MOLECULAR AND CELLULAR BIOLOGY, Oct. 1988, p. 4302–4313 Vol. 8, No. 10
0270-7306/88/104302-12$02.00/0
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Formation of an Inverted Duplication Can Be an Initial Step in Gene Amplification

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Received 7 March 1988/Accepted 25 July 1988

We have developed a gene transfer approach to facilitate the identification and isolation of chromosomal regions which are prone to high-frequency gene amplification. Such regions are identified by assaying for transformants which show high-frequency resistance to PALA and/or methotrexate by amplification of a vector containing the genes which encode the enzyme targets of these antiproliferative agents. We identified 2 of 47 transformants which displayed high-frequency amplification of the transfected genes, and in this report we describe the analysis of one of them (L46). Molecular analysis of the integration site in transformant L46 revealed that the donated genes were at the center of an inverted duplication which spanned more than 70 kilobase pairs and consisted largely of host DNA. The data suggest that integration of the transfected sequences generates a submicroscopic molecule containing the inverted duplication and at least 750 kilobases of additional sequences. The donated sequences and the host sequences were readily amplified and lost in exponentially growing cultures in the absence of drug selection, which suggests that the extrachromosomal elements are acentric. In contrast to the instability of this region following gene insertion, the preinsertion site was maintained at single copy level under growth conditions which produced copy number heterogeneity in L46. The implications of our results for mechanisms of genetic instability and mammalian gene amplification are discussed.

Gene amplification, a localized increase in DNA sequence abundance, is one mechanism by which a cell or organism can increase the level of a gene product required for continued cell function or survival. Examples of amplification have been described in organisms as diverse as bacteria (1), plants (42), insects (10), and humans (53). The numerous examples of gene amplification indicate that many regions of the genomes of these organisms are susceptible to molecular remodeling by this process (for reviews of amplification mechanisms, see references 3, 9, 20, 41, and 44).

To examine the molecular mechanisms of mammalian gene amplification, we have developed gene transfer systems which enable us to scan the genome for sites which facilitate amplification (48, 51). The generation of cell lines which amplify transfected drug resistance genes at frequencies higher than 10^{-3} facilitates the molecular characterization of the initial events of amplification. One example of the usefulness of this approach is provided by our previous studies with a transfected CAD gene which revealed previously unsuspected intermediates in the amplification process. These intermediates, called episomes, have been shown to be submicroscopic autonomously replicating circular precursors of double minute chromosomes (DMs), the typical cytogenetic structures associated with extrachromosomal gene amplification in mammalian cells (7, 8, 51). Importantly, the involvement of episomes as intermediates in the amplification of endogenous genes is indicated by the localization of amplified human dihydrofolate reductase (DHFR) sequences to ~650-kilobase (kb) extrachromosomal elements in HeLa BU25 cells (32) and c-*myc* sequences to ~120- to ~250-kb circular molecules in COLO320 and HL60 human tumor cell lines (47). The similar results obtained with the gene transfer system and the amplification of endogenous genes demonstrate that conclusions reached by analyzing the amplification of transfected genes should also apply to the amplification of endogenous genes.

In this paper we report the molecular and cellular characterization of one transformant, L46, which exhibited high-frequency amplification of the donated sequences. The results presented are consistent with a new mechanism for gene amplification. We show that insertion of a small genetic element led to the formation of an inverted duplication which spanned more than 70 kb and consisted mainly of host DNA. The genomic region containing the inverted duplication undergoes rapid fluctuations in copy number. The formation of inverted duplications has been implicated in the amplification of proto-oncogenes in some human tumor cell lines and the amplification of endogenous genes which engender drug resistance in cultured cell lines (16, 22, 31, 40). Our data extend the observations made in endogenous systems by showing that the formation of the inverted duplication can be a very early, perhaps initial, event in the amplification of some chromosomal regions.

MATERIALS AND METHODS

Construction of pLPDL. pLPDL (Fig. 1) was constructed as follows. (i) pLP (39; contains *Escherichia coli pyrB*, which encodes aspartate transcarbamylase, under the transcriptional control of the Moloney murine sarcoma viral promoter) was cleaved at unique *Bgl*II and *Sal*I sites 3′ of the *pyrB* gene and ligated to a 4.4-kb *Bgl*II-*Sal*I fragment derived from the plasmid in31su which contains suIII (an amber-suppressing *E. coli* tRNA gene [27, 30]). This plasmid, now called pLPL, was propagated in *E. coli* CC114, which has an amber mutation in the *lacZ* gene. When plated on X-Gal–IPTG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–isopropyl-β-D-thiogalactopyranoside) plates, pLPL transformants give rise to blue colonies. All recombinant plasmids containing suIII were propagated in CC114. The plasmid in31su and *E. coli* CC114 were generously provided by S.

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Goff. The final step in constructing pLPDL was insertion of a *Bam*HI fragment containing the DHFR expression module derived from pSV2-DHFR (45) into the unique *Bgl*II site located between *pyrB* and suIII. A clone containing the DHFR gene in the transcriptional orientation opposite to that of *pyrB* was chosen for use in these studies so that the simian virus 40 late polyadenylation signal would be placed downstream of *pyrB* (39; Fig. 1).

Cell lines, transfection, and drug selection protocol. DG44 is a Chinese hamster ovary (CHO) cell line which has a double deletion of the DHFR loci (46); it was kindly provided by L. Chasin. DG44 was propagated in Dulbecco modified Eagle medium–8% dialyzed fetal calf serum–nonessential amino acids–30 μM hypoxanthine–3 μM thymidine. KB-V1 (14) is a human carcinoma cell line which was propagated in minimal essential medium–8% dialyzed fetal calf serum–1 μg of vinblastine per ml; it contains a greater than 100-fold amplification of the *mdr-1* gene on 600- and/or 750-kb episomes and minute chromosomes (J. Ruiz, K. Choi, D. Von Hoff, I. Roninson, and G. Wahl, submitted for publication). KB-V1 was provided by I. Roninson. C5R500 is a CHO cell line which was maintained in Dulbecco modified Eagle medium–8% dialyzed fetal calf serum–500 μM *N*-(phosphonacetyl)-L-aspartate (PALA); it contains approximately 50 copies per cell of a 250-kb CAD episome (8). B5-4 is a Syrian hamster cell line which was propagated in the same medium as C5R500; it has a 50-fold intrachromosomal amplification of the CAD gene (50). The methotrexate resistant (Mtx^R) mouse cell line R50 was kindly provided by T. Tlsty and was grown in alpha-minimal essential medium–8% dialyzed calf serum–50 μM methotrexate; it has a 40- to 50-fold amplification of the DHFR gene, and the amplified genes have been localized to minute chromosomes (5).

A 9.2-kb *Pvu*I-*Sal*I fragment derived from pLPDL (Fig. 1) was transfected into DG44 cells by using a BTX electroporator (Biotechnologies and Experimental Research, Inc., San Diego, Calif.). Electroporation was done as follows. Cells (5×10^6) were suspended in 1 ml of shocking medium (0.3 M sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM Tris, pH 7.4). DNA at 5 μg/ml was mixed with the cells for 5 min, following which the cells were given 10 pulses of 50 ms at an amplitude setting of 700, 70 ms at an amplitude setting of 800, or 99 ms at an amplitude setting of 950. The cells were seeded at approximately 10^6/10-cm dish, allowed to recover for 1 day, and then selected for growth in medium lacking hypoxanthine and thymidine (medium which selects for cells expressing DHFR).

Cell transformants expressing the donated DHFR gene were cloned from a single colony and expanded for 1 to 2 weeks until the transformant population reached approximately 10^6 cells. At this time 10^4 cells from each transformant were plated in concentrations of PALA ranging from 0 to 1,000 μM, of methotrexate from 0 to 1,000 nM, and various combinations of both agents. The number of drug-resistant colonies was scored after 2 weeks of growth in selective media. A frequency of colony formation in PALA at least 100 times greater than that of DG44 or in methotrexate at least 100 times greater than that of wild-type CHO-K1 (diploid for DHFR) indicated that a transformant was a candidate for high-frequency amplification of the donated sequences.

