Cloning of a Non–c-*myc* DNA Fragment from the Double Minutes of a Human Colon Carcinoid Cell Line

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ABSTRACT: *The cell line COLO 320 DM, derived from an untreated human colon carcinoid tumor, was subcloned to obtain a population (Cl 11) with an average of 37 double minutes (DM) per cell. Fractionation of the chromosomes by differential centrifugation yielded a fraction enriched in DM. DNA isolated from the DM-enriched fraction was inserted into the Pst I site of pBR322. One clone, p446, representative of a number of similar clones, contained a region complementary to genomic unique sequences (region p446U). Southern blot analysis using COLO 320 DNA, and DNA from two other cell lines derived from the same biopsy, COLO 320 HSR and COLO 321 HSR, demonstrated amplification and rearrangement of sequences complementary to p446U when compared with 28 different tumor and normal cell lines, some of which contained DM or homogeneously staining regions (HSR). COLO 320 DM Cl 11 had approximately 110 copies per cell of the p446U sequence, or three copies per DM. COLO 320 HSR, which contained one HSR, had 35 copies per cell, while COLO 321 HSR, which contained two HSR, had 700 copies. In addition, p446U did not hybridize with insert sequences of recombinant plasmid pHM(E+H), which includes the human c-myc* coding region, 3 kb of upstream flanking sequences and 0.5 kb of downstream flanking sequences, or with an exon 3 probe, pMYC RI-CLA. Amplification of p446U was also not seen in cell lines containing amplified c-*myc* or N-*myc* genes. These results indicate that more than one sequence may be amplified in DM or HSR containing tumor cells, but that they need not be amplified together in other tumors.

INTRODUCTION

Double minutes (DM), small extrachromosomal fragments found in tumor cells, were first described in metaphase spreads from a patient with carcinoma of the lung [1]. DM, which are seen in a limited number of human tumor specimens, have been reported in a wide range of tumor types, such as several types of neural ectoderm derived tumors [2–6] as well as leukemia [7–9], medullary thyroid carcinoma [10], breast carcinoma [11], small cell lung carcinoma [12, 13], ovarian carcinoma [14–

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16], malignant melanoma [17, 18], stomach [19], and colon tumors [20–22]. In addition, a related chromosome phenomenon, known as the homogeneously staining region (HSR) has been reported in neuroblastoma [23–24], colon carcinoma [21], small cell lung carcinoma [12, 13], breast carcinoma [25], and malignant melanoma [18, 26, 27]. Evidence obtained from tumor growth in mice [28] and somatic cell hybridization experiments [6] suggest that DM and HSR are different manifestations of the same phenomenon. Occasionally, either DM or HSR are seen in different cells derived from the same tumor [21, 24]. The occurrence of DM and HSR has not been reported in normal cells.

A number of studies of induced drug resistance in tissue culture cells have indicated that DM and HSR are cytogenetic manifestations of gene amplification [23, 29–32]. DM and HSR from a variety of mouse and human tumor cell lines also have been shown to contain amplified copies of cellular DNA sequences [33–40]. Interestingly, some DM and HSR have been described recently, which contain amplified copies of cellular oncogenes, such as c-Ki-*ras* in Yl mouse adrenocatical tumor cells [37], c-*myc* in COLO 320 HSR cells [38], and N-*myc* in a number of neuroblastoma cell lines [39]. C-*myc* is also amplified in human lung cancer cell lines that contain DM or HSR [40].

We have begun to study the DNA sequences from naturally occurring DM because of their unique presence in tumor cells. We selected the cell line COLO 320 DM [21] derived from a human colon carcinoma because the patient did not undergo chemotherapy before removal of the tumor. Two sister cell lines, COLO 320 HSR and COLO 321 HSR, each containing HSR, were also isolated from the same biopsy. We report here the cloning of a DNA sequence from a DM-enriched chromosomal fraction. This sequence is highly amplified in the three biopsy derived cell lines, compared with all other cell lines tested, and does not appear to be related to the c-*myc* oncogene, which is also amplified in these cells.

MATERIALS AND METHODS

Cell Lines

The cell lines used in this study are listed in Table 1. Cell lines 1–7 were obtained from Dr. G. Moore (Denver General Hospital, Denver, CO), cell lines 8–11 from Dr. M. Herlyn (Wistar Institute, Philadelphia, PA), cell lines 12–13 from Dr. V. Cristafalo (Wistar Institute, Philadelphia, PA) cell line 18 from Dr. F. Gilbert, (Mount Sinai Medical Center, New York, NY), and cell lines 19–23 from Dr. J. Minna (NIH, Bethesda, MD). A Southern blot of lines 24–30 was provided by Dr. P. Melera (Sloan-Kettering Institute, Rye, NY). Line 31 was derived by Dr. C. Chern (Wistar Institute, Philadelphia, PA). All were grown in either RPMI 1640 or MEM, both supplemented with 10% fetal calf serum and 2 mM glutamine. The COLO 320 DM line was subcloned by plating approximately 100 cells in a Petri dish. The subclones were expanded and prepared for karyotype analysis by standard methods [41]. Twenty-five cells from each subclone were scored for the number of DM per cell. Cell lines not previously karyotyped were G-banded by the method of Seabright [42].

