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Chromosomal Destabilization during Gene Amplification

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Acentric extrachromosomal elements, such as submicroscopic autonomously replicating circular molecules (episomes) and double minute chromosomes, are common early, and in some cases initial, intermediates of gene amplification in many drug-resistant and tumor cell lines. In order to gain a more complete understanding of the amplification process, we investigated the molecular mechanisms by which such extrachromosomal elements are generated and we traced the fate of these amplification intermediates over time. The model system consists of a Chinese hamster cell line (L46) created by gene transfer in which the initial amplification product was shown previously to be an unstable extrachromosomal element containing an inverted duplication spanning more than 160 kilobases (J. C. Ruiz and G. M. Wahl, Mol. Cell. Biol. 8:4302-4313, 1988). In this study, we show that these molecules were formed by a process involving chromosomal deletion. Fluorescence in situ hybridization was performed at multiple time points on cells with amplified sequences. These studies reveal that the extrachromosomal molecules rapidly integrate into chromosomes, often near or at telomeres, and once integrated, the amplified sequences are themselves unstable. These data provide a molecular and cytogenetic chronology for gene amplification in this model system; an early event involves deletion to generate extrachromosomal elements, and subsequent integration of these elements precipitates a cascade of chromosome instability.

Chromosomal rearrangements are a hallmark of advanced tumor cells but occur rarely in normal cells. While chromosomal aneuploidy, expansive deletions, translocations, and localized increases in DNA sequence copy number (i.e., gene amplification) are commonly observed in many malignancies, gene amplification is most accessible to detailed molecular and cytogenetic investigation under controlled laboratory conditions. Therefore, we are investigating the molecular mechanisms of gene amplification to gain insight into the broader issue of the factors which lead to chromosome destabilization in malignancy and prevent it from occurring in normal cells.

Many studies reveal that gene amplification is a dynamic process in which the structures generated early are altered at the cytogenetic and molecular levels during cell propagation or drug selection. Amplified sequences are often detected in one of two types of chromosomal anomalies. These are paired acentric circular structures called (double) minute chromosomes (DMs) and expanded chromosomal regions (ECRs). Several recent studies indicate that DMs can originate from submicroscopic circular elements called episomes (5, 6, 37, 40, 50); however, in some cases, DMs may be generated without such precursors by a process which involves chromosome fragmentation (44). Studies with various genes in different cell lines suggest, and in one case directly show, that DMs integrate into a chromosome to form an ECR (5, 7, 28, 47, 49, 50; this study). It is reasonable to infer that when episomes or DMs are present, they represent early or initial amplification products, since extrachromosomal elements are transient intermediates in the amplification process because of their tendency to integrate (51) and because ECRs have not been observed to break down to generate episomes or DMs (5, 7). In light of data from two studies, however, the amplification of some loci might initiate with formation of unstable chromosomally amplified regions which then stabilize later as ECRs (15, 17, 40).

The DNA breakage and joining reactions which form amplification units leave unique sequence combinations or "novel joints" as their molecular fingerprints. Novel joints can be formed in the initial step of amplification, in which case they may directly reflect the molecular mechanisms involved, or they can be generated in subsequent steps by incidental reactions not directly related to the amplification process (15, 40, 45). For example, as initially shown by Fried and co-workers (12, 13, 35) and later established by others (19, 21, 22, 29, 34, 38, 41), amplification units are often arranged as inverted duplications. The inverted duplications represent early, if not initial, products of the amplification process in several examples (12, 21, 35, 38), and they can be stable over many generations. By contrast, other novel joints are highly unstable (15, 40). An unresolved issue is whether the molecular instability revealed by alterations within novel joints reflects the occurrence of coincident cytogenetic changes.

The experiments presented here were designed to analyze an amplification event over time in order to gain insight into two questions. First, is the chromosomal region which is amplified preserved or deleted when the initial amplification products are generated? Preservation of sequences would suggest conservative mechanisms such as rereplication (see Fig. 1A; 42, 43) or strand switching at nicked palindromes (21, 22, 34). Deletion would implicate nonconservative processes such as mitotic recombination or recombination across replication bubbles (see Fig. 1B; 5, 35, 38, 51). Second, since extrachromosomal elements can integrate into chromosomes, can integration precipitate further chromosomal instability and generate the highly abnormal chromosomes often detected in cells with amplified sequences? This question arises from the results of gene transfer studies which indicate that integration of transfected DNA molecules into some sites precipitates chromosome destabilization (13, 16, 38, 53). The creation of abnormal chromosomes subject to further rearrangement could explain how some

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novel joints associated with gene amplification change over time.

Investigation of the chromosomal consequences of producing an extrachromosomal element is facilitated in a cell containing a single copy of the selected locus so that the molecular and cytogenetic events are not obscured by the presence of additional identical copies. Since most cell lines employed for studies of gene amplification are at least diploid, we used electroporation to create a cell line containing a single copy of a dihydrofolate reductase (DHFR) minigene construct (pLDPL; 38, 52). The recipient CHO cell line has a double deletion of the endogenous DHFR genes to facilitate subsequent genetic and cytogenetic studies (cell line DG44; 38, 48). Several aspects of the amplification of the transfected sequences in one transformant, L46, indicate its usefulness for investigating the production of extrachromosomal elements. First, cells resistant to high concentrations of methotrexate were detected at a high frequency in the L46 population within 25 cell doublings (cd) of DNA introduction. This observation is readily explained by the presence of acentric extrachromosomal molecules containing the transfected sequences at this early time (38) (this paper). Unequal segregation of these elements at mitosis rapidly generates a population with substantial heterogeneity in the copy number and expression of the donated DHFR gene. Second, the amplification unit in L46 contains an imperfect inverted repeat consisting of the pLDPL vector at its center and more than 160 kilobases (kb) of host DNA flanking the insertion site. This structure presumably represents an initial molecular product of amplification since it was also detected within 25 cd of DNA introduction (the earliest time accessible to molecular analysis). These data, along with other evidence described previously (38), indicate that the inverted repeat and the extrachromosomal element were generated concurrently. Therefore, L46 provides an attractive system to analyze since we know that an extrachromosomal element was produced as a very early and probably initial product of amplification and that the molecular structure of the amplicon resembles that described for many endogenous genes.

MATERIALS AND METHODS

Cell lines, transfection, and drug selection protocol. L46 is a transformant created by electroporation of DG44 cells with the vector pLPDL, which contains the DHFR and *Escherichia coli pyrB* genes (38). L46 is propagated in Dulbecco modified Eagle medium–8% dialyzed fetal calf serum–nonessential amino acids. DG44, a CHO cell line containing a double deletion of the DHFR loci (provided by L. Chasin; 48), is propagated in Dulbecco modified Eagle medium–8% dialyzed fetal calf serum–nonessential amino acids–30 μM hypoxanthine–3 μM thymidine (complete medium). Cell lines derived from L46 which lack the transfected sequences are also propagated in complete medium.