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 (F-methotrexate) and incubation of viable cells with F-methotrexate previously described (17).

Construction of cosmid libraries. *Mbo*I genomic partials of 30 to 50 kb were generated and purified from L46 high-molecular-weight DNA by using the protocol described by Dillela and Woo (11) and then were ligated into the *Bam*HI cloning site of the cosmid vector pWE15 (12, 49). The library was packaged by using Gigapack Gold (Stratagene Cloning Systems, La Jolla, Calif.). The cosmid clones containing 30- to 44-kb inserts including the donated pLPDL sequences (including the amber-suppressing suIII tRNA gene) were identified by transducing the packaged DNAs into *E. coli* CC114, which has an amber mutation in the *lacZ* gene. Cosmids containing suIII generate blue Lac^+ colonies, while clones representing the rest of the genome generate white Lac^- colonies when the library is plated on X-Gal-IPTG-kanamycin plates.

Restriction mapping of the cloned inserts is facilitated by the organization of the cloning cassette in pWE15 (12). This cassette contains two *Not*I restriction sites flanking T3 and T7 bacteriophage promoters, which in turn border a unique *Bam*HI cloning site. Since *Not*I cleaves mammalian DNA infrequently, most inserts can be excised as a single *Not*I fragment. Therefore, opposite ends of the insert will be attached to either a T7 or T3 promoter. Partial digestion of such a fragment with an enzyme of choice produces a ladder of bands. Fractionation of partial digests by the conditions described below, followed by Southern blotting and hybridization (52) to oligonucleotides complementary to the T3 or T7 promoters (T3- and T7-specific oligonucleotides used for hybridization were generously provided by Stratagene Cloning Systems), produces a blot containing a ladder of bands ranging in size from that of the smallest restriction fragment containing either promoter to the size of the entire insert. The size (in kilobases) of each band corresponds to the distance of a given restriction site from the flanking T3 or T7 promoter. Cosmid DNA (100 to 300 ng) was digested to completion with *Not*I, partially digested with *Bam*HI or *Eco*RI, and then fractionated on agarose gels by one of two methods. Standard electrophoresis used a continuous electrical field at 1.4 V/cm for 60 h through a 0.6% Tris acetate-agarose gel. Field inversion gel electrophoresis was performed (6) at 5.4 V/cm for 19 h (pulse time, 0.76 s forward and 0.38 s reverse) through a 1% Tris borate-agarose gel by using a Programmable Power Inverter (MJ Research, Inc., Cambridge, Mass.).

Isolation of circular molecules from mammalian cells by alkaline lysis. Circular DNAs exceeding 15 kb were isolated by using alkaline lysis (8, 18). The fraction of circular molecules recovered in the supernatant was calculated as follows. Approximately 2×10^7 cells were divided into two aliquots. Total DNA from one aliquot was purified by using standard techniques, while the circular DNA was extracted from the second aliquot by alkaline lysis (8, 18). Various dilutions of each DNA preparation were applied onto nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, N.H.) in triplicate through a dot blot apparatus (Minifold I; Schleicher & Schuell) and hybridized with a probe specific for the amplified gene present in the particular cell line and also with a probe specific for mitochondrial and ribosomal gene DNA (34). After hybridization, the dots were punched out, and the counts per minute (cpm) in each dot was quantitated in a scintillation counter. The percent recovery of the circular molecules was calculated according to the formula (cpm in alkaline lysate/cpm in total DNA) × 100. A percent recovery of >5% (the usual background for chromosomal sequences

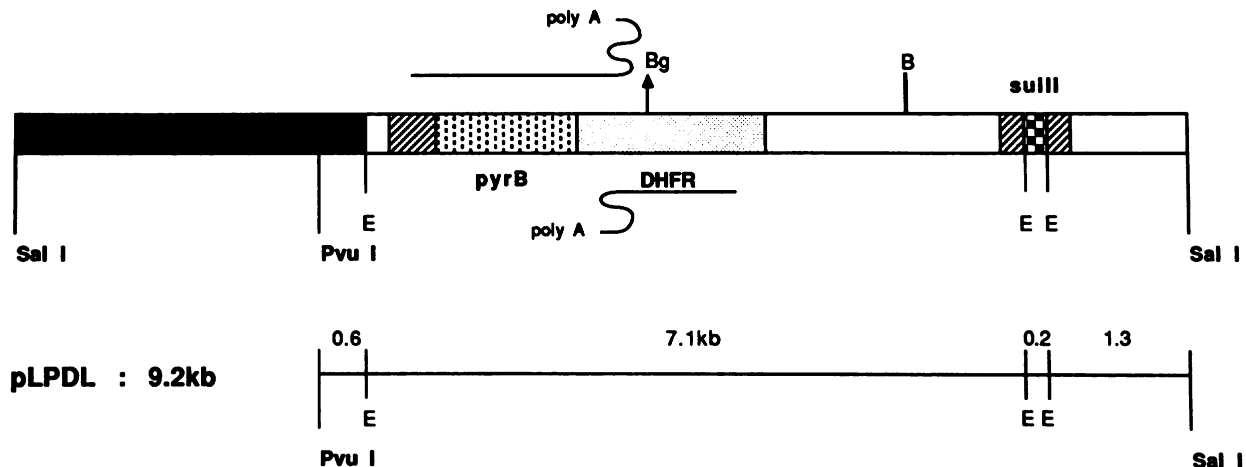

FIG. 1. Map of recombinant vector used in the gene transfer-gene amplification system. pLPDL is a 12.9-kb plasmid which contains the *pyrB*, DHFR, and sulII genes cloned between the *Eco*RI and *Sal*I sites of pBR322 (see Materials and Methods for details). A 9.2-kb *Pvu*I-*Sal*I fragment was purified and used for the transfection studies. A map of the *Eco*RI sites in the 9.2-kb *Pvu*I-*Sal*I fragment is presented as a reference for Fig. 5. E, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI. ▬, pBR322; ▨, Moloney long terminal repeat; ▥, *pyrB*; ▦, DHFR; ▩, sulII; ▭, Moloney murine leukemia virus or random mouse genomic sequences.

such as ribosomal genes) indicates that a gene is present on submicroscopic circular molecules.

Preparation of metaphase spreads and in situ hybridization. Metaphase spreads were prepared as previously described (8) and hybridized to a tritium-labeled *pyrB* probe (8, 21). Chromosomes were observed through a standard Zeiss microscope with an ×80 objective and photographed under bright field with a Nikon FX35A camera.

RESULTS

Isolation of cell transformants which display high-frequency amplification. The recombinant vector and mammalian cell line used for these studies have several features which facilitate the analyses of chromosomal regions prone to high-frequency amplification. (i) The recombinant vector pLPDL (Fig. 1) contains two minigenes, *E. coli pyrB* (39) and the mouse cDNA encoding DHFR (45). The overexpression of these genes by amplification can engender resistance to PALA and methotrexate, respectively, so that mutants can be selected for resistance to either or both agents. (ii) In the DHFR-deficient CHO cells (DG44; 46) used for transfection, expression of a single donated DHFR gene is sufficient to transform them to a $DHFR^+$ phenotype (P. Gaudray and J. Ruiz, unpublished observations). In addition, the level of DHFR expression in individual viable cells can be determined easily by using F-methotrexate and flow cytometry (17, 24). Since the level of F-methotrexate binding to the DHFR enzyme provides an estimate of relative gene copy number (24), we can use the fluorescence-activated cell sorter to obtain subpopulations of cells which contain amplified DHFR sequences. (iii) The recombinant vector (a 9.2-kb linear molecule; Fig. 1) is introduced into random locations in the mammalian genome by electroporation to increase the probability of generating transformants containing a single copy of the recombinant molecule (37). This is important because transformant lines which contain multiple copies of the transfected genes may show enhanced drug resistance relative to single copy level transformants and thus may be falsely identified as hyperamplifiable transformants. (iv) The bacterial selectable marker sulII (27, 30) is included in pLPDL to facilitate the isolation of the host sequences which flank the donated genes (see Materials and Methods).

