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Characterization of an Episome Produced in Hamster Cells That Amplify a Transfected CAD Gene at High Frequency: Functional Evidence for a Mammalian Replication Origin

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In a previous study (G. M. Wahl, B. Robert de Saint Vincent, and M. L. De Rose, Nature (London) 307:516–520, 1984), we used gene transfer of a CAD cosmid to demonstrate that gene position profoundly affects amplification frequency. One transformant, T5, amplified the donated CAD genes at a frequency at least 100-fold higher than did the other transformants analyzed. The CAD genes in T5 and two drug-resistant derivatives were chromosomally located. In this report, we show that a subclone of T5 gives rise to an extrachromosomal molecule (CAD episome) containing the donated CAD genes. Gel electrophoresis indicated that the CAD episome is approximately 250 to 300 kilobase pairs, and a variety of methods showed that it is a covalently closed circle. We show that the CAD episome replicates semiconservatively and approximately once per cell cycle. Since the CAD cosmid, which comprises most of the CAD episome, does not replicate autonomously when transfected into cells, our results indicate that either (i) the process which generated the episome resulted in a cellular origin of DNA replication being linked to the CAD sequences or (ii) specific rearrangements within the episome generated a functional origin. The implications of these results for mechanisms of gene amplification and the genesis of minute chromosomes are discussed.

One mechanism by which a cell can accumulate large amounts of a specific protein or RNA is by amplification of the corresponding gene (for a review, see reference 47). Amplified genes are located either within expanded chromosomal regions (referred to as homogeneously staining or abnormally banding regions) or on extrachromosomal autonomously replicating elements (called minute or double minute chromosomes; 13) Molecular and cytogenetic analyses of many amplified regions in mammalian tumor cells have demonstrated that the sizes of amplified regions vary but are generally larger than the target genes and are often more than 200 kilobase pairs (kbp). One explanation for the presence of large and variable amounts of flanking sequence in amplified regions is that amplification is mediated by specific *cis*-acting elements present in the flanking sequences. For example, it has been proposed that amplification occurs in units of replication, in which case each amplification unit should contain an origin of DNA replication (10, 25) and any other sequences required for replication. Therefore, the repeated unit found within the amplified array may actually represent the chromosomal "domain" under the control of such *cis*-acting elements.

We are interested in identifying and isolating *cis*-acting chromosomal elements which are involved in the process of gene amplification in mammalian cells. The study of gene amplification in procaryotes and *Drosophila melanogaster* suggests at least two classes of *cis*-acting sequences which should be directly involved in the amplification process. First, in bacteria, it has been demonstrated that homologous unequal exchanges between repetitive sequences such as rRNA genes can generate duplicated or amplified units at a high frequency (2). Interestingly, in some Syrian hamster mutants selected for CAD gene amplification, amplification of the flanking rRNA genes has been observed (41). These results are consistent with, but do not prove, the hypothesis that gene amplification can be mediated by unequal exchange between repetitive elements flanking the target CAD gene. In cases in which mitotic recombination involving unequal sister chromatid exchange mediates amplification, recombination hot spots could be important *cis*-acting sequences. A variant of mitotic recombination models may be envisioned in which replication intermediates are recombined to form amplified regions. A second, strikingly different model for gene amplification invokes multiple rounds of DNA replication in one cell cycle to generate the extra gene copies (43, 48). Subsequently, the rereplicated units are recombined into an intra- or extrachromosomal array of amplified sequences. The absolute requirement for DNA synthesis in this second model and potential involvement of replication intermediates in the first model implies that a second type of *cis*-acting sequence, namely, an origin of DNA replication, should be present within each unit of amplification. Experimental evidence for the involvement of DNA replication origins in amplification has been obtained in *D. melanogaster*, in which amplification of the chorion genes and approximately 100 kbp of flanking sequences occurs because of replication of this region. Replication is initiated at an origin immediately upstream of the chorion gene cluster in response to *trans*-acting developmental signals (15, 45, 46). There is also some indication that replication is involved in gene amplification in mammalian cells. For instance, amplification in situ of integrated polyomavirus or simian virus 40 requires a functional viral origin of replication and is dependent on the presence of the respective large T antigen from each virus (31, 37). In addition, in Chinese hamster ovary (CHO) cells containing a chromosomally amplified dihydrofolate reductase gene, there is indirect evidence suggesting that replication is initiated at a

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sequence approximately 14 kbp from the 3′ end of the dihydrofolate reductase gene in each amplified unit (10, 25).

To identify chromosomal regions which may initiate or resolve the amplification process, we previously introduced the CAD gene into random genomic locations by gene transfer (49; CAD is an acronym for the multifunctional protein containing carbamylphosphate synthetase, aspartate transcarbamylase, and dihydroorotase). We reasoned that insertion of a donated gene close to elements which initiate or resolve the amplification process would allow the inserted gene to be amplified at a frequency higher than if it were located far away from such elements. One transformant exhibited a high frequency of amplification of the transfected gene. It was speculated that the donated gene had inserted into or near a region controlling replication of the adjacent area or that insertion destabilized that region.

This report extends the analysis of the hyperamplifiable transformant described previously and demonstrates that some subclones contain an extrachromosomal element, composed mainly of sequences derived from the tandem array of donated CAD genes present in the original transformant. Like a minute chromosome, this element is circular and replicates autonomously, semiconservatively, and with kinetics which parallel the replication of chromosomal genes. Thus, it appears that this molecule contains a functional origin of DNA replication. In contrast to other minute chromosomes, this molecule is small (approximately 250 kbp) and experimentally manipulable by physical and molecular techniques. It therefore provides an excellent substrate from which to isolate the putative replication origin.

