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Genetics

Amplified human *MYC* oncogenes localized to replicating submicroscopic circular DNA molecules

(oncogene amplification/amplification mechanisms/replication origins/tumor prognosis)

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ABSTRACT **Amplification of genes can sometimes be detected by molecular hybridization but not by cytogenetic methods, suggesting that in some cases the units of amplification may be too small to be detected by light microscopy. The experiments reported here investigate whether submicroscopic amplification units are present in early passages of the human tumor cell lines HL-60 and COLO 320. The results show that such cells do contain submicroscopic, extrachromosomal, supercoiled circular molecules harboring *MYC* genes. The molecules in HL-60 are ≈250 kilobase pairs (kbp), while those in COLO 320 are 120–160 kbp. The extrachromosomal molecules in HL-60 are shown to replicate semiconservatively and approximately once in one cell cycle. We propose that these submicroscopic elements are precursors of double-minute chromosomes, the usual extrachromosomal manifestation of gene amplification, since both are structurally similar and replicate autonomously.**

The large number of oncogenes and drug-resistance genes subject to amplification indicates that many, possibly most, genomic regions are capable of undergoing regional increases in copy number (see refs. 1–3 for review). Cytogenetic analyses of many independently isolated cell lines containing different amplified genes show that amplified sequences localize to two types of abnormal chromosomal structures, referred to as double-minute chromosomes (DMs) and expanded chromosomal regions (also called homogeneously staining or abnormally banding regions). DMs are acentric extrachromosomal elements, which replicate approximately once per cell cycle and are believed to be circular (4, 5). Expanded chromosomal regions consist of amplification units arranged as both inverted and direct repeats (6–9). Size estimates for individual amplified units of different chromosomal regions range from 250 to >1000 kilobase pairs (kbp) (1–3).

While DMs and expanded chromosomal regions are generally visible by light microscopy, a submicroscopic extrachromosomal structure not previously known, which is supercoiled, circular, and replicates autonomously, has recently been implicated in mammalian gene amplification (10). Subsequent studies showed that these elements are precursors of DMs (11). Recently, ≈650-kbp elements were found in methotrexate-resistant human cells in which only a minority of cells contain DMs or expanded chromosomal regions (12).

It is possible that submicroscopic amplification products may be peculiar to cases involving drug selections in which chromosomal damage results from drug treatment or to those involving chromosomal destabilization caused by gene transfer. The experiments reported here were initiated to determine whether submicroscopic elements are also generated in the amplification of endogenous cellular oncogenes where the selective pressures for gene amplification were imposed within the tumor environment *in vivo*. The cell line HL-60 was chosen for our initial studies because previous work established that *MYC* amplification existed in the tumor cells prior to their establishment as a cell line (13).

Two observations suggested that the visible cytogenetic anomalies found in the HL-60 and COLO 320 neuroendocrine tumor cells analyzed here may be formed from precursors that are <1000 kbp and, hence, would not be visible by light microscopy. First, the DMs observed in single cells in these and other cell lines are usually variable in size and are sometimes barely detectable by light microscopy (14, 15). Second, estimation of genetic complexity showed that the amplified units in HL-60 and COLO 320 cells containing DMs or homogeneously staining regions (HSRs) are, respectively, ≈95 and ≈300 kbp (16). The results reported here show that some early passages of HL-60 promyelocytic leukemia cells and some subclones of COLO 320 (DM) neuroendocrine tumor cells do indeed harbor submicroscopic circular extrachromosomal elements.

MATERIALS AND METHODS

Cell Lines. An early passage (passage 36) of HL-60 promyelocytic leukemia cells (16) was kindly provided by S. Collins (Fred Hutchinson Cancer Center), and HL-60 late passage (>200 passages) was obtained from B. Sefton (Salk Institute). COLO 320 cells (17) containing DMs (passage 15) and HSRs (passage 11) were obtained from the American Type Culture Collection, and Raji cells [ref. 18; containing the 178-kbp circular Epstein–Barr virus (EBV)] were provided by C. Mulder (University of Massachusetts, Worcester). All human cell lines were grown in RPMI 1640 medium/10% fetal calf serum. Single cell subclones of COLO 320 DM and HSR cells were obtained by limiting dilution. T5S1-3 and C5R500 are Chinese hamster ovary (CHO) cell lines containing ≈50 copies per cell of a transfected, amplified CAD gene (CAD, carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydroorotase) (10, 19). In T5S1-3, the CAD genes are amplified intrachromosomally, while in C5R500 they localize to extrachromosomal supercoiled ≈250-kbp circular molecules (10). Both lines are grown in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum, 1× nonessential amino acids (GIBCO), and 500 μM *N*-phosphonacetyl-L-aspartate (a

