MOLECULAR AND CELLULAR BIOLOGY, July 1992, p. 2911–2918
0270-7306/92/072911-08$02.00/0

Vol. 12, No. 7

Molecular Structure and Evolution of Double-Minute Chromosomes in Methotrexate-Resistant Cultured Mouse Cells

PETER J. HAHN,* BARBARA NEVALDINE, AND JOHN A. LONGO

Department of Radiology, Division of Radiation Oncology, State University of New York Health Science Center, Syracuse, New York 13210

Received 16 August 1991/Accepted 3 April 1992

To determine whether microscopically visible double-minute chromosomes (DMs) are derived from submicroscopic precursors, we monitored the amplification of the dihydrofolate reductase (DHFR) gene in 10 independent isolates of methotrexate (MTX)-resistant mouse cells. At every other doubling in MTX concentration, the cells were examined both microscopically, to detect the presence of microscopically visible DMs, and by pulsed-field gel electrophoresis and hybridization to a DHFR-specific probe, to detect submicroscopic DMs. One of the cloned MTX-resistant isolates was examined in detail and was shown to originally contain amplified DHFR genes on circular DMs measuring 1 and 3 Mb in size; additionally, metaphase chromosome preparations from this cloned isolate were examined and were shown to contain microscopically visible DMs too large to enter a pulsed-field gel. During stepwise selection for increasing levels of MTX, the smaller DMs (not microscopically visible) were shown to be preferentially amplified, whereas the larger (microscopically visible) ones decreased in relative numbers. Rare-cutting *Not*I digestion patterns of total genomic DNA that includes the DMs containing the DHFR gene suggest that the DMs increase in copy number without any further significant rearrangements. We saw no evidence from any of the 10 isolates to suggest that microscopically visible DMs are formed from smaller submicroscopic precursors.

Genomic amplifications are mutations associated with drug resistance and tumor progression in mammalian cells (25, 29, 31). Amplifications result from chromosomal rearrangements that lead to multiple gene copies and overproduction of the product of the amplified gene. If the overproducing cell derives a growth advantage from this rearrangement, its descendants will come to dominate a population. Amplified genes have been associated with two types of microscopically visible aberrant chromosome structures: expanded chromosomal regions, referred to as homogeneously staining regions (HSRs) (18), and extrachromosomal acentric fragments, referred to as double-minute chromosomes (DMs) (for reviews, see references 7 and 26).

Highly amplified genes in HSRs have been studied extensively in hamster cells and have been found to consist of tandem repeats of DNA arranged as either head-to-head or head-to-tail repeats (14, 16, 17, 19, 22). The repeated units have been shown to be 200 to 500 kb in size (13, 17). Much less is known about the DNA in DMs beyond the observation that they appear to be circular DNA when examined with the electron microscope (12) or with pulsed-field gel electrophoresis (PFGE) (11, 20, 21, 28) and that their sizes range from 120 kb (30) to large enough to be visible microscopically (greater than 5 million bp).

The initial molecular events underlying the formation of either of these structures are at present unknown. Attempts to elucidate the mechanism of gene amplification have been hampered by the fact that amplification is a rare event (10^{-4} to 10^{-7} per cell per generation) involving relatively large segments of chromosomal DNA. The large size of the genomic region undergoing amplification has meant that molecular biological techniques, e.g., cosmid cloning and chromosome walking, have required tremendous amounts of labor to characterize these regions (8, 9, 16). Biedler and coworkers (2, 3) exploited the involvement of large regions of DNA by using cytogenetic studies of Giemsa-banded metaphase chromosomes from drug-resistant Chinese hamster lung cells. These authors reported that the amplified gene is frequently on HSRs at locations other than the normal location of the gene undergoing amplification. On the basis of these observations, they proposed that gene amplification involves a transient extrachromosomal step. Similarly, since many DNA-damaging agents such as X rays induce both chromosomal breakage and gene amplification, Hahn et al. (10, 11) and Ruiz and Wahl (23) proposed that, as a crucial intermediate in gene amplification, this transient extrachromosomal step involves a chromosomal deletion event followed by unequal segregation of the resulting acentric chromosome fragments.

Trask and Hamlin (27) and Windle et al. (32), using fluorescent in situ hybridization to investigate early events in dihydrofolate reductase (DHFR) gene amplification in CHO cells, have confirmed and extended Biedler's (2) observation that HSRs occur at sites other than the normal location of the DHFR gene. Trask and Hamlin (27) examined similar CHO cells and surprisingly found, in addition to the amplification event occurring at locations distant from the normal site of the DHFR genes (although frequently on the same chromosome), that the two original copies of the gene were still intact in their original locations. In contrast, Windle et al. (32), using a CHO cell line with a single DHFR gene, reported that the DHFR gene was frequently deleted from its native position in methotrexate (MTX)-resistant variants of this line that contained amplified copies of the DHFR gene. They proposed that a deletion event preceded the amplification event by facilitating unequal segregation of extrachromosomal acentric fragments.

* Corresponding author.

DMs have been proposed to arise by a similar mechanism. Carroll et al. (4) have reported that *N*-phosphonacetyl-L-aspartate-resistant Syrian hamster cells, which generally amplify carbamylphosphate synthetase-aspartate transcarbamylase-dihydroorotase genes as HSRs, on one occasion amplified these genes on 250-kb circular molecules. Some of these cells were subsequently shown to contain deletions of the chromosomal copy of the genes (originally introduced by transfection). Some also contained a 500-kb circular DNA molecule in addition to the 250-kb circle. These circular DNAs were shown to be capable of reintegration into new locations. On the basis of these observations, these authors proposed a general model for both DM and HSR formation. The first step is deletion or chromosome breakage followed by the formation of small (120- to 250-kb) circular autonomously replicating DNAs. The authors suggested that these structures, which they called episomes, are capable of enlarging over time to form DMs as well as reintegrating into new chromosomal locations to form HSRs.