MATERIALS AND METHODS

Cell culture. The isolation of cell lines T5, T5S13, T1, and T6 was described previously (49). T5C5 was isolated as a single-cell clone from T5 by limiting dilution in 96-well tissue culture dishes. C5R500 was selected as an *N*-phosphonacetyl-L-aspartate (PALA)-resistant colony from T5C5 by seeding at 10^3 cells per 10-cm (diameter) plate followed by selection in 500 μM PALA. T5C5, T1, and T6 were routinely passaged in Hams F12 supplemented with 10% regular fetal calf serum. T5S13 was propagated in Hams F12 supplemented with 10% dialyzed fetal calf serum and 200 μM PALA. C5R500 was maintained in Dulbecco modified Eagle medium supplemented with 10% dialyzed fetal calf serum, nonessential amino acids, and 500 μM PALA. Raji cells were kindly provided by Carol Mulder (University of Massachusetts, Worcester) and grown in RPMI containing 10% regular fetal calf serum.

Preparation of metaphase chromosome spreads and in situ hybridization. Metaphase chromosome spreads were prepared essentially as previously described (50), with the following minor modifications. After mitotic shake off, the cells were pelleted for 10 min at 1,000 × *g*. The first fixation step in Carnot fixative (3:1 methanol-glacial acetic acid) was for 30 to 60 min. After pelleting, cells were fixed one additional time for 15 min. The cell suspension was dropped onto glass slides subbed with Denhardt reagent (9) and suspended above a 37°C water bath.

In situ hybridizations were performed as previously described (50) except that the nick-translated probe was labeled with [^{3}H]thymidine to a specific activity of 10^8 cpm/μg. Approximately 5×10^5 cpm of probe was used per slide. The probe and herring sperm DNA carrier were denatured before use by incubation in 0.025 N NaOH at 37°C for 30 min; subsequently, HCl was added to 0.025 N, and the solution was added to the hybridization mixture. Approximately 25 μl of hybridization mixture was deposited onto each slide and covered with a cover slip. The slides were placed between layers of paper towels soaked in mineral oil to prevent dehydration and incubated at 42°C overnight. The cover slips were removed by immersing the slides in two changes of chloroform. The slides were then washed, dipped in 0.1% gelatin–0.1% chromium potassium sulfate–0.3 M ammonium acetate, covered with Kodak NTB-2 emulsion, exposed, developed, and stained exactly as previously described (50). Chromosomes were observed with a standard Zeiss microscope equipped with an 80× objective and photographed under a bright field with a Nikon FX35A camera.

Isolation of circular DNA by alkaline lysis. Large-scale isolation of circular DNA from CHO-derived cell lines and Raji cells was performed as described by Griffin et al. (22). We also modified the procedure to accomodate small samples of cells. For instance, 10^6 cells were lysed in a microfuge tube with 100 μl of lysis buffer (50 mM NaCl, 2 mM EDTA, 1% sodium dodecyl sulfate [SDS], pH 12.45). The lysate was then vortexed for 2 min at the highest speed and incubated at 30°C for 30 min. The solution was neutralized by adding 0.2 volume of 1 M Tris hydrochloride, pH 7, to obtain a final pH of approximately 8.5. Additions of 5 M NaCl (0.11 volume) and 10 mg of proteinase K per ml (0.01 volume) were as previously described (22). Phase separation after extractions with phenol and chloroform was achieved by centrifugation at 5,000 rpm in a refrigerated microcentrifuge. To ensure precipitation of small amounts of circular DNA, glycogen carrier (approximately 25 μg) was added before addition of 2 volumes of absolute ethanol. Recovery of the CAD episome by this procedure can range from approximately 10 to 80% of the estimated amount in the cells.

To prepare alkaline lysate DNA for cleavage by restriction enzymes, the ethanol-precipitated sample was suspended in 10 mM Tris hydrochloride–0.1 mM EDTA, pH 7 (TE), containing 100 μg of DNase-free RNase per ml and incubated at 37°C for 30 min. The sample was extracted once with phenol-chloroform and once with chloroform and then ethanol precipitated in the presence of 0.3 M sodium acetate. The final precipitate was dissolved in TE.

Cell lysis and gel electrophoresis. Preparation of whole-cell samples and gel electrophoresis were performed as described by Eckhardt (16) and Gardella et al. (18). Briefly, cell monolayers were gently trypsinized, and 2.5×10^5 to 10^6 cells or an alkaline lysate preparation from 10^6 cells was suspended in sample buffer containing 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA at pH 8.3 (TBE), 15% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), 0.01% bromophenol blue, and 3 mg of DNase-free RNase (Worthington Diagnostics, Freehold, N.J.) per ml added immediately before use. The comb used to form sample slots consisted of two rows of teeth, and the rows were separated by 0.3 cm. Each sample slot held 0.1 ml, and the gel box was approximately 21 cm long by 14 cm wide. Lysis buffer, consisting of 5% Ficoll, 1% SDS, 0.05% xylene cyanol green, and 1 mg of proteinase K per ml (added immediately before use) in TBE, was loaded into the wells closest to the cathode (i.e., the upper wells). The gels were 0.8% agarose dissolved in TBE. The samples were loaded into the second row of wells. Gels were run at 4°C for 3 h at 0.8 V/cm to allow the lysis buffer to migrate into the sample and then increased to 5.2 V/cm for an additional 6 to 8 h. In our experience, the most reproducible results were obtained with freshly prepared sample buffer and lysis buffer. Gels

were transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) and hybridized to nick-translated probes as previously described (33).

