Double Minutes Arise from Circular Extrachromosomal DNA Intermediates Which Integrate into Chromosomal Sites in Human HL-60 Leukemia Cells

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Abstract

Amplification of oncogenes has been found to be an important prognostic factor in behavior of patients' malignancies. In this study we have used new gel electrophoresis techniques to follow the location of amplified c-*myc* oncogene sequences in HL-60 promyelocytic leukemia cells. In passages 46–62 of the cells, the cells contain amplified c-*myc* sequences on submicroscopic circular extrachromosomal DNA (episomes). With increased passages in culture (passages 63–72) the cells lose the episome c-*myc* sequences with a shift of those sequences to double minutes. With additional passage in culture, the c-*myc* shifts from the double minutes to a chromosomal site der(5)t(5;17)(q11.2;q?11.2). Concomitant with the shift of the c-*myc* sequences into the chromosomal compartment is a phenotypic change of a shortened cell-doubling time. These studies provide the first molecular evidence of a progression from a submicroscopic location for amplified oncogene sequences to a chromosomal location for the amplified sequences.

This molecularly documented model can now be used to test various strategies to prevent incorporation of extrachromosomally located oncogene sequences into chromosomal sites. Prevention of integration of the oncogene sequences into chromosomal sites could modulate progression of patients' tumors. (*J. Clin. Invest.* 1990. 85:1887–1895.) **oncogene • c-*myc* • gene amplification • episomes • homogeneously staining regions**

Introduction

Gene amplification is a mechanism whereby transformed cells generate multiple copies of discrete regions of their genome. This amplification gives an increase in titers of gene product(s) coded for within the amplified regions (1, 2). Gene amplification has been demonstrated to be important in vitro and in vivo for resistance of patients' tumors to antineoplastic agents (via amplification of drug resistance genes) and for progression of patients' tumors (via amplification of oncogenes) (3–7).

There are two cytogenetic manifestations of gene amplification. These include double minutes (DMs)[1] and homogeneously staining regions (HSRs) (8, 9). Amplified drug resistance genes and amplified oncogenes have been localized to these structures.

DMs were first described by Spriggs et al. in 1962 (10). They are extrachromosomal chromatin bodies which lack centromeres and consequently have unequal separation at cell division. DMs have a wide variation in size even in a specific cell line. In addition, there is also a great variation in the number of DMs per cell (8, 9).

HSRs were first described by Biedler and Spengler in 1976 (11, 12). HSRs are an intrachromosomal abnormality in which regions of chromosomes stain in a uniform manner rather than in the usual differential banding pattern characteristic of G-band preparations. Within a cell line, HSRs tend to occur most commonly on specific chromosomes. Like DMs, HSRs appear to replicate early in S phase.

The origin of DMs is unclear. They have been considered as artifacts, microbial contamination, breakdown products of chromosomes, or fragments of HSRs (1, 13, 14). The origin of HSRs is also unclear. There is some evidence that suggest HSRs may be generated by unequal crossing over between sister chromatids (15, 16). Another mechanism involves saltatory replication whereby replication can be initiated at the same origin a number of times during one replication. Multiple initiations of replication generates an "onion skin" structure which together with recombination into the chromosome could generate duplicated sequences at the site of the specific gene (17). Several investigators have documented, using cytogenetic methods in a variety of systems, that HSRs appear to arise from a chromosomal integration of DMs (see discussion below).

In recent months some new molecular evidence has been reported which may give a clue as to the origin of both DMs and HSRs. In a Chinese hamster ovary (CHO) cell line resistant to the antineoplastic agent *N*-phosphonacetyl-L-aspartate (based on amplification of the CAD gene), Carroll and colleagues (18) have reported no chromosomal localization for the amplified CAD sequence but rather localization of the sequences on replicating circular submicroscopic supercoiled DNA. The same team has provided molecular and cytogenetic evidence that these circular DNAs (termed episomes) replicate and form double minutes in that CHO line (19). Episomes containing drug resistant genes have now also been documented to be present in human tumor cells with amplification

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[1] *Abbreviations used in this paper:* ABR, abnormal banding region; CAD, multifunctional protein that catalyzes the first three steps in *de novo* UMP biosynthesis; CHEF, contour-clamped homogeneous electric field (electrophoresis); DMs, double minutes; FIGE, field inversion gel electrophoresis; HSRs, homogeneously staining regions; TE, Tris-EDTA.

of the dihydrofolate reductase gene (20–22) and with amplification of the multidrug resistance gene (22, 23).

Most recently, we have reported that passages 36–46 of the HL-60 human promyelocytic leukemia line, which has amplified copies of the c-*myc* oncogene, has those copies of the gene located on extrachromosomal replicating supercoiled circular DNA (episomes) ~ 250 kbp in size (24). In the present study, we have used molecular and cytogenetic techniques to follow the fate of those episomal elements as the HL-60 cells are passaged in tissue culture. We have found that with increasing passage in culture the c-*myc*-containing episomes are replaced by c-*myc*-containing DMs. With additional time the c-*myc* sequences on the DMs move from their extrachromosomal site and integrate into the short arm of the der(5)t(5;17) chromosome. Coincidentally with chromosomal c-*myc* incorporation there is a shortening of the population doubling time, indicating a more rapidly dividing cell population. The present finding that an oncogene sequence can shift from an extrachromosomal compartment (where it has potential to be lost in the dividing cell, e.g., is unstable) to an intrachromosomal compartment (where it is not lost on cell division, e.g., is stable) provides a model in which to study methods to prevent that integration. Prevention of integration of an oncogene sequence into the chromosomal compartment could potentially modulate tumor growth.

