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Double Minute Chromosomes Can Be Produced from Precursors Derived from a Chromosomal Deletion

SUSAN M. CARROLL,[1] MARGARET L. DeROSE,[1] PATRICK GAUDRAY,[1]† CHARLEEN M. MOORE,[2] DONALD R. NEEDHAM-VANDEVANTER,[2] DANIEL D. VON HOFF,[2] AND GEOFFREY M. WAHL[1]*

Gene Expression Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037,[1] and University of Texas Health Science Center and Cancer Therapy and Research Center, San Antonio, Texas 78284[2]

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Recent experiments have shown that gene amplification can be mediated by submicroscopic, autonomously replicating, circular extrachromosomal molecules. We refer to those molecules as episomes (S. Carroll, P. Gaudray, M. L. DeRose, J. F. Emery, J. L. Meinkoth, E. Nakkim, M. Subler, D. D. Von Hoff, and G. M. Wahl, Mol. Cell. Biol. 7:1740–1750, 1987). The experiments reported in this paper explore the way episomes are formed and their fate in the cell over time. The data reveal that in our system the episomes are initially 250 kilobases, but gradually enlarge until they become double minute chromosomes. In addition, we show that episomes or double minute chromosomes can integrate into chromosomes. Our results also suggest that episomes can be produced by deletion of the corresponding sequences from the chromosome.

The genomes of organisms spanning the phylogenetic scale can achieve regional increases in gene copy number: a process referred to as gene or DNA sequence amplification (see references 5, 9, 19, 41, 45, and 46 for reviews). In mammalian cancer cells, gene amplification in vivo and in vitro can confer resistance to a wide range of drugs and may contribute to malignant progression (see reviews above and reference 2). Gene amplification in eucaryotes has typically been thought to be restricted to malignant cells and to a few examples in development (45). However, recent experiments show that gene copy number increases are the basis of resistance to pesticides in some plants and insects (34, 43) and to heavy metals in *Drosophila melanogaster* (36). In these cases, gene amplification must have occurred in the germ line, since the resistant phenotype is heritable.

Until recently, only two cytogenetic markers have commonly been associated with gene amplification in cancer cells: double minute chromosomes (DM; extrachromosomal, acentric, autonomously replicating elements) and intrachromosomally amplified regions (also referred to as homogeneously staining or abnormally banding regions). Both structures have been shown to harbor amplified oncogenes or drug resistance genes (see reviews above for references). Recently, extrachromosomal molecular structures consisting of 100 to 650 kilobase pairs (kb) of DNA have been associated with the amplification of drug resistance genes and proto-oncogenes in rodent and human cells (7, 31; D. D. Von Hoff et al., Proc. Natl. Acad. Sci. USA, in press). These molecules, which have been referred to as episomes, have been shown in some cases to be circular and to replicate semiconservatively and approximately once per cell cycle (7; Von Hoff et al., submitted).

Several pieces of evidence suggest a molecular linkage among episomes, DMs, and intrachromosomal amplified regions. For instance, the sizes of episomes are similar to estimates of the sequence complexity of DMs present in tumor cell lines which have amplified various oncogenes (24, 25). This might appear to be inconsistent with the data of Hamkalo et al. (18), who have calculated that the physical size of the smallest DM seen in the light microscope is approximately 1×10^3 to 5×10^3 kb. However, these results can be reconciled if a precursor-product relationship exists between the episomes and DMs. Such a hypothesis is also consistent with the observation that DMs within a cell show wide variation in size (18, 48). In addition, there appears to be a temporal order in the appearance of the extrachromosomal and chromosomal forms of gene amplification. Both in vivo and in vitro, DMs are often present in the earlier stages of amplification, whereas homogeneously staining regions are apparent in later stages (14, 28). These observations suggest that homogeneously staining regions may, in some cases, be formed from DMs.

In the work presented in this report, we investigated whether episomes, DMs, and intrachromosomally amplified regions are interrelated and the mechanism(s) by which episomes are generated. We were aided in these experiments by the availability of a CHO cell line created by introduction of cloned CAD genes (see Materials and Methods for a description of the CAD gene). This transformant, referred to as T5, contains the donated CAD genes integrated in a single chromosomal site as a pentamer arranged in a head-to-tail tandem array (51). Previous studies showed that in some subclones the integrated array was amplified near to or at the site of integration, whereas in others it was amplified as ca. 250-kb episomes and no DMs were visible initially (7). We have used these various subclones of T5 to show that episomes can be precursors of DMs and can integrate into chromosomes and that episomes are not produced at detectable frequencies from intrachromosomal amplified sequences. Furthermore, our results indicate that one mechanism which generates episomes produces a deletion of the corresponding chromosomal sequences. The data reported here provide the first molecular evidence that episomes are precursors of DMs and that they can integrate into chromosomes.

* Corresponding author.
† Present address: Laboratoire de Pathologie Cellulaire et Genetique, U.F.R. de Medecine, 06034 Nice Cedex, France.

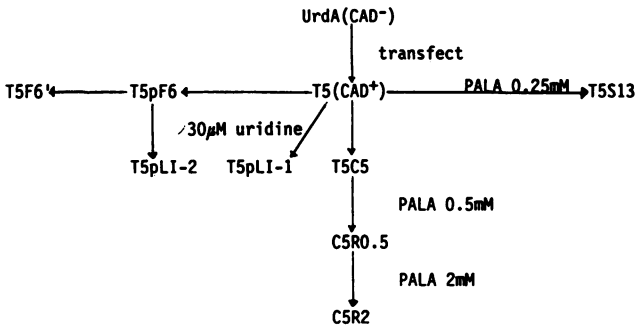

FIG. 1. Cell lines used in this work. The figure illustrates the ways in which the cell lines are related.