BrdU labeling, DNA preparation, and CsCl density gradients. Monolayers of exponentially growing log-phase cells were labeled with 5-bromodeoxyuridine (BrdU) as described by Mariani and Schimke (32). Cells were incubated for the indicated number of cell generations (determined by cell counting) in 30 μM BrdU (the same results were also obtained with 3 μM BrdU, which was far less toxic to the cells). Plates were rinsed and trypsinized, and the cells were pelleted by centrifugation at 1,000 × *g* for 3 min. The pellet was suspended in TE containing 0.1 mg of RNase per ml and incubated at 37°C for 5 min. Cells were lysed in the presence of 0.2 M Tris (pH 7.4)–50 mM EDTA–0.5% SDS and incubated with proteinase K (0.1 mg/ml) at 50°C for 60 min. The mixture was extracted once with phenol-chloroform and once with chloroform and then precipitated in the presence of salt with absolute ethanol. The precipitated DNA was dissolved in TE and sheared by passage five times through a 22-gauge needle. CsCl was then added to a density of 1.75 g/ml in TE to yield a single 5-ml gradient per plate. The gradients were run for 16 h at 45,000 rpm in a Beckman VTi 65 rotor. Twenty-four 200-μl fractions were collected, and the refractive index of every other fraction was checked. A 50-μl portion of each sample was denatured in 0.3 N NaOH at 65°C for 15 min, neutralized in 1 M ammonium acetate, and blotted to nitrocellulose (Schleicher & Schuell) in a dot blot apparatus. The nitrocellulose was hybridized as previously described (33), and the dots were punched out and counted in 2 ml of CytoScint (West Chem, San Diego, Calif.).

Replication assays. Replication of cosmid cCAD1, which contains the entire CAD gene (40), was assayed in a system similar to that described by Peden et al. (36). As a positive control for replication, pLT-1, a plasmid containing a modified polyomavirus genome (51), was included. CAD-deficient CHO cells (35) were transfected by calcium phosphate coprecipitation (21) with 500 ng of cosmid or plasmid per 10^6 cells. On various days after transfection, cells were harvested by trypsinization, and circular DNA was isolated by the alkaline lysis procedure. The DNA from one plate was digested with 10 Units of *Eco*RI and 3 Units of either *Dpn*I or *Mbo*I for 4 h at 37°C. To ensure that *Dpn*I digestion was complete, 100 pg of cloned methylated pUC13 was added to each digest. Absence of the 2.7-kbp linearized pUC13 molecule on Southern blots probed with nick-translated plasmid indicated complete digestion by *Dpn*I. The cleavage products were then fractionated on a 0.7% agarose gel, transferred to nitrocellulose (Schleicher & Schuell), and hybridized to nick-translated probe (33).

RESULTS

A clonal cell line stability transformed with CAD gives rise to progeny containing the CAD gene located on an extrachromosomal element. Results previously described (49) showed that gene position can significantly affect the frequency at which a transfected CAD gene is amplified. In these studies, CAD-deficient CHO cells (35) were transfected with a cosmid containing the CAD gene isolated from a Syrian hamster cell line (40). Transformants able to grow as uridine prototrophs were subsequently screened for their ability to resist PALA, an inhibitor of aspartate transcarbamylase (12). All resistant lines contained amplified transfected CAD genes, and one of them, T5, exhibited a 100- to 1,000-fold increased frequency of amplification of the transfected genes compared with the other transformants. Analysis of six PALA-resistant clones derived from T5 within a few weeks after the initial gene transfer experiment demonstrated that each clone contained the amplified copies of the CAD gene at the same chromosomal site. These results led to the conclusion that amplification occurred at or near the site of integration in this transformant.

Recently, the original T5 cell line was subcloned from single cells for genetic analyses. PALA-resistant clones were then selected from one particular subclone, T5C5. The parental subclone and one clone resistant to 500 μM PALA, C5R500, were analyzed by in situ hybridization with a CAD-specific probe. The amplified copies of the CAD gene in cell line C5R500 could not be localized to a single chromosome, and most of the grains of hybridization occurred in the spaces between chromosomes (Fig. 1B). In addition, the parental subclone, T5C5, also failed to show a chromosomal location and exhibited a much lower level of hybridization compared with that of C5R500 (Fig. 1A). This result is in contrast to the initial isolate of T5, in which the CAD gene was stably integrated and amplified intrachromosomally (Fig. 1C) and indicates that the single-cell clone, T5C5, and its amplified derivative, C5R500, contain the donated CAD genes as extrachromosomal elements. These extrachromosomal elements will subsequently be referred to as CAD episomes.

Detection of CAD episomes by gel electrophoresis. Although the CAD episome was easily observed by in situ hybridization, minute chromosomes were not observed in Giemsa-stained metaphase spreads of T5C5 or C5R500. This observation indicates that the size of the CAD episome in these cells is below the limit of detection (approximately 1,000 to 5,000 kpb) of the light microscope. To analyze the episome in greater detail, we used a gel system developed to analyze large bacterial plasmids (16) and the episomal form of Epstein-Barr virus (EBV; 18). The key feature of this method is that whole cells are gently lysed in the well of an agarose gel by migration of SDS and protease into the well at low voltage. The voltage is then increased, causing migration of circular DNA into the gel. The position of migration is approximately correlated with the logarithm of the molecular weight of covalently closed circular molecules (11). The majority of intact chromosomal DNA is retained at the well, and linear fragments of less than 164 kbp migrate near the xylene cynanol green dye front. Various subclones derived from T5 contained CAD sequences which migrated at the position of a covalently closed circular molecule (Fig. 2). T5C5 (lane b) contained a single discrete band migrating slightly higher than the 178-kbp EBV episome derived from Raji cells (lane a) and faster than the 436-kbp megaplasmid present in *Agrobacterium tumefaciens* C58 (data not shown). The same CAD-specific band was also present in C5R500 (lane c), with greater hybridization intensity corresponding to the increase in CAD gene copy number in this PALA-resistant line. In contrast, no hybridization was apparent in this region of the gel in T5S13 (lane d), a PALA-resistant mutant of the original T5 clone which contains intrachromosomally amplified CAD genes (49). Two other transformants, T6 and T1, (lanes e and f) isolated at the same time as T5 but not exhibiting the high-frequency amplification phenotype, also did not contain detectable levels of CAD sequences which migrate as circular molecules in this gel system. In addition, none of 20 recently isolated CAD transformants contained a CAD episome (P. Gaudray, J. Emery, and G. Wahl, unpublished data). Thus, production