However, data from cell lines that typically amplify genes primarily as DMs are not entirely consistent with this view. Pauletti et al. (20), using PFGE to separate X-ray-linearized large circular DNA, have reported that HeLa cells resistant to MTX have amplified a 650-kb circular DNA molecule that showed no tendency either to enlarge or to integrate into the genome during the course of the study. They proposed the term "amplisomes" to describe these structures to distinguish them from episomes, since the structures that they observed showed no tendency to enlarge or reintegrate. Similarly, Hahn et al. (11) have reported stable 1-, 1.5-, and 3-Mb circular DNAs in MTX-resistant mouse EMT-6 cells. However, in neither of these studies were the circular molecules detected at the very first amplification step, although the HeLa amplisomes were detected quite early, nor were any mapping studies performed, leaving open the possibility that these circular DNAs originated as smaller (or larger) units.

We have investigated 10 independently isolated mouse EMT-6 colonies that were each resistant to a first step of 0.15 μM MTX and which were subsequently exposed to successive doublings in MTX concentration. We report that these cells amplified the DHFR gene on variably sized (1- to 5-Mb) circular DNA molecules that increased in copy number without further detectable rearrangements.

MATERIALS AND METHODS

Cells and cell cultures. The isolation of the first-step 0.15 μM MTX-resistant mouse EMT-6 cell strains used for this study has been previously described (10). Briefly, 10 single colonies, 5 from a population subjected to 7.5 Gy of X irradiation immediately prior to selection and 5 from the unirradiated parent population, were isolated in 0.15 μM MTX and contained various degrees of amplification of the DHFR gene. In this study, all 10 isolates were subject to stepwise doublings in MTX concentration to a resistance level of 160 μM MTX (15), and at each second doubling, cells were analyzed for presence and size of DMs. *Saccharomyces cerevisiae* was kindly provided by Shiming Chen, and *Schizosaccharomyces pombe* embedded in low-melting-point agarose plugs were kindly provided by Michael J. Lane. Plasmid pSV2dhfr was obtained from the American Type Culture Collection.

Plug formation. DNA was prepared as described previously (1). Briefly, confluent cultures were trypsinized, washed, resuspended in 0.75% low-melting-point agarose (FMC) at 37°C, and poured into 200-μl molds (7 by 3 by 10 mm; referred to hereafter as plugs) at a concentration of 10^6 cells per plug. The plugs were placed at −20°C for 2 min to harden the agarose and then lysed immediately by placing the plugs into 0.5 M EDTA (pH 8)–1% sarcosyl–0.1% proteinase K (ESP) at 55°C for 24 h.

Electrophoresis conditions: whole DMs. Slices of the plugs were exposed to 40 Gy of X irradiation (Philips 320-keV industrial irradiator at 3 Gy/min) to linearize circular DMs (21, 28), placed into the wells of a gel (1% agarose in 1× Tris-borate-EDTA), and electrophoresed in a contour-clamped homogeneous electric field apparatus (5) with ramped pulse times of 6, 12, 24, 42, 66, and 96 min (1). The pulse times were programmed to cycle continuously in a series with 6 min in each direction followed by 12 min, etc. After the 96-min pulse times, the cycle started back at 6 min. Therefore, each cycle takes 492 min. The ramping of the pulse times is necessary to increase the range of molecules separated and eliminate discontinuities in the mobility of DNA as a function of molecular weight. These conditions result in a roughly log-linear (molecular weight-distance migrated) separation of DNA molecules with sizes between 0.2 to 7 Mb.

Restriction enzyme digestion. Prior to electrophoresis, plugs were redigested overnight with fresh ESP at 55°C and washed twice for 20 min with Tris-EDTA (TE), twice with TE plus 1 mM phenylmethylsulfonyl fluoride, and twice again with TE. Thin slices of the plugs were rinsed with restriction enzyme buffer and then digested with the restriction enzyme overnight at 37°C as described by the manufacturer. The plug slices were then placed at 55°C for 30 min after the addition of 200 μl of ESP.

Restriction fragment electrophoresis. Digested plug slices were placed into the wells of a 1% agarose gel and electrophoresed at 125 V with ramped pulse times of 60, 64, 68, etc., to 300 s, repeated continuously for 3 days.

Hybridization conditions. Following electrophoresis, the gels were stained with ethidium bromide, photographed, and blotted onto Hybond-N nylon membranes (Amersham). The membranes were hybridized (6) against pSV2dhfr to detect the DHFR-containing DMs and restriction endonuclease digestion products and were then autoradiographed (1, 11).

Cytogenetics. Colcemid-arrested metaphase chromosomes were prepared as previously described (11).

RESULTS

Experimental design. To determine whether DMs undergo significant rearrangements or size changes during gene amplification, 10 independent isolates of MTX-resistant mouse EMT-6 cells were grown in successive doublings of MTX concentrations (15). These cells were first isolated as 10 single colonies resistant to 0.15 μM MTX. At every other doubling in MTX, the cells were prepared for analysis by PFGE and metaphase cells were prepared for cytogenetic analysis. All were shown to have amplified the DHFR gene (11). The five isolates from the unirradiated EMT-6 cells were isolated from five different dishes but from the same starting population. Therefore, some of these isolates may represent multiple recoveries of descendants of a single original event. The five isolates from the irradiated population, on the other hand, received a radiation dose that increases the MTX resistance frequency in the survivors 100-fold, so that these isolates have, at most, only a 1% chance of being related to the cells from the parental unirradiated population. An important difference between

the irradiated and the unirradiated cells is that the initial events in the unirradiated population probably occurred long before the MTX selection was applied, whereas the X-ray-induced events occurred only 1 day prior to selection (i.e., the day of X-ray exposure).