Methods

Cell lines

Passage 46 of the HL-60 promyelocytic leukemia cell line (growing in RPMI media with 10% FBS) was provided in a T25 flask by Dr. Steve Collins at the University of Washington, Seattle, WA (25). This cell line contains approximately 16–32 copies of the c-*myc* oncogene (26). To establish subclones from the parental cell line, the cells were diluted so that approximately three to five cells were placed in each well of 96-well titer plates. The subclones were grown at 37°C in 5% CO_2 in RPMI medium enriched with 20% FBS. A total of 186 wells were initially seeded. The cells from 12 of these wells were selected for expansion. The subclones selected for expansion had the best percent recovery of episomal DNA by the alkaline lysate method described below. After several population doublings (see below), cells were transferred to six-well dishes and then to T-25 flasks. Growth medium was changed to RPMI + 10% FBS in the flasks and cells were diluted 1:4 with the fresh medium once per week. Each of the subclones was numbered and continued in passage. Two of the subclones (nos. 173 and 161) were selected for serial molecular analysis, again based on the greatest intensity of the c-*myc* signal (on Southern blots) for form I (supercoiled episome) in those subclones (see below).

As a control cell line, we utilized the C5R500 CHO cell line (18) kindly supplied by Dr. Geoffrey Wahl at the Salk Institute, La Jolla, CA. This cell line contains a 250-kbp episome containing the CAD gene.

Alkaline lysis

This technique was used to isolate the episomal DNA. Cells were removed from culture and washed with PBS. Cell counts were performed and 2.0×10^7 cells were lysed under alkaline conditions using vortexing at a pH of 12.45 for 2 min in 2 ml of 1% SDS, 50 mM NaCl, and 25 mM EDTA (18, 24). Samples were then incubated for 30 min at 30°C and then neutralized with 0.4 ml of 1.0 M Tris HCl, pH 7.0 (all chemicals from Sigma Chemical Co., St. Louis, MO). Lysates were treated with 0.3 ml of 5M NaCl and 14 μl of 20 mg/ml proteinase K (Sigma Chemical Co.) and incubated for 30 min at 37°C. Phenol (saturated with 0.2 M NaCl, 0.2 M Tris HCl at pH 8) (0.8 ml) was added, samples were mixed, and lysates were spun at 10,000 *g* for 10 min at 4°C. Aqueous phases were transferred to new tubes, 2.5 vol of 100% ethanol were added, and samples were stored at −20°C overnight. DNA was precipitated by centrifugation at 10,000 *g* for 10 min at 4°C, rinsed with 1 ml of 70% ethanol, spun-dried, and resuspended in TE buffer. Samples were made 5% with respect to glycerol for electrophoresis.

Preparation and irradiation of DNA in agarose blocks

Cells were harvested, rinsed with PBS, and suspended in PBS at a density of 2.5×10^7 cells/ml. Cell suspensions were mixed with an equal volume of molten (55°C) 1% low melting point agarose (SeaPlaque, FMC Corporation, Rockland, ME) in PBS and pipetted in 40-μl aliquots (5×10^5 cells) into a rubber-based block-forming mold (27). Blocks were allowed to harden and were placed in 15-ml culture tubes containing 2 ml of 1% Sarkosyl (Sigma Chemical Co.), 100 mM EDTA. Proteinase K (2 mg/ml) was added, the blocks were incubated for 24 h at 55°C, rinsed twice with 10 ml of TE, and stored in TE at 4°C. Blocks were placed in 100 μl of TE in 1.5-ml microfuge tubes, and irradiated by ^{137}Cs source (Gamma Cell 40, Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose of 1.2 Gy/min for 6 min before electrophoresis.

DNA electrophoresis

Alkaline lysate DNA was electrophoresed in 0.8% agarose (Ultra Pure, Bethesda Research Laboratories, Gaithersburg, MD) using electrophoretic gels run in 45 mM Tris borate, 1 mM EDTA, pH 8.3 (0.5 × TBE) buffer with recirculation at room temperature at a field strength of 1.7 V/cm for 24 h. Episomal DNA molecules embedded in agarose blocks and linearized by ^{137}Cs irradiation were electrophoresed by either field inversion gel electrophoresis (FIGE, resolving molecules below 1,000 kbp in size) or contour-clamped homogeneous electric field (CHEF, resolving molecules between 1,000 and 9,000 kbp in size) electrophoretic strategies (22, 28, 29). FIGE was performed using a pulse switcher (Hoefer Scientific Instruments, San Francisco, CA) at 7 V/cm with a 3:1 forward to reverse pulse ratio in 1% agarose gels at 14°C with buffer recirculation. Pulses were ramped linearly from 3 s forward to 60 s forward over 12 h. CHEF electrophoresis was performed using a CHEF-II DR apparatus (Bio-Rad Laboratories, Richmond, CA) in 0.6% agarose gels at 14°C with 60-min pulses for 158 h at 50 V. Molecular weights between 200 and 1,100 kbp were determined by electrophoresing the chromosomes of *Saccharomyces cerevisiae* (Beckman Instruments, Inc., Houston, TX). Linearized *Escherichia coli* chromosome was used as a 4,800-kbp marker (30). Gels were stained for 30 min in 1.0 μg/ml ethidium bromide and photographed with a red filter by transillumination with a 300-nm wavelength UV source.

DNA transfer and hybridization

DNA was transferred from agarose gels to nylon-66 membranes (Nytran, Schleicher & Schuell, Inc., Keene, NH) by alkaline capillary transfer. Gels were equilibrated in 0.5 M NaOH, 1.5 M NaCl (transfer buffer) for 30 min at room temperature and placed on top of two pieces of wetted 3MM chromatography paper (Whatman Inc., Clifton, NJ) draped into transfer buffer. Nylon-66 membrane was wetted in water and then transfer buffer, placed on top of agarose gels, and covered with two pieces of 3MM paper wetted in transfer buffer. Six inches of paper towels and a 500-g weight were placed on top, and transfers were allowed to proceed overnight. Membranes were baked 30 min at 80°C in vacuo. Membranes were hybridized with specific DNA probes labeled with ^{32}P by nick-translation kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) to a specific activity of > 10^8 dpm/μg as previously described (31). Hybridized membranes were matted with X-ray film (X-OMAT, Eastman Kodak Co., Rochester, NY) between two intensifying screens (Lightning Plus, DuPont Co., Wilmington, DE) and incubated at −70°C to generate autoradiograms.