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Abbreviations: DHFR, dihydrofolate reductase; CAD, the multifunctional protein that catalyzes the first three steps of *de novo* UMP biosynthesis; DM, double-minute chromosome; HSR, homogeneously staining region; rDNA, ribosomal RNA-encoding DNA; EBV, Epstein–Barr virus; CHO, Chinese hamster ovary.

specific inhibitor of CAD). B5-4 is a Syrian hamster cell line containing ≈200 intrachromosomally amplified CAD genes (20), many of which are presented as inverted repeats (6).

Isolation and Analysis of Large Circular DNA from Mammalian Cells. Circular DNA exceeding 100 kbp was isolated by alkaline lysis (10) and then either applied (21) directly onto Nitrocellulose (BA85; Schleicher & Schuell) through a dot blot apparatus (Minifold I; Schleicher & Schuell) or analyzed by electrophoresis through agarose gels under conditions designed to fractionate supercoiled circular DNA according to molecular weight (10). Blots were hybridized as described (21).

Replication of *MYC* Episomes in HL-60 Cells. Exponential-phase suspension cultures of early passage HL-60 cells were used. The cells were labeled with 3 μM BrdUrd plus 3 μM thymidine for either 0.5, 1, or ≈2 generations as determined directly by cell counting. Thymidine was essential to reduce the toxicity of BrdUrd to HL-60 cells. Even in the presence of equimolar thymidine and BrdUrd, growth retardation was observed after one generation. Replication of *MYC* episomes was then determined either from total high molecular weight DNA prepared by standard procedures (22), or from episome-enriched DNA prepared by the alkaline lysis method (10). DNA from 1–5 × 10^7 cells prepared by either method was sheared by 10 passages through a 27-gauge needle and then sedimented to equilibrium in 5-ml gradients (45,000 rpm in a Beckman Vti65 rotor at 15°C for 16 hr) of CsCl added to an initial density of 1.75 g/ml. Each gradient was fractionated into 24 200-μl aliquots, and the refractive index of every other fraction was determined. A 100-μl portion of each fraction was then denatured by adding NaOH to 0.3 M and heating at 65°C for 15 min, neutralized by adding an equal volume of 2 M NH_4OAc, and the samples when then applied to a dot blot apparatus (Schleicher & Schuell), hybridized with the nick-translated probes indicated, and washed under stringent conditions as described (21). The dots were excised with a paper punch and the cpm per fraction was determined in a liquid scintillation counter.

RESULTS

Submicroscopic Elements Containing *MYC* Genes in HL-60 Revealed by Alkaline Lysis. Lysis of mammalian cells at pH 12.45, followed by neutralization and phenol extraction at high salt concentrations, enables isolation of supercoiled DNA >100 kbp in the supernatant, while chromosomal DNA partitions at the phenol–aqueous interface. To determine whether MYC sequences might reside in circular elements recoverable by this method, duplicate DNA samples from early passage HL-60 cells were prepared by either the alkaline lysis method or by a $NaDodSO_4$ lysis method that enables the recovery of total cellular DNA. Fig. 1 shows that in six independent experiments, 8–52% of the total MYC sequences are recovered in the alkaline lysate supernatant. By contrast, only 1% of the MYC sequences are recovered in the alkaline lysate supernatant from late-passage HL-60 cells in which the MYC sequences are amplified intrachromosomally (6). However, recovery of MYC DNA in the extrachromosomal fraction of early passage HL-60 cells obtained from different laboratories is variable (data not shown). The same procedure applied to the isolation of the 178-kbp circular EBV genome from Raji cells (18) and the 250-kbp circular CAD episome from C5R500 CHO cells (10) produced yields of 31–100% and 31–54%, respectively. Fig. 1 also shows that the recovery of the circular mitochondrial DNA from HL-60 cells is only about half that from Raji cells, suggesting that it