Isolation of DM and DM DNA

COLO 320 DM Cl 11 cells were synchronized by overnight incubation in medium containing 0.1 μg/ml Velban (Eli Lilly and Co., Indianapolis, IN). Mitotic cells were detached by gently shaking the culture flasks. In all experiments, the mitotic index

Table 1 Cell lines analyzed

Cell line	Cell type	DM or HSR
1) COLO 320 DM Cl 11	Colon carcinoid	DM
2) COLO 320 HSR	Colon carcinoid	HSR
3) COLO 321 HSR	Colon carcinoid	HSR
4) COLO 205	Colon adenocarcinoma	—[a]
5) COLO 399	Colon adenocarcinoma	DM
6) COLO 324	Melanoma	DM
7) COLO 415	Melanoma	DM
8) SW 480	Colon adenocarcinoma	DM
9) SW 620	Colon adenocarcinoma	DM
10) SW 1116	Colon adenocarcinoma	ND
11) SW 1222	Colon adenocarcinoma	ND
12) MRC 5	Normal fibroblast	—
13) WI-38	Normal fibroblast	—
14) GM1500	Multiple myeloma	—
15) HELA S3	Cervical adenocarcinoma	—
16) DAUDI	Burkitt lymphoma	—
17) K562(S)	Chronic myelogenous leukemia	—
18) NB-16	Neuroblastoma	DM
19) NCI-H60	Small cell lung carcinoma	DM
20) NCI-H69	Small cell lung carcinoma	DM
21) NCI-H82	Small cell lung carcinoma	HSR
22) NCI-H146	Small cell lung carcinoma	—
23) NCI-H209	Small cell lung carcinoma	—
24) SK-N-BE2C	Neuroblastoma	HSR
25) SMS-MSN	Neuroblastoma	DM
26) SMS-KAN	Neuroblastoma	DM
27) CHP-234	Neuroblastoma	DM
28) NAP	Neuroblastoma	DM
29) MC-IXC	Neuroblastoma	DM
30) F-ALF	Normal fibroblast	—
31) HT1080 MTX	Fibrosarcoma–MTX resistant	—

[a]One DM seen in 74 cells counted.

was approximately 50%. The mitotic cells were pelleted, put on ice for 30 minutes, treated with 0.075 M KCl hypotonic solution for 30 minutes at room temperature, and then spun down. The DM were prepared by a modification of the Barker and Stubblefield [43] differential centrifugation technique. The cells were resuspended in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM EDTA, 1.0 mM spermidine, and lysed by four passes through a 23-gauge needle. The lysate was layered over a pad of 20% sucrose in isolation buffer and centrifuged at 650 × g for 10 minutes to remove the intact nuclei and whole cells. The supernatant was diluted 1:1 with isolation buffer and centrifuged at 16,000 × g for 30 minutes to pellet the chromosomes. The supernatant containing the DM was centrifuged in a SW-27 rotor at 70,000 × g for 60 minutes. An aliquot of the resulting DM pellet was fixed in methanol, placed on a slide, and stained with the DNA specific fluorochrome Hoechst 33258 [44]. DNA was isolated from the rest of the pellet by digestion with 50 μg/ml proteinase K for 3–6 hours at 37°C followed by extraction with phenol-chloroform and ethanol precipitation.

Cloning of DM DNA

DM DNA was digested to completion with restriction endonuclease Pst I. The DNA was then electrophoresed through a 0.8% agarose gel in 40 mM Tris-HCl, pH 8.0, 5 mM Na acetate, 2 mM EDTA [45]. The gel region between 3.0 and 7.0 kb was cut out, crushed through a syringe, and the DNA eluted in 0.5 M NH_4 acetate, 10 mM Mg acetate, 0.1% SDS, 1 mM EDTA (46) at 37°C overnight. The DNA was precipitated from the eluate with ethanol and dissolved in distilled water.

All experiments were carried out under the NIH guidelines for recombinant DNA research. Plasmid pBR322 was digested to completion with Pst I followed by bacterial alkaline phosphatase. The digested pBR322 (300 ng) and DM DNA (300 ng) were mixed and ligated with T4 ligase in 66 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM spermidine, 15 mM dithiothreitol, 4 mM ATP and 200 μg/ml gelatin at 12°C for 16 hours. The ligated plasmids were transfected into *E. coli* HB101 by calcium phosphate precipitation. The bacteria were grown on 1.5% agar plates containing 15 μg/ml tetracycline.

Bacterial colonies were replica plated and screened by the method of Sippel et al. [47] as modified by Dolby et al. [48]. The filters were prehybridized with 40% formamide, 4 × SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate), 1 × Denhardt's [49] solution 0.5% SDS, 200 μg/ml *E. coli* DNA at 37°C for 16 hours. The DM DNA fraction was nick translated by the method of Rigby et al. [50]. The labeled DNA was heat denatured, added to a hybridization mix to give the same components as the prehybridization buffer, and hybridized to the filters at 37°C for 3 days. The filters were washed at 37°C in buffers containing 50% formamide, 0.2% SDS, and 1 × Denhardt's with decreasing concentrations of SSC. Finally, the filters were washed at 65°C in 0.2% SDS containing decreasing concentrations of SSC. The filters were air-dried and exposed to Kodak X-Omat XRP-5 x-ray film at −70°C.