DNA probes

Nylon membranes were probed for c-*myc* sequences using a commercially available probe (Oncor, Gaithersburg, MD) for the third exon of human c-*myc*. The PET 5 probe (a gift from Dr. Geoff Wahl) was used to probe for mitochondrial DNA.

DM and karyotype analysis

HL-60 cells in log phase of growth were harvested by a modification of the trypsin-hypotonic-colcemid (THC) technique of Hozier and Lindquist (32). Cells were incubated in 10 ml of a 1:9 0.25% trypsin/0.075 M KCl solution containing 0.1 μg/ml colcemid (Gibco Laboratories, Grand Island, NY) for 1 h at 37°C. After fixation with 3:1 methanol/acetic acid, cells were dropped on clean wet slides and dried on a 60°C slide warmer. Slides for DM analysis were stained with 2% Giemsa (Harleco, EM Science, Gibbstown, NJ). Slides for karyotype analysis were aged at room temperature for 7–10 d or heated at 60°C for 72 h before trypsin-Giemsa banding.

50 metaphase spreads from each passage were examined for the presence of DMs. The DMs were categorized by size on a scale of 1–5 (1, just barely visible with 100× oil immersion; and 5, just smaller than the width of a chromatid). The number of pairs in each size category was also recorded. A minimum of 20 metaphase spreads from each passage was also analyzed for karyotyping after trypsin-Giemsa banding. The cytogeneticist was blinded to the molecular probing results for each passage.

HL-60 in situ mapping of c-myc adapted from technique of Pinkel et al. (33)

Metaphase spreads. HL-60 cells in log phase growth were incubated for 20 min in 10 μg/ml ethidium bromide at 37°C. Colcemid (0.1 μg/ml) was added, cells were incubated an additional 20 min, and cells were pelleted. Cell pellets were suspended in 37°C 0.075 M KCl, incubated for 20 min, and pelleted again. Cell pellets were suspended in fixative (fresh 3:1 methanol/glacial acetic acid), incubated at room temperature for 20 min, pelleted, and resuspended in fixative. Suspended cells were stored overnight at 4°C.

Metaphase spreads were made by pelleting cells, resuspending in fresh fixative, and dropping suspended cells on clean microscope slides. Slides were air-dried and stored for 10 d at room temperature to age.

Chromosome denaturation. Metaphase spreads on microscope slides were treated with 150 μl of 100 μg/ml RNase (Sigma Chemical Co.) in 2× SSC, pH 7, under a coverslip (24 × 50 mm) at 37°C in a moist chamber for 1 h. Slides were rinsed in four changes of 2× SSC, pH 7, at room temperature, dehydrated by incubating for 2 min in 70%, 85%, and 100% ethanol (consecutively), and dried with an air jet. Metaphases were treated with 150 μl of 0.5 μg/ml proteinase K (Sigma Chemical Co.) in 20 mM Tris HCl, 2 mM $CaCl_2$, pH 7.5, for 7.5 min at 37°C under a coverslip, washed in 2× SSC, fixed in 4% paraformaldehyde (in 50 mM $MgCL_2$, 1× PBS, pH 7.5) for 10 min, and rinsed in 2× SSC.

Chromosomes were denatured by immersing slides in 70% formamide (Sigma Chemical Co.), 2× SSC, pH 7, for 2 min at 70°C. Slides were quickly transferred to 70% ethanol for 1 min, followed by 85% ethanol and then 100% ethanol, also for 1 min, and then dried by air jet.

Biotin labeling of c-myc probe. 1 μg of pSVc-*myc* (a plasmid containing a simian virus promoter along with c-*myc* sequence) was labeled with biotin by incubation with 2.5 μl of dCTP, dATP, and dGTP from the Boehringer Mannheim nick kit, in addition to 5 μl of 10× nick buffer and 1 μl of biotin-16-dUTP (Boehringer Mannhein). Tritiated dATP (1 μl; 250,000 cpm) was also added to follow biotin incorporation. The nick volume was made up to 45 μl, and 5 μl of nick enzyme was added. The reaction proceeded at 15°C for 90 min and was stopped by addition of 10 μl of 0.1 M EDTA. DNA was separated from unincorporated dNTPs by chromatography on BioGel P-60 resin (BioRad Laboratories) in TE, pH 7.5. Fractions were scintillation counted (^3H window), and 400 μl of labeled DNA (2.5 μg/ml) was collected for hybridization to HL-60 chromosomes.

Hybridization. Deproteinated and denatured HL-60 metaphase chromosomes were hybridized with biotin-labeled pSVc-*myc* probe by incubation at 37°C at a concentration of 100 ng of labeled probe/ml of hybridization mix (10% dextran sulfate, 50% formamide [Sigma Chemical Co.], 2× SSC, and 50 μg/ml denatured herring testes DNA [Sigma Chemical Co.]) under a glass coverslip. Labeled probe and carrier DNA were denatured by making them 0.1 N with respect to NaOH and incubating at 37°C for 10 min before addition to the hybridization mix. Hybridization mix (30 μl per slide) was placed on slides warmed to 37°C, and coverslips (24 × 50 mm) were placed on top and sealed with rubber cement. Hybridizations were allowed to proceed overnight at 37°C in a tissue culture incubator, and slides were washed with three changes of 50% formamide, 2× SSC, pH 7, at 45°C for 2 min each, once in room temperature 2× SSC for 2 min, and then placed in 0.1 M sodium phosphate, 0.1% NP-40, pH 8.0 (PN buffer) until ready for staining.