Analysis of DHFR expression by flow cytometry. The DHFR enzyme levels in individual cells were estimated by flow cytometry by using the conditions for synthesis of fluoresceinated methotrexate and incubation of viable cells with fluoresceinated methotrexate as described previously (14).

Preparation of DNAs for polymerase chain reactions (PCR). Up to 10^5 cells were pelleted into microfuge tubes for DNA isolation (25). Cells were suspended in 10 μl of phosphate-buffered saline plus 40 μl of H_2O and were incubated at 95°C for 10 min. Proteinase K (10 μl from a 10-mg/ml stock) was added, and the samples were incubated at 65°C for 30 min and then incubated at 95°C for 10 min. DNA (24 μl) was added to 25 μl of 2× PCR buffer (1× PCR is 67 mM Tris hydrochloride [pH 8.8], 6.7 mM $MgCl_2$, 3.3 mM $(NH_4)_2SO_2$, 5 mM β-mercaptoethanol, 6.7 μM disodium EDTA, and 10% dimethyl sulfoxide), 1.5 μg of oligonucleotide primers, and 4 units of TaqI polymerase (Stratagene Cloning Systems, La Jolla, Calif.). Primer sequences were as follows: *pyrB* primer pairs were 5′-TGCGCCGCTTCTGACGATGA and 5′-GCT GTCGCCAGCACCAGATT, while the CHO *tk* primer pairs were 5′-GAAGTCCTCGGTACAGCCTG and 5′-GCTGGC TCTAGTCTGGGCTA. The reaction mixtures were placed into a programmable cyclic reactor (Ericomp Inc., San Diego, Calif.), and a PCR (39) was performed for 35 cycles (denatured for 1 min at 92°C, reannealed for 1 min at 60°C, and elongated for 3 min at 70°C). One-fifth of the reaction was fractionated through a 10% polyacrylamide gel, stained with ethidium bromide, and photographed.

Preparation of metaphase spreads and in situ hybridization. Metaphase spreads were prepared on glass slides as described previously (6) and hybridized (26, 36) to biotinylated pLPDL, cL5 (a cosmid which contains pLPDL sequences and ~20 kb of host DNA flanking the L46 integration site; 38), and cDCAD42 (a cosmid containing the CHO CAD gene) sequences. The probes were generated by nick translation by standard conditions (Enzo Diagnostics, Inc., New York, N.Y.). Each hybridization contained 20 ng of nick-translated probe and 5 to 10 μg of CHO carrier DNA per slide. The most crucial factors for high signal and low background were found to be both probe size and carrier size. The experiments presented employed size ranges for both from 100 to 500 base pairs. The slides were mounted in "antifade" medium (36) containing 0.25 μg of propidium iodide per ml to stain the chromosomes. Chromosomes were visualized through a ×100 Planapo objective (Carl Zeiss) and photographed directly if the signal was strong. Otherwise, the signal intensity was increased by antibody amplification as described previously (36).

RESULTS

Chromosomal deletion generated the extrachromosomal element in L46. Several mechanisms could account for the concurrent production of an inverted repeat and the extrachromosomal element containing it (for recent reviews, see references 42, 43, 45, 46, and 51). These mechanisms fall into two general conceptual categories. The first category consists of conservative mechanisms (e.g., rereplication [42, 43] or strand switching at nicked palindromes [chromosomal spiral model; 22]) which generate extrachromosomal elements by processes which preserve at least one copy of the native locus at the native chromosomal position (see Fig. 1A for an example involving rereplication; see references 22 and 45 for diagrams of the chromosomal spiral model). The second category, by contrast, contains nonconservative mechanisms (e.g., intrachromosomal recombination [35, 38, 51]) which lead to the deletion of the native locus to form such extrachromosomal molecules (Fig. 1B).

The two classes of amplification mechanisms make distinctly different predictions which can be tested readily by using the L46 cell line. The conservative model predicts that each L46 cell should contain the transfected pLPDL gene transfer module (which has a DHFR and a *pyrB* gene) at the original chromosomal integration site in addition to extrachromosomal elements. By contrast, the nonconservative model predicts that the chromosomal site should be missing

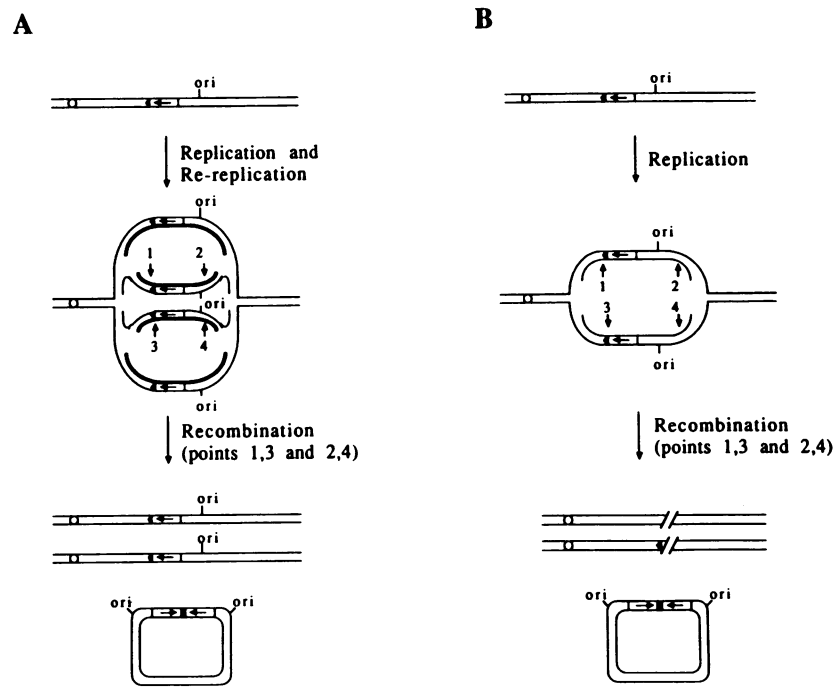

FIG. 1. Models for the formation of acentric elements containing an inverted duplication. (A) Conservative amplification mediated by rereplication. One unscheduled round of replication is shown. Rereplicated strands are indicated in the middle diagram by thick lines, but they have been omitted from the bottom diagram for simplicity. Recombination within the resulting duplexes at sites 1,3 and 2,4 generates a circular molecule containing an asymmetric inverted duplication (site 1 is to the left of the black triangle, site 3 is to the right). Note that the chromosomal locus remains intact. The figure is drawn to represent the molecular structure of the inverted duplication isolated from L46 (38). A second inverted duplication should also be created at sites 2,4, but it has not been characterized as yet. (B) Nonconservative amplification mediated by chromosomal deletion. The example shown depicts recombination in a replication intermediate. Recombination at sites 1,3 and 2,4 concurrently generates an acentric molecule containing an asymmetric inverted duplication in addition to a chromosomal deletion. Only one of the possible ways of rejoining the broken chromosome is shown for simplicity. For both panels A and B, ori refers to an origin of replication.

and that all transfected pLPDL sequences should be localized to extrachromosomal elements. If the nonconservative model is correct, then random segregation of extrachromosomal elements at mitosis should generate some daughter cells devoid of all transfected sequences through the loss of all of their acentric elements. On the other hand, the conservative model predicts that all daughter cells should retain at least the chromosomal copy of the donated sequences and cells devoid of these sequences are not expected. Since a mutant with no chromosomal copies of the DHFR gene was used to create L46 (38, 48), segregants devoid of transfected DHFR sequences should be detected readily.