Forty-seven $DHFR^+$ transformants were analyzed for the capacity to generate $PALA^R$ and/or Mtx^R colonies at a high frequency. A frequency of drug resistance for a $DHFR^+$ transformant at least 100 times greater than the frequency of colony formation for DG44 or wild-type CHO-K1 under identical selective conditions indicated that the transformant might be capable of high-frequency amplification of the transfected genes. Approximately 10^4 cells from each $DHFR^+$ transformant and from DG44 or CHO-K1 were plated in concentrations of PALA ranging from 0 to 1,000 μM, of methotrexate ranging from 0 to 1,000 nM, and in combinations of both agents. The number of drug-resistant colonies was scored after 2 weeks of growth in selective media. Of 47 $DHFR^+$ transformants, 2 (transformants G32 and L46) exhibited high-frequency drug resistance by amplification of the donated genes (see Table 1 for a summary of their amplification characteristics). Importantly, these studies were performed at <25 cell doublings after the generation of the initial $DHFR^+$ cell. This result suggests that the high-frequency amplification phenotype was generated at or near the time of gene introduction.

We have concentrated our analysis on transformant L46, since 3% of L46 cells resist 1 μM methotrexate (~10 times the 50% lethal dose [LD_{50}]) after one selection step; this frequency of Mtx^R is 1,000- to 10,000-fold greater than the frequency of Mtx^R for CHO-K1 at ~10 times the LD_{50} (LD_{50} of CHO-K1 is ~50 nM methotrexate). Southern analysis indicated that the copy number of pLPDL was increased 50 to 100-fold in L46 mutants selected to resist 1

TABLE 1. Amplification characteristics of G32 and L46

Cell line	Frequency of drug-resistant cells at ~10 times the LD_{50} in: PALA	Methotrexate	Fold amplification
DG44	$<10^{-4}$	$<10^{-5}$	ND[a]
G32	2×10^{-2}	7×10^{-4}	3–5
L46	1.6×10^{-2}	3×10^{-2}	50–100

[a] ND, Not determined.

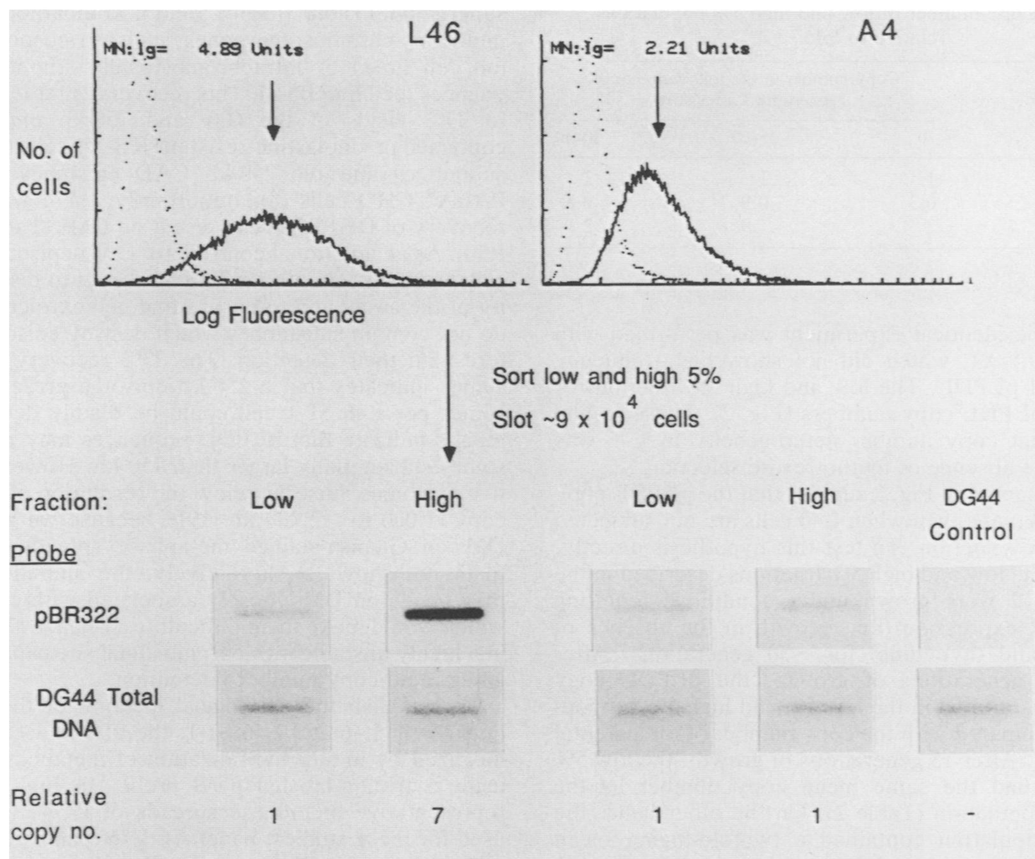

FIG. 2. L46 shows pLPDL copy number heterogeneity in exponentially growing cultures. L46 and A4 cultures were incubated for 18 h in the presence of 10 μM F-methotrexate–30 μM hypoxanthine–3 μM thymidine (solid line) to measure the level of DHFR enzyme in individual cells or in the presence of 10 μM F-methotrexate–15 μM methotrexate–30 μM hypoxanthine–3 μM thymidine (dotted line) to measure the level of nonspecific F-methotrexate binding in these cells (17). For each transformant, cells with the lowest 5% and highest 5% fluorescence levels were sorted on the fluorescence-activated cell sorter into separate tubes and DNA from 9×10^4 cells was slotted directly onto nitrocellulose through a slot blot apparatus (34). The blot was hybridized to a 0.6-kb *PvuI*-*EcoRI* pBR322 fragment to quantitate pLPDL copy number. The amount of DNA loaded in each slot was internally standardized by hybridizing the blot with radiolabeled total DG44 DNA. The copy number relative to the low 5% fraction was calculated by densitometric analysis of the autoradiograms.

μM methotrexate in a single step (Fig. 5A, lanes 1 and 2; six of six Mtx^R L46 derivatives contained amplified pLPDL sequences).

Copy number heterogeneity of the donated genes occurred in L46 cultures not exposed to drug treatment. Two models can be envisioned for high-frequency drug resistance in L46. (i) Drug treatment induces amplification of the chromosomal region containing the pLPDL sequences, or (ii) variants exist in L46 populations which contain elevated numbers of pLPDL sequences prior to drug selection. These two models can be distinguished because in the absence of selection, model 1 predicts that all of the cells should have the same low level of DHFR enzyme. By contrast, model 2 predicts that some proportion of the cells will have elevated levels of DHFR enzyme.

To distinguish between these models, exponentially growing L46 cultures were incubated with F-methotrexate in the presence of hypoxanthine and thymidine so as not to select for DHFR amplification (17, 24). The cells were then analyzed in the flow microfluorometer in order to measure the level of fluorescence (i.e., DHFR enzyme content) on a cell-by-cell basis. The histograms in Fig. 2 (top panel) show F-methotrexate binding profiles in cell lines L46 and A4 (a pLPDL transformant which did not exhibit high-frequency PALA^R or Mtx^R). Included in the F-methotrexate histograms are controls in which L46 and A4 are incubated in the presence of both F-methotrexate and methotrexate to indicate the background of nonspecific F-methotrexate binding (17; dotted line in Fig. 2). The flow microfluorometer analysis indicated that L46 had a twofold-higher mean fluorescence than transformant A4 did (4.9 U versus 2.2 U; Fig. 2) and appeared to have a more heterogeneous distribution of fluorescent cells (i.e., higher coefficient of variation).

