Evidence for Linear Extrachromosomal Elements Mediating Gene Amplification in the Multidrug-Resistant J774.2 Murine Cell Line

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ABSTRACT: Previous studies from our laboratory have demonstrated specific cytogenetic alterations accompanying development of colchicine resistance in the J774.2 murine cell line and in two sublines (J7.CI-30 and J7.CI-100) [1]. Although gene amplification is not observed in the parental J774.2 cell line, a ~35-fold amplification of the gene for p-glycoprotein (mdr) was noted in the J7.CI-30 subline (770-fold CLC®) and a ~70-fold amplification in the J7.CI-100 subline (2500-fold CLC®). In this study, we analyzed the localization and organization of the mdr gene. In the colchicine-resistant (CLC®) J7.CI-30 subline, the p-glycoprotein domain was observed to reside on differently sized extrachromosomal elements. Our results indicate not only circular extrachromosomal elements but also linear extrachromosomal elements. By means of pulsed-field gel electrophoresis (PFGE), the sizes of the extrachromosomal elements were shown to be >2,500 kilobasepairs (kb), 800 kb, and 400 kb. In contrast, the J7.CI-100 subline was characterized by the presence of homogeneous staining regions (HSRs). We have noted that with increasing colchicine resistance the extrachromosomal elements are replaced by HSRs. Our findings of linear elements that appear to be precursors of HSRs may offer a new way to interpret different theories of extrachromosomal gene amplification. The J7.CI-30 cell line presents a unique system to analyze further the formation and structure of extrachromosomal elements.

INTRODUCTION

DNA amplification is known to occur in developmentally regulated genes [2]. Strong evidence also shows that amplified cellular oncogenes play a causative role in tumor initiation, progression, or both [3, 4]. Furthermore, gene amplification is frequently associated with acquisition of the multidrug-resistant (MDR) phenotype [5, 6]. Amplified sequences have been localized in double minutes (dmin) and homogeneously staining regions (HSRs) [7, 8].

Despite the importance of dmins and HSRs to the process of gene amplification, little is known about the underlying molecular mechanism of their formation. Studies to date indicate that the structure of amplified DNA domains can be generated by a variety of mechanisms and that the detailed events may vary in different cell types. In past years, several models for the generation of extrachromosomal elements and HSRs have been proposed. Recent models hypothesize a mechanism involving multiple replications at a single origin, leading to an "onion skin"-like structure [4, 9]. Multiple initiations of replication have been noted in amplification events of chorion genes in Drosophila [10]. Schimke et al. suggested that perturbation of cell cycle patterns may result in chromosome fragmentation and generation of various recombination events [11]. Sen et al. suggested that prematurely condensed S-phase chromosomes are precursors of extrachromosomal elements [12]. Chromosomal regions that are prematurely condensed while undergoing DNA replication are believed to be liberated from chromosomes, eventually forming HSRs. Based on this model, large linear DNA structures may be precursors for dmins. Wahl et al. proposed that dmins stem from smaller circular extrachromosomal elements [13]. These investigators showed that formation of submicroscopic extrachromosomal elements represents one of the earliest events in gene amplification. These small extrachromosomal elements are believed to enlarge in time to the size of microscopically detectable bodies (dmin), which eventually integrate into chromosomes to generate HSRs.

With the J774.2 cell line, we identified a unique system to analyze further the underlying mechanisms that lead to formation of extrachromosomal elements and HSRs. Based on our recent studies of mdr gene amplification in murine J7.CI-30 cell lines, we analyzed the structure and location of the amplified DNA domain. In the (CLC®) J7.CI-30 subline, we identified several extrachromosomal elements of different sizes. All the extrachromosomal elements have been shown to contain the amplified p-glycoprotein domain. Our study of the J7.CI-30 cell line suggests that not
only circular but also linear structures form a subset of the smaller extrachromosomal elements. Linear extrachromosomal elements of 42 (kb) kilobases long have previously been reported in yeast [14]. Circumstantial evidence also indicates linear dmins in neuroblastic cells [15]. Because linear molecules may simply represent linearization of native DNA circles, we included several control experiments. First, we demonstrated that no significant breakage is caused by DNA preparation. Second, we excluded the possibility that cell density accounts for the linear extrachromosomal elements. Third, the presence of large extrachromosomal structures that appear to be intact in the nonirradiated cells was used as an internal control for random breakage.