Microscopically visible DMs. The first-step isolates resistant to 0.15 μM MTX all showed the presence of microscopically visible DMs (Fig. 1). To determine whether the number of microscopically visible DMs increased with increasing levels of MTX resistance, we prepared metaphase spreads of EMT-6 isolate A cells resistant to 0.15, 0.6, and 2.4 μM MTX, as well as wild-type EMT-6 sensitive cells, and counted the number of DMs present in 100 metaphase cells from each (Fig. 2). In the cells resistant to 0.15 μM MTX, DMs were visible in most of the metaphases. Surprisingly, however, further increases in MTX concentration led to a decrease in the relative number of visible DMs (Fig. 2C and D). This is most easily seen by comparing the number of metaphases in which no DMs were seen. In the parental MTX-sensitive EMT-6 cells, no DMs were seen in the great majority of metaphases, whereas in the isolate resistant to 0.15 μM MTX, there were very few metaphases in which DMs were not seen. With increasing levels of resistance to MTX, the distribution of microscopically visible DMs actually decreased (and approximated the distribution in the sensitive population) and did not increase the average number of DMs per metaphase that might be expected for gene amplification. Since we knew that EMT-6 cells resistant to very high levels of MTX frequently contain submicroscopic DMs, this finding suggested that the DHFR gene might be amplifying primarily on these submicroscopic DMs.

Since size is the only known difference between the microscopically visible DMs and the submicroscopic circular DNAs resolvable on pulsed-field gels, and since these size ranges are overlapping, we will refer to all of these structures by the older cytogenetic term "double-minute chromosome" rather than "episome" or "amplisome" to denote those DNAs resolvable by PFGE. However, "acentric circular chromosome fragment" would probably be more precise.

PFGE of X-ray-linearized submicroscopic DMs. Submicroscopic DMs can frequently be visualized following PFGE and Southern hybridization with a probe specific for the amplifying region (29). This technique requires that the circular DMs be linearized by some means (generally X rays), because circular DMs do no enter a pulsed-field gel (21, 28). Figure 3 shows the evolution of submicroscopic DMs in isolate A (one of the isolates from the unirradiated population) that were resolved by PFGE. We observed two molecular species 1 and 3 Mb in length following X-ray linearization. However, in contrast to the cytogenetic results showing that the DMs decreased in number with increasing resistance to MTX, the submicroscopic DMs seen as bands in Fig. 3 increased in hybridization intensity, with the smaller 1-Mb DM increasing much more than the larger 3-Mb DM. At the first step (0.15 μM MTX), these DMs are not readily apparent, and PFGE of the X-ray-linearized DMs alone would be insufficient to demonstrate that they are present at this step (this is shown below; see the *Not*I digests in Fig. 4). However, two MTX concentration doublings later (0.6 μM MTX), the DMs of approximately 1 and 3 Mb in size are readily apparent. Two further doublings later (2.4 μM MTX), the bands corresponding to the DMs have increased in hybridization intensity but still have not changed size. Note that these bands migrate slightly faster than do the corresponding ones from the cells resistant to 0.6 μM MTX, indicating that even though there is slightly less DNA in this lane, more intense DHFR-specific hybridization is observed (see discussion below). There also has been a greater relative increase in the hybridization intensity of the smaller 1-Mb DM compared with the larger 3-Mb DM.

Attempts to find smaller DMs by using a higher radiation dose of 300 Gy combined with PFGE conditions specific for smaller DNAs or to find larger DMs by using a lower radiation dose of 2 Gy combined with lower voltages and conditions specific for resolving DNA up to 12 Mb were unsuccessful (data not presented). However, we cannot rule out the possibility that other size classes of DMs were present or were present prior to our analysis. The presence of microscopically observable DMs suggests that there were larger DMs that could not be detected by PFGE. However, the hybridization pattern in Fig. 3 obtained after irradiation of the DNA from isolate A is not consistent with the presence of HSRs or with the amplification of the DHFR gene on DNA larger than 1 Mb. An X-ray dose of 40 Gy would linearize less than half of the 1-Mb DMs (1), and most of the DMs would remain at the origin of electrophoresis as unbroken circles. This would look very similar to the pattern from the DNA in isolate A resistant to 2.4 μM MTX in Fig. 3. Amplified DHFR genes on DNA molecules such as very large DMs or HSRs, when treated with 40 Gy, would look like the pattern of the EMT-6 control DNA or the isolate A resistant to 0.15 μM MTX (Fig. 3); i.e., a majority of DNA migrates as large but randomly broken molecules (greater than 2 Mb). Figure 3 is most consistent with the interpretation that the majority of DHFR genes reside on the 1-Mb DM in the cells resistant to 2.4 μM MTX.

A technical problem associated with DNA size determination by PFGE, which makes analysis more difficult, is that electrophoretic mobility can be significantly decreased by local DNA concentration overloading. This presents a unique problem for studies in which amplified DNA sequences are compared; DNA may be overloaded in one sample and not in another even though the same total amount of DNA was loaded in both cases. Additionally, because these high-molecular-weight DNA samples must be prepared in agarose plugs to prevent shearing, it is much more difficult to control the total amount of DNA loaded into any particular lane than if the samples were liquid. There is both uneven distribution of the cells embedded in the agarose and a greater degree of difficulty in controlling total sample volume. With restriction enzyme digestion, some regions within the agarose are less accessible than others, which makes it difficult to achieve complete digestions. Some DNA is invariably nonspecifically trapped in the agarose plugs and unavailable for analysis. All of these factors complicate direct comparisons between two similar samples in adjacent lanes. Therefore, multiple comparisons are required to draw firm conclusions about molecular sizes.