Slide staining. All subsequent manipulations were performed in a room with very low light levels. Slides were removed from PN buffer, drained briefly, and 75 μl of 5 μg/ml fluorescein-labeled avidin (Vector Laboratories, Inc., Burlingame, CA) in PN buffer containing 5% nonfat dry milk (PNM buffer) was added, followed by a glass coverslip (24 × 50 mm). The slide was incubated for 20 min at room temperature in a moist chamber, and then washed in three changes of PN buffer for 2 min each. Slides were drained, and 75 μl of 5 μg/ml biotinylated goat anti–avidin (Vector Laboratories, Inc.) in PNM buffer was incubated under a coverslip for 20 min at room temperature. Slides were washed in three changes of PN buffer, and cycles of avidin, anti–avidin, and then avidin labeling separated by PN buffer washes were repeated again. Slides were washed once again in three changes of PN buffer, drained well (but not dried), and stained with 22 μl of antifade containing 0.2 μg/ml propidium iodide covered with a no. 1 glass coverslip (24 × 50 mm).

Chromosomes could be viewed immediately by fluorescent scope at 64 power under oil using the appropriate filter (FITC). Metaphases were photographed with Ecktachrome 400 slide film (Eastman Kodak Co.) with ∼ 15-s exposures.

Borate banding. After fluorescent staining and photography, chromosomes were banded by soaking slides extensively in borate buffer and then staining in Giemsa (Harleco) diluted 1:3 in borate buffer. Chromosome morphology is very poor after proteinase K and denaturation/hybridization, but banding patterns could be determined for some metaphases.

Results

Fate of episomal DNA. As discussed above we have previously documented that passages 36–62 of HL-60 cells have c-*myc* sequences located on supercoiled extrachromosomal DNA ∼ 250 kbp in size (episomes). The parent HL-60 line and the series of 12 subclones of the HL-60 were reexamined to document that they indeed had c-*myc* sequences located on episomal DNA (Fig. 1). As can be seen in this figure all 13 cell lines had evidence of an episome containing c-*myc* sequence in early passages (48–62) of the cells.

Two of these subclones (nos. 173 and 161) were followed in serial passages for the presence of c-*myc* on episomes. As can be seen in Fig. 2, there was a decline in the amount of c-*myc* located on episomal DNA from an average of 400 density units in passage 63 cells down to essentially no c-*myc* present on episomal DNA by passage 73. Fig. 3 provides molecular evidence for this finding in one of the subclones (clone no. 173). Thus, with increasing passage number, the amount of c-*myc* present on episomal DNA was declining even though the total c-*myc* present in the cell line was staying about the same (13-fold amplification). Thus the c-*myc* sequences must have been shifting out of the episomal compartment into some other compartment.

As has been discussed in the introduction, Carroll and colleagues (19) had noted that the CAD gene present as episomal DNA in CHO cells appeared to be precursors for DMs when

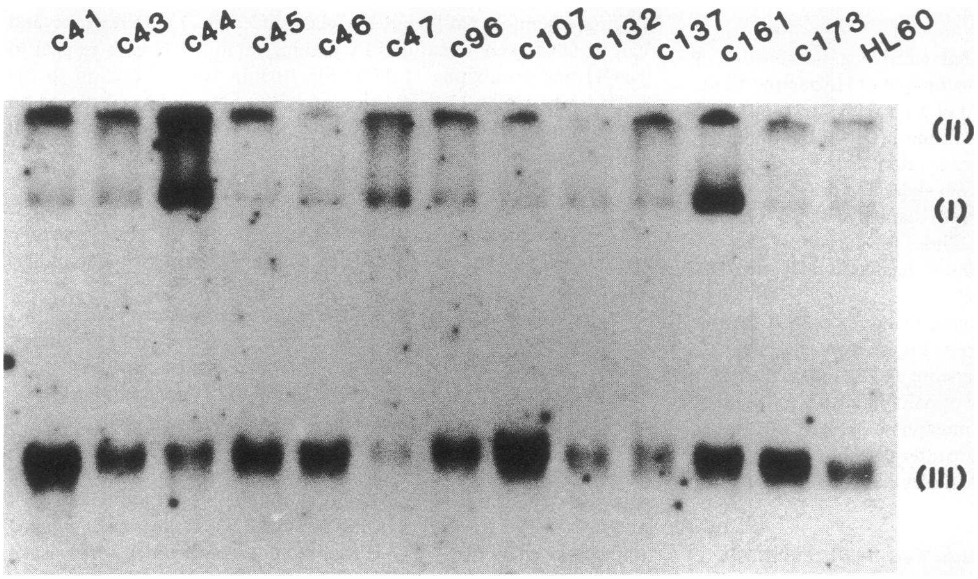


Figure 1. Parent line HL-60 and 12 subclones were examined to determine whether or not they contained episomal c-*myc*. All lines were processed using an alkaline lysate technique (see text) to isolate circular extrachromosomal DNA. The alkaline lysate material was run on a conventional gel 5–6 V/cm for 17 h at room temperature. Southern transfers from the gel were hybridized with a c-*myc* probe. All subclones demonstrated evidence of episomal DNA (different amounts because different number of cells of each clone were processed). The episomal DNA was present in three different forms including form I (supercoiled), form II (open circular which cannot migrate into the gel under the standard electrophoresis technique used), and form III which represents the linearized form of the circle. A reprobe of this blot with an actin sequence did not detect the episomal molecule, indicating the bands noted are specific for the c-*myc* sequence.