Our study of the J7.C1-100 subline indicates that the extrachromosomal elements are replaced by HSRs as the cells become more colchicine resistant. This aspect of our results is in good agreement with Wahl’s model showing that smaller extrachromosomal elements are precursors of dmin which in turn can integrate into chromosomes to form HSRs. The presence of linear extrachromosomal elements in the J7.CI-30 cell line can more easily be explained by alternative models, however (e.g., prematurely condensed chromosomes). Thus, our results indicate a novel mechanism that combines different aspects of recently proposed models for extrachromosomal gene amplification.

MATERIALS AND METHODS

Cell Culture

The parental J774.2 cell line and the colchicine-resistant sublines (J7.CI-30 and J7.CI-100) were maintained as previously described [16, 17]. The colchicine-resistant sublines were derived by a stepwise increase of colchicine concentration. All cell lines were tested for mycoplasmal contamination.

Cytogenetic Analysis

Cells in exponential growth were harvested after 1 hour of colchicine treatment. Chromosomes were prepared as previously described [18].

DNA Preparation, Gel Electrophoresis, and Southern Hybridization

Cells were embedded in agarose plugs to a final concentration of 5 × 10⁶ cells/ml. DNA was prepared essentially as previously described [19]. To linearize extrachromosomal DNA circles, agarose plugs were placed in microcentrifuge tubes and exposed to gamma irradiation (137Cs source). Pulsed-field gel electrophoresis (PFGE) was performed using a modified contour clamped homogenous electric field (CHEF) system [20]. Gels (0.8% agarose) were run in 0.5 × TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) at 12°–14°C with varying switching times. Transfer and hybridization have been previously described [21]. DNA probes were labeled with 32P by the random primer method of Feinberg and Vogelstein [22]. The probes used for this study were pgpl and pgp2 (provided by V. Ling, Ontario Cancer Center Institute, Toronto, Canada) which hybridize to mdr1a and mdr1b, respectively [23–25]. After hybridization, the filters (zeta probe) were washed twice in 2 × SSC/0.1% sodium dodecyl sulfate (SDS) for 20 minutes each at room temperature, and twice in 0.1 × SSC/0.1% SDS for 30 minutes each at 60°C.

RESULTS

Identification of Extrachromosomal Elements

Molecular and cytogenetic analysis on J774.2, a mouse macrophage-like cell line, and two CLC sublines was performed to determine the organization of the amplified p-glycoprotein domain. The J7.CI-30 subline, which exhibits a 35-fold mdr amplification, was analyzed for the presence of extrachromosomal DNA. When metaphase spreads from the J7.CI-30 subline were analyzed, extrachromosomal structures were observed in only a few metaphases spreads. Our cytogenetic analysis showed not only dmin bodies, but also single minutes in the J7.CI-30 cells. In addition, the J7.CI-30 cell was screened with PFGE for the presence of extrachromosomal elements. DNA was deproteinized in agarose plugs, separated by PFGE, and hybridized with pgpl and pgp2 which identify the mouse mdr1a and mdr1b genes, respectively. After hybridization of nonirradiated cells, a strong signal at 800 kb and a weaker signal at 400 kb were observed, indicating linear extrachromosomal DNA (Fig. 1A). After irradiation with 6 × 10² rad, a signal >2,500 kb was detected in addition to the signals at 800 and 400 kb. This signal at >2,500 kb most likely represents DNA from the dmin identified in the metaphase spread. No difference was noted between probes pgp1 and pgp2. Our results indicate extrachromosomal structures of different sizes containing amplified p-glycoprotein domains in the J7.CI-30 cell.

In the J7.CI-100 cell line that exhibits a ~70-fold mdr amplification, PFGE analysis did not indicate the presence of extrachromosomal DNA. After hybridization with pgp1 and pgp2, a “smear,” but no discrete band, was noted in the PFGE blot (Fig. 1C). These results are consistent with the karyotypic analysis. As previously described, HSR were identified in most cases and localized to a derivative 7 chromosome [der (7HSR)]. Hence, the dmin and the small extrachromosomal elements observed in the J7.CI-30 subline (~35-fold amplified) are replaced by HSR in the J7.CI-100 subline (~70-fold amplified). The replacement of extrachromosomal elements by HSR is consistent with the notion that dmin and smaller extrachromosomal elements are replaced by HSR as the cells become more colchicine resistant. This aspect of our results is in good agreement with Wahl’s model showing that smaller extrachromosomal elements are replaced by HSR as the cells become more colchicine resistant.