MATERIALS AND METHODS

Description of the CAD gene and cell lines analyzed. The CAD gene encodes a multifunctional protein containing the following activities: carbamyl-phosphate synthetase, aspartate transcarbamylase, and dihydroorotase. *N*-phosphonacetyl-L-aspartate (PALA) inhibits aspartate transcarbamylase (23).

Cell lines T5 and T5S1-3 were previously described by Wahl et al. (51), and T5C5 and C5R0.5 (previously referred to as C5R500) were described by Carroll et al. (7). The following discussion and Fig. 1 indicate how the cell lines used in this paper are related. The parental cell line Urd$^-$A is a Chinese hamster ovary cell mutant defective in de novo pyrimidine biosynthesis (38). T5 was isolated as a single-cell clone containing a chromosomally integrated tandem array of 5 donated Syrian hamster CAD genes (40, 51). T5C5 is a single-cell clone derived from T5 in which the CAD genes exist as 250-kb episomes. C5R0.5 is a single-cell clone derived from T5C5 which is resistant to 0.5 mM PALA and contains approximately 250 CAD genes per cell in an exclusively episomal form. C5R2 is a population derived from C5R0.5 by growth of the latter in 2 mM PALA. T5pF6 was derived from one frozen aliquot of the original T5 clone. The original T5 clone had undergone several rounds of freeze-thaw and passage prior to being frozen and stored as T5pF6. T5pLI-2 was derived as a population from T5pF6 by growth of T5pF6 in medium containing 30 μM uridine. T5F6′ was derived as a population from T5F6 by growth of the latter for approximately 5 to 6 months in medium containing regular fetal calf serum. T5LI-1 was derived as a single-cell subclone from a different frozen aliquot of the original T5 (i.e., not T5pF6) by growth of T5 in medium containing 30 μM uridine.

The cell lines were grown in either Dulbecco modified Eagle medium or Ham F12. Dialyzed fetal calf serum (with or without PALA) was used to establish selective conditions for the CAD gene. Nonselective conditions for CAD are defined as growth in medium plus regular fetal calf serum (which contains low levels of uridine) with or without the addition of exogenous uridine.

The following scenario describes mathematically how variability in cell populations can be achieved. Assume that there are 10^5 Y1 cells with a division time of 24 h and 1 Y2 cell with a 10% growth advantage (i.e., division time, 21.6 h). For the single Y2 cell to increase to 50% of the number of Y1 cells, it would take 156 days, calculated as follows:

$$Y2/Y1 = 1/2 = [1 \times e^{1.1\,(\ln 2)\,t}]/[10^5 \times e^{(\ln 2)\,t}]$$

and then solving for t, $1.1\,(\ln 2)\,t - (\ln 2)\,t = 5 \ln 10 - \ln 2$, hence $t \approx 156$ days.

Gel electrophoresis. Gel electrophoresis of whole cells lysed in situ (11, 12) or DNA isolated by alkaline lysis (16) was performed as previously described (7). Horizontal 0.8% agarose gels containing two parallel rows of wells for cell samples and lysing buffer were used. In brief, 10^6 cells or partially purified circular DNA molecules were loaded into the anode-proximal well of the gel in sample buffer containing RNase. For cell samples, lysis buffer containing sodium dodecyl sulfate (sds) and proteinase K was loaded into the cathode-proximal well. Electrophoresis was carried out at 0.8 V/cm for 3 h (to lyse the cells) and then at 5.2 V/cm for 6 h (to fractionate the DNA).

Restriction enzyme-digested DNA was fractionated on 0.7% agarose gels in TAE buffer at 35V for 16 h (29). Methods for Southern blotting, nick translation, and hybridization have been described previously (32).

Chromosome analysis. Metaphase chromosome spreads and in situ hybridization were performed as described previously (7, 52). To visualize DMS it was necessary to stain spreads with 4% Giemsa for 45 min as described by Howell et al. (20). For karyotype analysis, metaphase chromosomes were G banded by the methods of Worton et al. (53).

RESULTS

DMs can be generated from submicroscopic precursors. In model systems of gene amplification, DMs are typically detected in cells which have undergone multiple rounds of selection for resistance to increasing drug concentrations. Therefore, if CAD episomes are precursors of DMs, one would predict that the episomes should enlarge over time and after selection with PALA, a specific inhibitor of the CAD protein. Eventually, episomes should attain a size which can be detected microscopically (i.e., greater than 1,000 to 5,000 kb [18]). Using a gel electrophoresis assay, we have reproducibly observed this tendency for an increase in CAD episome size in PALA-resistant subclones derived from T5C5 (Fig. 2) (see Materials and Methods and Fig. 1 for a detailed description of cell lines).