The flow microfluorometer profile revealed possible evidence of heterogenous DHFR copy number in L46. The following experiment was performed to test directly whether amplified variants preexist in L46 populations. The cells with the lowest and highest fluorescence levels (the low 5% and high 5% fractions) were purified in the fluorescence-activated cell sorter, and the pLPDL copy number in each fraction was quantitated (34). The data in Fig. 2 show that L46 exhibited an approximately sevenfold range in pLPDL copy number between the low and high 5% fractions (the pLPDL copy number of an L46 exponentially growing population was approximately three to four times higher than that of the low 5% fraction). In addition, the increase in pLPDL copy number in the high 5% fraction correlated with an increased resistance of these cells to methotrexate (data

TABLE 2. Copy number of low and high 5% populations relative to L46

Cell population	Copy number at the following no. of generations after sort:		
	0	15–20	30–40
L46	1	1	1
Low 5%	0.3	0.9	0.8
High 5%	2	1.9	2

not shown). The identical experiment was performed with the transformant A4, which did not show high-frequency amplification of pLPDL. The low and high 5% fractions of A4 had equal pLPDL copy numbers (Fig. 2). These results demonstrate that copy number heterogeneity in L46 was generated in the absence of methotrexate selection.

The data presented in Fig. 2 suggest that the pLPDL copy number can fluctuate even when L46 cells are not subjected to methotrexate selection. To test this hypothesis directly, 10^4 cells from the low and high 5% fractions described in the legend to Fig. 2 were grown under conditions requiring minimal DHFR expression (i.e., growth in the absence of hypoxanthine and thymidine) for ~30 generations. After ~15 and ~30 generations of growth, the pLPDL copy number was quantitated in the low 5% and high 5% subpopulations and compared with the copy number of the parental L46 population. After 15 generations of growth, the low 5% subpopulation had the same mean copy number as the parental L46 population (Table 2). On the other hand, the high 5% subpopulation contained a twofold-higher mean copy number than did the L46 population even after 30 generations of growth (see Discussion). However, amplified pLPDL sequences from one Mtx^R L46 clone (L46 S1-1) were lost in the absence of methotrexate selection (97% of the amplified pLPDL sequences were lost after 24 weeks of growth without methotrexate with hypoxanthine and thymidine) (Fig. 3). Therefore, cells containing a relative copy number of one (e.g., the low 5% fraction) could generate cells with amplified pLPDL sequences after 15 to 20 generations of growth, and cells with amplified pLPDL sequences (e.g., the L46 S1-1 cell line) could generate cells with a low copy number. These experiments confirm the hypothesis that pLPDL sequences undergo rapid copy number changes in L46 cultures grown in the absence of methotrexate selection.

Are the donated sequences in L46 contained on extrachromosomal elements? The observation that the pLPDL sequences in L46-derived lines were subject to rapid gain and loss is consistent with the idea that pLPDL sequences reside on acentric extrachromosomal elements, such as DMs or submicroscopic circular episomes (8, 25). To determine whether the pLPDL sequences in L46 cells can be localized to episomes, an alkaline lysis procedure was used to enable isolation of extrachromosomal circular molecules (8, 18, 47). This procedure involves lysis of cells at pH 12.45, neutralization, and phenol extraction at a high salt concentration and results in circular molecules partitioning into the supernatant and chromosomal molecules precipitating at the phenol-water interphase. If episomes contain pLPDL sequences in drug-resistant L46 cells, then the fraction of pLPDL sequences recovered in the alkaline lysis supernatant should exceed that of chromosomally repeated sequences such as ribosomal genes. Only 3% of pLPDL sequences could be isolated from Mtx^R L46 S1-1 cells in the alkaline lysis supernatant (Table 3). This yield is comparable with that of multicopy chromosomal genes, such as ribosomal sequences (all cell lines) or intrachromosomally amplified CAD sequences (cell line B5-4). This recovery is far less than the 16 to 39% yields of the 600- and 750-kb *mdr-1* episomes contained in vinblastine-resistant KB-V1 cells (J. Ruiz et al., submitted) and the 250-kb CAD episomes contained in PALA^R CHO cells (8). Importantly, there was only a 2% recovery of DHFR genes present on DMs (Table 3, cell line R50). As an additional control, 10^6 CAD-episome-containing cells were mixed with 10^7 L46 S1-1 cells to test the sensitivity of the assay and to ensure that cell extracts of L46 S1-1 do not contain substances which destroy episomes or interfere with their detection. The 23% recovery of CAD episomes indicates that a 250-kb episome present at ~5 episomes per L46 S1-1 cell could be readily detected. These results indicate that pLPDL sequences may reside on episomes substantially larger than 750 kb. However, the putative episomes must be below the resolution of light microscopy (1,000 to >2,000 kb [19]), because we have not seen DMs in Giemsa-stained metaphase spreads on L46 S1-1 (data not shown). Alternatively, the amplified sequences may reside on DMs loosely associated with chromosomes, which would make them difficult to visualize (2), or possibly in a highly unstable intrachromosomal site capable of undergoing rapid copy number fluctuation.

To establish the cytological location of the pLPDL sequences in L46 and L46 S1-1, the pLPDL sequences were localized by in situ hybridization of metaphase spreads by using a tritium-labeled *pyrB* probe. In Fig. 4 are shown representative metaphase spreads of DG44 (host cell line used for these studies; panel A), L46 (panels B and C), and L46 S1-1 (panels D, E, and F). We observed no consistent chromosomal location for the donated pLPDL sequences in L46 or L46 S1-1 (24 metaphases analyzed per cell line). All three cell lines contained an average of 8 to 10 individual silver grains distributed throughout the metaphase spread (e.g., panels A, B, and D). However, the proportion of metaphase spreads which contained clusters of silver grains differed for each line. For example, 3% (1 of 17) of DG44

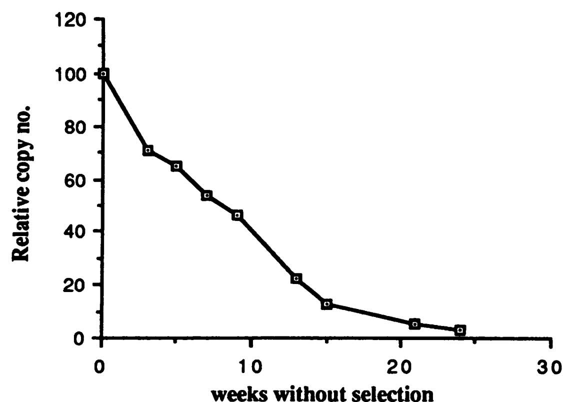

FIG. 3. Amplified pLPDL sequences in Mtx^R line L46 S1-1 were unstable. L46 S1-1 is a line selected to resist 1 μM methotrexate in one step and contains a 50- to 100-fold amplification of pLPDL relative to L46. At week 0, aliquots of 10^5 L46 S1-1 cells were pelleted and stored at −20°C for original copy number controls. L46 S1-1 was propagated in medium containing 30 μM hypoxanthine and 3 μM thymidine for 24 weeks. At 2- or 3-week intervals, 10^5 L46 S1-1 cells were pelleted and stored at −20°C. The pLPDL copy number for each time point was quantitated and normalized to week 0 L46 S1-1 copy number as described in the legend to Fig. 2.