Approximately 10^4 L46 cells obtained 35 cd posttransfection were expanded for an additional 10 cd under conditions which require minimal DHFR expression (i.e., in the presence of glycine, hypoxanthine, and thymidine, subsequently referred to as complete medium) to enable DHFR$^-$ cells to segregate (Fig. 2). This L46 population contained an average of 3 to 4 inverted duplications per cell prior to growth in complete medium, and it represents the earliest population containing sufficient cells for molecular and cytogenetic studies. These early passage cells were analyzed in order to minimize the accumulation of cells containing secondary amplification products. Subsequent to growth in complete medium, the L46 population was incubated with fluoresceinated methotrexate and cells containing the lowest fluorescence levels (i.e., the lowest DHFR levels) were obtained by using the fluorescence-activated cell sorter (14). The sorted population was then single cell cloned, by limiting dilution, into microdilution dishes. When each clone contained 10^3 to 10^4 cells, the cells were removed by trypsinization and divided into two samples. One sample was seeded into a well of a 6-well dish for propagation, and the remainder was pelleted into microfuge tubes for detection of transfected sequences by the PCR (the *E. coli pyrB* gene contained in the transfection module was used as the substrate for PCR amplification; see Materials and Methods). PCR amplification of the endogenous CHO thymidine kinase (*tk*) gene was performed simultaneously to provide an internal control for reaction efficiency.

The two models presented above predict two different outcomes to this experiment. If the conservative mechanism is correct, each single-cell clone should generate a PCR product specific for the transfected *pyrB* sequences (~275 base pairs) and the endogenous *tk* sequences (~300 base pairs). On the other hand, the nonconservative model predicts that random segregation of the extrachromosomal elements which harbor the amplified sequences in L46 should generate some single-cell clones devoid of the transfected *pyrB* sequences. Such cells should generate only the *tk*-specific fragment after PCR. Some clones are expected to contain *pyrB* sequences since the conditions employed for fluorescence-activated cell sorting cannot quantitatively purify cells with no DHFR expression from those expressing a single transfected DHFR gene.

The ethidium bromide-stained gel obtained from a typical PCR experiment is shown in Fig. 3A. The last three lanes

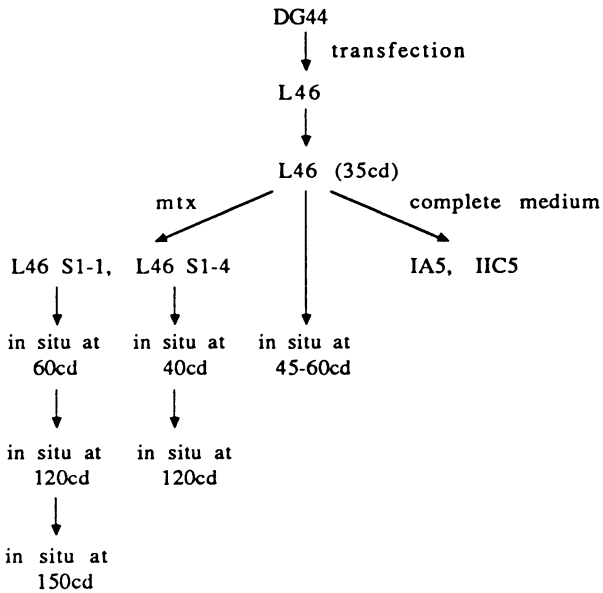

FIG. 2. Origin of cell lines used in this study. The DHFR⁻ CHO cell line DG44 was transfected with pLPDL DNA to generate the single-cell clone L46. At 35 cd posttransfection, L46 was either selected with methotrexate (mtx) to obtain single-cell clones resistant to methotrexate (clones L46 S1-1 and L46 S1-4) or was propagated in complete medium (i.e., the presence of glycine, hypoxanthine, and thymidine) for 10 cd before obtaining single-cell clones which had lost the transfected sequences (clones IA5 and IIC5). In situ hybridization analysis was performed on L46 cells 45 to 60 cd posttransfection and on L46 S1-1 and L46 S1-4 clones 40 to 150 cd post-methotrexate selection.

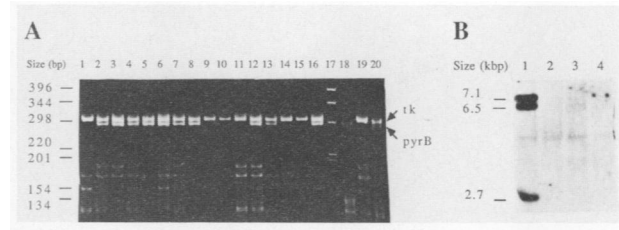

FIG. 3. A population of L46 cells contains variants with a loss of the transfected sequences. (A) L46 cells were fractionated on the fluorescence-activated cell sorter to obtain a population with the lowest DHFR expression (~2.4% of the population). Single-cell clones were then prepared from this population by limiting dilution into microdilution wells. DNA from approximately 10^3 to 10^4 cells from each clone was incubated with *pyrB*- or *tk*-specific oligonucleotides or both and *Taq*I DNA polymerase and was amplified by 35 cycles of PCR. One-fifth of the reaction was fractionated through a 10% polyacrylamide gel and stained with ethidium bromide. Lanes: 1 through 16, products of PCR amplification obtained from various L46 single-cell clones from the sorted population; 17, 1-kb-ladder size standard (Bethesda Research Laboratories, Inc.); 18, 7 ng of L46 DNA incubated with *pyrB*-specific oligonucleotides; 19, 7 ng of L46 DNA incubated with *tk*-specific oligonucleotides; 20, 7 ng of L46 DNA incubated with *pyrB*- and *tk*-specific oligonucleotides. (B) High-molecular-weight DNA (10 μg) was cleaved with *Eco*RI, fractionated through a 0.7% Tris-acetate-EDTA (TAE) agarose gel, transferred to nitrocellulose, and hybridized with a pLPDL probe prepared by oligonucleotide labeling (11). The DNA samples analyzed in the indicated lanes are from the following sources: 1, L46; 2, DG44; 3 and 4, clones identified by PCR to contain a loss of *pyrB* sequences (3, IA5; 4, IIC5).