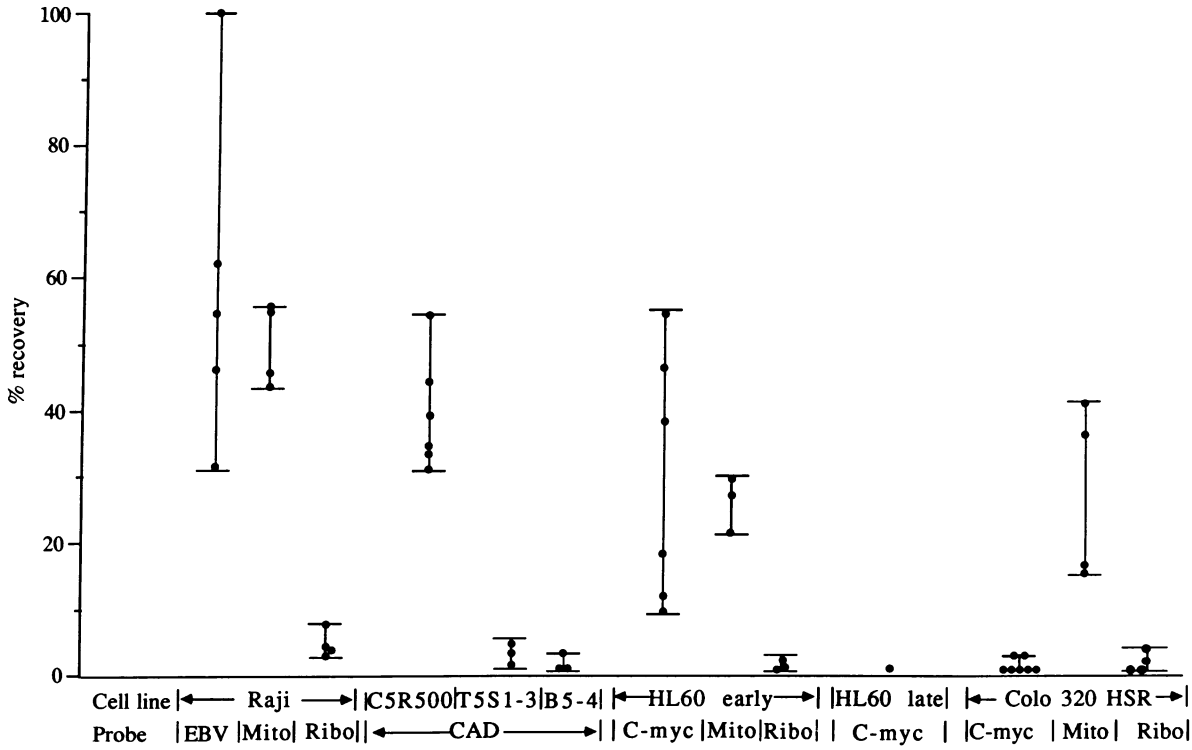

FIG. 1. Circular MYC sequences in HL-60 cells revealed by alkaline lysis. Approximately 10^7 cells of the indicated cell lines were divided into two equal aliquots and the DNA from one half was isolated by the $NaDodSO_4$/phenol method (i.e., total DNA), while that from the other half was isolated by alkaline lysis (i.e., circular DNA). Equal amounts of DNA isolated by either method were then applied to nitrocellulose filters through a dot blot apparatus (Minifold I; Schleicher & Schuell) and hybridized (21) with probes specific for MYC sequences, mitochondrial DNA (Mito), rDNA (Ribo), or the CAD gene. The dots were excised with a paper punch and the cpm of probe bound was determined in a liquid scintillation counter. The percentage of the total sequences hybridized to each probe in the alkaline lysate was then calculated by dividing the cpm in the alkaline lysate by the cpm in the total DNA. Each dot represents the value determined in an independent experiment.

is more difficult to obtain intact circular DNA from the HL-60 cells. By contrast, the recovery of naturally highly repeated chromosomal sequences such as ribosomal RNA-encoding DNA (rDNA) or chromosomally amplified CAD genes (10, 19) is generally <3%. Therefore, the recovery of MYC sequences from early passage HL-60 cells that contain few or no DMs is in the same range as the isolation of 178- to 250-kbp submicroscopic circular elements in Raji and CHO cells and is far higher than the recovery of repeated chromosomal sequences.