TABLE 3. Amplified sequences in L46 S1-1 cannot be isolated by alkaline lysis

Cell line (no. of expts)	Fold amplification	Form amplification	% Recovery with probe[a]:				
			CAD	DHFR	*mdr-1*	rDNA	Mito
C5R500 (3)	250× CAD	250-kb circular episome	39			3	46
KB-V1 (7)	100× *mdr-1*	600- and 750-kb circular episomes, DMs			16	4	48
R50 (5)	40–50× DHFR	DMs		2		1	58
B5-4 (3)	50× CAD	Intrachromosomal array of inverted duplications	4			5	44
L46 S1-1 (3)	150–200× pLPDL	Inverted duplications, location unknown		3		3	46
L46 S1-1 (10^7 cells) + C5R500 (10^6 cells)			23	2		4	43

[a] % Recovery = (counts per minute in alkaline lysate DNA preparation/counts per minute in total DNA preparation) × 100. rDNA and Mito, Ribosomal- and mitochondrial-specific probes, respectively.

metaphase spreads contained a cluster of silver grains, while 33% (8 of 24) of L46 metaphase spreads (panel C) and 67% (16 of 24) of L46 S1-1 metaphase spreads contained clusters of silver grains (panels D, E, and F). L46 S1-1 had between one and four grain clusters per metaphase spread near the ends or the sides of different chromosomes. Curiously, 3 of 24 L46 S1-1 metaphase spreads contained a ring chromosome; however, the ring did not appear to have a high copy number of pLPDL sequences (panel F; see Discussion). The observations that the donated pLPDL sequences in L46 and L46 S1-1 show no defined chromosomal location and that a number of metaphase spreads show randomly distributed grain clusters are consistent with the hypothesis that the majority of pLPDL sequences reside on extrachromosomal elements loosely associated with the ends or sides of chromosomes (2).

pLPDL sequences in L46 lie at the center of a ≥35-kb inverted duplication. The host sequences which flank the pLPDL insertion sites in L46 and which presumably confer the high-frequency amplification phenotype were isolated by molecular cloning (see Materials and Methods) and analyzed by Southern blotting. DNA from L46, from the Mtx^R line L46 S1-1, and from two recombinant cosmids, cL5 and cL6, which contain the pLPDL integration site and ~20 kb of flanking host DNA, was cleaved with *Eco*RI and hybridized with a pLPDL probe (Fig. 5A). This analysis revealed that L46 contained a 7.1-kb fragment corresponding to the internal *Eco*RI fragment in pLPDL (Fig. 1) and 6.5- and 2.7-kb novel fragments. Each of these fragments were amplified 50- to 100-fold in L46 S1-1 (lane 2). Each cosmid contained the 2.7-kb novel fragment (lanes 3 and 4) and either the 7.1-kb (cosmid cL6) or 6.5-kb (cosmid cL5) *Eco*RI fragments. Figure 5B shows the result of rehybridizing the blot shown in Fig. 5A with radiolabeled *pyrB* sequences. The internal 7.1-kb and the novel 6.5-kb fragment both hybridized with *pyrB*. Hybridization of this blot with the DHFR or pBR322 probe revealed that the 6.5-kb novel fragment also contained both of these sequences (data not shown).

Restriction mapping of three recombinant cosmids containing the pLPDL integration site (cosmids cL5, cL6, and cL8) led to the conclusion that they overlap by ~4 kb (Fig. 6A). While the host sequences in each cosmid had identical restriction maps, the pLPDL sequences were readily distinguished. Cosmid cL5 contained 6.5- and 2.1-kb fragments which hybridized with *pyrB* (Fig. 5B, lane 3; the 6.5-kb fragment also hybridized with pBR322 [data not shown]), while cosmid cL6 contained 7.1- and 2.2-kb fragments which hybridized with *pyrB* (Fig. 5B, lane 4; the 2.2-kb fragment also hybridized with pBR322 [data not shown]). These data suggest the existence of an inverted duplication in which the center consists of one intact and one truncated pLPDL and an adjacent stretch of more than 20 kb of host DNA. We have not been able to isolate cosmid clones containing more than 6 kb of the inversion (e.g., Fig. 6A, cosmid cL8), probably because phage, plasmids, and cosmids containing extensive inverted duplications are difficult to propagate in *E. coli* (29, 31).

The map of the hypothesized inverted duplication derived from the cosmid mapping data predicts the deletion of 1.8 kb of pLPDL sequences from one arm of the inverted duplication (the arm containing the 6.5-kb *Eco*RI fragment). As a consequence of the asymmetry, this model predicts the existence of 10.8-kb *Bam*HI and 6.2-kb *Bgl*II fragments which should hybridize with a pBR322 probe and which would span the center of the inverted duplication (Fig. 6B and C). To determine whether the 10.8-kb *Bam*HI and 6.2-kb *Bgl*II fragments are present in L46, high-molecular-weight DNA from L46 was restricted with *Bam*HI, *Bgl*II, or *Eco*RI and analyzed by Southern blotting using radiolabeled pBR322 as a probe. The autoradiogram shown in Fig. 6C confirms the presence of 10.8-kb *Bam*HI (lane 1) and 6.2-kb *Bgl*II (lane 2) fragments in L46 genomic DNA which hybridized with pBR322 (the 6.5-kb *Eco*RI fragment is shown in lane 3). Further Southern analysis of L46 genomic DNA digested with several other restriction enzymes also revealed the presence of fragments which were also predicted to span the center of the inverted duplication (data not shown). Thus, these data support the hypothesis that gene insertion in L46 is associated with the formation of a large inverted duplication containing the donated pLPDL sequences at the center.

Gene insertion appears to create a site in L46 subject to copy number fluctuation. The restriction pattern diagnostic for the inverted duplication was seen at the earliest time of analysis of L46. This observation suggests that the formation of the inverted duplication was coincident with or occurred soon after insertion of the pLPDL module into the chromosome. In addition, the observation that the inverted duplication could be amplified at least sevenfold in the absence of methotrexate selection in L46 cells (Fig. 2) suggests that gene insertion has created a structure prone to copy number fluctuation (see Discussion for models for inverted duplication formation and copy number fluctuation). However, an alternative model is that pLPDL may have fortuitously integrated into a genomic region which is unstable when the cells are put under appropriate selective conditions (4, 10).

If pLPDL integration destablizes this genomic region, then the relative copy number of the chromosomal region contained the inverted duplication in L46 parental cultures should be six- to eightfold higher than that in DG44, G32, or G32 drug-resistant mutants under standard growth conditions for each line (i.e., L46 had a mean of three to four