(lanes 18 through 20) contain controls in which DNA isolated from L46 (grown under conditions requiring expression of at least one DHFR gene) was incubated with *pyrB* (lane 18), *tk* (lane 19), or *pyrB*- and tk-specific (lane 20) primer pairs. The results show that 6 of 16 L46-derived single-cell clones contained no *pyrB*-specific fragment (e.g., compare lane 1 with lane 2). In total, 11 out of 38 L46-derived single-cell clones analyzed in several independent experiments did not produce a *pyrB*-specific fragment under conditions which resulted in robust amplification of the *tk* positive control. In order to confirm these results, DNA samples from two clones determined to be devoid of the transfected sequences by the PCR assay (clones IA5 and IIC5 in lanes 1 and 9, respectively) were digested with *Eco*RI and analyzed by Southern blotting with radiolabeled pLPDL sequences as the probe. The autoradiogram shows that these two clones did lose all of the donated sequences during nonselective growth (Fig. 3B, lanes 3 and 4). The absence of the 2.7-kb fragment, which derives from the junction between the donated and host sequences (Fig. 3B, lane 1; 35), shows that the deletion extends into the CHO DNA flanking the insertion site. Since 2.4% of the L46 population was obtained after cell sorting and 11 of 38 clones were devoid of transfected sequences, we estimate that 0.7% of the L46 population had lost all of the donated sequences after growing for 10 cd under the conditions employed.

Localization of transfected sequences in L46 by using fluorescence in situ hybridization. In situ hybridization of chromosomes with probes generated from pLDPL or cloned CHO sequences which flank the insertion site in L46 (cosmid clone cL5; 38) was used to localize the transfected sequences in L46. Figure 4 shows the results of an in situ hybridization analysis with biotinylated DNA probes detected with fluoresceinated avidin (26, 36). Figure 4 shows a representative DG44 metaphase spread in order to indicate the level of background fluorescence by using the pLPDL probe. Analysis of early-passaged L46 cells (passage 12 or 16; ~45 to 60 cd posttransfection; Fig. 2) revealed no chromosomal or extrachromosomal hybridization in 74% of the metaphases (62 of 84 metaphases; Fig. 4D), but 20% (17 of 84) of the metaphases showed hybridization signals consistent with the presence of one copy of pLPDL sequences (i.e., two adjacent paired dots of fluorescence in a single chromosome) at different chromosomal sites. These sites were in the middle of five different chromosomes (7 of 84 metaphases; see Fig. 4E for one example) or at the end of a single telocentric chromosome (10 of 84; see Fig. 4F for an example). A short ECR was detected at the end of a submetacentric or telocentric chromosome in 6% of the metaphases (5 of 84; data not shown).

In order to identify where in the DG44 genome the pLDPL vector integrated, we isolated genomic sequences flanking the integration site in cosmids and then hybridized DG44 metaphases with these cosmids. One clone, cL5, consists of 20 kb of flanking DG44 DNA in addition to pLPDL sequences. A representative metaphase is shown in Fig. 4C. The pattern of cL5 hybridization indicates that DNA flanking the insertion site consists of moderately repetitive elements which are dispersed throughout the genome. These repetitive elements are sufficiently concentrated in several chromosomal regions to generate bands of fluorescence. The hybridization of cL5 to multiple chromosomal regions is not the result of nonspecific hybridization of repetitive DNA, since other cosmids with repetitive DNA generate discrete hybridization signals for single copy genes (Fig. 4B). Other sequences flanking the insertion site also contain highly