Colonies that were hybridization-positive were grown in Tris-L broth, and minipreps of the plasmids were isolated [51] and analyzed by Pst I digestion. Large preparative batches of plasmid were isolated by the cleared lysis method followed by banding in ethidium bromide-CsCl gradients [52].

Blot Analysis of Human DNA

Total cellular DNA was prepared from all tissue culture lines by the proteinase K method, as outlined above. The DNAs were digested to completion with Pst I or Eco RI and then run on an 0.8% agarose gel. The DNAs were transferred to nitrocellulose with 20 × SSC or to diazobenzyloxymethyl (DBM) paper with 1 M sodium acetate, pH 4 [53]. Nitrocellulose blots were prehybridized, after baking, first in 5 × Denhardt's then 50% formamide, 4 × SSC, 1 × Denhardt's, 300 μg/ml salmon sperm DNA. Hybridization of nick translated DNA was carried out in 50% formamide, 4 × SSC, 1 × Denhardt's, 300 μg/ml salmon sperm DNA, 0.5% SDS at 37°C for 3 days. These blots were washed extensively in 50% formamide, 1 × Denhardt's, 0.2% SDS with decreasing concentrations of SSC at 37°C followed by 0.2% SDS with decreasing concentrations of SSC at 65°C. The DBM blots were prehybridized at 42°C with 50% formamide, 5 × SSC, 5 × Denhardt's, 50 mM sodium phosphate (pH 6.5), 300 μg/ml salmon sperm DNA, and 1% glycine. Nick translated DNA was hybridized at 42°C for 3 days, in 50% formamide, 5 × SSC, 1 × Denhardt's, 20 mM sodium phosphate (pH 6.5), 300 μg/ml salmon sperm DNA, 0.2% SDS. The blots were washed extensively in 2 × SSC, 0.2% SDS at 37°C and 0.1 × SSC, 0.2% SDS at 37°C and 65°C, air-dried and exposed at −70°C as previously described.

DNA sequence amplification was estimated by dot blot analysis. Triplicate sam-

ples of cellular DNA (5 μg/dot) were denatured in the presence of NaI [54] and loaded directly onto nitrocellulose filters. Increasing dilutions of plasmid p446, starting at 10 ng, were mixed with 5 μg of mouse DNA and loaded onto the same filters. The filters were hybridized with [^{32}P]-nick translated p446U. Under the conditions used, described above, the p446U insert does not hybridize to the mouse DNA. After exposure to x-ray film, the individual dots were cut out and the radioactivity measured by liquid scintillation counting. A unique 2.2-kb sequence (p446U) would make up 7.33×10^{-7} of a haploid genome of 3.0×10^{9} base pairs. Based on 5-μg aliquots of total cellular DNA, 5 ng of plasmid p446 (containing 1.3 ng of p446U insert) would correspond to 360 copies of a 2.2-kb sequence. Gene copy number of the p446U homologous sequences in the DNA was calculated from the plasmid reconstruction curves.

RESULTS

DM Isolation

In order to maximize the number of DM available for isolation, the parental COLO 320 DM cell line (Fig. 1A) was subcloned and the subclones were analyzed for the number of DM per cell (data not shown). There was a wide range of variability in the average number of DM per cell, both within and among these cell lines. Clone 11 was chosen for analysis because it had the highest average number of DM per cell, and none of the cells analyzed were without DM.

The cells were synchronized by a 16-hour exposure to Velban and a DM-enriched chromosome fraction was obtained by differential centrifugation. An aliquot of the final DM pellet was stained with the DNA specific fluorochrome, Hoescht 33258 (Fig. 1B). Many DNA containing DM-like structures (arrows) were seen. Intact metaphase chromosomes were not present in this preparation. Dot blot analysis of the parental COLO 320 DM Cl 11 and DM-enriched fraction DNA, using the subsequently cloned DNA as probe, indicated an approximate fourfold enrichment of the DM sequences by this method (data not shown). This four fold DM sequence enrichment is similar to that seen by Tyler-Smith and Bostock [55], using sucrose gradients for chromosome fractionation.

Cloning of the DM DNA Sequences

Plasmids were constructed from the DM-enriched DNA fraction. A 4.0-kb insert was found in eight of the 22 plasmids (36.4%) identified as possibly containing DM DNA sequences. Subsequent Southern blot experiments showed cross-hybridization of a repeat-free sequence (see below) in seven of these eight recombinants. Plasmid p446 was studied further. Double digests of p446 are shown in Figure 2A. The DNA fragments were hybridized to total genomic DNA from GM1500, a cell line derived from a multiple myeloma that contains a normal 46,XY karyotype, or plasmid BLUR 8, which contains the human Alu family interspersed repeats [56]. In both cases, only the 3′ half of the p446 insert contained sequences homologous to repetitive DNA. The results from the BLUR 8 experiments are shown in Figure 2B. Thus, the 5′ half of p446 (which is denoted p446U in Pst I/Bam HI digests) contains unique human DNA, whereas, the 3′ half of p446 (denoted p446L in Pst I/Bam HI digests) contains Alu family sequences. A map of p446 (Fig. 3) was constructed using various restrictions enzymes. Digestion of p446L with various enzymes, followed by Southern blot analysis using BLUR 8 as a probe, showed the presence of Alu family sequences in the small Acc I-BglII fragment (data not shown).