the cells were passaged under selective pressure with the drug *N*-phosphonacetyl-L-aspartate. To determine if the c-*myc* sequence in the HL-60 cells may have transferred to DM DNA we examined metaphase spreads of each passage of subclones 173 and 161 to determine whether or not DMs were present. As can be seen in Fig. 4, the percentage of HL-60 cells with DMs increased from about 20% at passage 60 to about 75% by passage 68. The average number of DMs per cell also increased between passages 60 and 68 (see Fig. 4). However, with further following of these cells, by passage 74 the DMs essentially disappeared from the HL-60 cell (see Fig. 4). The karyotype was also noted to change with increasing cell passage. Chromosome analysis of passages 60–63 revealed a modal chromosome number of 45 with the following karyotype: 45,X,−X, −5,−17,+18, 9p−,10p+,14q−, 16+,16q+, +der(5)t(5;17) (q11.2;q?11.2), +DM (stem line 1). By passage 70, a second stem line was apparent showing the following: 44,X,−X, −5,−8−17, −17,+18,9p−, 10p+,14q−,16q+, 16q+,+der(5) t(5:17) (q11.2; q?11.2) +der(5) t(5;17) (q11.2; q?11.2) (stem line 2). This accounted for 60% of cells and showed no DMs.

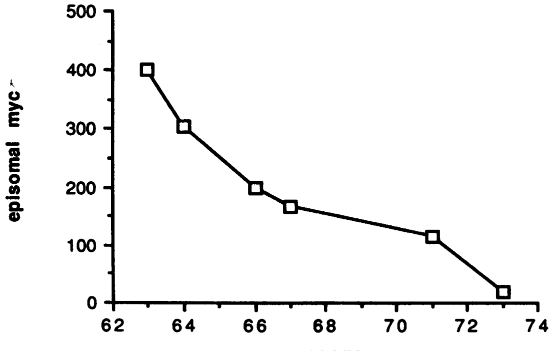


Figure 2. HL-60 subclones were passaged as described in the text and assayed for the amount of c-*myc* present on episomal DNA using the alkaline lysate technique and standard gel electrophoresis technique described above. The amount of c-*myc* present as episomal DNA was quantitated by densitometry of films done from Southern transfer from the electrophoresis gels. The amount of c-*myc* present on episomal DNA declined from 400 density units on passage 63 cells down to nearly undetectable amounts of c-*myc* present on episomal DNA by passage 73.

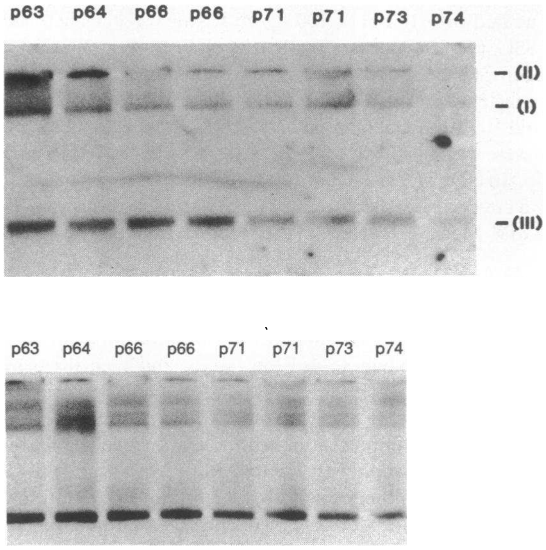


Figure 3. (*Top*) Decline in the amount of c-*myc* present on episomal DNA for one representative subclone (no. 173). The cell line was followed from passage 63 to passage 74 with alkaline lysates done as described in text and gel electrophoresis performed on the lysates. After Southern transfer, the membrane was then hybridized with a c-*myc* probe. Note the decline in c-*myc* signal of all three forms of the episomal DNA (forms I, II, and III) with increased passage of the subclones. Also note the good reproducibility of the assay when two different alkaline lysate procedures were performed on the same passage (passages 66 and 71). (*Bottom*) Same blot washed and reprobed with pET5 mitochondrial DNA sequence to document amount of alkaline lysate DNA loaded in each lane.

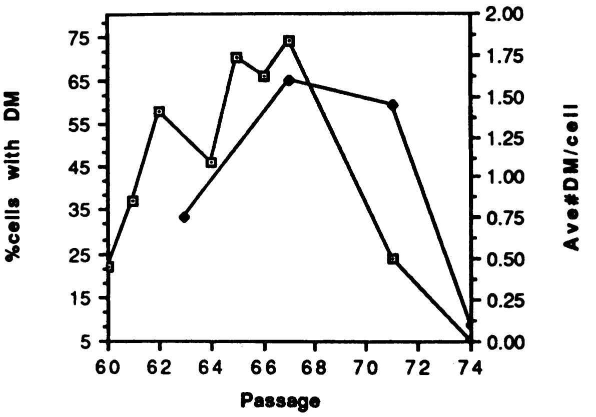


Figure 4. HL-60 cells passaged in culture were evaluated for the percentage of cells with double minutes (□) (50 metaphase spreads examined; see Methods for details). The percentage of cells with double minutes increased from 20% to 75% from passage 60 to 68 but decreased by passages 71 and 74. The average number of double minutes per cell followed a similar pattern (■).

The duplicated der(5) appeared to have a slightly larger short arm region in some cells. This duplicated chromosome may have arisen by a non–disjunction event. By passage 75, 90% of cells were from the stem line 2 and practically no DMs were seen. These surprising results were studied at the molecular level to ascertain what was happening to the c-*myc* sequences.