***Not*I digestion of DMs.** To determine whether major rearrangements had taken place in the region of the DHFR gene during amplification, we digested DNA from both MTX-resistant and -sensitive cells with the rare-cutting restriction enzyme *Not*I (Fig. 4). The *Not*I band containing the DHFR gene in the wild-type sensitive EMT-6 cells (more easily visible in Fig. 4B than Fig. 4A) is approximately 1 Mb. In the 0.15 μM MTX-resistant isolate A, additional *Not*I bands of approximately 0.5 and 0.68 Mb appear, and these are amplified without further rearrangements as the cells become resistant to higher levels of MTX. Novel *Not*I bands in 0.15 μM MTX-resistant isolate B (the second-fastest-growing isolate) are also evident (Fig. 4B, rightmost lane), and these

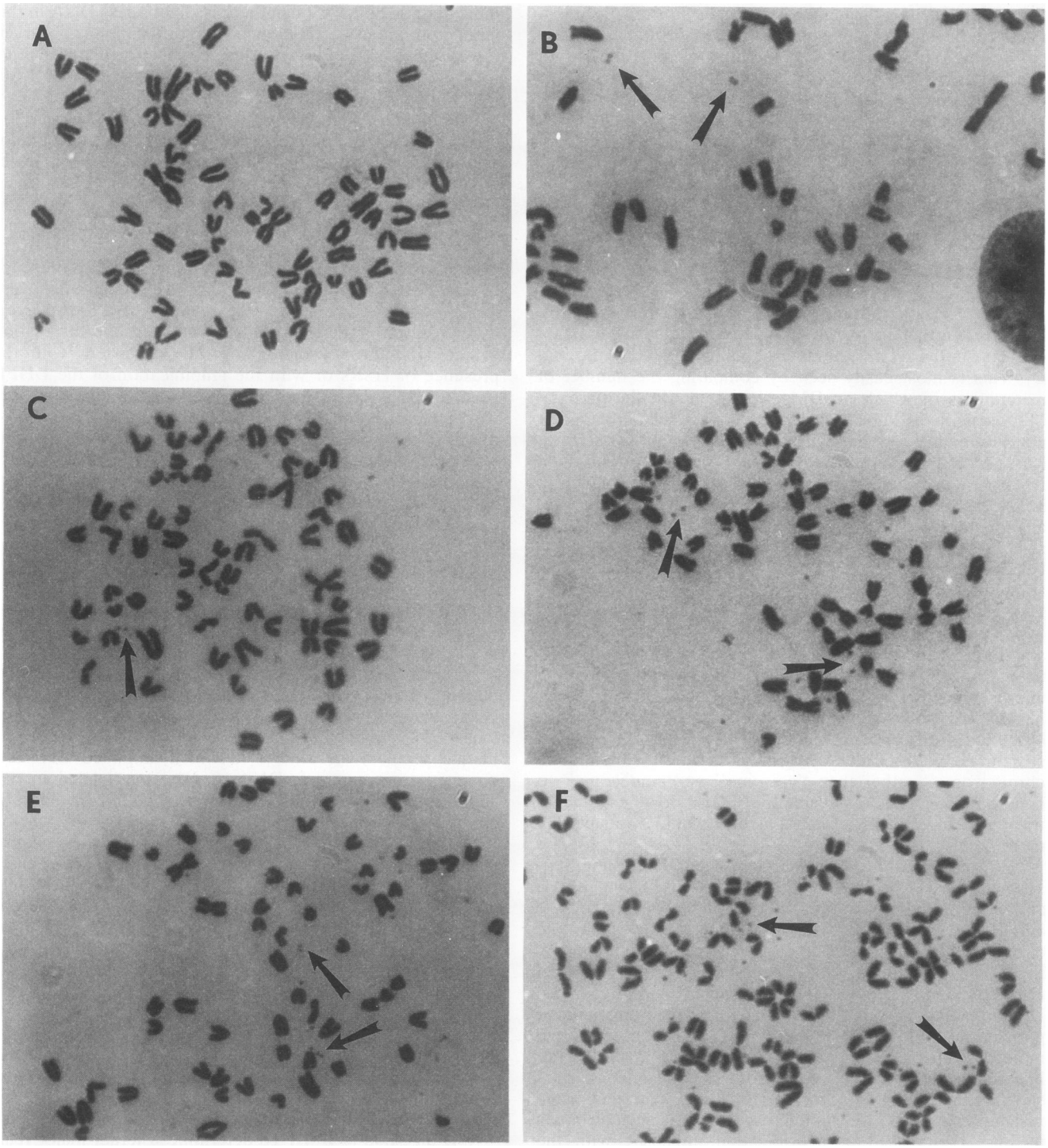

FIG. 1. Metaphase chromosomes from EMT-6 cells. (A) A metaphase cell from the MTX-sensitive population from which the MTX-resistant cells were selected. (B to F) Metaphase chromosomes from first-step MTX-resistant EMT-6 cells. These cells are all from a population expanded from a single colony (isolate A) isolated in 0.15 μM MTX. The arrows denote paired DMs. Also visible are many other minute chromosomes that may either be small doublets or single members of pairs that have separated during the sample preparation since they have no centromeres to keep them together.

are similar but distinct from the new bands evident in isolate A.