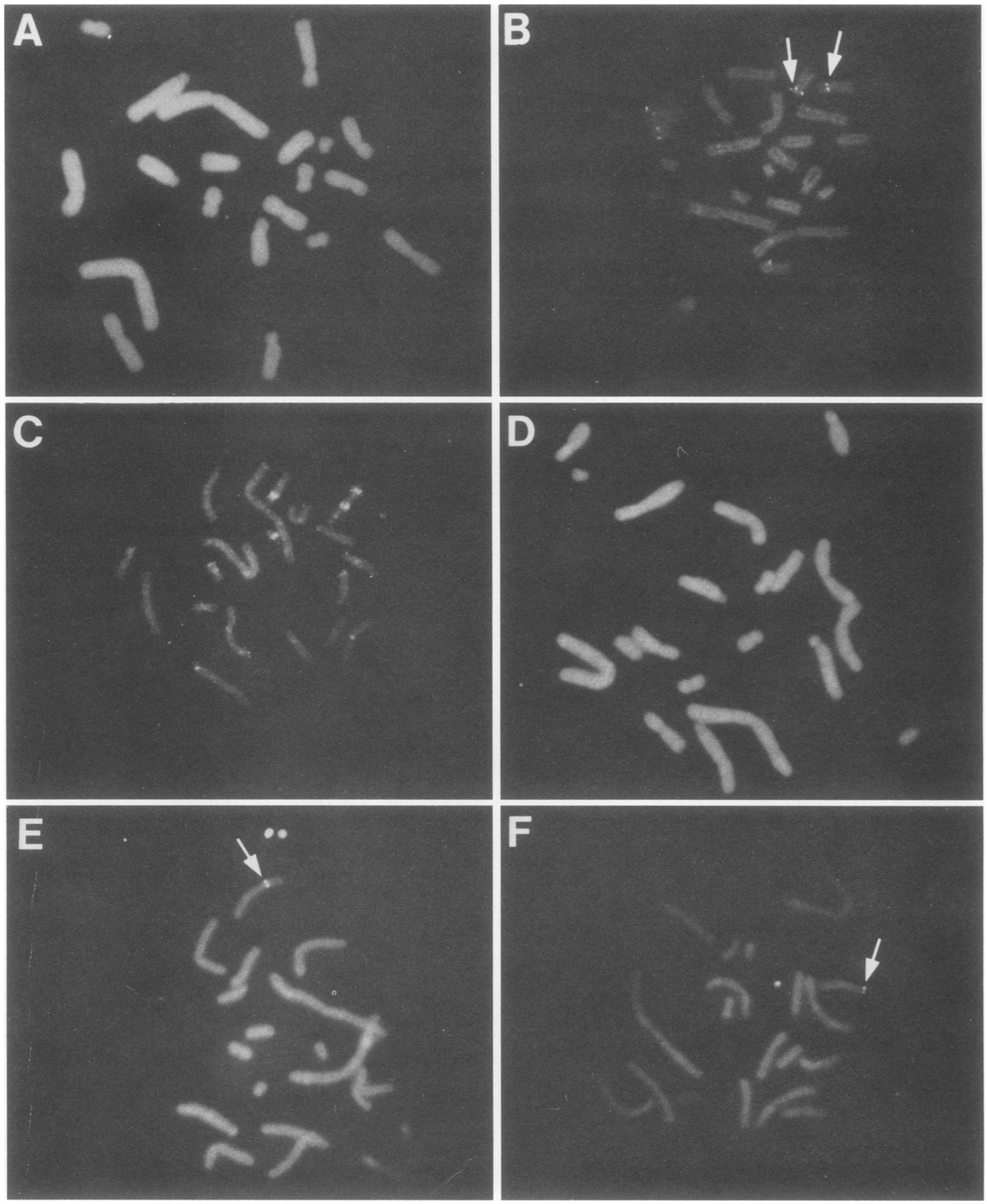

FIG. 4. Localization of pLPDL sequences in L46 early-passage cells. Metaphase spreads were prepared from the indicated cell lines and hybridized with the indicated biotinylated probes as described in Materials and Methods. The sites of hybridization were revealed by binding fluoresceinated avidin. Representative fluorescence photomicrographs are shown for cell line DG44 probe pLPDL (A); cell line DG44, probe cDCAD42 (B); cell line DG44, probe cL5 (C); cell line L46, probe pLPDL (D, E, and F). Panel A indicates the negligible level of nonspecific hybridization under the conditions employed, while Panel B shows the pattern and intensity of signal expected for a typical single-copy gene the CHO CAD gene) present on two autosomes (designated by arrows) with a cosmid probe. (The autosomes are not the same size because of the large number of chromosome rearrangements in these heavily mutagenized CHO cells). Panel C shows that CHO sequences derived from the integration site are highly repeated throughout the genome. The arrows in panels E and F designate the only sites of significant hybridization (the intensities of the signals and patterns of hybridization are approximately the same as those found for a single-copy gene shown in panel B). Note that the 10-kb pLPDL probe detects 20 kb of unique sequence, since each amplified unit contains an almost complete inverted duplication of this structure. The CAD gene cosmid probe has an insert of ~35 kb, part of which is highly repetitive and is eliminated by the prehybridization with CHO carrier DNA. Thus, these two probes should generate signals of roughly equal intensity when pLDPL hybridizes to a single homologous inverted duplication and when the cosmid hybridizes to a single copy of the CAD gene.

repetitive sequences and consequently cannot be used to localize the insertion site. The data reveal that integration occurred within a region enriched for dispersed, highly repetitive elements, but the presence of such sequences prevents us from elucidating the site at which integration occurred.

Generation of unstable chromosomally amplified structures following integration of extrachromosomal elements. McClintock described how a large inverted duplication within a chromosome could serve as a focal point for recombination events to generate dicentric chromosomes (see reference 31 for a discussion). Since the extrachromosomal elements in L46 contain at least one inverted duplication (38), integration of multiple copies of such structures could lead to many potential substrates for recombination and consequent chromosomal instability. We investigated, therefore, whether chromosome rearrangements involving the integrated extrachromosomal elements were commonly observed in methotrexate-resistant cells containing multiple copies of such sequences.

We selected L46 cells (passage 9, 35 cd posttransfection; Fig. 2) to resist a high concentration of methotrexate in a single step. The resistant cells represent preexisting variants which accumulated many copies of the extrachromosomal elements during growth under conditions routinely employed to passage L46. Two independently derived clones resistant to 1 and 2 μM methotrexate (clones L46 S1-1 and L46 S1-4, containing a 50- to 100-fold amplification of pLPDL, respectively; Fig. 2) were analyzed by fluorescence in situ hybridization. The methotrexate-resistant clones were analyzed soon after isolation (within 40 cd after exposure of the cells to methotrexate) or after several months in culture (more than 150 cd after drug selection).

The results show that the initially extrachromosomal elements frequently integrate in the chromosomes of methotrexate-resistant cells and that abnormal structures containing substantial levels of amplification are generated (the following text refers to the panels of Fig. 5). We observed acentric elements harboring multiple pLPDL sequences in 6% of the metaphases (9 of 149; see arrowheads labeled "p", Fig. 5B, C, and D). However, the amplified pLPDL sequences in the majority of the metaphases were detected in various types of rearranged chromosomes. For example, 51% (86 of 149) of the metaphases contained pLPDL sequences in ECRs at the ends of various chromosomes (Fig. 5A, E, F, and G) and 25% (37 of 149) contained amplified sequences in dicentric (12 of 149; Fig. 5B, C, and F) or ring chromosomes (25 of 149; Fig. 5D). Most of the ring chromosomes contained a few copies of the amplified sequences (15 of 25; data not shown); the example shown in Fig. 5C was one of three cases in which the amplified sequences comprised half of the chromosome. In addition, we observed a high proportion of metaphases (26%; 38 of 149) in which bands of amplified sequences were present at multiple sites within the same chromosome (Fig. 5E, F, and G). Clustering of amplified sequences at the ends of several chromosomes in a single metaphase spread has been observed repeatedly (e.g., Fig. 5G), as has the presence of heterogeneously sized acentric extrachromosomal elements (Fig. 5B, C, and D).

The abnormal chromosomes described above were observed at all stages of propagation subsequent to drug selection (i.e., whether analyzed 40 or 150 cd doublings after drug selection). Two dicentric chromosomes were observed in 84 L46 metaphases obtained from cells which were neither sorted nor exposed to methotrexate selection. In addition, the abnormal chromosomes shown in Fig. 5 were detected (with the exception of that shown in Fig. 5E) in the descendants of a single parental cell. The existence of a multitude of abnormal chromosomes containing the amplified sequences in individual cells within a single clone strongly indicates that the initial chromosome containing the reintegrated sequences was unstable and evolved into the observed structures.

Another example of chromosomal instability resulting from the integration of extrachromosomal elements is the rapid development of a broad distribution of chromosome number in methotrexate-resistant L46 cells (Fig. 6). By contrast, the modal chromosome numbers are the same in L46, two independently derived deletion variants of L46, and the DG44 cells used for electroporation (Fig. 6).

DISCUSSION

Deletion and gene amplification. The results reported here implicate chromosomal deletion in the genesis of the extrachromosomal elements which are the precursors of gene amplification in the system analyzed. The similarities between the early molecular products resulting from the amplification of many endogenous genes and those observed in this model system make it tempting to speculate that deletion can also initiate the amplification of some endogenous genes. The chronology of molecular and cytogenetic observations of the early steps of amplification in this system leads us to propose that a concerted process such as recombination within a replication bubble (Fig. 1B) concurrently generated an extrachromosomal molecule capable of autonomous replication, inverted repeats at the recombination junctions, and a deletion of the corresponding sequences from the chromosome.