The data presented above are compatible with the idea that some of the amplified *MYC* genes reside in submicroscopic circular elements in the early passage HL-60 cells used for these experiments. However, some amplified *MYC* genes in some sublines of later passages of HL-60 cells are present in inverted repeats within expanded chromosomal regions (i.e., HSRs; see ref. 6), suggesting that the molecules detected are not circular, but rather are renatured inverted repeats generated by the alkaline lysis procedure. This possibility is eliminated since late passage HL-60 and COLO 320 (HSR) cell lines that contain amplified *MYC* genes in inverted duplications (6) was only 1–3%, as was the recovery of amplified CAD genes present, in part, as inverted duplications in the Syrian hamster cell line B5-4 (ref. 20; see Fig. 1). The recovery of CAD sequences in this case was also between 1% and 3%. In addition, only 1% of the dihydrofolate reductase (DHFR) sequences from a mouse cell line containing amplified DHFR sequences in DMs fractionated into the alkaline lysate supernatant (J. Ruiz and G.M.W., unpublished data; cell line R50 provided by T. Tlsty, University of North Carolina). These results show that the recovery of MYC sequences from early passage HL-60 cells by the alkaline lysis method is significantly higher than the recovery of intrachromosomally amplified sequences that are arranged as inverted repeats or amplified sequences contained in DMs and strongly indicates that some of the MYC sequences are contained in submicroscopic extrachromosomal circular elements in the HL-60 cells analyzed here.

***MYC* Is Amplified in ≈250-kbp Supercoiled Circular Molecules in Early Passage HL-60 Cells.** If the MYC sequences in early passage HL-60 cells are supercoiled circles less than ≈750 kbp, then they should be detectable by electrophoresis of alkaline lysates through agarose gels under conditions that fractionate supercoiled circular DNA (10) according to the logarithm of molecular weight (e.g., 7.5/cm; 0.7% agarose). Under these conditions, some nicked circular molecules are trapped at the well, and some circular and linear DNA molecules migrate near the dye front (e.g., see ref. 23). Electrophoresis of alkaline lysates derived from early passage HL-60 cells revealed three intense bands that hybridize with a MYC probe: one band was at the well of the gel, possibly reflecting very large circles, or circles impaled by agarose threads (e.g., see ref. 24); one band was at the position of a 250-kbp supercoiled circular molecule; and one migrated at the dye front, corresponding to open circular or sheared DNA (Fig. 2, lanes 1, 2, and 5). Importantly, the alkaline lysates prepared from Raji and CHO C5R500 cells that provided 178-kbp (EBV) and 250-kbp (CAD) supercoiled circular size standards, revealed an analogous pattern of three bands. An alkaline lysate prepared from a COLO 320 (HSR) cell line containing approximately the same level of *MYC* amplification as the early passage HL-60 cells revealed only faint hybridization at the position of fragmented DNA (Fig. 2*B*, lane 7). These data show that some of the c-myc sequences in early passage HL-60 cells reside in ≈250 kbp supercoiled circular molecules (i.e., *MYC* episomes).

***MYC* Episomes in HL-60 Cells Replicate Semiconservatively and Approximately Once in One Cell Cycle.** If the *MYC* episomes are analogs of the previously described CAD episomes, which are precursors of DMs (10, 11), then they should replicate autonomously and hence contain cellular replication origins. To determine whether the *MYC* episomes replicate, the kinetics of BrdUrd incorporation were determined by a density-shift experiment patterned after that of Meselson and Stahl (25). Asynchronous HL-60 cells were labeled for either ≈0.5 or ≈1.0 generation in the presence of BrdUrd and thymidine, and then extrachromosomal sequences were isolated by alkaline lysis. Alternatively, total cellular DNA was prepared for the analysis of the replication of chromosomal sequences. The DNA was sheared, fractionated on CsCl density gradients, and the DNA in each gradient fraction was analyzed for MYC or chromosomal DNA sequences. Fig. 3 shows that the MYC sequences at 0.5 generation were distributed ≈30% LL (unreplicated) and 70% HL (one strand replicated), and no MYC sequences were detected at the position of HH DNA (both strands replicated). At 1.0 generation, the proportion of MYC sequences in LL DNA was decreased to ≈5%, that of HL was increased to ≈95%, and no HH DNA was evident. Duplicate samples analyzed for rDNA sequences as a marker of chromosomal replication showed a distribution of 25% LL, 75% HL, and no detectable HH DNA at ≈0.5 generation, and ≈5% LL, 95% HL, and no detectable HH DNA at ≈1 generation. While the cell lethality due to prolonged exposure