The CAD episomes in an early passage of the PALA-resistant cell line C5R0.5 (C5 indicates that this subclone was derived from T5C5; R0.5 indicates that this clone is resistant to 0.5 mM PALA) migrated in essentially the same

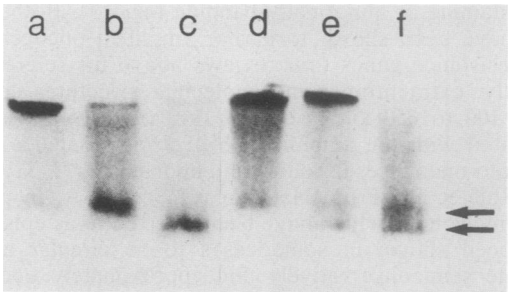

FIG. 2. Migration of extrachromosomal CAD genes in different PALA-resistant cell lines. The Southern blot was probed with the 8.6-kb, CAD specific *Bam*HI fragment 102 (37). The lower arrow indicates the position of migration of the originally described 250-kb CAD episome (7), and the upper arrow indicates the position of a more slowly migrating band of extrachromosomal CAD genes. Lanes: a, C5R2-long term (see Materials and Methods for description of all cell lines); b, C5R2; c, C5R0.5; d, equal mixture of C5R2-long term and C5R2; e, equal mixture of C5R2-long term and C5R0.5; f, equal mixture of C5R2 and C5R0.5.

fashion as those derived from the PALA-sensitive parent T5C5 (not shown). In both, the episomes were approximately 250 kb (Fig. 2, lane c). As described previously, each 250-kb episome consists of the donated tandem array of 5 CAD genes, and additional sequences, presumably derived from the host cell, which enable them to replicate autonomously (7). By contrast, the majority of CAD episomes in early passages of another PALA-resistant cell line, C5R2, migrated as if they were significantly larger than those in C5R0.5, whereas a minority of the CAD episomes in C5R2 were still about 250 kb (Fig. 2, lane b). The CAD episomes in C5R2 cells passaged for a long period (i.e., >6 months: referred to as C5R2-long term) displayed very heterogeneous migration and ranged in size from those initially found in C5R2 to molecules apparently too large to enter the gel under the electrophoretic conditions used (Fig. 2, lane a). When the gel samples contained the various cell lines mixed in equal cell numbers prior to lysis, the patterns seen after electrophoresis reflected the contributions from each independent cell line (Fig. 2, lanes d to f). This mixing experiment demonstrates that the migration differences in the CAD episomes found in these cell lines reflect real differences in molecular size or shape and are not due to lane-specific electrophoretic differences. It is noteworthy that there is hybridization in all lanes between the 250-kb region and the wells of the gel, and discrete bands above a diffuse background are occasionally present. The hybridization between the 250-kb band and the gel origin could reflect the presence of CAD episomes larger than 250 kb, catenanes, topologically different replication intermediates, or a combination of these structures. Hybridization at the well might reflect episomes which have integrated into chromosomes, episomes too large to enter the gel, or both.

DMs appeared in PALA-resistant derivatives of T5C5 coincident with the increases in CAD episome sizes described above. Analysis of 50 to 100 metaphase spreads of each cell line revealed DMs in the subclones resistant to PALA (Fig. 3C and D) and none in T5C5 (Fig. 3B). In addition, DMs were never observed in the Urd$^-$A cell line used for the initial transformation (Fig. 3A) or in T5S1-3 (not shown), a PALA-resistant T5 derivative in which the donated CAD genes are amplified intrachromosomally (51). The sizes of the DMs in individual cells in lines such as C5R0.5 and C5R2 also vary over a broad range Fig. 3C and D). The parallel increases in episome sizes measured by the gel electrophoresis assay, in the proportion of PALA-resistant cells containing DMs, and in the sizes of the individual DMs, lead us to conclude that the submicroscopic (250-kb) CAD episomes are precursors of microscopically visible DMs in these cell lines.

Chromosomally amplified CAD genes do not produce CAD episomes. Previous studies proposed that chromosomally amplified sequences may recombine to generate extrachromosomal sequences (3). We could test this possibility by using this system, since the PALA-resistant cell line T5S1-3 has a 50-fold chromosomal amplification of the donated CAD gene tandem array, and CAD episomes can be fractionated away from chromosomal DNA. In the experiment shown in Fig. 4, the yield of CAD episomes from 10^7 T5S1-3 cells was compared with that from 10^7 C5R0.5 cells, which contain approximately 50 CAD episomes per cell. No evidence of CAD episomes was found in T5S1-3 cells under conditions in which one CAD episome per cell in 1 to 10% of the population could have been detected. Since numerous experiments of this type have not revealed evidence of CAD episomes in T5S1-3, we conclude that CAD episome production from this chromosomally amplified region is either extremely rare or does not occur under the cell growth conditions used.

Formation of CAD episomes is associated with loss of the integrated sequences. Two other general models for episome formation can be envisioned. First, episomes could be formed by a process involving DNA re-replication within a single cell cycle followed by recombination or extrusion of circular molecules (42). Second, a recombination event which excises the donated array along with a replication origin could produce replication-competent circular molecules. A prediction of the latter model is that episome production should be associated with a deletion of the corresponding chromosomal sequences. Although maintenance of chromosomal sequences is predicted by the simplest form of the re-replication model, deletions may take place if recombination events occur in the appropriate DNA strands during overreplication (42).

Since there is a single site of integration in T5, we could determine whether episome production was associated with a loss of the integrated sequences. T5 cells containing an average of one or a few copies of the CAD episome were grown for several months under nonselective conditions (i.e., in medium containing uridine so that expression of the CAD gene is not required) to allow cells which had lost the episome to overgrow the population (7). These cells were then assessed for the presence or absence of the integrated CAD array.

In the first experiment, one colony emerging from nonselective growth conditions (designated T5LI-1) was isolated, grown into mass culture, and analyzed for CAD sequences by Southern blotting and for the presence of CAD episomes by the in situ cell lysis-gel electrophoresis method described previously (7). T5LI-1 contained neither CAD episomes (data not shown) nor the chromosomally integrated donated CAD sequences (Fig. 5B). Probes spanning the length of the CAD gene were hybridized to the same blot and failed to reveal bands corresponding to the donated sequences, although fragments corresponding to the single-copy endogenous CHO CAD genes were readily apparent (Fig. 5B and data not shown).