Several models other than that presented above could be proposed to account for the observations presented here, but we feel that they are less likely to pertain. For example, it is conceivable that an unstable chromosomal intermediate was created initially and that it broke down very rapidly to generate a deletion and an extrachromosomal element (17, 40). Since we have not been able to identify cells undergoing the amplification process within a few cell doublings of the initial event, we cannot rigorously exclude this possibility. We disfavor such a mechanism, however, because it requires at least two independent events (i.e., the first generates a unit of intrachromosomal amplification and the second excises the chromosomal structure), while the model proposed here involves only a single concerted process. Another alternative is that integration of the donated sequences created an unstable chromosome which produced extrachromosomal molecules by a conservative process, and this chromosome was subsequently lost at a high frequency by nondisjunction. This model is almost certainly excluded, since the number of chromosomes is the same in DG44, L46, and two independently isolated deletion variants of L46 (Fig. 6), and karyotype analysis of both deletion variants failed to reveal consistent chromosomal changes relative to DG44 or L46 (data not shown). Furthermore, since the number of chromosomes is the same in each of these cell lines, one would have to postulate that two chromosome nondisjunctions occurred in each deletion variant to account for their wild-type chromosome content (i.e., the first eliminates the chromosome containing the transfected sequences and the second results in the random gain of a second chromosome to bring the total number back up to that of DG44 and L46). A more extreme alternative is that the donated sequences integrated into a preexisting extrachromosomal element.

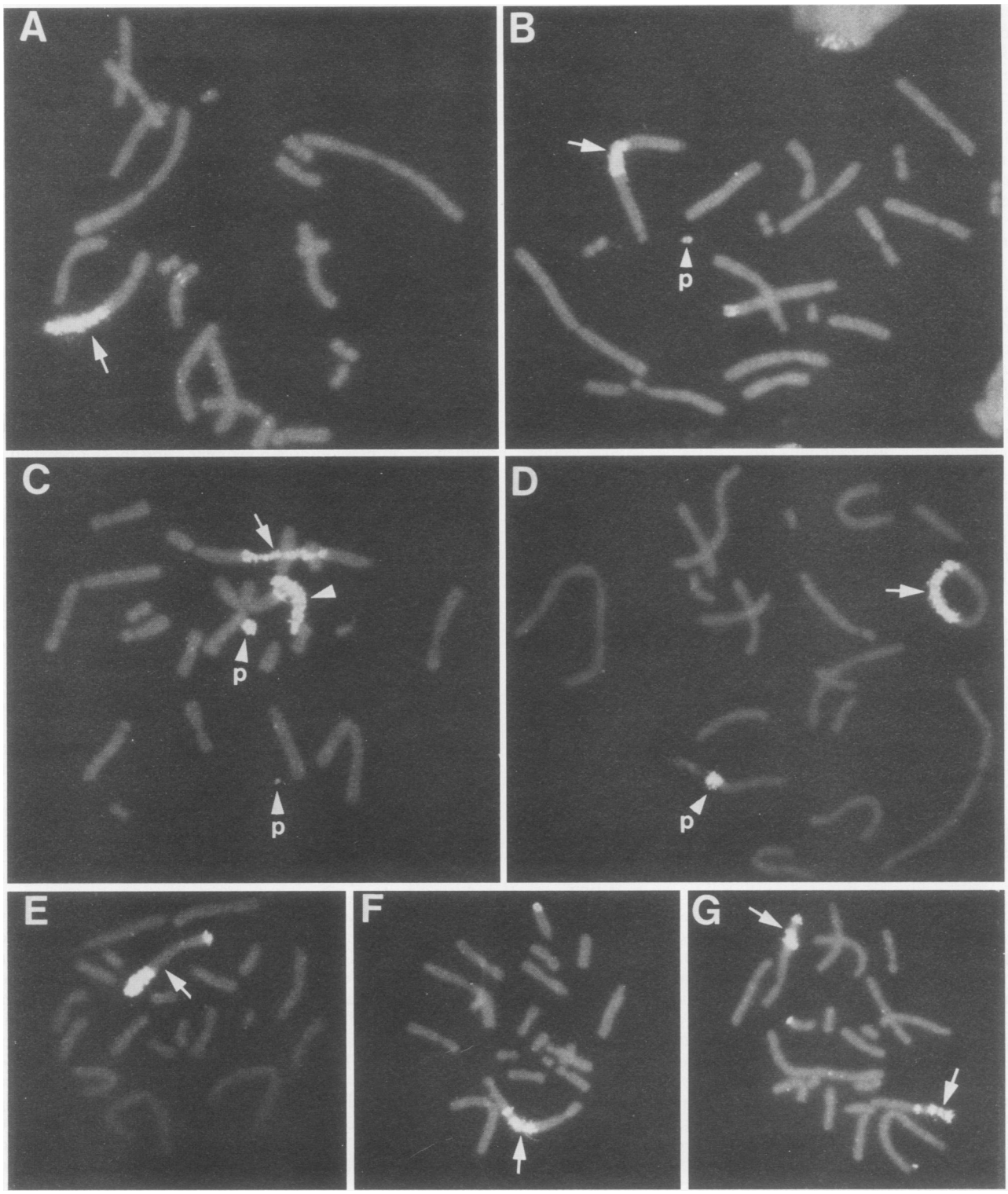

FIG. 5. Localization of amplified pLPDL sequences. Two single-cell clones (L46 S1-1 and L46 S1-4) containing amplified sequences were analyzed at the indicated approximate number of cell doublings following methotrexate administration. Sites of amplified sequences in the indicated cells were detected by fluorescence in situ hybridization as described in Materials and Methods. (A) L46 S1-1 (120 cd posttransfection); (B) L46 S1-1 (120 cd postselection); (C) L46 S1-1 (120 cd postselection); (D) L46 S1-1 (60 cd postselection); (E) L46 S1-4 (40 cd postselection); (F) L46 S1-1 (120 cd postselection); (G) L46 S1-1 (120 cd postselection). The arrows emphasize the chromosomes referred to in the text. The arrowheads denoted p in panels B to D signify acentric elements of varying sizes commonly found in the methotrexate-resistant cells and observed occasionally in unselected L46 (e.g., reference 38). The unlabeled arrowhead in panel C emphasizes a long acentric chromosome which bears a striking resemblance to CM chromosomes described previously (27).