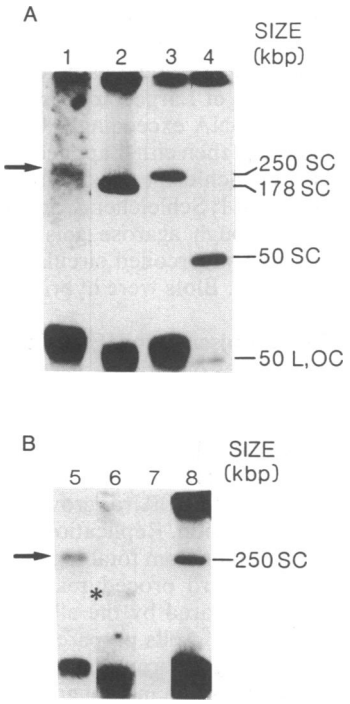

FIG. 2. Characterization of circular molecules in HL-60 and COLO 320 cells by gel electrophoresis. Alkaline lysates were prepared from 10^7 cells from the indicated cell lines, fractionated by electrophoresis on agarose gels at high voltage (7.5 V/cm) to separate supercoiled circular molecules by molecular weight (10, 23), transferred to nitrocellulose, and hybridized with probes specific for MYC (lanes 1, 5, 6, and 7), EBV (lane 2), or CAD (lanes 3, 4, and 8). Lanes: 1 and 5, alkaline lysates HL-60 cells; 2, from Raji cells containing the 178-kbp EBV episome; 3 and 8, from C5R500 CHO cells that contain the 250-kbp CAD episome; 4, the 50-kbp CAD cosmid (22); 6, alkaline lysates from COLO 320 (DM) clone 10; 7, from COLO 320 (HSR). Hybridizations were done with 2×10^6 cpm per ml of each probe with 10% (wt/vol) dextran sulfate, overnight, with standard incubation and stringent washing procedures (21). Autoradiography was for 24–48 hr at −70°C with two Lightning Plus intensifying screens and Kodak XR-5 film. The *MYC* episome in early passage HL-60 cells (lanes 1 and 5) is clearly different in size from that detected in COLO 320 clone 10 (asterisk). SC, L, and OC, supercoiled, linear, and open circular DNA, respectively.

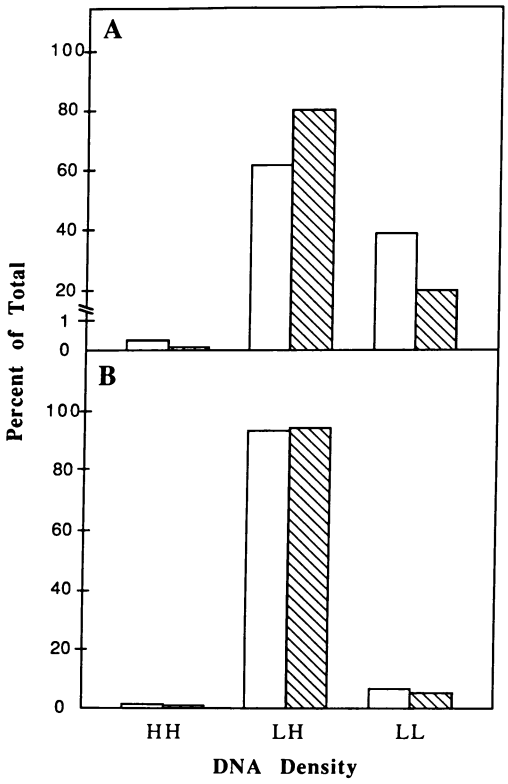

FIG. 3. *MYC* episomes in HL-60 cells replicate semiconservatively and approximately once in one cell cycle. HL-60 cells were grown for either ≈0.5 (*A*) or ≈1 (*B*) generation in our equimolar mixture of thymidine and BrdUrd. Total or circular DNA was then isolated by the $NaDodSO_4$/phenol or alkaline lysis methods, sheared, and centrifuged to equilibrium on CsCl gradients. The gradients were fractionated, applied to nitrocellulose through a Minifold 1 dot blotter, and hybridized with nick-translated (≈2×10^6 cpm per ml) probes specific for MYC or rDNA. After stringent washing, the cpm in each fraction was determined by counting in a liquid scintillation counter. Results similar to those shown were obtained in three independent experiments. The yield of rDNA sequences in the alkaline lysate was undetectable in the experiment shown, indicating that there was negligible contamination of the circular DNA with chromosomal sequences. □, MYC; □, rDNA.

