estimation of c-*myc* copy number in COLO 320 cells. Various amounts of *Sst* I-digested DNA from the cells were probed with the v-*myc Pst* I fragment, as in the experiment of Fig. 3*A*. Note that the ratio of 2.7-kbp and 1.4-kbp fragments is changed in the DM cells as compared with HSR and HL-60 cells and that there is an additional amplified fragment at 3.3 kbp.

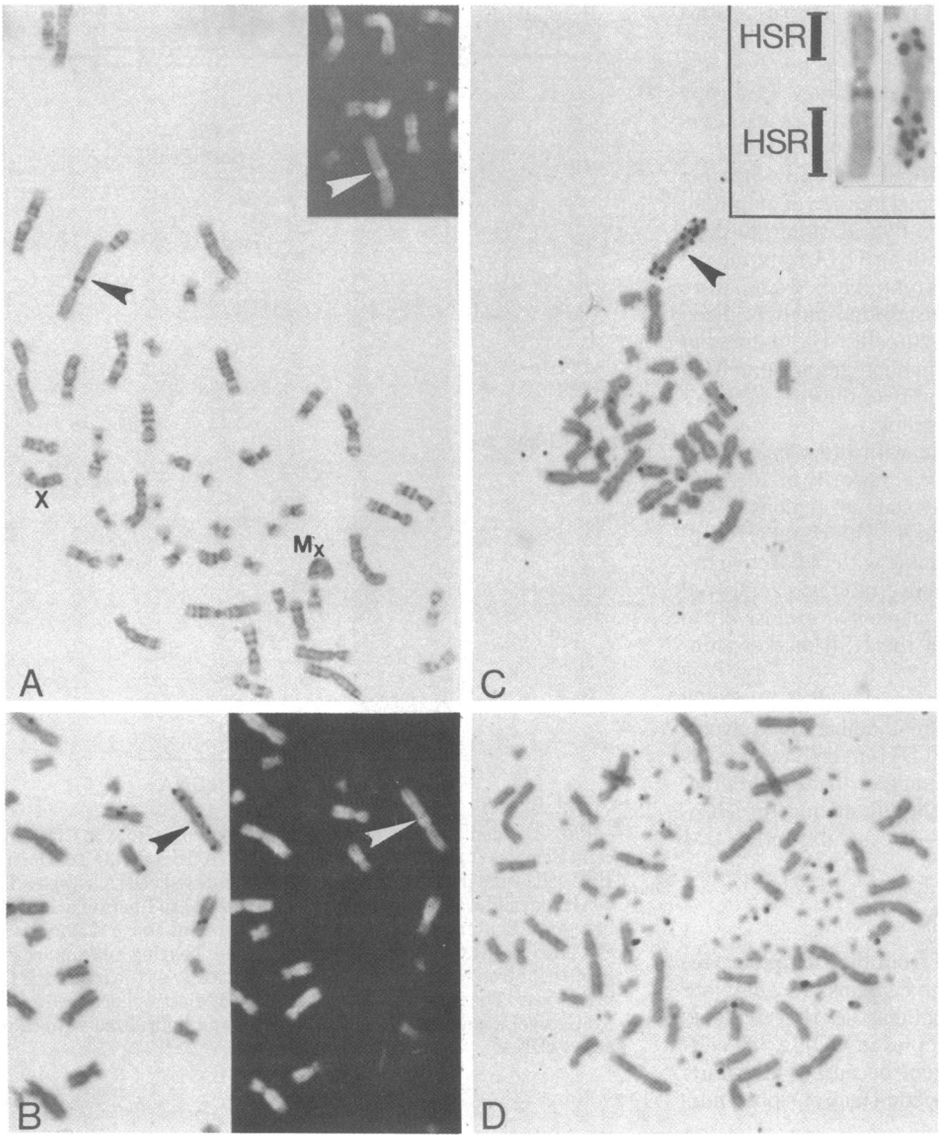

FIG. 4. Location of c-*myc* in COLO 320 metaphase spreads. (*A*) G-banding of COLO 320 HSR metaphase spread. Arrowhead points to the HSR marker chromosome, which may have been derived from an X chromosome (22). (*Inset*) A Q-banded preparation, in which the dark G-band in the proximal portion of the marker chromosome appears brightly fluorescent. X, Intact X chromosome; M_x, new marker chromosome involving the short arm of an X chromosome. (*B*) *In situ* autoradiograph and subsequent Q-banding of HSR metaphase chromosomes hybridized with the 3.5-kbp *Bam*HI–*Eco*RI v-*myc* probe. (*C*) A partial HSR metaphase spread hybridized with ^{3}H-labeled unique-sequence fragment of the human c-*myc* locus. (*Inset*) Correspondence between the autoradiographic signal and the G-banding pattern of the HSR marker chromosome. Note that the signal is confined to the homogeneously staining regions in both short and long arms. (*D*) *In situ* autoradiograph of a DM metaphase spread hybridized with the human probe under the same conditions as the metaphase shown in *C*. Although some grains do occur on DM chromosomes, the result here remains inconclusive as to the localization of c-*myc*.

mal c-*myc* (unpublished data), (*ii*) amplification has not affected typical restriction fragments of *myc* pseudogenes (14, 28), (*iii*) the size of *myc* mRNA from the tumor cells is 2.7 kb as described for the normal gene (13, 27, 30, 31), and (*iv*) unlike the *myc* pseudogenes, cDNA copied from the RNA of COLO 320 cells displays homology to both identified exons of c-*myc*. In addition to the seemingly normal c-*myc* locus, however, COLO 320 DM cells have amplified copies of an altered c-*myc* locus; smaller amounts of this altered locus are also present in the COLO 320 HSR cells.