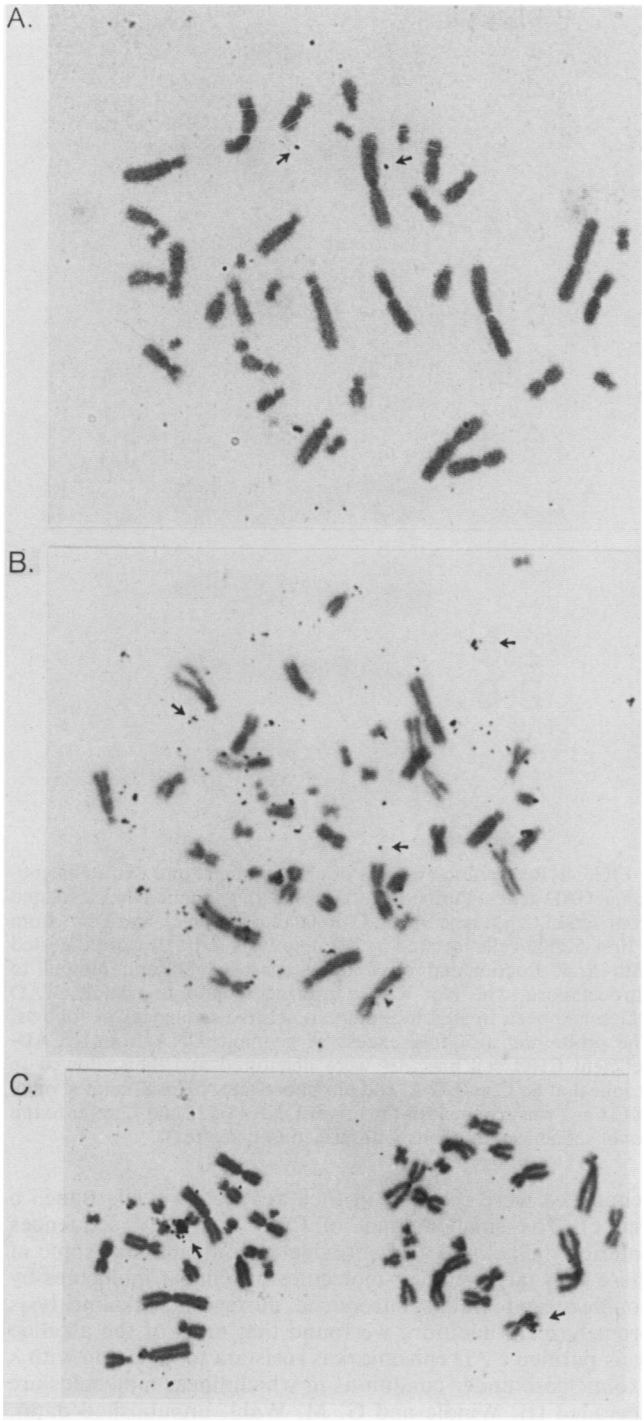

FIG. 1. Localization of the donated CAD genes in cell lines derived from T5. Metaphase spreads of cell lines T5C5, a single cell clone derived from T5 (panel A); C5R500, a PALA-resistant line derived from T5C5 (panel B); and T5-1, a PALA-resistant line derived from the initial isolate of T5 (panel C), were hybridized in situ with labeled 102 fragment as described in Materials and Methods. Panel C shows two individual chromosome spreads. Exposure times were 2 to 3 weeks at −70°C. Arrows indicate sites of hybridization.

of the CAD episome is specific for transformant T5 and only some of its subclones.

Characterization of the structure and organization of the CAD episome. The electrophoretic behavior of the CAD episome suggested that it was a covalently closed circular molecule. We applied several other analytical methods to support this conclusion. First, it was determined whether the CAD sequences in C5R500 could be isolated by an alkaline lysis procedure developed to purify the circular form of EBV away from chromosomal DNA (22). In this procedure, cells are lysed at high pH, resulting in denaturation of linear duplex DNA while the strands of covalently closed circular molecules remain intertwined. Upon neutralization and phenol extraction at high salt concentration, the single-stranded DNA which results from denaturation of duplex DNA partitions at the interphase, while covalently closed circular DNA remains in the aqueous phase. The CAD sequences could be recovered almost quantitatively from C5R500 by the alkaline lysis procedure although nicking and shearing could cause the yield to vary between experiments. By contrast, no CAD sequences were recovered from T5S13 by this procedure. These results are consistent with the conclusion that most, if not all, of the CAD sequences in C5R500 are extrachromosomal covalently closed circular molecules. Figure 3 presents a comparison of the migration of the episomes obtained either by lysis of cells in the wells of an agarose gel or by alkaline lysis in the gel system described above. The alkaline lysate (lane c) and native (lane d) forms of the CAD episome displayed similar mobilities, as did the corresponding preparations of EBV episomes from Raji cells (lanes a and b).