The 0.68-Mb *Not*I band from isolate A is most likely due to cleavage of the DNA from the 1-Mb DM. By comparing the *Not*I digests of isolate A in Fig. 4, it is evident that the DNA containing the 0.68-Mb *Not*I band has amplified to a much greater extent than has the DNA containing the 0.5-Mb band. Similarly, in Fig. 3 (see Fig. 5 also), it is evident that the 1-Mb DM has amplified to a much larger extent than has the 3-Mb DM and in parallel with the

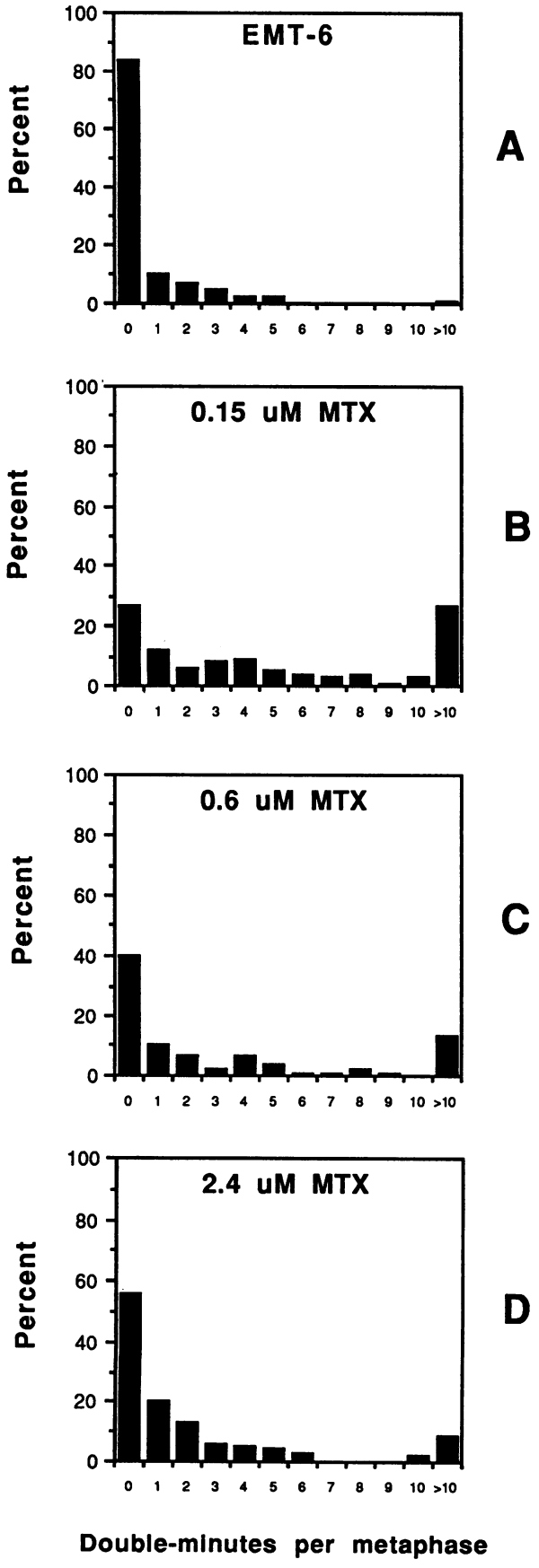

FIG. 2. Histograms of the distributions of microscopically visible DM pairs in the MTX-sensitive and -resistant EMT-6 cells shown in Fig. 1 and from the same MTX-resistant population (isolate A) grown in increasing levels of MTX. (A) MTX-sensitive EMT-6; (B to D) MTX-resistant EMT-6 isolate A resistant to 0.15, 0.6, and 2.4 μM MTX. Approximately 100 metaphase chromosome spreads were observed for each population, and individual minute chromosomes were assumed to be pairs of small DMs.

increase in the copy number of the 0.68-Mb *Not*I band. Therefore, the 0.68-Mb *Not*I band probably comes from the 1-Mb DM. Likewise, the 0.5-Mb *Not*I band is probably cleaved from the DNA of the 3-Mb DM, since neither of these DNAs amplifies to a large extent.

The normal EMT-6 chromosomal DHFR gene resides on a 1-Mb *Not*I band (Fig. 4) that is approximately the same size as the 1-Mb DHFR-containing DM (Fig. 3). Therefore, the amplified 1-Mb band from isolate A could be due either to partial cleavage of the amplified 1-Mb DM or to complete *Not*I cleavage of an amplified DHFR gene with a *Not*I digestion pattern similar to that of the normal chromosomal gene and in which the amplification unit is much larger than 1 Mb (e.g., an HSR).

Since the DHFR gene on the 1-Mb DM resides on a 0.68-Mb *Not*I restriction fragment that is more than half the total size of the circular DM, this DM is most likely composed of either single-copy DNA or a simple inverted repeat. The DHFR gene occupies approximately 30 kb of chromosome, so if these genes were amplifying within the DMs, we could detect this amplification as an increase in the size of the DM. The *Not*I digestion patterns indicate that DMs do not undergo any detectable rearrangements during this process despite the months of growth in the presence of toxic levels of the DNA-damaging agent MTX. This finding is quite different from the observation in drug-resistant hamster cells containing HSRs that the DNA undergoes complex rearrangements (17, 24).

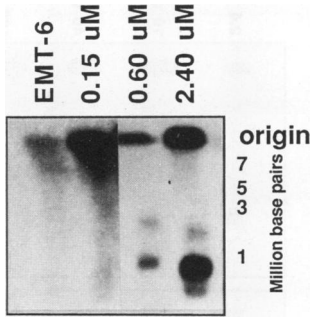

FIG. 3. Southern hybridization of DMs from isolate A linearized with 40 Gy of X rays prior to PFGE. The DNAs analyzed were from the same samples analyzed in Fig. 2. The EMT-6 cells were from a sensitive population; isolate A was isolated in 0.15 μM MTX as a single colony and then exposed to successive doublings in MTX. The probe was pSV2dhfr, so only the position of the DHFR gene can be seen. The two leftmost lanes (EMT-6 and isolate A resistant to 0.15 μM MTX) were from an autoradiograph exposed overnight at −70°C, whereas the lanes on the right (isolate A resistant to 0.60 and 2.4 μM MTX) are from an autoradiograph of the same membrane but exposed overnight at room temperature to reduce the signal intensity. Since these genes are amplified to increasing amounts, it is not possible to obtain a single autoradiograph of the membrane with all lanes visible in the same exposure.