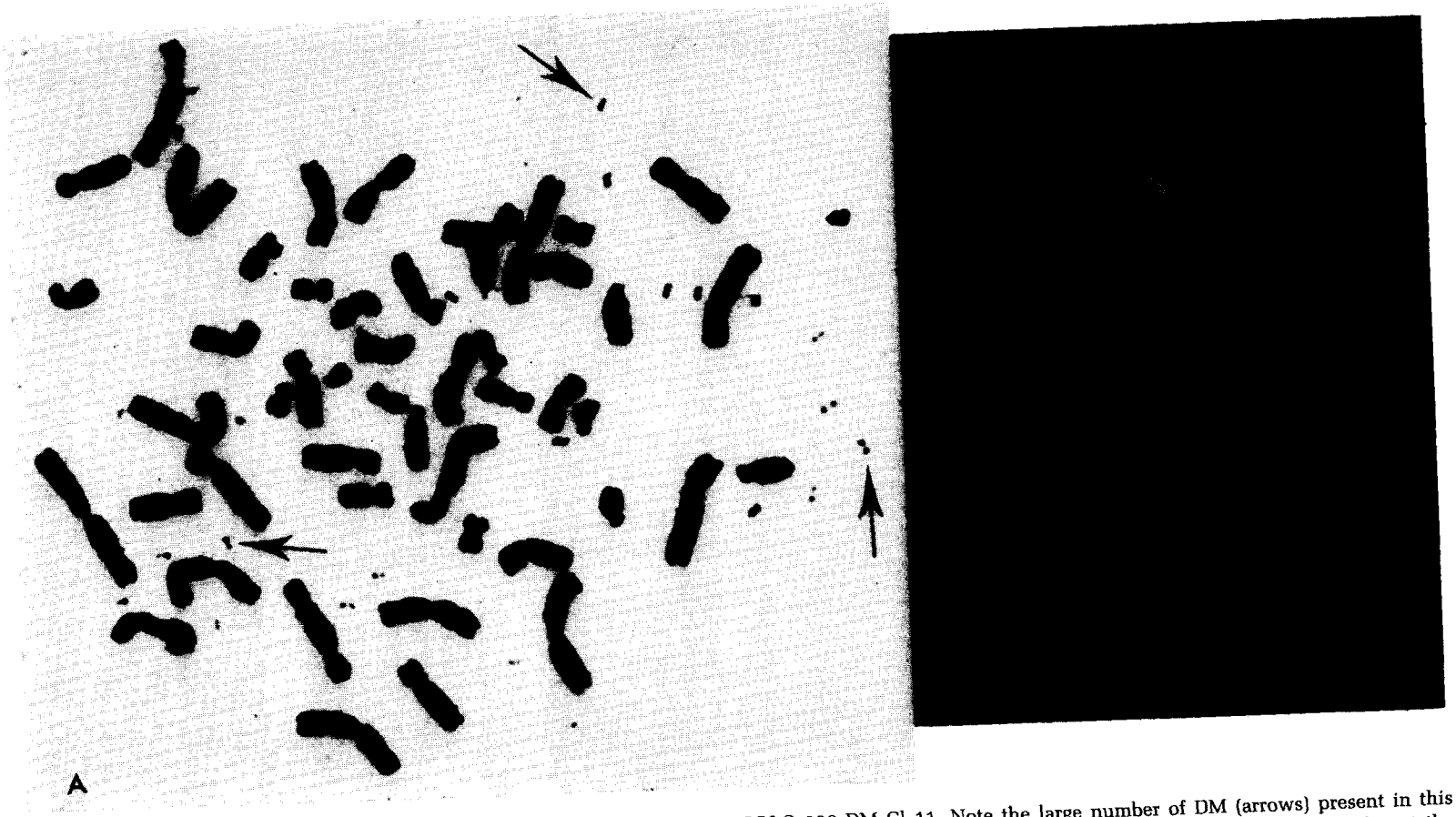

Figure 1 (A) Conventional Giemsa stained chromosome preparation from COLO 320 DM Cl 11. Note the large number of DM (arrows) present in this cell. (B) DM-enriched fraction obtained by differential centrifugation. Mitotic-arrested COLO 320 DM Cl 11 cells were subfractionated and an aliquot the final pellet was stained with the DNA-specific fluorochrome Hoechst 33258. Many DM-like structures are seen along with some chromosome fragments. (Bar = 3μM)