The similar behavior of the EBV episome, a molecule

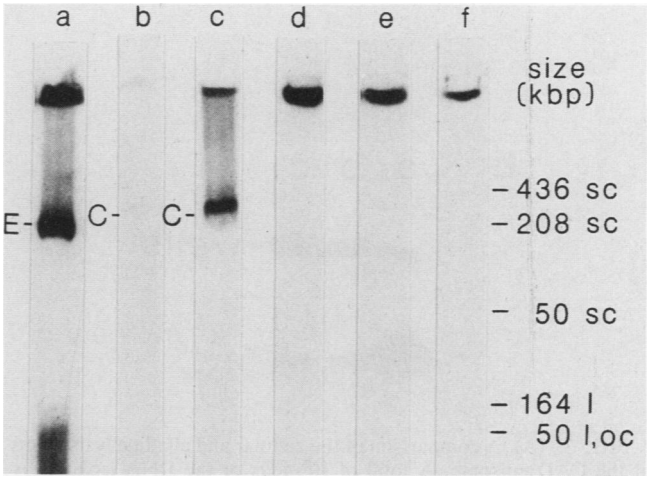

FIG. 2. Detection of extrachromosomal CAD genes in various cell lines. Cells (10^6) were loaded into the lower well of a 0.8% agarose gel in sample buffer containing RNase. Lysis buffer containing SDS and proteinase K was loaded in the upper well. Electrophoresis was at 0.8 V/cm for 3 h and then at 5.2 V/cm for 6 h (see Materials and Methods for details). The gel was transferred to nitrocellulose, and the blot was hybridized (i) with a nick-translated probe specific for a segment of the CAD gene which is devoid of repeats (102 fragment; reference 43) and then (ii) with plasmid pA2437, which contains an insert derived from EBV. pA2437 also hybridizes to the plasmid sequences presented in the CAD episome (40, 51). Lanes: a, Raji cells; b, T5C5, c, C5R500; d, T5S13; 3, T6; f, T1. Markers of known size and shape are indicated on the right. Abbreviations: sc, supercoiled circle; oc, open circle; l, linear; E, EBV episome, C, CAD episome.

known to be covalently closed and circular, and the CAD episome with regard to electrophoretic migration and isolation by alkaline lysis indicates that the CAD episome is also a covalently closed circle. A faster-migrating CAD and EBV species was sometimes observed in alkaline lysis preparations and probably reflects conversion of supercoiled molecules to nicked and broken circles. However, the data presented thus far do not rule out the less likely possibility that the CAD episome is actually a hairpin generated by inverted repeats known to be present in amplified regions (17). To investigate this possibility, we compared the ability of alkaline lysis-prepared CAD episome and EBV episome to be trapped in agarose, a method previously demonstrated to differentiate between linear and circular molecules (44). In this method, DNA is mixed with molten agarose and pipetted into the well of an agarose gel, and the DNA-agarose mixture is allowed to solidify before electrophoresis. Linear molecules migrated unimpeded into the gel during electrophoresis, but the solidified agarose imprisoned circular molecules. Greater than 90% of the linear, 164-kbp T4 DNA (Fig. 3b, lane a) migrated out of the agarose plug into the gel, while 60 to 70% of both the CAD and EBV

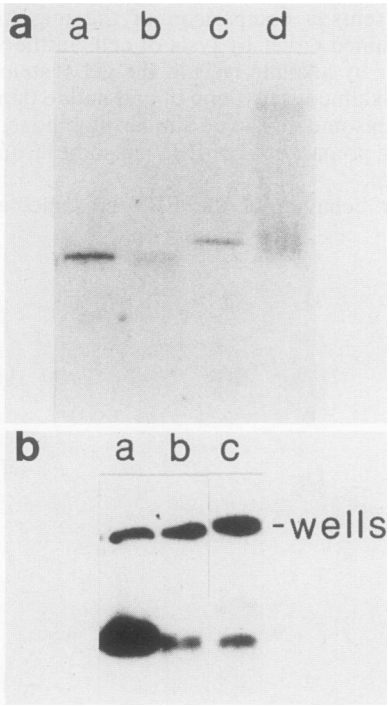

FIG. 3. (A). A comparison of the cellular and alkaline lysis forms of the CAD episome. A total of 10^6 cells or the DNA isolated by alkaline lysis from an equivalent number of cells was electrophoresed as described in the legend to Fig. 1 and Materials and Methods. The Southern blot was probed with nick-translated 102 fragment and pA2437, which contains EBV-derived sequences. Lanes: a, alkaline lysate from Raji cells; b, Raji cells lysed in the gel; c, alkaline lysate from C5R500 cells; d, C5R500 cells lysed in the gel. (B) Comparison of the ability of agarose to trap a large linear DNA and the episomes from C5R500 and Raji cells. T4 DNA (100 ng), a linear molecule of 164 kbp (lane a), and DNAs prepared by alkaline lysate from 10^6 C5R500 cells (lane b) and 10^6 Raji cells (lane c) were mixed with molten agarose to a final agarose concentration of 0.8%. The samples in liquid agarose were loaded into the wells of a 0.3% agarose gel, allowed to solidify, and electrophoresed at 1 V/cm for 16 h. The gel was transferred to nitrocellulose and hybridized with nick-translated pA2437 and T4 probes.