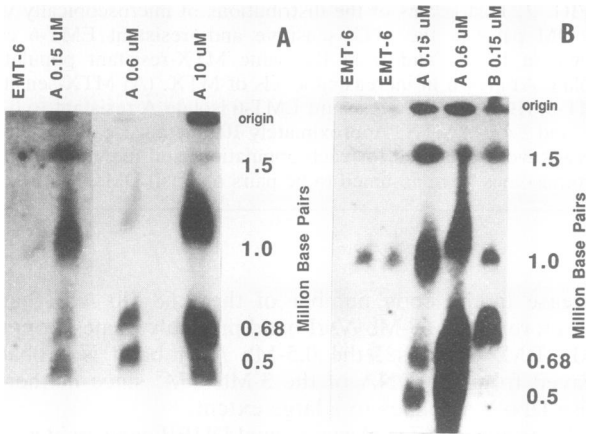

FIG. 4. (A) Southern hybridization of *Not*I-digested DNA from the samples analyzed in Fig. 3, except that the highest level of resistance is to 10 μM MTX instead of 2.4 μM MTX. The membranes were probed with pSV2dhfr. As for Fig. 3, it was necessary to expose the autoradiography film overnight at −70°C for both the EMT-6 sensitive DNA and DNA from the cells resistant to 0.15 μM MTX, whereas the DNA from the cells resistant to further increases in MTX required only a room temperature exposure. Note that the linearized DM has approximately the same electrophoretic mobility (1 million bp) as does the EMT-6 parental DHFR-containing *Not*I fragment. (B) A similar *Not*I digest in which the EMT-6 *Not*I band is more apparent. All samples are from the same exposure (note that the 0.6 μM MTX-resistant sample is now difficult to distinguish), and the *Not*I digestion pattern for isolate B (B 0.15 μM) is shown to be different from the pattern for isolate A.

DMs—further amplification steps. As the MTX level increased further, there was very little change in the relative proportion of the 1- and 3-Mb DMs in isolate A, nor was there any obvious change in their sizes (Fig. 5A). There was an increase only in the relative hybridization intensity.

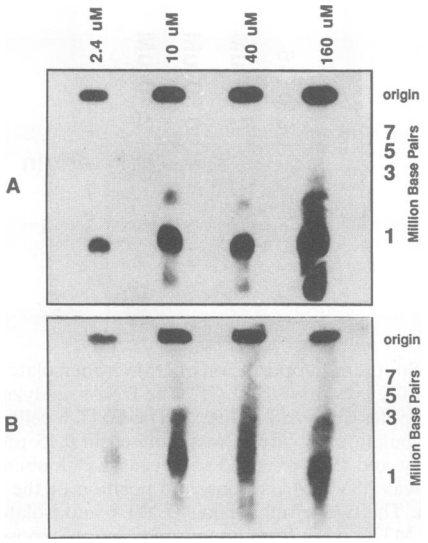

FIG. 5. PFGE of DMs in MTX-resistant EMT-6 isolate A and isolate B during stepwise selection for resistance to further increasing levels of MTX. The DNA from samples of cells resistant to 2.4, 10, 40, and 160 μM MTX were prepared in agarose plugs and given a dose of 40 Gy of X rays immediately prior to electrophoresis to linearize the DMs. The membrane was probed with pSV2dhfr.

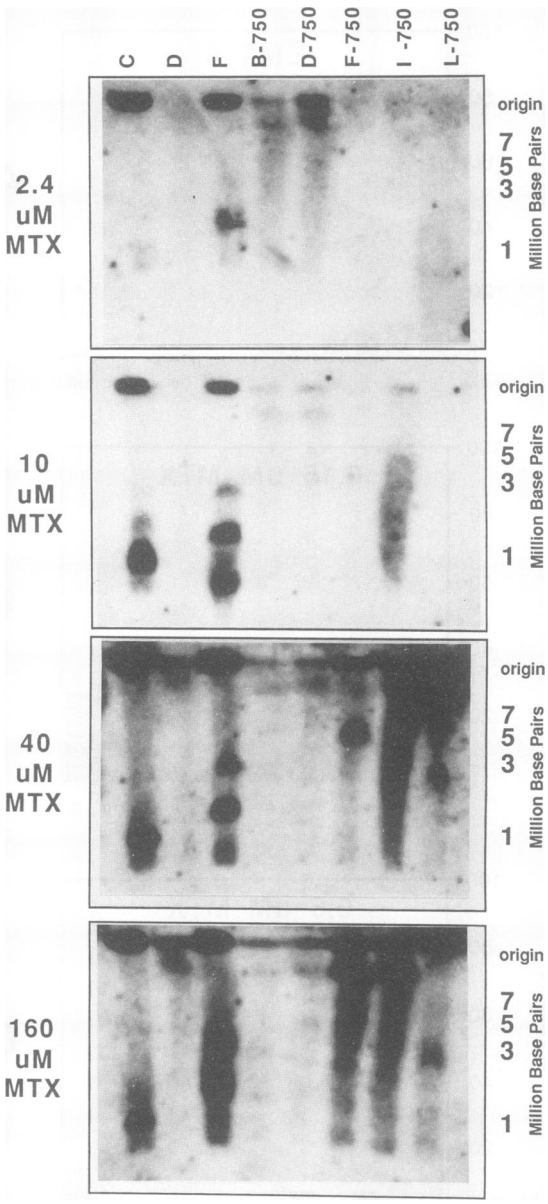

FIG. 6. PFGE of DMs from the other eight isolates compared at 2.4, 10, 40, and 160 μM MTX resistance linearized with 40 Gy of X rays. The 750 following the letter designation indicates that the isolate was originally cloned as a single colony growing in 0.15 μM MTX from a population that had received 750 cGy (7.5 Gy) of X rays immediately prior to selection.