To determine whether c-*myc* sequences were located on the double-minute DNA we utilized a new gel technique suited for isolation of large (1–9 mbp)-sized DNA. DMs visible on light microscopy are thought to be at least 1–2 mbp in size. The new gel technique is called CHEF electrophoresis (28, 29). This technique was performed on passages of the HL-60 cells known to contain DMs (passages 64–72) and cells in which DMs could no longer be detected cytogenetically (passage 74). As can be seen in Fig. 5, there are distinct c-*myc* containing species at sizes of 1.5 mpb and at approximately twice that size. These are the appropriate sizes for double-minute DNA (30). Double-minute DNA running in this position has also been described for other cell lines (30). Also of note in Fig. 5 is that the signal intensity for the double-minute DNA signal appears to decrease with increasing passage number. However, the intensity of signal in the zone of compression increases in intensity. The zone of compression contains very large DNA molecules possessing c-*myc* sequences. These probably represent chromosomal incorporation of the c-*myc* sequence. To explore that possibility we have performed in situ hybridizations on these later passage cells. As can be seen in Fig. 6, c-*myc* has integrated into the short arm of the duplicated derived chromosome 5,der(5)t(5;17)(q11.2;q?11.2) in the cells. This localization was documented in every single one of the 50 different metaphases examined. Thus, with both molecular and cytogenetic techniques, it appears that with continued passage of the HL-60 cells, c-*myc* sequences integrate into chromosomal DNA. It is of note that Wolman and colleagues (34) have previously documented that amplified c-*myc* was localized to the long arm of a marker chromosome M3q+, which appears to be similar to the der(5) described here.

Fig. 7 summarizes the c-*myc* sequence data from the alkaline lysate and the CHEF techniques. As can be seen in this figure, as the amount of c-*myc* in the episome decreases, there is an increase in c-*myc* sequences in double-minute DNA. As the amount of c-*myc* sequences on the DMs decrease the amount of c-*myc* sequences present on chromosomal DNA increases. We have now followed these cells with karyotype examination and CHEF gels for 26 additional passages (up to passage 100) and to date have found no evidence of transfer of the c-*myc* sequences back into the double minute or episomal compartments.

Effect of movement of c-myc on cell-doubling time. The populations doubling times of the HL-60 cells were followed with increasing passage number. As can be seen in Table I, as the cells are passaged the population doubling time becomes progressively shorter. By the time the chromosomal integration of c-*myc* has taken place, the doubling time has fallen to 44.1 h.

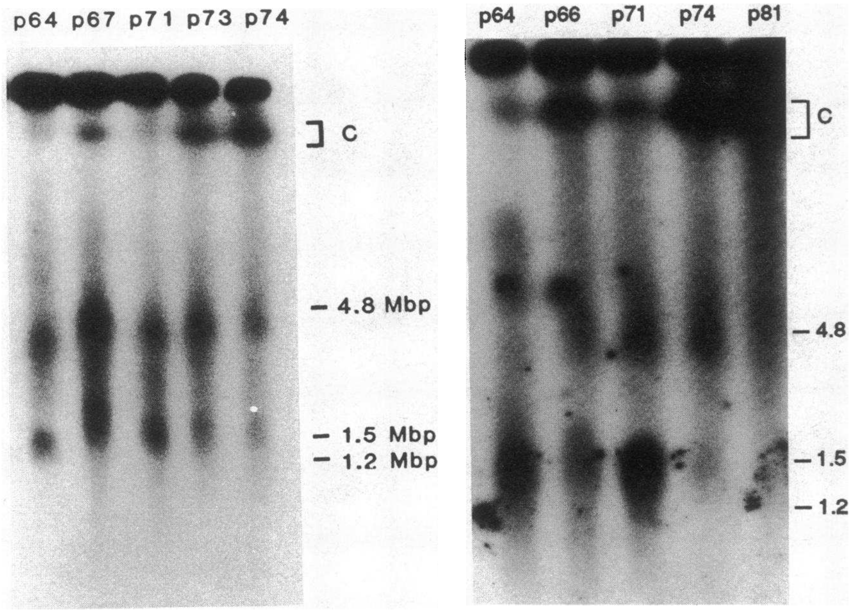
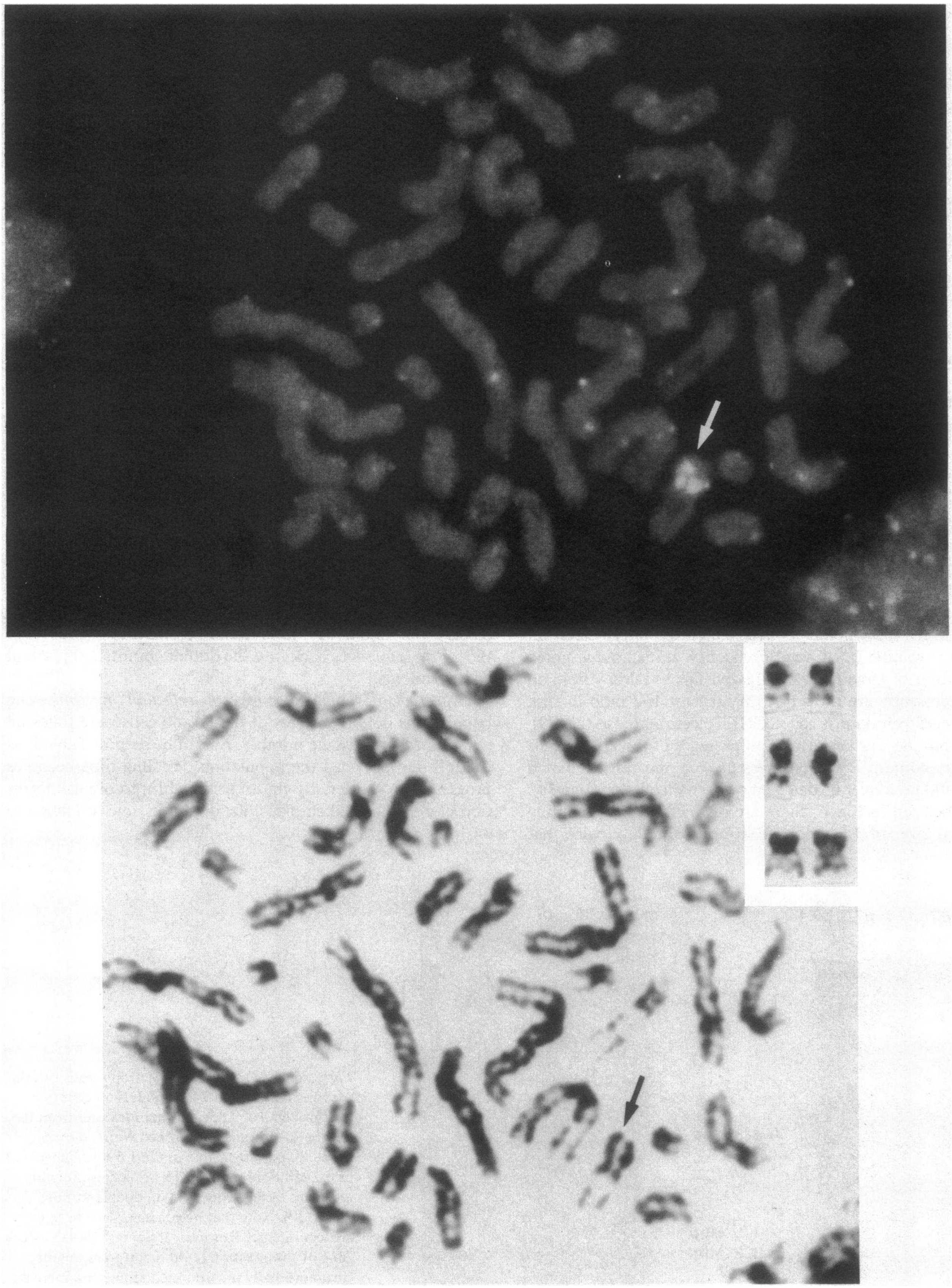