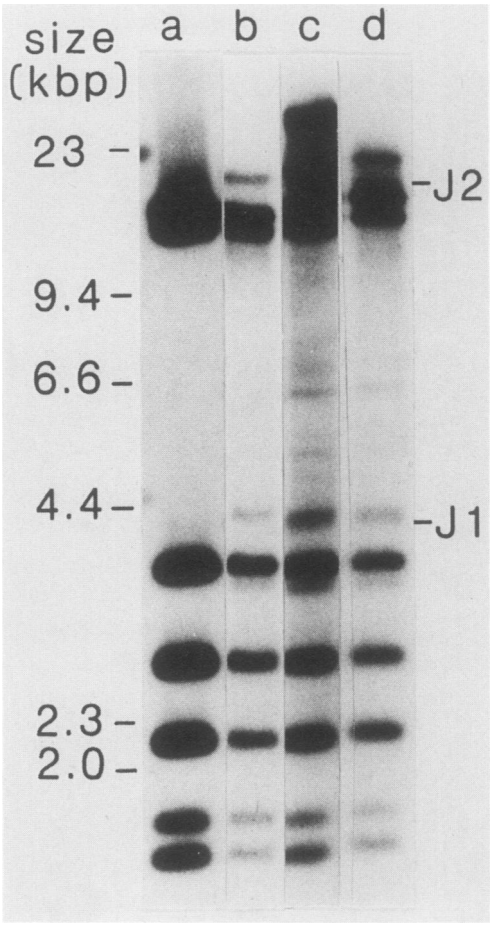

FIG. 4. Restriction analysis of chromosomal and extra chromosomal CAD genes. Purified cCAD1 (lane a), genomic DNAs isolated from T5S13 (2 μg; lane b) and C5R500 (2 μg; lane c), and DNA from ~10^6 C5R500 cells isolated by alkaline lysis (lane d) were digested with *Sst*I, fractionated on a 0.7% agarose gel, and blotted to nitrocellulose. The blot was hybridized to nick-translated cCAD which had been treated to remove repetitive sequences as follows. The probe and a 50-fold excess of genomic DNA from a CAD-deficient CHO cell line (35) were denatured and then allowed to reanneal at 68°C in 0.12 M sodium phosphate, pH 6.8, until a value of $C_0t = 1$ was reached for the driver DNA (3). J1 and J2 refer to the novel sequences previously described (49; see text).

sequences were retained in their respective wells (lanes b and c). The small amount of CAD and EBV sequences entering the gel was probably due to conversion of some of these very large circular molecules into linear molecules by double-strand breaks introduced during the alkaline lysis procedure. In addition, we found that most of the alkaline lysis-purified CAD episome was resistant to digestion with λ exonuclease under conditions in which linear molecules are degraded (B. Windle and G. M. Wahl, unpublished data). Taken together, our data provide compelling evidence that the CAD episome is a covalently closed circular molecule.

Arrangement of CAD sequences in the CAD episome. To understand the mechanism by which the CAD episome was generated, it was necessary to know whether the sequence arrangement of the CAD genes within the episome was different from that present in chromosomally amplified CAD sequences in other T5 subclones. Figure 4 shows a Southern blot hybridization analysis of an *Sst*I digest of total cellular DNA from various cell lines derived from the original T5

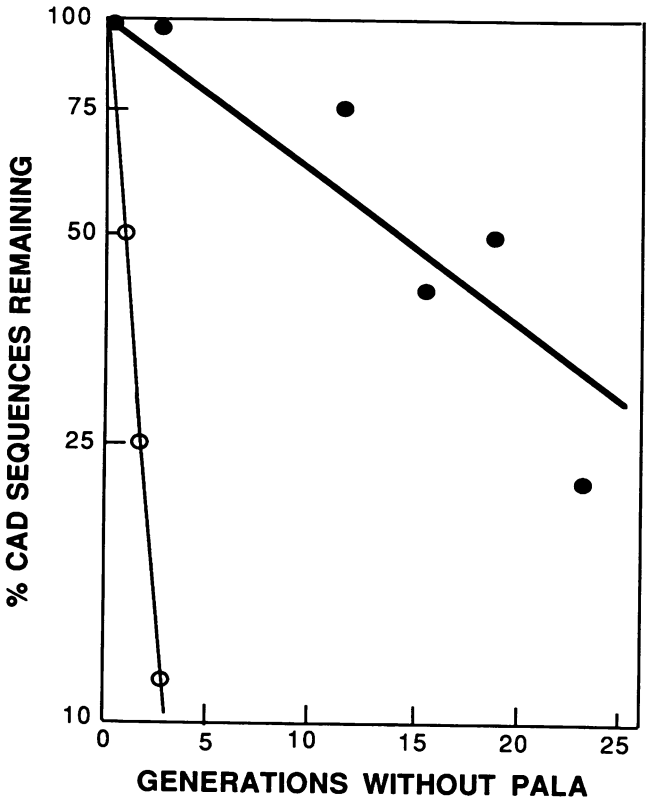

FIG. 5. Loss of the CAD episome from C5R500 cells propagated in the absence of PALA. C5R500 cells were grown in medium without PALA. At the times indicated, cell samples were frozen. All samples were run on the same gel by the procedure described in the legend to Fig. 1 and Materials and Methods. The gel was blotted to nitrocellulose and hybridized to nick-translated 102 fragments. The intensity of hybridization of the band corresponding to the CAD episome was estimated by densitometry scanning of the autoradiogram. Closed circles (●) represent actual loss of the CAD episome, and open circles (○) represent predicted loss by dilution of a non replicating molecule.

clone and episomal DNA isolated by the alkaline lysis method from C5R500. To analyze the sequence composition and arrangement in the intrachromosomal (T5S13) and extrachromosomal (C5R500) CAD genes, the blot was probed with nick-translated cCAD1 (the cosmid which was used for the initial transfection and contains the entire CAD gene; 40), from which repeated sequences were removed (3). This analysis demonstrated that all of the fragments present in cCAD1 (lane a) were also present in both the intrachromosomal (lane b) and extrachromosomal (lane c) CAD genes, indicating that no major reorganization of CAD sequences occurred upon generation or amplification of the extrachromosomal element. In addition, the hybridization patterns of the digested alkaline lysate form of the extrachromosomal molecule (lane d) indicated that it contained the same CAD fragments and that they were not rearranged.