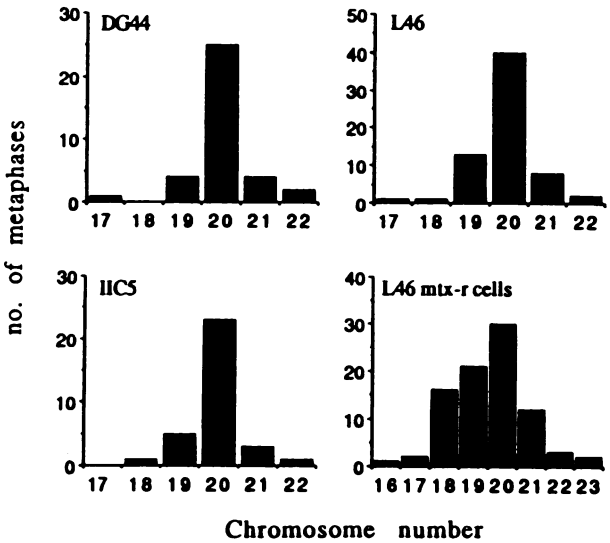

FIG. 6. The number of chromosomes in DG44, L46, and IIC5 are the same, but L46 methotrexate-resistant cells display considerable aneuploidy. Metaphase chromosomes were prepared from the indicated cell lines, stained in 4% Giemsa, photographed, and counted. A minimum of 36 metaphases was counted for each cell line. The chromosome counts were as follows: DG44, 20 ± 0.84 ; L46, 19.9 ± 0.8; IIC5, 19.9 ± 0.7; and L46 methotrexate-resistant (mtx-r) cells, 19.6 ± 1.3.

This scenario is also exceedingly unlikely, since the DNA content of such an element would comprise less than 1/3,000 of the genome (i.e., it would have to be $<2 \times 10^6$ base pairs or else it would have been visible in the light microscope), and L46 was detected as ~1 of 25 transformants (38). In addition, if we assume that the frequency of generating such elements is the same as that of the first step of gene amplification (10^{-4} to 10^{-6}), then only 1 to 100 cells in the population of 10^6 cells employed for transfection could have contained such an element. Thus, the probability of integration into such an element should be ~$1/10^8$ to $1/10^9$ of that expected for integration into chromosomal DNA.

Consideration of the data available leads us to propose that integration of the transfected sequences in L46 led to the destabilization of an adjacent chromosomal region extending at least 160 kb (the length of the region known to be amplified thus far). Recombination within this structure is then proposed to generate both an extrachromosomal element and a corresponding deletion. Excision of the donated sequences at or near the time of DNA integration, followed by unequal segregation of the acentric, replication-competent structure expected to be generated by such an event, would produce a cell population with the characteristics observed in L46; a sizeable fraction of this population would contain multiple copies of the donated sequences within an inverted repeat containing donated and host sequences, and the initial integration site would be absent from most or all of the cells in the population. An intriguing possibility is that the strong enhancer in the pLPDL vector activated an adjacent (although not necessarily nearby) DNA replication origin, stimulated it to fire at an inappropriate time, and led to the formation of the proposed recombination substrate. The importance of enhancers for the functioning of DNA replication origins in many DNA viruses (for a review, see reference 9), the inferred distribution of replication origins in mammalian cells (10), the ability of the extrachromosomal molecules to replicate (e.g., see references 6, 38, and 50), and the frequent occurrence of transformants like L46 in which unstable extrachromosomal elements are generated as an apparent consequence of DNA integration (5, 6, 16, 38, 53) are all consistent with such a speculation.

Extrachromosomal elements integrate rapidly. Heterogeneously sized extrachromosomal elements were readily detected in methotrexate-selected L46 cells but rarely in cells of a population grown in complete medium (38; Fig. 5, this report, and unpublished data). In a typical experiment, in situ hybridization of an unselected L46 population containing an average of 3 to 4 inverted duplications per cell revealed that 74% of the spreads had no hybridization, 20% had single-copy hybridization in several chromosomes, and 6% had integration of 5 to 10 copies. These experiments employed conditions which revealed the presence of single-copy chromosomal genes in 100% of the metaphases. Since the copy number of the L46 population cannot be accounted for solely by those cells with integrated sequences, we infer that the majority of the cells must have contained submicroscopic elements which could not be detected by in situ hybridization conditions employed. It is conceivable that such small structures are readily eliminated from the spreads by the mechanical forces generated during slide preparation.

The presence of donated sequences in several different chromosomal sites in a sizeable fraction of the L46 cell population suggests that integration of the putative extrachromosomal elements occurred substantially earlier than the 45 cd time point analyzed. This result suggests an alternative interpretation of the commonly held belief that amplification in CHO cells generally occurs by intrachromosomal expansion. Our data suggest that amplification in CHO cells can initiate with (submicroscopic) extrachromosomal molecules which integrate extremely rapidly to generate intrachromosomally amplified structures. This view is consistent with the initial instability of amplified endogenous DHFR genes in CHO cells, their subsequent stabilization at an intrachromosomal site (23; B. Windle, B. Draper, and G. M. Wahl, unpublished data), and the difficulty of detecting extrachromosomal structures in CHO cells containing amplified sequences after passaging such cells for many generations prior to analysis.

Chromosomal destabilization following integration. The existence of a large inverted repeat in the L46 amplicon and the types of abnormal chromosomal structures observed in this system (Fig. 6) lead us to propose the molecular pathways shown in Fig. 7 to explain the chromosome instability in this system. We propose that the integrated amplicons containing inverted repeats provide focal points for recombination. Such recombination could occur in two ways. As proposed earlier (24; Fig. 7A), integration within a telomere could interfere with telomere function and thereby prevent DNA replication from proceeding to the end of the chromosome. Subsequent to replication, both sister chromatids would be left with uncapped ends which could join to one another to form a dicentric chromosome. As originally shown by McClintock (e.g., references 30 and 31), dicentric chromosomes are physically broken at anaphase when the two centromeres are pulled towards opposite centrosomes. This generates two new chromosomes lacking telomeres and opens up the possibility for additional bridge-breakage-fusion cycles until the frayed ends generated by the process are capped by telomeres. The initial chromosomes generated by this model should be identical because they derive from the fusion of two sister chromatids. Indeed, we have seen symmetrical dicentric chromosomes containing the transfected sequences in L46 and L46 methotrexate-resistant mutants (Fig. 5B and