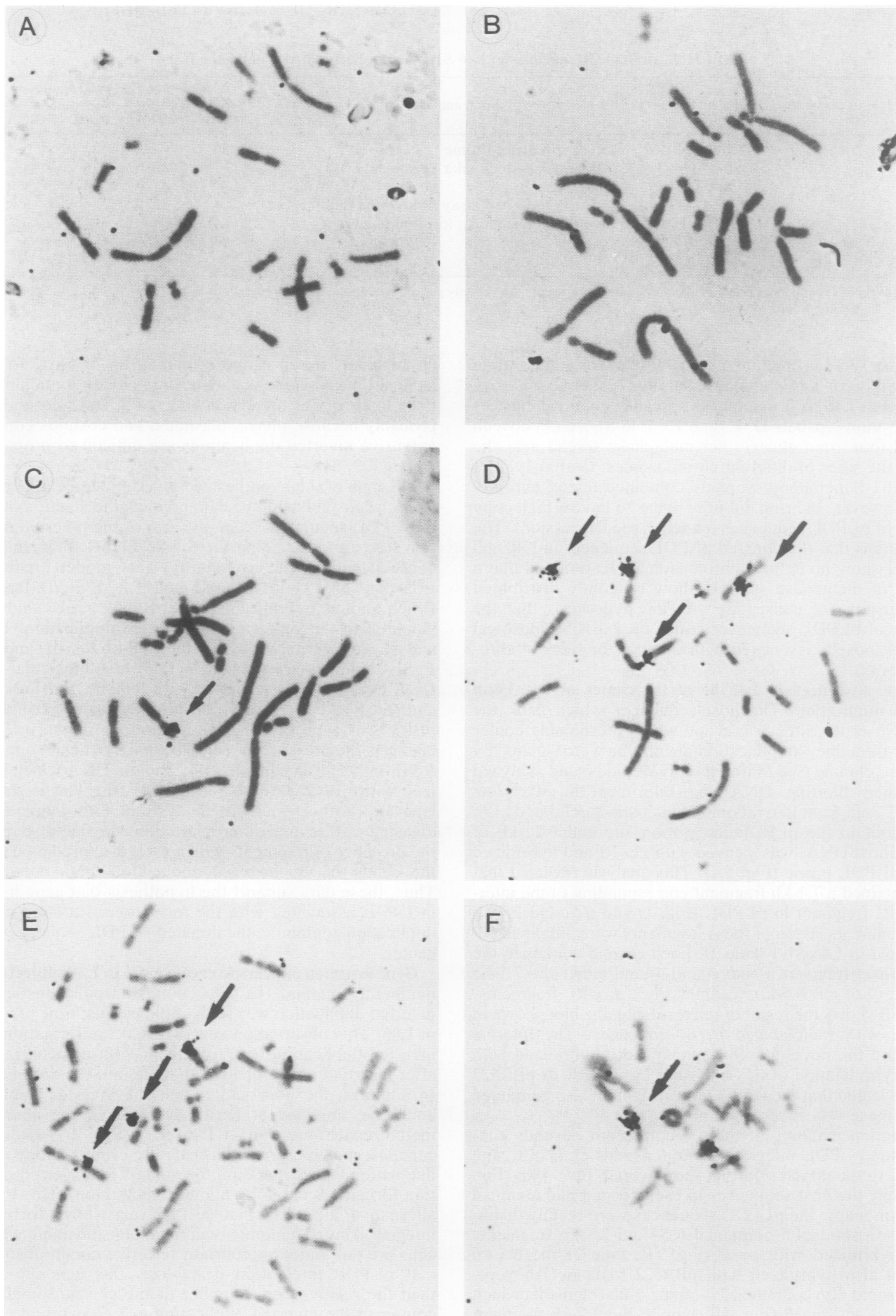

FIG. 4. Localization of the donated pLPDL sequences in metaphase spreads of DG44, L46, and L46 S1-1 by in situ hybridization. Metaphase spreads were hybridized to a tritium-labeled *pyrB* probe (7, 21) and exposed for 2 weeks at −70°C. Photographs were made of 22 DG44, 24 L46, and 24 L46 S1-1 metaphase spreads. (A) DG44; (B and C) L46; (D, E, and F) L46 S1-1.

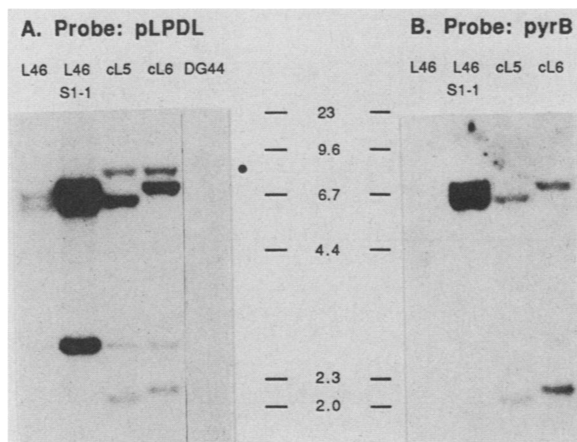

FIG. 5. Restriction analysis of DNA isolated from cell lines L46 and L46 S1-1 (resistant to 1 μM methotrexate) and two recombinant cosmids spanning the integration site in L46. ●, Position of pWE15. (A) *Eco*RI-restricted DNA was fractionated through a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to radiolabeled pLPDL sequences. Lanes (from left to right): 1, L46 (10 μg); 2, L46 S1-1 (5 μg); 3, cosmid cL5 (1.5 ng of cL5 mixed with 5 μg DG44); 4, cosmid cL6 (1.5 ng of cL6 mixed with 5 μg of DG44); 5, DG44 (5 μg). (B) The same blot used in panel A was washed and rehybridized with radiolabeled *pyrB* sequences. In lane 1 (L46), the 7.1- and 6.5-kb fragments can be detected after a longer exposure. Sizes are shown in kilobases.

inverted duplications per cell; Fig. 2). Alternatively, if the preintegration site can be made unstable during growth under selective conditions (e.g., growth under conditions requiring expression of at least one DHFR gene), then this chromosomal region should be increased in another pLPDL transformant (e.g., G32) grown under the same conditions (in G32 the pLPDL sequences were integrated into a different genomic site than in L46). To distinguish between these two possibilities, we isolated a genomic fragment located approximately 20 kb from the pLPDL-host junction site (Fig. 6B, probe A) and compared the copy number of this genomic sequence in DG44, transformants L46 and G32, and G32 drug-resistant mutants. Note that this experiment does not test whether an inverted duplication of the chromosomal region containing probe A is present in DG44 or G32 cell lines; it only assesses whether this chromosomal region is prone to amplification in isogenic cell lines which do not contain a pLPDL molecule inserted into this region. The copy number of the 13-kb *Eco*RI fragment recognized by probe A was at least 5-fold greater in L46 than in G32 when both were grown under conditions which required DHFR expression (Fig. 7, compare lanes 3 and 4). Probe A also hybridized with an unlinked 10.9-kb *Eco*RI fragment, which provided an internal standard for the amount of DNA loaded per lane. The copy number in G32 was approximately the same as that seen in DG44 (the host line used for transfection; lane 1). In addition, the selection of transformant G32 with either PALA or methotrexate to obtain mutants which amplified the donated vector did not result in the amplification of the region containing probe A (lanes 5 and 6). The results support the hypothesis that the preintegration region in L46 is stable under the same growth conditions which produce copy number fluctuations in this region subsequent to gene insertion. We infer from these results that gene insertion in L46 leads to the formation of a large inverted duplication and a structure which exhibits rapid fluctuations in copy number.

DISCUSSION

We have used gene transfer to identify regions of the eucaryotic genome which are prone to gene amplification. In a previous study, we identified 1 of 25 CAD gene transformants which gave rise to high-frequency PALA resistance due to amplification of the donated sequences (8, 51). In the present study, we developed a new gene transfer system which uses the pLPDL vector (Fig. 1) to facilitate the molecular and cellular analyses of hyperamplifiable gene transformants. Similar to our previous results, the results of this study showed that 2 of 47 transformants displayed high-frequency drug resistance by amplification of the donated sequences. Thus, even though a different recipient cell line, gene transfer module, and gene introduction procedure were used, approximately 4% of the transformants displayed a hyperamplifiable phenotype. We interpret these results to indicate that 4% of the genomic sites sampled by gene integration will impart this phenotype. However, it is not clear whether similar or different mechanisms for amplification are used in independent hyperamplifiable transformants.

In transformant L46, 3% of the cells could resist 1 μM methotrexate (~10 times the LD_{50}) after one selection step. Quantitation of pLPDL copy number in subpopulations of L46 cells never exposed to methotrexate revealed that some cells contained at least a sevenfold amplification of the donated pLPDL sequences (Fig. 2). Although one copy of the DHFR gene under the control of the simian virus 40 early promoter is sufficient to complement the defect in the DHFR-deficient line used in these studies (P. Gaudray and J. Ruiz, unpublished observations), cells with amplified pLPDL sequences accumulated in a population grown under minimal selective conditions (i.e., in the absence of hypoxanthine and thymidine). Thus, it appears that cells with higher pLPDL copy number have a selective advantage. If L46 cells with higher pLPDL copy number have a selective advantage in medium lacking hypoxanthine and thymidine, then this may explain why a subpopulation with high pLPDL copy number (the high 5% fraction; Fig. 2) did not readily generate cells with a lower pLPDL copy number (Table 2).