In a second experiment, a different population of T5 cells (referred to as T5pF6) containing an average of one CAD episome per cell was also grown under nonselective conditions for 1 to 2 months. The population which emerged is referred to as T5pLI-2. As with T5LI-1, no CAD episomes were detected in T5pLI-2 (data not shown). Total cellular DNA from this population of cells also contained no detectable donated Syrian hamster CAD sequences (Fig. 5A, compare lanes 1 and 2). T5pLI-2 was subsequently grown in the absence of uridine (i.e., selective conditions for the presence of functional CAD genes) to determine whether any of the cells in the population still contained functional donated CAD genes. None of 10^3 plated cells survived this selection. From both of these experiments, we infer that the vast majority of the population which had lost the episome under nonselective growth conditions also did not contain any of the chromosomally integrated sequences. Our inability to detect any residual chromosomally located CAD sequence in T5LI-1 and T5pLI-2 suggests that the deletion event actually occurred in the flanking DNA. This is consistent with our previous conclusion that the CAD episome contains host sequences which direct its replication (7).

The data described above lead us to propose that a recombination event removed the CAD sequences from the chromosomal insertion site in T5, thereby generating the

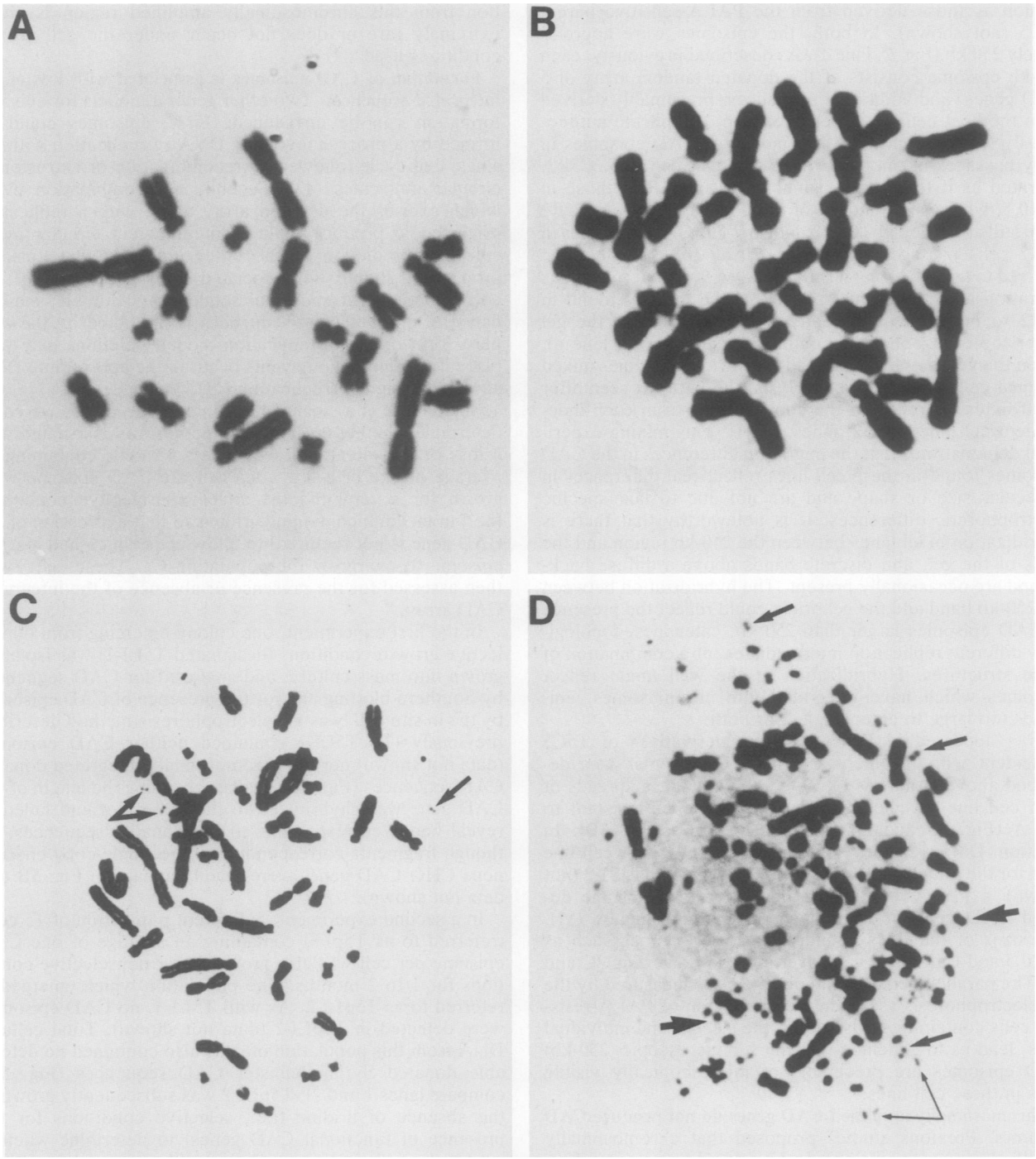

FIG. 3. Presence of DMs in PALA-resistant cell lines containing extrachromosomal CAD genes. (A) Urd$^-$A (38); (B) T5C5 (7); (C) C5R0.5; (D) C5R2. See Fig. 1 and Materials and Methods for a complete description of all cell lines. Small, medium, and large arrows indicate examples of typically observed small, medium, and large DMs. DM chromosomes were observed in approximately 25% of the spreads of C5R0.5 and 35% of the spreads of C5R2. These two cell lines are tetraploid, in contrast to the diploid complement of chromosomes found in UrdA$^-$ and T5 (in addition, see Fig. 6). Spreads (50 to 100 per cell line) were scored positive for DMs only if they contained DMs as obvious as those shown in panels C and D.