Isolate B (Fig. 5B) was similar but more heterogeneous; therefore, it was difficult to determine the stability of the DMs. However, a 1-Mb DHFR-containing DM dominates this population at higher levels of resistance (160 μM MTX lane in Fig. 5B). There is also a *Not*I band in isolate B very similar in size to the *Not*I band from the 1-Mb DM in isolate A (Fig. 4).

We also examined the other eight isolates to determine whether they had developed DMs and, if so, when. At 2.4 μM MTX (Fig. 6), only one of the eight, isolate F, had detectable levels of X-ray-linearized DMs, but five of the eight eventually showed the emergence of DMs small enough to enter a pulsed-field gel during the course of the

experiment. They ranged in size from slightly less than 1 Mb to approximately 5 Mb (Fig. 6). The other three, isolates D, B-750, and D-750, never developed detectable DMs and presumably contained amplified genes on structures too large to enter the gel (larger than 7 Mb). These three isolates exhibited the lowest levels of amplification and the slowest growth rates (data not shown).

In no case was there evidence to suggest significant changes in size of the submicroscopic DMs over time in any of the isolates, although some of them had heterogeneous populations which made it difficult to say that such changes did not occur in those lines.

DISCUSSION

This study was designed to determine whether DMs become smaller, become larger, or stay the same size during their amplification. Our results suggest that at least for this cell line, gene amplification is relatively simple: DMs increase in numbers without significant alterations. The results reported here support and extend the observations of Pauletti et al. (20) that the DHFR amplisomes in HeLa cells increased in copy number as the cells became more resistant to MTX. In the cultured mouse tumor EMT-6 cells, DMs are present in MTX-resistant cells isolated as first-step DHFR-amplified cells, and subsequent selection for resistance to increasing levels of MTX favors a copy number increase in the smaller-sized DMs. Our results do not support the proposal of Wahl and colleagues (4, 31) that episomes are precursors of DMs.

The relationship between the microscopically visible DMs and the submicroscopic circular DMs that are distinguishable by PFGE is unclear. The observation that visible DMs were present in the initial resistant population but did not amplify to the same extent as did the smaller ones in the more highly resistant populations indicates that the smaller DMs are not precursors to larger microscopically visible ones. The simplest explanation is that they represent different parts of a continuum of sizes and that smaller DMs amplify more frequently or more readily than do larger ones. This explanation is supported by the data for isolate A; the 1-Mb DM amplified much more extensively than did the 3-Mb DM, whereas the microscopically visible DMs actually decreased in copy number. It is possible, however, that rapid selection for increasing resistance favors the smaller DMs but that stable maintenance at high levels of MTX might eventually favor larger units composed of multimers of the smaller units, as suggested by the Wahl model (31). This could simply be due to a more equal partitioning of larger units and a greater gain/loss rate of smaller ones. Maintaining these cells long enough at the high levels of MTX resistance might select for larger microscopically visible DMs and eventually HSR-containing variants.

These studies were specifically designed to characterize secondary processes in gene amplification and do not directly address the question of the mechanism of the origin of these structures. The fact that even in the first-step isolate A there were already at least three different molecular species, the 1-Mb, 3-Mb, and microscopically visible DMs, suggests that we are not looking at the immediate products of the initial gene amplification event. Rather, it is likely that these DMs arose in the first few cell divisions following the initial event, although perhaps many generations prior to placing the cells under selection for their presence. It is important to note that a significant percentage of the normal EMT-6 cells have microscopically observable DMs. If these are random pieces of chromosomal DNA, then some will have the DHFR gene on them. Selection for MTX resistance will therefore select for these cells. Similarly, it is likely that there are smaller ones as well, and it is possible that all three different molecular species present in isolate A at the first step of analysis were present prior to selection. We have previously isolated MTX-resistant variants of EMT-6 cells from the same parent population with 1- and 3-Mb DMs (10), and these may represent independent isolations of descendants of the same mutation.

DHFR gene amplifications behave like classic mutations, i.e., those spontaneously generated or induced by mutagens such as X rays and revealed by selection. These results are consistent with our previous suggestion that the initial gene amplification event is similar to a gene deletion-type mutation. Selection, however, is for the cells harboring the deleted piece of chromosome now maintained as a circular acentric fragment rather than for the cell that lost the fragment.

ACKNOWLEDGMENTS

We thank Robert West, David Mitchell, and Robert Painter for critical analysis of the manuscript.

This work was supported by Public Health Service grant CA4688004 from the National Institutes of Health.