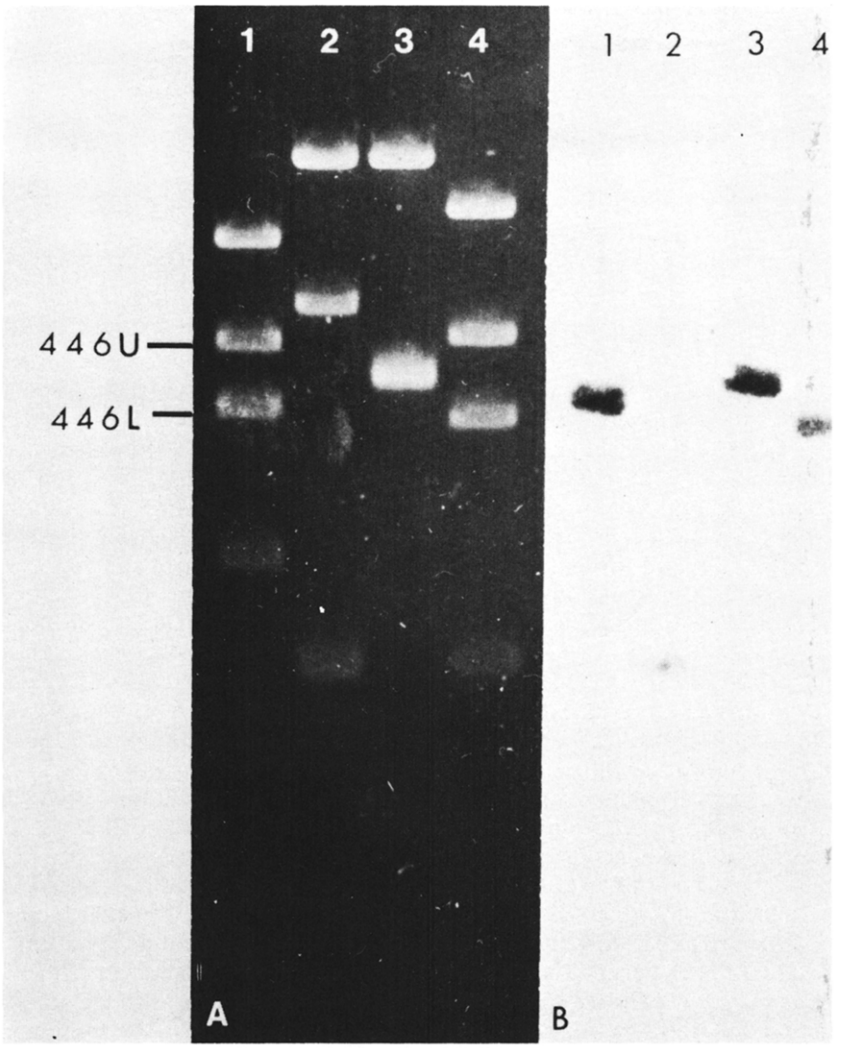

Figure 2 Southern blot analysis of plasmid 446 for repeated sequences. Plasmid 446 (2 μg) was digested with Pst I followed by (1) Bam HI, (2) BglII, (3) Bst EII or (4) Hind III, run on an 0.8% agarose gel and stained with ethidium bromide (A). The DNA was transferred to DBM paper and hybridized to [^{32}P]-labeled BLUR 8 (Alu family) insert (B). Note the presence of the repetitive sequence in the band of Pst I/Bam HI digest denoted 446L. Band 446U did not contain repeated sequences. The other two bands in this digest are pBR322 sequences.

Blot Analysis Using p446U as a Probe

Thirty-one cell lines (Table 1) were examined for sequences complementary to the p446U probe. Pst I digests of COLO 320 DM Cl 11 and its two sister cell lines showed varying amount of amplification of p446U sequences (Fig. 4A) at 4.0 and 3.2 kb. The other 28 total cell DNA did not show amplification of the p446U sequence. In all cell lines digested with Pst I, a single band of 4.3 kb was seen. The Eco RI digests of COLO 321 HSR showed amplification of bands at 19.0 and 8.5 kb, compared with a single 8.5-kb band of greatly decreased intensity in the other tumor and control cell lines (Fig. 4B). Interestingly, MC-IXC cells (Fig. 4B, lane 7) in

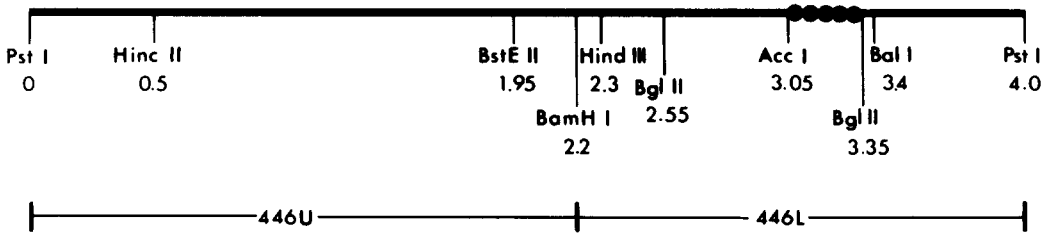

Figure 3 Restriction map of the 446 insert. The Alu family sequence is present in the AccI-BglII fragment of 446L (●●●).

which c-*myc* is amplified approximately 30-fold [57] did not show significant amplification of the p446U sequence. In addition, an 8.5-kb band was seen in Eco RI digested MC-IXC DNA hybridized to p446U, however, a 13.5-kb band was seen in a similarly digested DNA hybridized to c-*myc* sequences [57]. Two of the five small cell lung carcinoma lines studied here, NCI-H60 and NCI-H82, also have amplified c-*myc* sequences [40], but all five lines show a single 4.3-kb band of similar intensity after Pst I digestion and hybridization to p446U (data not shown).