Figure 5. (*Left*) CHEF gel electrophoresis run on passages 64 through 74 of the HL-60 cells (see text for details). After Southern transfer from the gel, the membrane was probed with a c-*myc* probe. Bands are visible in the 1.5 mbp range and in the < 4.8 mbp range. These signals probably represent c-*myc* sequences on double minute DNA (30). Note that by passage 74 these signals are less intense. However, the c-*myc* signals in the zone of compression (*c*), thought to represent chromosomally incorporated sequences, increase in intensity over passages 73 and 74. (*Right*) Same experiment repeated with intermediate (passage 66) and later passage cells (passage 81) demonstrating same trend.



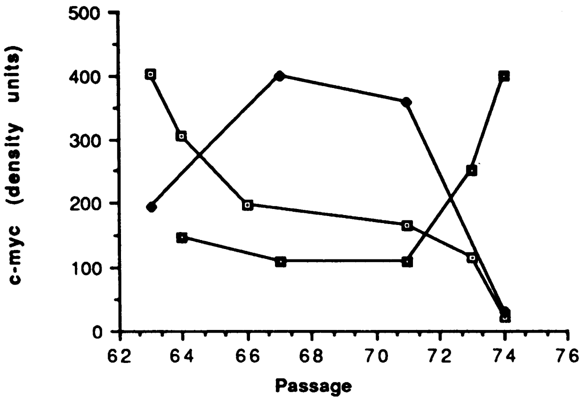


Figure 7. Composite of c-*myc* sequence localization data. Note that c-*myc* sequence (determined from densitometry from electrophoresis gels) is located in the episomal compartment in early passages of the cells (□). It shifts to the double minute compartment with maximal localization there between passages 67 and 71 (♦). It finally shifts to the chromosomal compartment by passage 74 (■). The amount of c-*myc* (*y*-axis) is expressed in densitometry units.

Table I. Cell Population Doubling Time for the HL-60 Cells in Serial Passage

Passage number	Cell population doubling time
	h
9* (ref 44)	55–60
63	48
71	45.4
75	44.1

* See Gallagher et al. (44).

Discussion

Molecular and cytogenetic evidence has been presented to support the concept that extrachromosomal DNA (episomes) containing c-*myc* sequences in earlier passages of the human HL-60 cell line are replaced in later passages of the line by larger structures identifiable as DMs. Evidence is also presented to indicate that with further passage in culture these c-*myc* sequences appear in chromosomal sites. Thus the c-*myc* sequences appear to evolve from the episomal compartment to a double minute compartment (both of these being extrachromosomal DNA) and finally into an intrachromosomal compartment (see Fig. 7 for a summary). What is quite surprising is the rapidity with which this transfer to different compartments occurs (over a period of 10 passages [with cell passages done two times per week = a period of 35 d]). One possible mechanism for this rapid change is that the population with the chromosomally incorporated c-*myc* becomes the predominant population because the generation time for that population is shorter than for the population of cells which have episomes or DMs. Biedler and colleagues (35) have previously noted that human neuroblastoma cell lines with homogeneously staining regions have shorter generation times than do double minute–containing cell lines. Another possible explanation for the rapid change in the population is a parallel, but not simultaneous evolution of episomes to DMs and to HSRs. Our cytogenetic evidence is also compatible with that possibility.