Molecular characterization of the genomic region containing the transfected pLPDL sequences at the hyperamplifiable site in L46 revealed that the vector sequences were at the center of an inverted duplication spanning at least 70 kb (recent experiments using field inversion electrophoresis indicate that the inverted duplication spans at least 160 kb). This is an intriguing observation because large inverted duplications have recently been reported in several examples of endogenous gene amplification. For example, some of the amplified units containing c-*myc* proto-oncogenes in two human tumor cell lines (16), CAD genes in one $PALA^R$ Syrian hamster cell line (40), DHFR genes in one Mtx^R CHO cell line (31), *aprt* locus in one spontaneous *aprt* mutant CHO line (35), and adenylate deaminase genes in one coformycin-resistant Chinese hamster line (22) are arranged as inverted duplications. One interpretation of the occurrence of inverted duplications in gene amplification is that this type of structure is formed as an early step in the process (see below for further discussion). While there is only circumstantial evidence consistent with this hypothesis for the amplification of endogenous genes, the data reported here show that the inverted duplication in L46 was present at the earliest time that a molecular analysis could be performed. Since L46 displayed high-frequency Mtx^R <25 cell doublings after the formation of the original $DHFR^+$ cell, we hypothesize that the insertion of pLPDL into the genome in

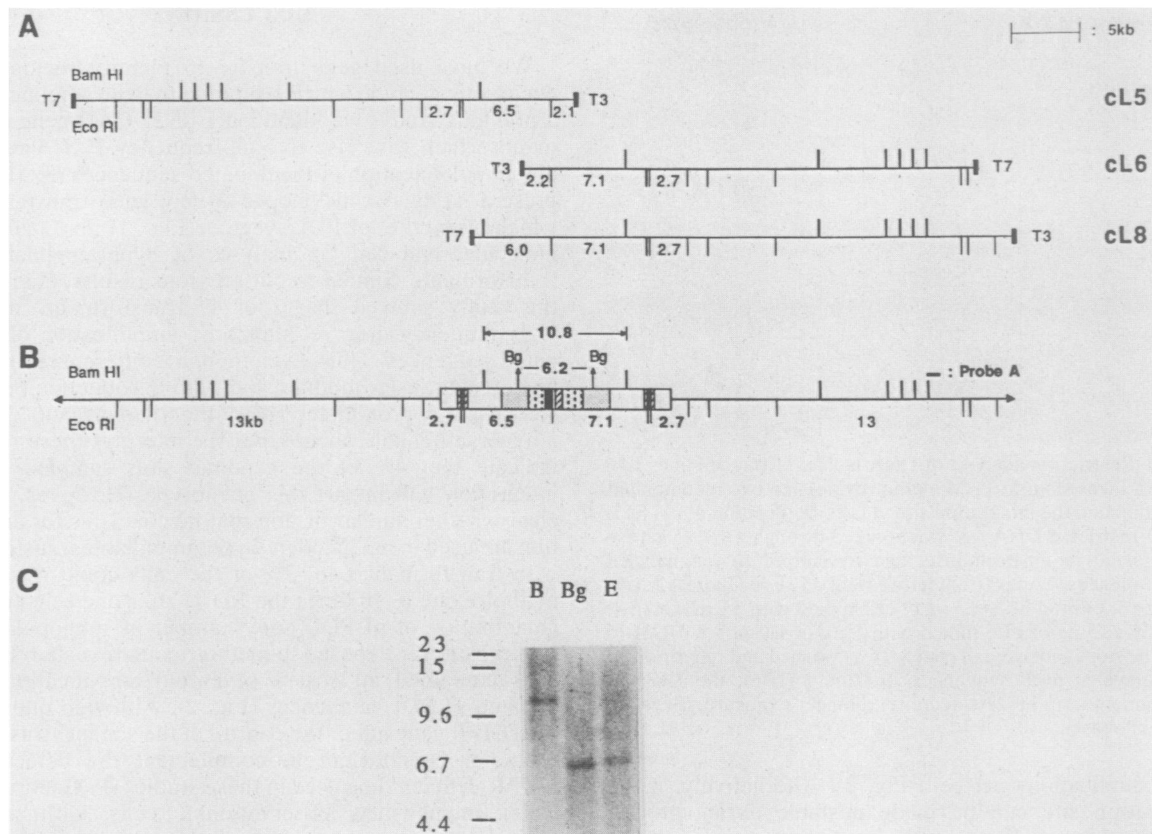

FIG. 6. Donated pLPDL sequences form an inverted duplication. (A) The restriction sites of *Bam*HI and *Eco*RI in cosmids cL5, cL6, and cL8 were determined by indirect end labeling (see Materials and Methods). The sizes of the restriction fragments generally were rounded to the nearest 0.5 kb. (B) Map of the gene integration site in L46 based on the cosmid mapping data. The inverted duplication model predicts the existence of 10.8-kb *Bam*HI and 6.2-kb *Bgl*II (Bg) fragments. Probe A is a 0.7-kb *Bam*HI-*Rsa*I fragment located approximately 20 kb from the pLPDL-host DNA junction which has no highly repetitive sequence elements. (C) Approximately 1.25 μg of high-molecular-weight L46 DNA was restricted with *Bam*HI (B), *Bgl*II (Bg), or *Eco*RI (E), fractionated through a 0.7% agarose gel, and transferred to nitrocellulose. The blot was then hybridized with a 0.6-kb *Pvu*I-*Eco*RI pBR322 fragment which was radiolabeled without purifying the fragment from the agarose (13). Sizes are shown at left in kilobases.

L46 results in the formation of a structure containing an inverted duplication of the donated sequences along with the host sequences flanking one side of the original integration site (see below for models for the generation of an inverted duplication). We feel that the structure containing the inverted repeat is acentric because L46 cultures generate copy number heterogeneity at a high frequency and because the amplified sequences in one Mtx^R clone (L46 S1-1; Fig. 3) are highly unstable in the absence of selection. The inability to isolate the presumed extrachromosomal elements by alkaline lysis suggests that they either are circular molecules larger than 750 kb (Table 3) or are not circular. However, fractionation of undigested L46 S1-1 DNA by field inversion electrophoresis revealed that amplified pLPDL sequences do not reside on linear molecules <2,200 kb (data not shown). The in situ hybridization results are consistent with the presence of submicroscopic extrachromosomal elements which are loosely associated with chromosomes (Fig. 4). Analysis of the insertion site in transformant G32, which also showed high-frequency amplification of the donated sequences, did not reveal an inverted duplication in 40 kb of DNA surrounding the integration site. While it is possible that an inverted duplication is located outside the region analyzed, these data indicate that high-frequency amplification does not require the formation of such structures directly at the insertion site.

There are several molecular models which could account for the formation of an inverted duplication initiated by gene insertion. Any model must account for the observations that the inverted duplication in L46 was probably formed coincident with gene insertion and that copy number heterogeneity (manifested by high-frequency Mtx^R) was seen <25 cell doublings after the generation of the original L46 cell. We will present two models. The first model is based on the breakage-fusion-bridge cycle proposed by McClintock (33) and later adapted by Kaufman et al. for gene transfer (26). In this model, DNA insertion leads to chromosome breakage which results in the generation of an acentric fragment along with a centric chromosome containing the donated sequences. After replication, the broken ends of the sister chromatids of the centric chromosome fuse to form a dicentric chromosome in which the donated sequences lie at the center of the fusion. The dicentric chromosome will break at a random site during anaphase if each centromere is pulled to an opposite pole. The newly broken chromosome then could reenter a fusion-bridge-breakage cycle, become stabilized by the addition of telomeric sequences to the broken end, or form a ring chromosome by joining of the ends. While this model accounts for the formation of the inverted duplication, it still does not answer the question of how copy number heterogeneity is generated rapidly in L46. To account for such heterogeneity, one must postulate that the intrachro-

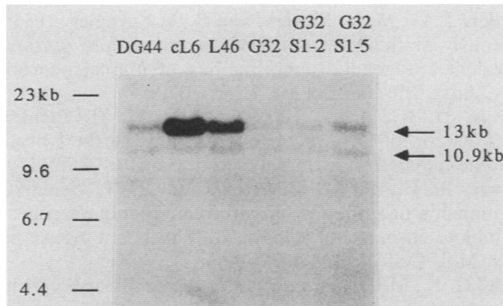

FIG. 7. Evidence in support of insertional destabilization in L46. *Eco*RI-restricted DNA (10 μg) was fractionated through a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to radiolabeled probe A sequences which are located 20 kb from the gene insertion site in L46 (probe A fragment was subcloned into pBluescript [Stratagene Cloning Systems]). This genomic fragment was chosen as a probe because it was the only fragment derived from host DNA near the insertion site which did not contain repetitive sequence elements. Probe A hybridized to 10.9 and 13-kb *Eco*RI fragments. The 13-kb fragment was contained within the inverted duplication in L46, while the 10.9-kb fragment was unlinked to the gene insertion site in L46 and thus could be used as an internal control for the amount of DNA loaded per lane. Lanes (from left to right): 1, DG44; 2, cosmid cL6 (1.2 ng mixed with DG44); 3, L46; 4, G32; 5, G32 S1-2 (mutant selected to resist 1 mM PALA in one step); 6, G32 S1-5 (mutant selected to resist 1 μM methotrexate in one step).

mosomally located inverted duplication is unstable and by some mechanism is capable of generating copy number fluctuation (22).