The extent of amplification of the p446U sequence in the COLO 320 DM Cl 11, COLO 320 HSR and COLO 321 HSR DNAs was determined by dot blot analysis. DNA samples were directly dot blotted onto nitrocellulose and hybridized to p446U. Triplicate dots were cut out and analyzed by liquid scintillation counting. A dilution series of plasmid p446, starting with 10 ng, in 5 μg of mouse DNA was blotted and hybridized simultaneously. The 5 ng of plasmid p446, containing 1.3 ng of p446U insert (Fig. 5, dot 5), corresponds to 360 copies per cell (see MATERIALS AND METHODS). Calculation of the hybridization of total cellular DNA, compared with the linear portion of the reconstruction curve indicates COLO 320 DM Cl 11 contains approximately 110 copies of the 446U sequence per cell. If it is assumed that all copies of the p446U sequence reside in the DM, and because an average of 37 DM were seen per cell, each DM contains three copies of this sequence. The difference in amplification between the two HSR containing lines was striking. COLO 320 HSR, which has one HSR per cell, has 35 copies, but COLO 321 HSR which has two HSR has 700 copies of the p446U sequence. The length of the individual HSR, however, is comparable in both cell lines [21]. The formal possibility exists that some of the p446 sequences were not localized to the HSR, but were scattered in the genome as reported by Tyler-Smith and Bostock [55]. Thus, a cell line with twice the number of HSR contains 20 times the number of homologous sequences. The extent of amplification in each of the cell lines is consistent with the estimated relative intensities of the hybridization of p446U seen in these cells as measured by densitometry (Fig. 4A).

Table 2 Estimate of 446U sequence amplification

Cell line	Estimated gene copies	DM or HSR/Cell	Copies/DM or HSR
COLOR 320 DM Cl 11	110	37.2	3
COLO 320 HSR	35	1.0	35
COLO 321 HSR	700	2.0	350

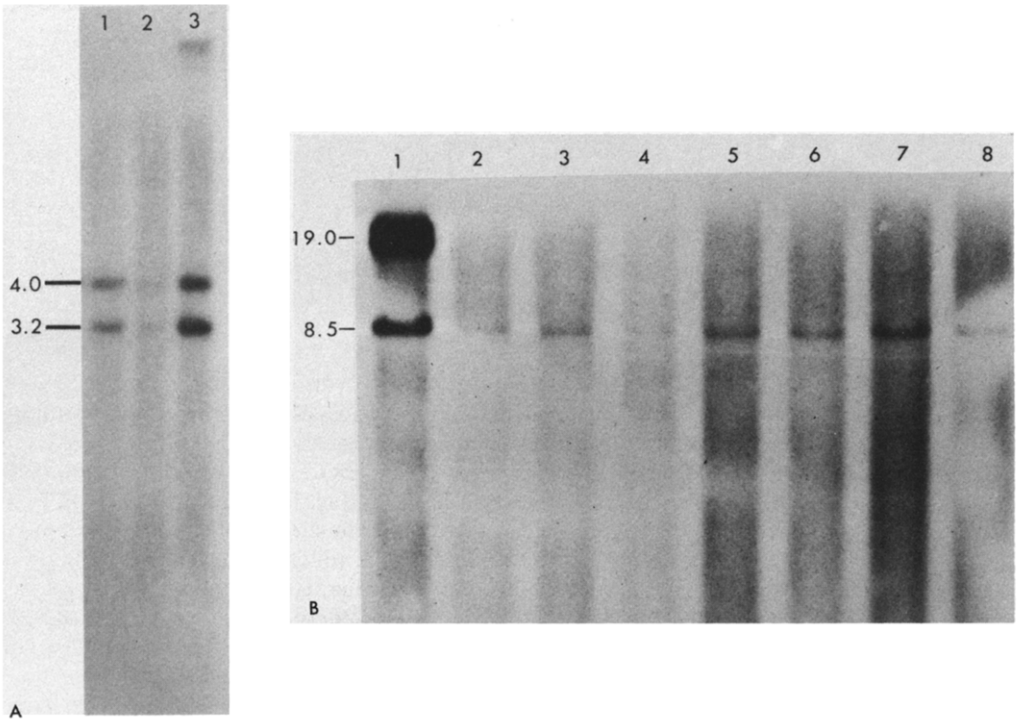

Figure 4 (A) Southern blot analysis of total cellular DNA. DNA (10 μg) from (1) COLO 320 DM Cl 11, (2) COLO 320 HSR and (3) COLO 321 HSR were digested to completion with Pst I, run on a 0.8% agarose gel and transferred to DBM paper. The DNAs were hybridized to [^{32}P]-labeled 446U. Note the bands at 4.0 and 3.2 kb in all three cell lines. (B) DNA (10 μg) from (1) COLO 321 HSR, (2) SK-N-BE2C, (3) SMS-MSN, (4) SMS-KAN, (5) CHP-235, (6) NAP, (7) MC-IXC and (8) F-ALF were digested to completion with Eco RI, run on a 0.8% agarose gel and transferred to nitrocellulose. The DNAs were hybridized to [^{32}P]-labeled 446U. Note the intense bands at 19.0 and 8.5 kb in the COLO 321 HSR cells while all other cells show a single band at 8.5 kb.