Structural Analysis of Extrachromosomal Elements

To analyze the structure of extrachromosomal elements in the J7.CI-30 cell line, we exposed the cells to gradually increasing gamma irradiation. Although the size of the 800-kb signal was not affected by gamma radiation of up to 75 × 10⁶ rad, there was an increase in signal intensity at low radiation doses (Fig. 2). This increase was observed consistently when J7.CI-30 cells were exposed to low doses of gamma irradiation. These findings indicate circu-
**Figure 1**  (A) Pulsed-field gel electrophoresis (PFGE) analysis of extrachromosomal elements in the J7.C1-30 cell line. High-molecular-weight DNA was gamma-irradiated as indicated, size-fractionated by PFGE, and subsequently hybridized with the pgp1 probe. The blots were exposed for 8 hours. The running conditions were as follows: 125 V, 180-second pulse time, 28-hour run time, temperature 14°C, 0.8% agarose in 0.5 TBE. The size of the fragments were estimated with yeast chromosomes (Beckman). After gamma irradiation at 600 rad, a hybridization signal at $>2,500$ kb is evident. The exposure time was 4 days to identify the 400-kb signal. (B) Ethidium bromide stain of the PFGE gel. DNA was gamma-irradiated as indicated and size-fractionated. The running conditions were as in A. (C) Analysis by PFGE of amplified p-glycoprotein domains in the J7.C1-100 cell line. DNA from the C1-100 subline was gamma-irradiated as indicated, size-fractionated by PFGE, transferred, and hybridized with pgp1. The running conditions were as in A. In contrast to the J7.C1-30 subline, no extrachromosomal elements were identified.

**Figure 2**  PFGE analysis of extrachromosomal elements in the J7.C1-30 cell line. DNA was gamma irradiated as indicated, fractionated by PFGE, transferred and probed with pgp1. The running conditions were as described in the legend to Fig. 1A. The strongest signal was noted at 2,000 rad of gamma irradiation. The increase in signal intensity at low irradiation doses indicates circular elements that are linearized by irradiation. After a dose of 15,000 rad, the signal at 800 kb was lost due to fragmentation.
 Extrachromosomal Elements in J774.2

Figure 3 (A) Pulsed-field gel electrophoresis (PFGE) analysis of extrachromosomal circular elements in Raji cells. DNA was isolated from Raji cells containing 180-kb Epstein-Barr virus (EBV) episomes. After gamma irradiation of 0 to 80,000 rad, the DNA was fractionated by PFGE, transferred, and hybridized with an EBV-specific probe. The running conditions were as indicated in the legend to Fig. 1A. No discrete band was noted in nonirradiated cells and in cells irradiated with 6,000 rad, whereas a strong signal at 180-kb was detected when DNA was exposed to higher doses of gamma irradiation. (B) Analysis by PFGE of extrachromosomal elements in J7.C1-30 cells. Cells were harvested at different levels of confluencies. Lane A: Cells were grown to approximately 20% confluency (1 day). Lane B: Cells were grown to 100% confluency (2 days). Lane C: Cells were grown for 2 days beyond confluency. After size fractionation, the DNA was transferred and probed with pgpl. The running conditions were as described in the legend to Fig. 1A.

lar elements that are linearized by irradiation, thereby causing the increase in intensity of the 800-kb signal. Hence, our results indicate circular fragments in the J7.C1-30 cell line.

As shown in Fig. 2, however, PFGE analysis also disclosed apparently linear extrachromosomal DNA in nonirradiated J7.C1-30 cells. The finding of extrachromosomal elements migrating as linear molecules is at variance with Wahl's model of gene amplification which postulates only circular elements as dmin precursors. To analyze the structure of these smaller extrachromosomal elements further, we performed the following experiments.