to BrdUrd prevented accurate determination of BrdUrd incorporation after a second round of replication, we note that a similar pattern of incorporation was seen for rDNA and MYC sequences after ≈2 generations of labeling (data not shown). These data indicate that BrdUrd is incorporated into *MYC* episomes in a manner consistent with semiconservative replication, but the *MYC* episomes appear to replicate later than the rDNA sequences, on the average. Since no HH DNA was observed at either 0.5 or 1 generation, and the amount of LL DNA decreased as the incubation time approached one cell doubling, the data also indicate that most, if not all, of them replicated only once during the single cell cycle analyzed.

***MYC* Episomes in COLO 320 Cells.** The results described above encouraged us to analyze COLO 320 (DM) cells for submicroscopic *MYC* episomes. Analysis of an uncloned population obtained directly from the American Type Culture Collection failed to reveal any *MYC* episomes in alkaline lysates derived from $>10^7$ cells. However, the COLO 320 (DM) cells had grown for an undefined period of time *in vivo* and then were passaged in culture before being frozen. By analogy with our observation that submicroscopic episomes increase in size over time during *in vitro* culture (11), if submicroscopic episomes had been present initially, most would have increased in size to form DMs by the time we obtained the cells. Therefore, we prepared seven independent single cell subclones of the COLO 320 (DM) and COLO 320 (HSR) cell lines. We found that each of the seven DM subclones contained very similar levels of *MYC* amplification (29 ± 6 copies per haploid genome), in agreement with published values (26). However, there was considerable variation in the median number and size of DMs in each subclone (median, 0–27 DMs per cell). No HSR subclone contained DMs, and the average -fold amplification of *MYC* genes was virtually identical to that in the DM subclones (31 ± 10 copies per haploid genome). All DM subclones were analyzed for the presence of *MYC* episomes, but only two of them were found to contain such molecules. Subclone 10 had the lowest median number of DMs, while subclone 3 had the highest median number, but in the latter the vast majority of DMs were very small. Gel analysis of an alkaline lysate of 10^7 cells from COLO 320 (DM) subclone 10 reveals a faint band of *MYC* hybridization at the position of a supercoiled circular molecule with a size of ≈120 kbp and a very intense band of hybridization corresponding to nicked and/or linearized episomes. The yield of extrachromosomal MYC sequences from COLO 320 DM is higher than from HL-60, as is expected, since COLO 320 DM has twice the MYC copy number of HL-60. The size of the *MYC* episome in COLO 320 (DM) clone 3 is ≈160 kbp (data not shown).

No evidence of *MYC* episomes was found in any COLO 320 HSR subclone as indicated by the lack of *MYC* hybridization at the position of supercoiled molecules or at the dye front (Fig. 2, compare lanes 6 and 7). These results indicate that *MYC* episomes and expanded chromosomal regions containing amplified *MYC* sequences are rarely, if ever, found in the same cell.

DISCUSSION

The results presented here document the existence of submicroscopic circular extrachromosomal molecules harboring amplified *MYC* genes in two human tumor cell lines derived from patients who had not undergone chemotherapy or radiation therapy (16, 17). Since other results indicate that such molecules can be precursors of DMs (11), and DMs containing *MYC* genes in HL-60 cells were observed previously in tumor cells not subjected to *in vitro* culturing (16), we propose that the submicroscopic elements were generated *in vivo* under the conditions that presumably select for increased expression of *MYC* genes. Furthermore, the submicroscopic molecules containing amplified *MYC* genes were detected only in some early passages of HL-60 cells and in some subclones of COLO 320 (DM), but not in the initial uncloned population of COLO 320 (DM) cells, in COLO 320 (HSR) cells, or in late passage HL-60 cells that have intrachromosomal amplification of *MYC* genes present in part as inverted repeats (6). These results are similar to those we obtained in a gene transfer model system (10, 11), leading us to propose that the submicroscopic *MYC* molecules represent an early stage of gene amplification in these two tumor cell lines and that they are precursors of DMs. However, our data cannot address the issue of whether these elements represent the initial products of gene amplification, since the tumor cells grew for an undocumented number of generations *in vivo* prior to their establishment as a cell line. These results and others concerning CAD (10, 11) and *DHFR* (12) gene amplification strongly indicate that the formation of submicroscopic elements may be a general early event in the amplification process, independent of the type of selective pressure imposed. In light of statistical analyses indicating that amplification of specific oncogenes can provide significant indicators of a poor prognosis in human breast cancer and neuroblastoma (27, 28), it will be important to determine whether patients whose tumor cells are at an early stage of the