CAD episome. However, they are also consistent with the alternative interpretation that growth of T5 cells under nonselective conditions results in the loss of the chromosome containing the inserted sequences. Therefore, we analyzed the chromosome composition of the cell lines which had lost the CAD episome to determine whether they had also undergone a selective loss of the single metacentric chromosome, [t(z3:6)] (D. Needham-Vandevanter and C. Moore, unpublished data), which originally contained the donated CAD genes in T5 (51). Karyotypic analysis of T5pF6, T5LI-1, and T5pLI-2 by G banding shows that T5pF6 and T5pLI-2, which are directly related to one

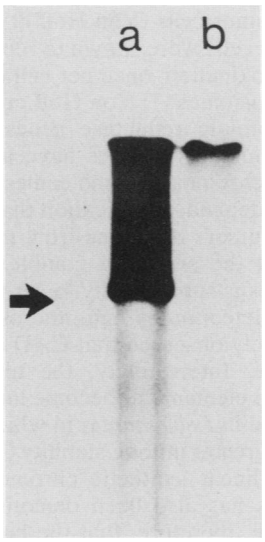

FIG. 4. Lack of extrachromosomal CAD molecules in a cell line containing an intrachromosomal amplification of the donated CAD sequences. Extrachromosomal circular CAD molecules were isolated by the alkaline lysis procedure as described previously (7). The yields from 10^7 cells of C5R0.5 (lane a) and T5S1-3 (lane b), (51) were electrophoresed as described in Materials and Methods, and the Southern blot was hybridized to fragment 102 (37). The arrow indicates the position of the ca.250-kb CAD episome. Although an overexposure of the autoradiogram is shown, no episomal material is visible in T5S1-3.

another, have identical karyotypes, with a modal number of 20 chromosomes (Fig. 6; the chromosomes are ordered according to the nomenclature used in reference 53). T5LI-1 differs from T5pF6 and T5pLI-2 in that it has a modal number of 21 chromosomes, resulting from the presence of an additional copy of CHO chromosome 6. This is not unexpected, since it was derived from a different population of T5 cells, and CHO cells are known to exhibit fluctuations in chromosome number (15). These results indicate that the loss of CAD sequences in T5LI-1 and T5pLI-2 following growth of their respective parental populations under unselective conditions did not occur by preferential loss of [t(z3:6)] or any other single chromosome. Analyses of banded karyotypes also failed to reveal evidence of large chromosomal rearrangements (M. DeRose and G. Wahl, unpublished data). Taken together, these observations are consistent with the proposal that a submicroscopic chromosomal deletion generated the CAD episome in these T5-derived cell lines.

CAD episomes can integrate into various chromosomal locations. In one cell population derived from T5pF6 after growth under nonselective conditions, we observed considerable CAD-specific hybridization to high-molecular-weight DNA, but consistently failed to observe CAD episomes or DMs (data not shown). This suggested that episomes originally present in T5pF6 cells might have integrated into a chromosome(s) in one or more cells which then became the dominant members of the population. In support of this idea, in situ hybridization to metaphase spreads of this population (referred to as T5pF6′; Fig. 7A and B) revealed CAD-specific hybridization near or at the telomere of one medium-sized subtelocentric chromosome. This chromosome is clearly different from metacentric chromosome [t(z3:6)], which harbored the original site of CAD gene integration in T5 (C. Moore and D. Needham-Vandevanter, unpublished data). It is highly unlikely that the subtelocentric chromosome containing the new integration site represents a rearrangement of chromosome [t(z3:6)], since an apparently normal [t(z3:6)] is also present in all T5pF6′ cells examined (data not shown).

We obtained additional evidence for a reintegration event by analyzing the restriction pattern of the CAD genes in T5pF6′. Total genomic DNA from T5pF6′ was restricted with *Sst*I and hybridized with nick-translated cCAD1 (the cosmid used to generate T5 [51]) from which repeated sequences had been depleted (Fig. 7C, lane 2). One novel band detected with this probe derives from a 10-kb region near the 5′ end of the CAD gene (Fig. 7C, lane 5; defined by clone λ200 [37]). This novel fragment is amplified in three PALA-resistant derivatives of T5pF6′ (data not shown). We infer that the new band represents a junction between host sequences and CAD episome sequences. Although integration is expected to produce at least two novel junctions, this fragment was the only new one observed after hybridization with the entire CAD cosmid or subclones derived from it (Fig. 7C; data not shown). Other novel junctions were probably obscured by the high background created by the repeated sequences present in probes from this region of the CAD gene (37). The CAD sequences in T5pF6′ are clearly derived from CAD episome sequences, since they contain the novel fragments J1 and J2, which result from rearrangements in the CAD cosmid in the original T5 clone and which serve as molecular fingerprints for T5-related lines (7). Interestingly, CAD gene amplification does not occur at a high frequency in T5pF6′, suggesting that some feature of