We have previously described two novel restriction fragments, J1 and J2 (Fig. 4), associated with insertion of the cosmid into the chromosome. Molecular cloning demonstrated that J1 is actually an inversion within one CAD cosmid of the five that integrated in T5 (J.-M. Clement, P. Gaudray, and G. Wahl, unpublished data) rather than a joint between the donated and adjacent chromosomal sequences. The ratio of either J1 or J2 to the unrearranged CAD sequences in the original T5 clone was 1 to 5, and the same ratio of sequences was found in the CAD episome. This observation indicates that the CAD episome contains approximately five copies of the 50-kbp CAD cosmid and that the chromosomally and extrachromosomally amplified sequences in T5 subclones share a common molecular ancestor. Since the amount of DNA contained within the molecule that was not derived from the CAD cosmid is not precisely known at present, we conclude that the molecule contains at least 250 kbp of DNA. This is in accord with the electrophoretic migration described above, which indicates that the episome is significantly smaller than 436 kb and slightly larger than 178 kb.

Autonomous replication of the CAD episome. The presence of the CAD episome in subclones of T5 presented the intriguing possibility that this molecule could replicate autonomously and that it should, therefore, contain a cellular origin of DNA replication. Two analyses demonstrated that the extrachromosomal molecule replicates autonomously. First, we determined the kinetics of loss of the CAD episome from C5R500 in the absence of selection. This line, which is resistant to 500 μM PALA, contains approximately 200 copies of the CAD gene, which is equivalent to an average of 40 extrachromosomal molecules per cell, each of which contains five CAD genes. After passage for 25 generations in the absence of drug, C5R500 retained 25 to 30% of the original level of the CAD episome (Fig. 5). If the episomes were not replicating, simple dilution as a consequence of cell division would have decreased the number of CAD episomes to less than 1% of the original level in only seven generations (Fig. 5).

To provide biochemical evidence for CAD episome replication and to determine whether it replicates semiconservatively in a cell cycle-regulated fashion, the kinetics of BrdU incorporation were measured. A density shift experiment patterned after the classic Meselson and Stahl experiment (34) was performed. C5R500 cells were labeled for approximately one or two generations with BrdU, and total DNA was isolated, sheared, and then analyzed by isopycnic centrifugation in CsCl gradients. The data in Fig. 6A are representative of many experiments and show that most of the CAD episome sequences were of hybrid density after one generation of labeling. In addition, there was a shift to approximately 50% of the molecules being of hybrid density and approximately 50% being labeled in both strands after two generations. The small amount of unlabeled DNA seen after one generation was variable between experiments and probably reflects the contribution from a small portion of noncycling cells in the asynchronous population used for these experiments. The data are in good agreement with the predicted density shifts for DNA which replicates semiconservatively. The density labeling pattern of the CAD episome was also compared with that of ribosomal RNA genes since ribosomal RNA genes are present at about the same copy number in the genome as are CAD genes in CAD episomes, and they are known to replicate early in each S phase (14). The results shown in Fig. 6B demonstrate that the CAD episome and chromosomal ribosomal RNA sequences have virtually indistinguishable labeling patterns. Taken together, these experiments show that the CAD episome not only replicates semiconservatively but obeys replication controls similar to those obeyed by a known chromosomal sequence.

An origin of DNA replication is not present in the CAD cosmid. As described above, the CAD episome consists of