amplification process as manifested by the presence of extrachromosomal submicroscopic elements have better prognoses than those at later stages in which amplification is characterized by microscopic cytogenetic anomalies.

The idea that submicroscopic elements are early products of gene amplification and may be precursors of both DMs and intrachromosomal units of gene amplification is supported by observations from other systems. For example, early stages of methotrexate resistance due to *DHFR* gene amplification have been described in which only a small percentage of the cells contained DMs (29), implying that those without DMs contained elements below the resolution of light microscopy. In addition, DMs within a single cell are often highly variable in size (e.g., see ref. 4, 13, and 14), suggesting that small DMs could be precursors of larger ones. The frequent observation that cell lines containing DMs *in vivo* are replaced by cell lines containing the same sequences amplified intrachromosomally after passage *in vitro* has generally been interpreted in terms of chromosomal integration of DMs, followed by overgrowth of DM-containing cells by those with intrachromosomal amplification under *in vitro* growth conditions (30). Recently, we have shown that submicroscopic extrachromosomal elements are precursors of DMs and that these elements can reintegrate into chromosomes and subsequently amplify from the new site (11).

The sizes we have determined for the submicroscopic episomes in HL-60 (250 kbp) and two subclones of COLO 320 cells (120–160 kbp) are significantly different from those reported previously in which in-gel renaturation was used to obtain estimates of size of the amplified region (HL-60, ≈95 kbp; COLO 320, ≈300 kbp; see ref. 13). Note that the size estimates made here are based on the physical parameter of gel migration, while measurements made by in-gel renaturation estimate "genetic complexity." Since the physical size we determined for the *MYC* episomes in HL-60 is about twice that of the genetic complexity estimated by in-gel renaturation, it is possible that each episome contains a duplication (or higher multimer) of each amplified unit. Interestingly, a mechanism proposed by Passananti *et al.* (7) to explain *MYC* amplification in HL-60 and COLO 320 cells involves a chromosomal excision to generate replicating circular molecules containing an inverted repeat of MYC sequences. The physical size of such molecules should, therefore, be approximately double that of their genetic complexity. While our data are compatible with some of the predictions of this model, it remains to be determined whether *MYC* episomes contain an inverted repeat and whether they are formed by excision. In the absence of such information, and the proof that the first product of amplification is a circular molecule with the properties described above, additional models such as those invoking rereplication–recombination (31) or recombination of replication intermediates (32) are also compatible with the data and cannot be excluded.

The fact that the previous estimates for the size of the amplified unit in COLO 320 cells far exceed those made here suggest that sequences unlinked to the *MYC* gene, and therefore not present in *MYC* episomes, are amplified in these cells. Consistent with this idea, we have found no correlation between the number of *MYC* copies and the number and sizes of DMs in any of the subclones analyzed, suggesting that most of the DMs may not harbor MYC amplification regions.

It has been suggested that functional chromosomal substructures such as replication domains comprise each unit of amplification because large expanses of DNA are coamplified with the selected gene (1, 3). We have now described three instances in which the early products of amplification are within the size range expected for mammalian replicons (33). Importantly, in the two cases analyzed for replication, both were shown to replicate in a manner similar to that of chromosomal DNA sequences. These results indicate that one or more functional DNA replication origin must reside within each submicroscopic element, and they further strengthen the view that in some cases the unit of amplification may comprise part or all of the unit of replication (1, 3). The ability to isolate and analyze the submicroscopic elements should provide a means for elucidating the mechanisms of their formation at the molecular level and for obtaining functional mammalian replication origins.

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