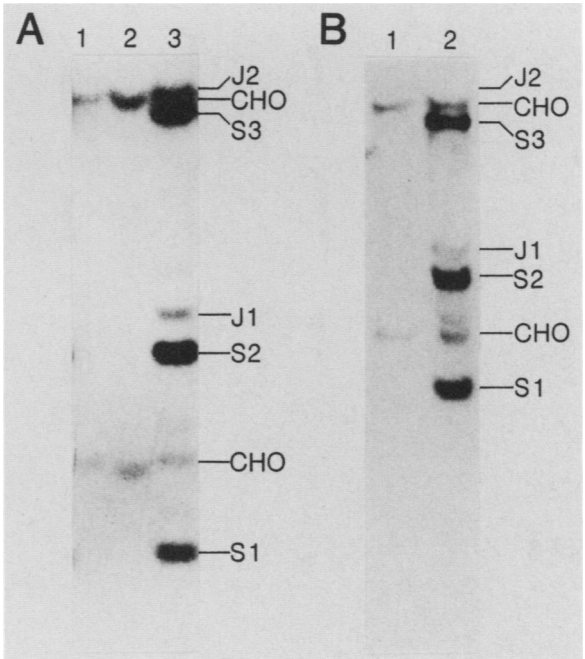

FIG. 5. Restriction analysis of genomic DNA from T5-derived cell lines which have lost the CAD episome. Cell lines T5LI-1 and T5pLI-2 were derived, respectively, from T5 and T5pF6 by growth under nonselective conditions as described in Materials and Methods. Approximately 10 μg of genomic DNA was digested with *Sst*I and fractionated on a 0.7% agarose gel, and the Southern blot was hybridized to 102 fragment (37). The fragments indicated on the right of the autoradiograms are derived from the donated Syrian hamster CAD genes (S1, S2, S3, J1, and J2) or from the endogenous CHO CAD genes (CHO1 and CHO2) (7, 51). Panels A and B are autoradiograms from different gels. (A) Lanes: 1, Urd$^-$A; 2, T5pLI-2; 3, T5C5. (B) Lanes: 1, T5LI-1; 2, T5pF6.

the new insertion site or arrangement of the integrated sequences suppresses their ability to amplify at high frequency. The intensity of hybridization of the CAD sequences is substantially higher in unamplified T5pF6′ cells than in T5pF6 (standardized against the endogenous CHO CAD genes), indicating that either more than one copy of the CAD episome or a CAD episome multimer integrated in T5pF6′.

DISCUSSION

In this report, we show that DMs can be generated from autonomously replicating submicroscopic precursors. We refer loosely to the precursors as episomes (6), since they originate from a chromosomal site, can replicate as autonomous elements, and, as shown here, can integrate into new chromosomal sites. We also show that the cells which contain episomes have lost the corresponding chromosomal sequences. This has led us to propose that in some cases an early step in gene amplification is a recombination event which deletes a chromosomal region containing a replication origin along with adjacent genes which confer a selective growth advantage. Increases in gene copy number can then occur rapidly as the acentric elements partition unequally at mitosis.

The generality and importance of episomes as intermediates in eucaryotic gene amplification are substantiated by data obtained with other systems. For example, circular 120- to 250-kb elements containing c-*myc* proto-oncogenes have been detected in some early passages of human HL60 promyelocytic leukemia cells and in subclones of C0L0320 neuroendocrine tumor cells (Von Hoff et al., in press). The episomes in HL60 cells were shown to replicate semiconservatively and approximately once per cell cycle, as has been shown for CAD episomes (7; Von Hoff et al., in press). The amplified dihydrofolate reductase genes in some methotrexate-resistant human cell lines have also recently been localized to extrachromosomal molecules of approximately 650 kb (31). An independent indication that DMs are formed from smaller precursors has come from molecular analyses which reveal that the sequence complexity of DMs in a variety of cell lines is substantially lower than the minimum size required for detection by light microscopy (24, 25).

We have routinely observed that CAD episomes increase in size over time. Interestingly, the tendency for small extrachromosomal elements to become larger over time has also been observed in *Leishmania*, in which larger elements appear to display greater mitotic stability (13). The increased stability of large linear synthetic chromosomes compared with smaller ones has also been demonstrated for yeasts (35). We speculate, therefore, that the heterogeneous sizes of DMs often observed in mammalian cells (Fig. 3) (18, 48) may be due to the formation of DMs from episome precursors and that the eventual dominance of DMs is due to an increased mitotic stability of larger extrachromosomal elements.

Although the episome or its larger derivatives can be maintained extrachromosomally for an undefined number of cell doublings, in one instance we have observed that some extrachromosomal elements integrated into a chromosome. This apparently resulted in a growth advantage for this cell,