Other investigators have previously presented cytogenetic evidence that HSRs appear as DMs disappear. Biedler et al. (35) first presented the concept of a reciprocal relationship between the appearance of HSRs and the disappearance of DM-containing cells in two human neuroblastoma cell lines (SK-N-MC and NAP). Levan et al. (36–38) noted the appearance of "CM" (c-minus) chromosomes or abnormal banding regions (ABRs) and disappearance of DMs in the mouse SEWA ascites tumor line. Cowell (40) has observed that an in vitro transformed mouse salivary gland epithelial cell line had DMs observed in 100% of cells at early passage. After 17 in vitro passages a subpopulation of cells appeared that had no DMs but contained an HSR on chromosome 5 (39, 40). Similar findings have been reported by George and Powers (41) in a mouse adenocortical cell line and by Quinn et al. (42) in a human neuroendocrine tumor cell line. Most recently, Trent and colleagues (43) have described a newly biopsied human malignant melanoma that had an HSR on chromosome 7 in only 1 of 110 cells whereas 99 of the 110 cells had multiple DMs. When the tumor was established in culture, by passage 9 in vitro, 100% of the cells had an HSR on chromosome 7 and no DM-containing cells were observed. All of these prior studies as well as the present study suggest a selection for HSR- or ABR-bearing cells with increasing passages in culture.

Other workers have recognized a variety of changes in the cytogenetics of the HL-60 cell line over time. Gallagher and colleagues (44) noted the tumor from the patient and the cell line (passages 6–55) had only "occasionally observed" cells with DMs. Other abnormalities included deletion of chromosomes X, 5, 8, and 17 with the appearance of a submetacentric marker chromosome (M3).

Norwell and colleagues (45) examined karyotypes of HL-60 cells frozen in 1978 and found in their cells (passage number not given), that "nearly all metaphases contained numerous DMs." In HL-60 cells frozen 1 year later (1979), they noted a normal chromosome 8 had been replaced with an 8q+ chromosome with an abnormal banding pattern (45). Of particular note, DMs were not observed in any of the cells. These findings were also noted in HL-60s kept in continuous passage since 1978. Thus they presented indirect evidence that c-*myc* was amplified in the HL-60 leukemia line originally in DMs

Figure 6. In situ hybridizations were carried out on passage 74 cells (see text for details). (*Upper panel*) In this representative metaphase with hybridization with the c-*myc* probe, there is increased signal noted over an area of a marker 3 chromosome (*arrow*); (*lower panel*) by borate banding patterns (*arrow*), this signal appears to be a chromosome derived from chromosomes 5 and 17 (der5p;17q). Some signal on chromosome 8 was noted in some metaphase spreads but there was clearly no increased hybridization in the native gene locus. (*Lower panel inset*) G-banding of partial karyotypes of three cells showing two copies of the der(5)t(5;17)(q11.2;q?11.2). The right chromosome in each pair is suggestive of increased p-arm material above the centromere.

which progressed to c-*myc* amplification on an abnormal banding region on 8q. This finding of amplification of c-*myc* on an ABR on chromosome 8 was of particular note because later the native locus of c-*myc* was found to be 8q24 (46–48).

Wolman and colleagues (34) studied approximately passage 50 of the HL-60 line. They noted a modal chromosome number of 45 with losses of no. 5, no. 17, and X, and a gain of no. 18 and the presence of a number of markers including an M3q+. They found no cells with multiple minutes and noted fewer than 1% of metaphases had possible DMs. The M3q+ was interpreted as a translocation t(5p;17q). The ABRs on M3q+ were found to be the site of the amplified c-*myc*. They concluded the breakpoint on chromosome 17 in 17q21 was the area where the c-*myc* DNA sequences were inserted. Basically their work documented that, if DMs do integrate into a chromosome, "it is not obligatory that the native region of the gene be involved in the integration event" (34). They postulated that the mobile form of the c-*myc* will seek a region susceptible to breakage or "take advantage of ongoing breakage and reunion events." Our data indicate that the c-*myc* sequences on the double minutes incorporated into a duplicated chromosome, supporting the concept that it make take a duplication event to get incorporation.

Misawa et al. (49) recently examined HL-60 cell lines (passages not designated) one of which had the ABR on chromosome 8 (with no DMs), reported by Nowell et al. (45), while the other had DMs but no abnormality of chromosome 8. In situ hybridization studies demonstrated localization of c-*myc* to the ABR in the one cell line and to the DMs in the other. These data were consistent with the concept that these chromosomal abberations were interchangeable.

As noted above, gene amplification has been described for both oncogenes and drug resistance genes in patients' tumors. The two cytogenetic manifestations of gene amplification are double minutes and homogeneously staining regions (also known as expanded chromosomal regions or abnormally banding regions). Since DMs do not have a centromere they segregate randomly at each mitosis and consequently are lost from the population over time unless there is some selective pressure to give cells with amplified sequences on DMs a growth advantage. On the other hand, if amplified sequences are located on expanded chromosomal regions (HSRs), they segregate normally during mitosis and are probably not lost even in the absence of selection (1, 11, 23).

In the present study we have identified a time in the passage of HL-60 human promyelocytic leukemia cells during which the c-*myc* sequences are located on extrachromosomal sites (first on episomes, then later on DMs). It is conceivable these c-*myc* sequences might be vulnerable to loss while they are on these extrachromosomal sites. Loss of the c-*myc* sequences could be associated with a less aggressive biologic behavior. However, once the c-*myc* sequences are located on the chromosome it is unlikely the oncogene sequences can be eliminated.

Several strategies have been proposed to eliminate extrachromosomal DNA gene sequences. Therefore, knowing that oncogene sequences or drug resistance gene sequences are on extrachromosomal DNA makes a strategy for elimination of the extrachromosomal sequences (for therapeutic reasons) a possibility (20, 23, 24, 50). Since it has recently been demonstrated that human tumors taken directly from patients more often have DMs present than HSRs, this strategy may have some clinical application (Benner, S. E., G. M. Wahl, and D. D. Von Hoff, manuscript submitted for publication).

Our work has not addressed the question of how the episomes arise. They are present in the earliest passages of the line. Wahl (23) has proposed that some insult to the cell causes perturbation of DNA synthesis which leads to stalled replication loops, excision of which result in the formation of episomes. As of now, there is no definite proof this is the case. However, it is an area of active investigation.

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