REFERENCES

1. **Ahn, S. Y., B. Nevaldine, and P. J. Hahn.** 1991. Direct measurement of pulsed-field gel electrophoresis of induction and rejoining of X-ray induced double-strand breaks in cultured mouse cells. Int. J. Radiat. Biol. **59:**661–675.
2. **Biedler, J. L.** 1982. Evidence for transient or prolonged extrachromosomal existence of amplified DNA sequences in antifolate-resistant, vincristine-resistant, and human neuroblastoma cells, p. 39–45. *In* R. T. Shimke (ed.), Gene amplification. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Biedler, J. L., T. D. Chang, K. W. Scotto, P. W. Melera, and B. A. Spengler.** 1988. Chromosomal organization of amplified genes in multidrug-resistant Chinese hamster cells. Cancer Res. **48:**3179–3187.
4. **Carroll, S. M., M. L. Derose, P. Gaudray, C. M. Moore, D. R. Needham-Vandevanter, D. D. Von Hoff, and G. M. Wahl.** 1988. Double minute chromosomes can be produced from precursors derived from a chromosomal deletion. Mol. Cell. Biol. **8:**1525–1533.
5. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234:**1582–1585.
6. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
7. **Cowell, J. K.** 1982. Double minutes and homogeneously staining regions: gene amplification in mammalian cells. Annu. Rev. Genet. **16:**21–59.
8. **Federspiel, N. A., S. M. Beverley, J. W. Schilling, and R. T. Schimke.** 1984. Novel DNA rearrangements are associated with dihydrofolate reductase gene amplification. J. Biol. Chem. **259:**9127–9140.
9. **Giulotto, E., I. Saito, and G. R. Stark.** 1986. Structure of DNA formed in the first step of CAD gene amplification. EMBO J. **5:**2115–2121.
10. **Hahn, P., W. F. Morgan, and R. B. Painter.** 1987. The role of acentric chromosome fragments in gene amplification. Somatic Cell Mol. Genet. **13:**597–608.
11. **Hahn, P., B. Nevaldine, and W. F. Morgan.** 1990. X-ray induction of methotrexate resistance due to dhfr gene amplification. Somatic Cell Mol. Genet. **16:**423–423.
12. **Hamkalo, B. A., P. J. Farnham, R. Johnston, and R. T. Schimke.** 1985. Ultrastructural features of minute chromosomes in a methotrexate-resistant mouse 3T3 cell line. Proc. Natl. Acad. Sci. USA **82:**1126–1130.

13. **Hamlin, J. L., and C. Ma.** 1990. The mammalian dihydrofolate reductase locus. Biochim. Biophys. Acta **1070:**107–125.
14. **Hyrien, O., M. Debatisse, G. Buttin, and B. R. de Saint Vincent.** 1988. The multicopy appearance of a large inverted duplication and the sequence at the inversion joint suggests a new model for gene amplification. EMBO J. **7:**407–417.
15. **Kingston, R. E.** 1987. Amplification using dihydrofolate reductase, p. 9.9.1–9.9.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
16. **Looney, J. E., and J. L. Hamlin.** 1987. Isolation of the amplified dihydrofolate reductase domain from methotrexate-resistant Chinese hamster overy cells. Mol. Cell. Biol. **7:**569–577.
17. **Ma, C., J. E. Looney, T. H. Leu, and J. L. Hamlin.** 1988. Organization and genesis of dihydrofolate reductase amplicons in the genome of a methotrexate-resistant Chinese hamster ovary cell line. Mol. Cell. Biol. **8:**2316–2327.
18. **Nunberg, J. H., R. J. Kaufman, R. T. Schimke, G. Urlaub, and L. A. Chasin.** 1978. Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. Proc. Natl. Acad. Sci. USA **75:**5553–5556.
19. **Passananti, C., B. Davies, M. Ford, and M. Fried.** 1987. Structure of an inverted duplication formed as a first step in a gene amplification event: implication for a model of gene amplification. EMBO J. **6:**1697–1703.
20. **Pauletti, G., E. Lai, and G. Attardi.** 1990. Early appearance and long term persistance of the submicroscopic extrachromosomal elements (amplisomes) containing the amplified DHFR genes in human cell lines. Proc. Natl. Acad. Sci. USA **87:**2955–2959.
21. **Ruiz, J. C., K. H. Choi, D. D. Von Hoff, I. B. Robinson, and G. M. Wahl.** 1989. Autonomously replicating episomes contain *mdr*1 genes in a multidrug-resistant human cell line. Mol. Cell. Biol. **9:**109–115.
22. **Ruiz, J. C., and G. M. Wahl.** 1988. Formation of an inverted duplication can be an initial step in gene amplification. Mol. Cell. Biol. **8:**4302–4313.
23. **Ruiz, J. C., and G. M. Wahl.** 1990. Chromosomal destabilization during gene amplification. Mol. Cell. Biol. **10:**3056–3066.
24. **Saito, I., R. Groves, E. Giulotto, M. Rolfe, and G. R. Stark.** 1989. Evolution and stability of chromosomal DNA coamplified with the CAD gene. Mol. Cell. Biol. **9:**2445–2452.
25. **Stark, G. R., M. Debatisse, E. Giulotto, and G. M. Wahl.** 1989. Recent progress in understanding mechanisms of mammalian DNA amplification. Cell **57:**901–908.
26. **Stark, G. R., and G. M. Wahl.** 1984. Gene amplification. Annu. Rev. Biochem. **53:**447–491.
27. **Trask, B. J., and J. L. Hamlin.** 1989. Early dihydrofolate reductase gene amplification events in CHO cells usually occur on the same chromosome arm as the original locus. Genes. Dev. **3:**1913–1925.
28. **van der Blick, A., C. R. Lincke, and P. Borst.** Circular DNA of 3T6R50 double minute chromosomes. Nucleic Acids Res. **16:** 4841–4851.
29. **VanDevanter, D. R., V. D. Piaskowski, J. T. Casper, E. C. Douglass, and D. D. Von Hoff.** 1990. Ability of circular extrachromosomal DNA molecules to carry amplified MYCN protooncogenes in human neuroblastomas in vivo. J. Natl. Cancer Inst. **82:**1815–1821.
30. **Von Hoff, D. D., D. R. Needham-VanDevanter, J. Yucel, B. E. Windle, and G. M. Wahl.** 1988. Amplified human MYC oncogenes localized to replicating submicroscopic circular DNA molecules. Proc. Natl. Acad. Sci. USA **85:**4804–4808.
31. **Wahl, G. M.** 1989. The importance of circular DNA in mammalian gene amplification. Cancer Res. **49:**1333–1340.
32. **Windle, B., B. R. Draper, Y. Yin, S. O'Gorman, and G. M. Wahl.** 1991. A central role for chromosome breakage in gene amplification, deletion formation, and amplicon integration. Genes Dev. **5:**160–174.