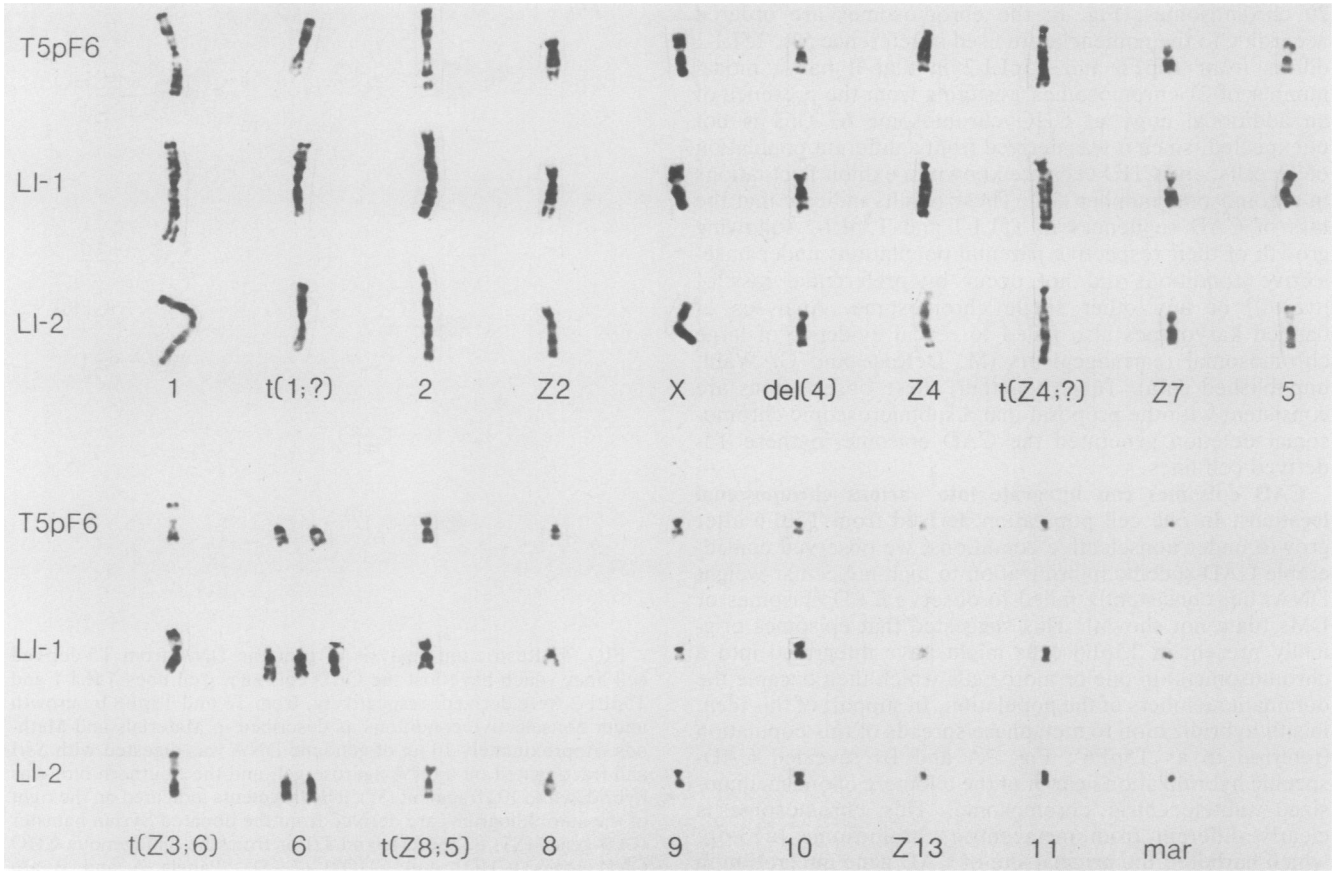

FIG. 6. G-banded karyotypes of T5pF6, T5LI-1, and T5pLI-2. Twenty karyotypes per cell line were examined. The chromosomes are named by the system of Worton et al. (53). mar: Marker chromosome. Chromosome [t(z3;6)] is the site of the original integration in T5.