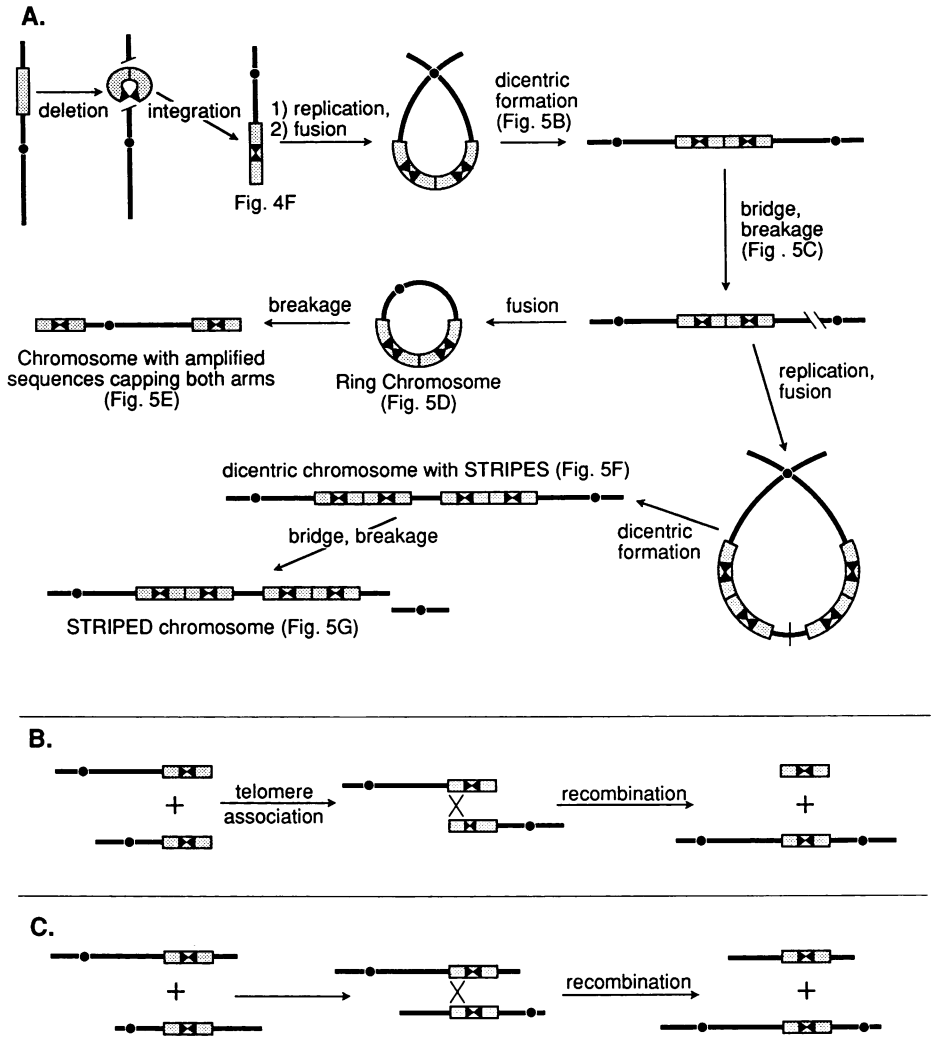

FIG. 7. Models for the generation of abnormal chromosomes precipitated by integration of extrachromosomal elements. (A) Replication destabilization model. The first model proposes that integration of the extrachromosomal element interferes with normal telomere structure and results in uncapped chromatids being produced after replication. Fusion of the two chromatids creates a dicentric chromosome, and then bridge-breakage-fusion cycles generate the abnormal chromosomes diagrammed in the figure and documented in the indicated panels of Fig. 6. The model is essentially the same as that of Kaufman et al. (24), except that we propose that deletion initiates the formation of the extrachromosomal elements, and it is the integration of these elements which initiates the process of chromosomal destabilization. (B and C) Recombination destabilization model. The second model proposes that integration of an acentric molecule containing a long inverted repeat generates a substrate for homologous recombination with the same structure integrated into a homologous or nonhomologous chromosome. Alignment of the two chromosomes with their centromeres on opposite sides of the inverted repeat, followed by recombination anywhere within the inverted repeat, produces a dicentric chromosome and acentric fragments, both of which contain amplified sequences. Panel B depicts recombination of two telomere proximal sites, and panel C depicts recombination at sites far removed from telomeres. This model readily explains how dicentric chromosomes containing two dissimilar "arms" (e.g., Fig. 5C and F) can be created. The other abnormal chromosomes in Fig. 5 can be generated by multiple bridge-breakage-fusion cycles, as shown in Fig. 7A.

data not shown). In addition, we have observed several examples in which dicentric chromosomes with amplified sequences appear to have been trapped during the bridge phase just prior to breakage (Fig. 5C). Figure 7A reveals how subsequent rounds of the bridge-breakage-fusion process can generate the other abnormal structures observed in this system.

We have also observed integrated sequences near the telomeres of two different chromosomes in a single cell, integrated sequences within chromosomes far from telomeres, and many dicentric chromosomes in which both arms are so dissimilar that they almost certainly derive from the fusion of two non-sister chromosomes. While it is possible to use the scheme described by Kaufman et al. (24) to explain the genesis of some asymmetric dicentric chromosomes, the existence of the inverted repeats in the amplicon suggests another intriguing possibility. We propose that the long regions of homology provided by the inverted repeats could allow two dissimilar chromosomes to pair with their centromeres positioned on opposite sides of the inverted repeat (Fig. 7B and C). Recombination within the extensive homology provided by the inverted repeats would generate asymmetric dicentric chromosomes (e.g., Fig. 5C and F), acentric fragments (e.g., Fig. 5C), and other abnormal structures. This model requires that multiple sites of integration occur in a single cell. Indeed, we frequently observed multiple sites

of hybridization in methotrexate-resistant L46 cells (e.g., Fig. 5A, B, F, and G).

Several observations support the feasibility of both schemes. First, telomere-telomere interactions have been observed in mitotic cells of many species (for reviews, see references 3, 8, 18, and 20). This may derive from the increased concentration of telomeres in a region just below the nuclear membrane in interphase and prophase nuclei (1, 8, 20) or because telomeres have extensive regions of Z-DNA which tend to self associate (2). Second, McClintock has presented evidence that dicentric chromosomes can be produced by the fusion of broken chromosomes through their ends which lack telomeres (31). Third, recombination of two homologous chromosomes can occur at a region which is present as an inverted duplication in one of them (31). Thus, it is conceivable that the integration of the extrachromosomal sequences at telomeres enables promiscuous joining or recombination to occur because of the tendency of telomeres to associate. On the other hand, intrachromosomal integration of amplicons containing inverted repeats could make it possible for dicentric or other abnormal chromosomal structures to be generated as a consequence of recombination events occurring within chromosomes as well.

An important implication of this work is that amplification begins with the production of unstable extrachromosomal structures, and their subsequent integration initiates a wave of chromosome instability. Consistent with the results reported here, we and others (5, 6, 23, 28, 50) have implicated unstable elements in the initial stages of amplification involving oncogenes in tumors and genes engendering drug resistance in vitro. The types of abnormal chromosomes described here have also been observed in the amplification of endogenous genes (e.g., references 4, 23, 27, 32, 33, and 54), suggesting that the mechanisms underlying their formation may be similar to those proposed here. It will be important to determine, therefore, whether the molecular and cytogenetic chronology reported here, from deletion to chromosome destabilization, is observed in examples of endogenous gene amplification involving selective conditions which prevail in vivo and drug selections imposed in vitro.

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