A second model (Fig. 8) links the formation of an inverted duplication with the production of an episome which generates the copy number fluctuation observed in L46. In this model, adapted from Passananti et al. (36), gene insertion occurs adjacent to a replication origin. Following the initiation of replication, recombination occurs across the replication fork and within the donated sequences (Fig. 8, x). This recombination event will produce acentric and dicentric chromosomes which both contain an inverted duplication with its center at the crossover point. The dicentric chromosome of recombination event x could enter the bridge-breakage-fusion cycle discussed above. To form an episome with an inverted duplication, an additional recombination event must take place at position y in Fig. 8. The recombination products generated by recombination at both x and y will be an episome containing two replication origins and an inverted duplication in addition to an acentric and a dicentric chromosome, each of which contains an inverted duplication at the point of crossover. Random segregation of the autonomously replicating episome would lead to copy number fluctuation and an apparent high rate of pLPDL amplification. In L46 the episome may be substantially larger than 750 kb (Table 3). We have found a ring chromosome in some L46 and L46 S1-1 cells (e.g., Fig. 4F) and a dicentric chromo-

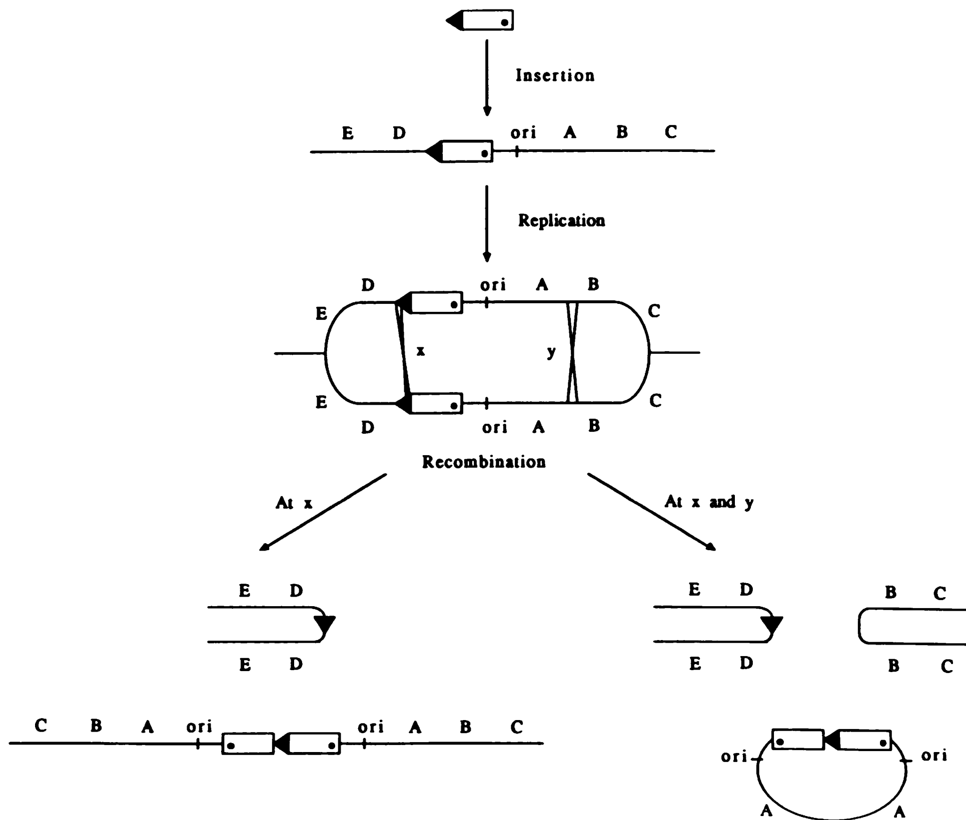

FIG. 8. Model for the formation of an inverted duplication after gene insertion. Gene insertion occurs adjacent to a replication origin. During replication two possible recombination events may occur. A recombination event at x (within the donated sequences) leads to the formation of acentric and dicentric chromosomes which each contain an inverted duplication at the crossover point (the donated sequences will be present on one of the abnormal chromosomes). Recombination events at x (within the donated sequences) and y lead to the formation of similar chromosomal products, except that the donated sequences will reside on an episome containing two replication origins (ori) and an inverted duplication of the donated genes and flanking host DNA.

some in others (data not shown). Since we did not find either structure in the parental DG44 cells, we infer that they may be the expected cytogenetic products predicted by either model. In situ hybridization experiments reveal no consistent chromosomal location of the amplified sequences in L46 S1-1 and suggest that most of the amplified pLPDL sequences are located extrachromosomally (Fig. 4). However, these data do not allow us to exclude models which invoke the generation of extrachromosomal elements from intrachromosomal inverted duplications (22). Genetic and in situ hybridization experiments using a fluoresceinated detection system (28) should enable us to determine whether pLPDL sequences are contained chromosomally in L46 or whether all of the donated sequences have been excised to form extrachromosomal elements.

The observation that insertion of a 9.2-kb genetic element (pLPDL) could lead to the formation an extrachromosomal molecule >750 kb which contained an inverted duplication provides a new way of envisioning the types of events which may initiate the amplification process. Other observations substantiate the idea that perturbations of some regions of the eucaryotic genome can cause large-scale reorganization of neighboring chromosomal regions. For example, DNA insertion subsequent to DNA transfection has been reported to be associated with genomic reorganization (38), including formation of inverted duplications (15, 36). Insertion of viral sequences has also been reported to be associated with the formation of an inverted duplication spanning several hundred kilobases (28). Owing to the large-scale rearrangements associated with insertion of the small pLPDL module, it is tempting to speculate that the insertion of other small genetic elements, such as retroviral DNA or polydispersed circular DNAs (43), also could precipitate the formation of unstable structures which lead to copy number fluctuation. We also note that chromosomal translocation can lead to reorganization of adjacent chromosomal regions. Molecular analysis of the sequences near the translocation breakpoints occurring in one patient with ataxia-telangiectasia revealed that translocation probably led to the formation of an inverted duplication near one of the breakpoints (23). In light of these data, it is reasonable to propose that some chromosomal translocations associated with the amplification of endogenous genes may have served as initiators of the amplification process. The analysis of the hyperamplifiable transformant L46 has lead to a novel way of envisioning how the amplification process may be initiated in endogenous systems; the study of additional hyperamplifiable transformants, such as G32, may enable a greater understanding of the multiple mechanisms and sequences involved in the amplification process.

ACKNOWLEDGMENTS

We thank J. Trotter for his expert assistance with the flow cytometry experiments; S. Kjemtrup for technical assistance; and S. Carroll, R. Kelly, J. Meinkoth, G. Nonet, S. O'Gorman, and B. Windle for reading the manuscript.

This work was supported in part by Public Health Service grant GM27754 from the National Institutes of Health and by a grant from the G. Harold and Leila Y. Mathers Charitable Foundation.

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