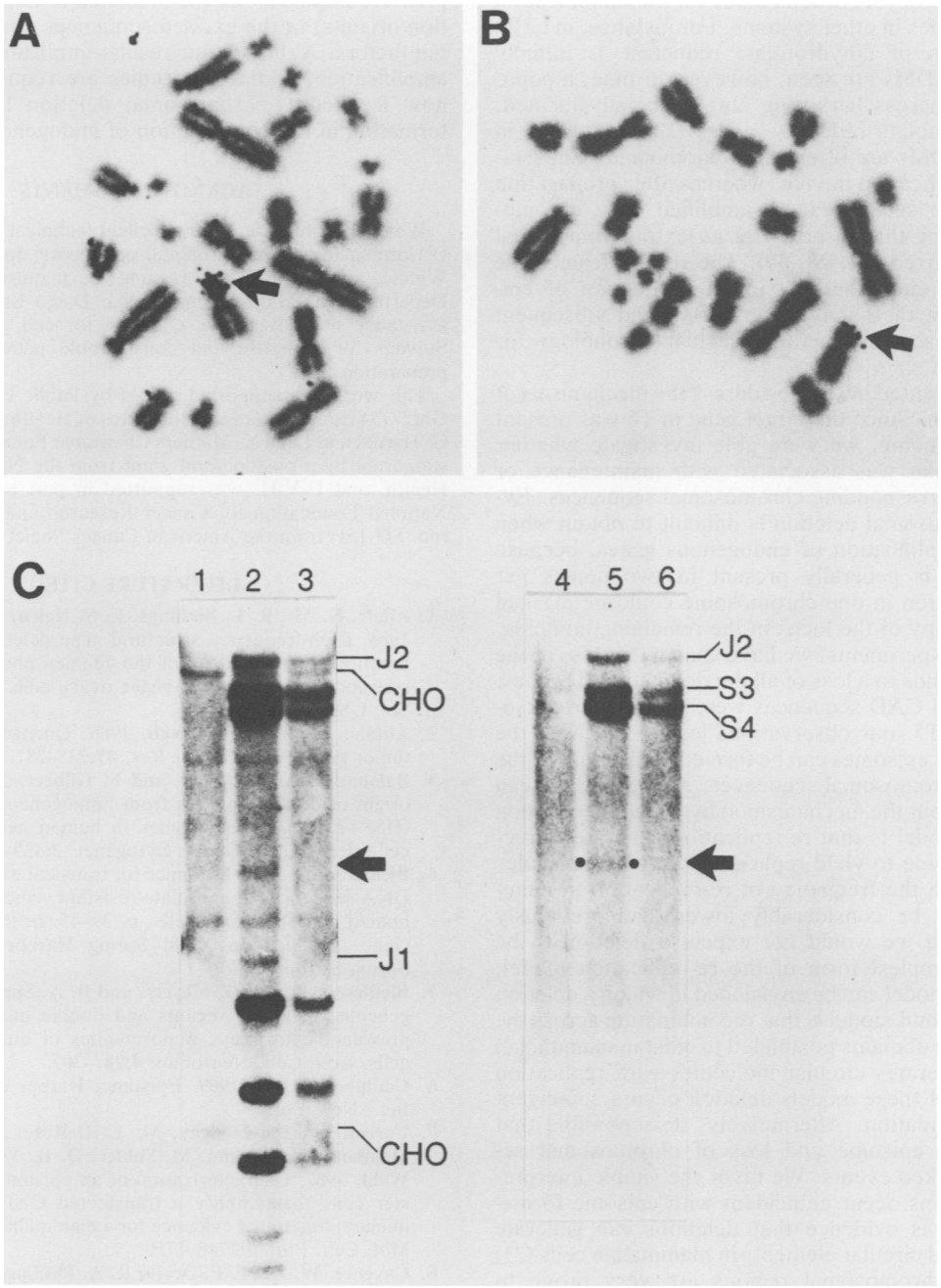

FIG. 7. Reintegration of the CAD episome. (A and B) Metaphase chromosome spreads of T5pF6′ were hybridized with ^{3}H-labeled 102 fragment. Exposure times were 2 to 3 weeks at −70°C. Arrows indicate sites of hybridization. Panels A and B are two different spreads of T5pF6′. (C). Genomic DNA (10 μg) from T5pF6′, Urd^{-}A, and T5C5 was digested with *Sst*I, and the Southern blot was hybridized with cCAD1, from which most repetitive sequences had been removed (lanes 1 to 3). The same blot was washed free of probe and was reprobed with λ200 (37) from which most repetitive elements had been removed (lanes 4 to 6). The arrow indicates the position of the novel fragment in T5pF6′. Lanes: 1 and 4, UrdA^{-}; 2 and 5, T5pF6′; 3 and 6, T5C5.

which allowed its progeny to eventually overgrow the population (see Materials and Methods). A model for amplification involving integration of elements has previously been proposed by Biedler (4). Although integration is likely to occur by nonhomologous recombination in most cases, the recent observations that the frequency of homologous insertion is dramatically increased with increasing lengths of homology and is not diminished by the presence of repetitive sequences (47) suggest that episomes containing more than 100 kb of homology could also integrate at the native locus. Nonhomologous integration of extrachromosomal elements could generate the complex patterns of gene amplification which have been observed in many cell lines (see, e.g., reference 26), whereas homologous integration could give the appearance of amplification at or near the site of the native gene.

Our observations concerning the amplification of a transfected gene parallel observations made on the amplification of endogenous genes in other systems. For instance, in CHO cells amplification of dihydrofolate reductase is initially unstable and few DMs are seen; however, in time, a population of cells emerges harboring chromosomally located, amplified dihydrofolate reductase genes (22). Likewise, in tumor cell lines DMs are often the predominant manifestation of gene amplification in vivo, whereas after propagation in vitro the same sequences are amplified on a chromosome(s), suggesting that insertion of an extrachromosomal element has occurred (14, 28, 39). The results from these diverse systems imply that the initial production of episomes, followed by their conversion to DMs and subsequent integration, represent a general molecular chronology for gene amplification.

The results presented here also address the mechanisms of episome formation. Since the target gene in T5 was present only once per genome, we were able investigate whether episome production was associated with maintenance or deletion of the corresponding chromosomal sequences. Evidence for chromosomal deletion is difficult to obtain when analyzing the amplification of endogenous genes, because the target locus is generally present in two copies per genome and deletion in one chromosome could be masked by an identical copy of the locus in the remaining homolog. In two different experiments, we have shown that loss of the episome corresponds to a loss of all the donated CAD genes. Since the donated CAD sequences were intially chromosomally located in T5, our observations lead us to favor the interpretation that episomes can be formed by deletion of the corresponding chromosomal sequences. At present, we can only speculate about the mechanism(s) by which the deletion occurred. One model is that re-replication generates structures that recombine to yield replicating circular molecules (30, 42). Although the frequency of re-replication in mammalian cells may be considerably lower than previously reported (17), and we would not expect a deletion to be created in the simplest form of the re-replication model, variations of the model can be envisioned in which a deletion does occur. A second model is that recombination across the looped replication domains postulated to exist in mammalian cells (33, 50) generates circular molecules with replication origins. In both of these models deletion occurs coincident with episome formation. Alternatively, it is possible that formation of the episome and loss of chromosomal sequences are unlinked events. We favor the simple interpretation that deletions occur coincident with episome formation, since there is evidence that deletions can generate extrachromosomal circular elements in mammalian cells (21) and that some chromosomal regions are very prone to deletion (1, 44).

The potential involvement of deletions in the formation of amplified sequences is intriguing given the frequent occurrence of large deletions in mammalian cells. For instance, recombination events in the Duchenne muscular dystrophy gene occur frequently, and some take place between sequences separated by 100 to 300 kb (10). Intrachromosomal nonhomologous recombination can also generate large (ca. 90-kb) deletions, resulting in thalassemia (49), and interstitial deletion is a common mechanism for the loss of tumor suppressor genes associated with malignant transformation (see, e.g., references 8 and 27). These observations encourage the speculation that similar mechanisms could generate deletions leading to allele loss and decreased function on the one hand or gene amplification and overexpression on the other. The outcome might be determined by the selective pressure imposed and the presence or absence of a replication origin(s) in the excised sequences, among other potential factors. Although our results implicate deletion in gene amplification, additional studies are required to determine how frequently chromosomal deletion leads to episome formation in the amplification of endogenous genes.

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