First, circular molecules may be linearized in the process of DNA preparation and during gel electrophoresis. To exclude this possibility, we stained nonirradiated DNA by ethidium bromide after separation by PFGE (Fig. 1B). The ethidium bromide stain of the total DNA did not indicate disproportionally high levels of randomly broken DNA that could account for the hybridization signals at 800 and 400 kb. In addition, using the same procedure as for the preparation of J7.CI-30 DNA, we isolated DNA from Raji cells, which contain circular 180-kb episomes. After hybridization with an Epstein-Barr virus (EBV)-specific probe, we noted no discrete band in nonirradiated cells, indicating intact DNA circles that do not enter the PFGE gel (Fig. 3A). As a positive control, DNA from Raji cells was exposed to increasing doses of gamma irradiation. After irradiation, a signal at 180 kb was detected, indicating linearized DNA molecules. These results demonstrate that no significant DNA breakage results from DNA preparation or fractionation.

Second, linearization of circular DNA molecules may occur during cell culture, e.g., due to cell death. To test the influence of cell density on linearization of DNA circles, harvested cells at different confluencies. We also included an experiment in which cells were grown for several days beyond confluency. In none of the experiments was the hybridization signal significantly altered, indicating that cell density did not cause significant DNA breakage of extrachromosomal elements in the J7.CI-30 cell line (Fig. 3B).

Third, the simultaneous presence of extrachromosomal structures of different size offers a unique internal control for random breakage. Large circles are believed to be more susceptible to breakage than smaller extrachromosomal circles, but we never observed linearization of the large DNA circles of ≥2,500 kb in nonirradiated cells. These results indicate that random DNA breakage is unlikely to cause the linear DNA fraction at 800 kb observed in nonirradiated cells. Consequently, the signal in nonirradiated cells strongly indicates native linear fragments. This internal control is even more conclusive in light of the likely sequence similarity between dmin and smaller extrachromosomal structures.
DISCUSSION

We identified linear extrachromosomal elements that contain an amplified mdr domain. Recent studies show the presence of linear plasmid-like structures in yeast and linear dmin in neuroblastoma cells [14, 15]. According to our findings, large circles such as dmin might be formed by polymerization of varying numbers of linear elements. Such a concept could readily explain the size heterogeneity common in populations of extrachromosomal elements. Surprisingly, the linear elements appeared to be relatively stable as long as the J7Cl-30 cells were grown in the presence of colchicine (data not shown). This stability might be conferred by telomeric sequences since they have been demonstrated in linear extrachromosomal elements in yeast [26]. Because telomeric sequences have been shown to be rich in origins of replication, telomeric regions could account for the autonomous replication of linear elements. Recent studies demonstrated a mechanism for the de novo synthesis of telomeric sequences [27].

Furthermore, our study provides further evidence that the small extrachromosomal elements are precursors of dmin, which in turn can integrate into chromosomal DNA from HSR. Our findings are consistent with results of several other studies of extrachromosomal elements and HSR. Specifically, using the CAD gene, Carroll et al. demonstrated that dmins can be produced from smaller precursors which were derived from small chromosomal deletions [28]. Further evidence that small extrachromosomal elements are involved in the formation of dmin was provided by Von Hoff et al. who described the formation of dmin from smaller elements in human leukemia cells [29]. In addition, in several human cell lines, amplified genes have been localized on extrachromosomal structures [30, 31]. Numerous examples also show that the location of the amplified domain differs from the location of the amplified genes in nonamplified cells [5, 32]. These findings, together with the notion that HSR can be formed at multiple chromosomal sites, supports the idea that dmin are precursors of HSR. Additional experimental evidence for the integration of dmins into chromosomes was provided by Von Hoff et al. who described the formation of dmin amplification de novo [34].

In this analysis, we present a unique system that offers further insight into the molecular mechanism leading to extrachromosomal structures. In part, the findings are consistent with the amplification model outlined above which postulates that HSR are derived from dmin and smaller extrachromosomal elements. The finding of linear structures can be more readily interpreted in light of alternative theories. However, findings that prematurely condensed chromosomal regions can be precursors for extrachromosomal elements are in good agreement with the idea of large linear molecules being liberated from chromosomes to form dmin. Linear structures may represent early intermediates of extrachromosomal elements that are released from the genomic DNA by an excision or recombination mechanism.

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