Is c-*myc* an Oncogene in Man? The pathogenic properties of the *myc* oncogene have been described only in fowl. In chickens, v-*myc* of avian myelocytomatosis viruses characteristically induces liver carcinomas, endotheliomas or carcinomas of the kidney, myelocytomatosis, and occasional mesotheliomas (33, 34). By contrast, activation of the chicken c-*myc* by avian leukosis virus has been implicated in the genesis of bursal lymphomas (2, 3), which also are caused by naturally occurring variants of myelocytomatosis virus (35).

In human beings, several recent findings associate alterations in the c-*myc* oncogene with malignant neoplasms. First, the human c-*myc* locus is expressed to high levels in at least a few tumors of diverse origins (26, 27). Second, amplification of c-*myc* in association with enhanced expression of the gene has now been described in the cells of two human tumors—the promyelocytic leukemia HL-60 (28, 29) and the APUDoma COLO 320 (present data). Third, the t(8;14)(q24;q32) chromosomal translocation, characteristically occurring in Burkitt's lymphoma and acute B-cell lymphocytic leukemias (36, 37), is in some cases associated with a rearrangement that translocates c-*myc* from its normal position at 8q24 to chromosome 14, next to the constant domain region of immunoglobulin heavy chain locus at 14q32 (38, 39).

What Is the Nature of the Amplified Chromosomal Unit Containing c-*myc*? In addition to tumor-specific chromosomal translocations, tumor cells frequently display other forms of chromosomal abnormalities: DM chromosomes and HSRs. Despite their abundance, very little is known about the genetic information included in DM and HSR DNA in tumors. We now show that the c-*myc* oncogene is repeated along the HSR of an abnormal chromosome in COLO 320. Given the size and copy number of the c-*myc* locus and the large size of the HSR in COLO 320, we presume that DNA other than c-*myc* makes up the bulk of the HSRs. Perhaps the extent of the amplified chromosomal unit in COLO 320 cells is defined by the boundaries of the replicon in which c-*myc* resides.

Although we have no direct evidence that the DMs of COLO 320 contain c-*myc*, this is a likely possibility given previous evidence that HSRs apparently arise from chromosomal reintegration of amplified DNA in DMs (6, 10) and our finding of amplified c-*myc* in the HSR of COLO 320. No primary tumor material is available from the colon carcinoma that gave rise to COLO 320, but the occurrence of DM chromosomes in early passages of tumor explants (22) and their stable retention in the DM line suggest that gene amplifications were present in the original tumor.

Copies of c-*myc* Have Been Translocated to an X Chromosome in COLO 320 HSR Cells. The apparent translocation of amplified c-*myc* from chromosome 8 to the X chromosome in COLO 320 contrasts with previous claims for amplifications selected by metabolic inhibitors. HSRs bearing amplified genes for dihydrofolate reductase (6) and for carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase (40) may have arisen by incorporation of DM elements at the chromosomal sites that carry the unamplified versions of the genes, as if the reinsertion of amplified DNA might be facilitated by homologous recombination. The structure of the chromosome containing HSRs in COLO 320 is now so distorted that karyotypic identification cannot be decisive. There are residual traits of the X chromosome, however, and earlier karyotypes more clearly identified the affected chromosome as X (22). It appears that HSRs now have expanded to occupy the bulk of both chromosomal arms. In any event, the translocation of amplified DNA from one chromosome to another is for the moment a novelty that cannot be explained readily by homologous recombination.

Why Is c-*myc* Amplified? Gene amplification may be a spontaneous feature of DNA replication in mammalian cells (6). Strong selective pressures are generally required to bring the amplification to view. We cannot account for the amplification of c-*myc* in COLO 320, nor can we identify selective pressures that might have caused DNA amplification to persist as both DM chromosomes and HSRs. The amplified c-*myc* in COLO 320 is in two forms: one resembles the normal c-*myc* locus, the other has been affected by rearrangement of DNA upstream of c-*myc* (unpublished data). Because the rearrangement is apparent in close to half of the amplified c-*myc* loci in COLO 320 DM (see Fig. 3, for example), it seems likely to have preceded amplification. Perhaps the rearrangement induced enhanced expression of c-*myc*, initiated an early stage of tumorigenesis, conferred a selective growth advantage on the cells expressing c-*myc* in excess, and favored further enhancement of expression by gene amplification. It also is possible that amplification of c-*myc* does not have a role in the oncogenic events that led to the colon carcinoma but instead reflects a hitherto unrecognized amplification that occurs during growth or differentiation in some cell lineages. Such a process may have been frozen by the malignant transformation that engendered the original COLO 320 cells. The role of c-*myc* in the genesis of human tumors remains uncertain, but the provocative finding of amplified c-*myc* in chromosomal anomalies characteristic of numerous cancers encourages further study.

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