Comparison of p446 and c-*myc* Sequences

A previous report has indicated that the c-*myc* protooncogene is amplified in COLO 320 HSR cells [38]. We did not, however, see amplification of the p446U sequence in cells other than COLO 320 DM Cl 11, COLO 320 HSR and COLO 321 HSR, even though some of these cell lines have been shown to have amplified c-*myc* sequences

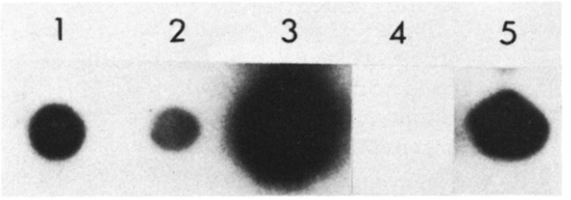

Figure 5 Dot blot analysis of amplified sequences in total cellular DNA. DNA (5 μg) from (1) COLO 320 DM Cl 11, (2) COLO 320 HSR, (3) COLO 321 HSR, (4) mouse DNA and (5) mouse DNA with 5 ng plasmid 446 were denatured in the presence of NaI and loaded directly into nitrocellulose filters. The DNAs were hybridized to [^{32}P]-labeled 446U and the sequence copy number was estimated as outlined in MATERIALS AND METHODS.

(see above). To further demonstrate a lack of homology between p446 and c-*myc*, these sequences were analyzed by the Southern blot procedure using either sequence as a probe. As seen in lane 1 of Figure 6A the plasmid pHM(E+H), which contains an 8.2-kb insert with the complete c-*myc* gene did not cross-hybridize with the p446U probe, even though this probe did hybridize strongly to the p446U band of a Pst I/BAM HI digest of plasmid p446 (Fig. 6A, lane 2). Conversely, both the 8.2-kb c-*myc* insert and the pBR322 sequences hybridized with a [^{32}P]-labeled c-*myc* exon 3 sequence probe, pMYC RI-CLA (Fig. 6B, lane 1), whereas, only the pBR322 sequences from the double digested p446 plasmid hybridized with the c-*myc* probe (Fig. 6B, lane 2). The same result was obtained regardless of whether the experiment was carried out under stringent or non-stringent conditions.

DISCUSSION

The three colon carcinoid cell lines we have worked with provided an opportunity to study gene amplification in human tumor cells. It is well known that, at least in vitro, gene amplification can be induced in a variety of normal and abnormal cells by anticancer drugs, such as methotrexate. The biopsy, however, was derived from a patient who did not undergo chemotherapy before surgery. Thus, it appears that these DM and HSR arose as a cause of, or in response to, tumorigenesis. The p446 sequence does not appear to be related to dihydrofolate reductase, the target enzyme of methotrexate. The HT1080 MTX cell line we studied is resistant to 400 μM MTX and shows a 100-fold increase in the amount of DHFR enzyme activity. The p446U sequence is not amplified in these cells.

In studies of drug-induced gene amplification, it is well established that commonly derived DM and HSR contain the same sequence. DM and HSR sequence homology was found in sublines of a mouse adrenocortical tumor [33, 34]. In the human colon tumor cells the HSR apparently are involved in a number of complex chromosome rearrangements such that they have differing numbers of copies in the two HSR cell lines and appear on different marker chromosomes. The cytologic appearance of the HSR is also distinguishable, however, their length appears to be comparable in both cell lines (see Fig. 6A and 6B in ref. 21). The DM-derived p446 sequence shows amplification in both HSR containing lines, in spite of their differing physical characteristics.

The specific histologic type of tumor from which these cell lines have been derived has been difficult to ascertain. They are defintely not adenocarcinomas, as seen by their morphology and lack of binding to anticolorectal adenocarcinoma monoclonal antibodies (M. Herlyn, personal communication). The cells do react with antineuroblastoma monoclonal antibodies (R. Kennett, personal communication). The cells appear to have secretory granules and produce catecholamines [21], however, dopa-decarboxylase is not present (A. Gazdar, personal communication), making it difficult to categorize these cells as apudomas. Without this histologic definition of the cells it is not possible to observe if cells of this same histologic type always amplify p446 sequences. From this study, however, it is clear that DM and HSR containing tumors of other types have not amplified p446 sequences.

Some of the cell lines lacking p446 amplification did contain amplified c-*myc* or N-*myc* oncogene sequences, so it was not likely that p446 was a clone of either of these genes. However, c-*myc* became of particular concern when, during the course of this work, Alitalo et al. [38] demonstrated that c-*myc* was amplified in COLO 320 DM and COLO 320 HSR cells. These authors indicated that c-*myc* was amplified 16- to 32-fold in COLO 320 HSR cells, similar to the approximate 35-fold amplification of p446U sequences seen in these cells. C-*myc* coding or flanking sequences did not cross hybridize with p446, even under non-stringent conditions. In addition, Eco RI-digested MC-IXC DNA gave a different sized DNA fragment when

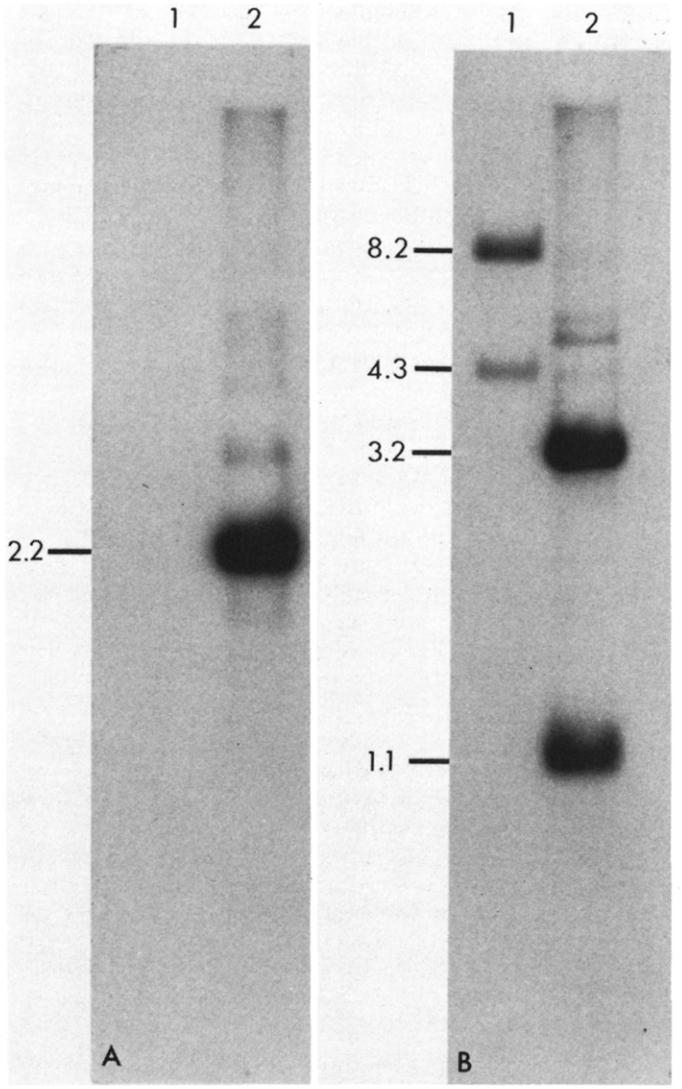

Figure 6 Southern blot analysis of plasmids pHM(E+H) and p446. DNA (1 μg) from either (1) pHM(E+H) digested with Eco RI and Hind III or (2) p446 digested with Pst I and Bam HI was run on an 0.8% agarose gel and transferred to nitrocellulose. The DNAs were hybridized to [^{32}P]-labeled 446U (A) or pMYC RI-CLA (B), the exon 3 sequence of c-*myc*. Note the lack of cross-hybridization of these probes, except in the homologous pBR322 sequences.

hybridized with p446U or c-*myc* [57]. Therefore, p446 and c-*myc* genes are different. More than one sequence is amplified in these COLO cells. Recent evidence indicates that the HSR in IMR-32 (neuroblastoma) cells was derived from sequences from two or more domains, separated by thousands of kilobases, of chromosomal segment 2p [58]. In addition, a DNA sequence, amplified in IMR-32 cells but different from N-*myc*, has been shown to be expressed in one retinoblastoma and a variety of neuroblastoma cell lines. This second sequence is both amplified and expressed to different degrees than the N-*myc* gene in these cells [59]. The possibility exists that p446 may be a second gene similar to that seen in the neuroblastoma system. Whether or not the p446 sequence is expressed is presently under investigation. Preliminary analysis (Hubbell, unpublished data) indicates that p446 sequences are expressed in COLO 320 HSR cells, but not in Daudi cells, the latter of which express high levels of c-*myc* [60].

Estimates of sequence copy number imply that there are three copies of the p446 sequences per DM. A rough estimate of the amount of DNA in a DM is around 3000

kb [43]. Thus, the total repeat unit could be as high as 1000 kb. A 3000-kb repeat unit has been estimated for the HSR in IMR-32 cells [35]. A 1000-kb repeat unit has also been calculated in mouse cells resistant to MTX [55]. In mouse cells, the DHFR gene is approximately 42 kb [61]. The large size of the amplified unit also indicates that more than one gene is present in a DM.

Restriction analysis of total cell DNA for the presence of the p446 sequence indicates that mutational changes in the 446 sequence of these cells occurred previous to or at an early stage of the amplification event. After Pst I digestion, the unamplified DNAs are found in a band at 4.3 kb, whereas, the DM and HSR DNAs show bands at 4.0 and 3.2 kb. Reorganizations of these DNA sequences are also seen in Eco RI digests in which most of the amplified sequences are found in 19-kb fragments compared with the 8.5 kb seen in other tumor and normal cells. Similar changes were not seen in the DM and HSR of mouse adrenocortical tumors [33, 34]. Genetic changes within amplified DNA were found in a study of MTX-resistant mouse cells [55]. These DNA rearrangement occurred near the ends of the DHFR gene [62].

Certain facts in the present work lead us to consider a different mechanism. There are no Eco RI sites within the p446 sequence, yet the wildtype spacing between the Eco RI sites in which the p446 sequence resides is apparently preserved in some of the amplified units. Assuming this preservation, the 446 sequence cannot be terminal within the repeat unit. Because essentially no 446 sequences with the wildtype 4.3-kb spacing are amplified, the mutational changes within it do not result from abberrencies at the terminus of the amplification unit. We suggest that in this case, at least two and probably many more mutational changes preceeded the large scale multiplication of the amplified unit leading to DM and HSR.

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