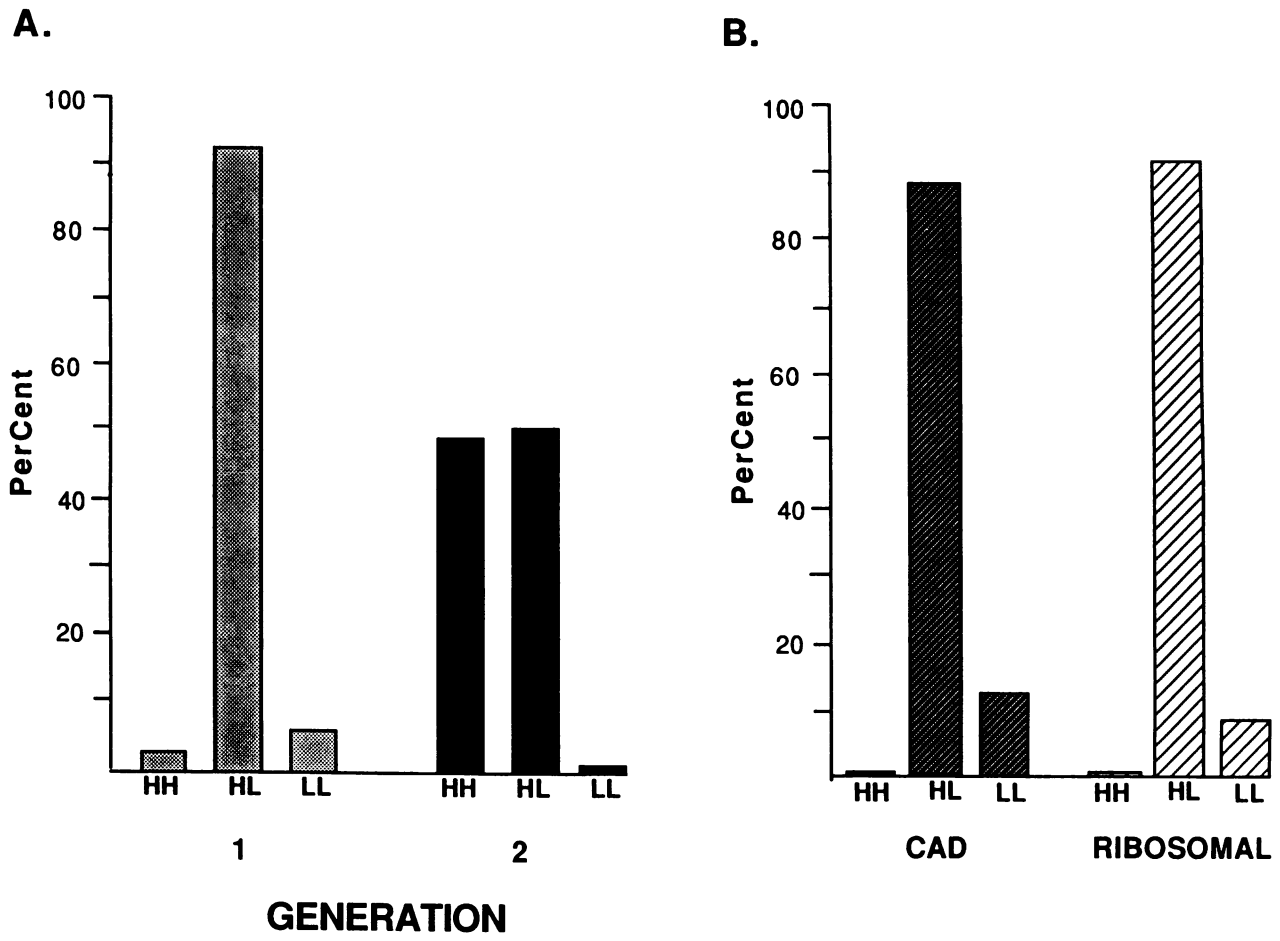

FIG. 6. Replication of the CAD episome as determined by BrdU labeling. (A) C5R500 cells were grown for one (light shading) or two (dark shading) generations in the presence of BrdU. Total genomic DNA was isolated, sheared, and fractionated on a CsCl gradient as described in Materials and Methods. Fractions were dot blotted to nitrocellulose, hybridized to nick-translated CAD-specific 102 fragment, and counted by liquid scintillation spectrometry. Percent refers to the portion of total counts found in each fraction (HH, heavy-heavy; HL, heavy-light; LL, light-light). (B) DNA was isolated from C5R500 cells, grown in BrdU for one generation, and treated as described above. The dot blots were hybridized to either 102 fragment (dark shading) or a fragment complementary to the ribosomal genes (light shading).

two components: (i) sequences derived from the cosmid used from the original transfection and (ii) an unknown amount of sequence derived from rearrangements within the cosmid, from the host genome or both. Therefore, an origin of replication could be present in either of these two components. To test whether an origin is present in the CAD cosmid sequences, we determined whether the CAD cosmid, cCAD1, could replicate in vivo in a transient assay system (36). In this system, replication is detected by an alteration in the methylation state of transfected molecules in the following way. Propagation of cloned molecules in bacteria which contain the *dam* methylase results in methylation of the adenine residue in the sequence GATC at the N6 position. The enzyme *Dpn*I cleaves at the sequence GATC only when the adenines in both strands are methylated. Since mammalian cells do not contain an enzyme which adds N6 adenine methyl groups, plasmid molecules which undergo one round of replication are converted to a hemimethylated form. Thus, replication is assayed by conversion of a sequence from *Dpn*I sensitivity to *Dpn*I-resistance.

The uridine auxotrophic CHO cell line (35), which was the parent of T5 and can sustain replication of the CAD episome, was transfected with cosmid cCAD1. To provide a positive control for replication, cells were also transfected with a plasmid (pLT-1) which contains a modified polyomavirus genome (51) and is capable of replication in hamster cells (see below). At various times after transfection, extrachromosomal circular molecules were isolated by the alkaline lysis procedure, digested with a restriction enzyme to linearize the plasmid molecules, and also digested with *Dpn*I to assess the methylation state of the transfected molecules. Since replication is indicated by the presence of *Dpn*I-resistant molecules, a small amount of cloned plasmid DNA (pUC13) was added to each digest to ensure that the presence of *Dpn*I-resistant bands was due to replication and not incomplete enzyme digestion. After digestion, the fragments were separated on an agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated probe complementary to plasmid sequences shared by both the internal control and test plasmids.

The results of a typical replication experiment are shown in Fig. 7. pLT-1 replicated in hamster cells as indicated by the presence of *Dpn*I-resistant bands of the appropriate size (e.g., compare lanes b and a [the latter is a control which was digested with *Eco*RI alone]). In contrast to the result obtained with pLT-1, all of the molecules isolated from cells

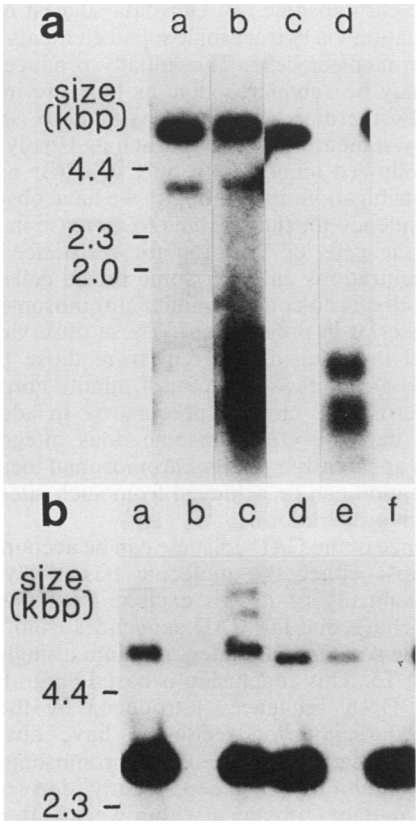

FIG. 7. Replication of cCAD1 and pLT-1. (a) Approximately 10^6 CAD-deficient CHO cells (35) were transfected in monolayer with 500 ng of pLT-1 (lanes a and b) or 500 ng of cCAD1 (lanes c and d). Extrachromosomal DNA was isolated after 3 days by alkaline lysis. The yield from one plate was divided into two parts and digested with *Eco*RI alone (lanes a and c) or *Eco*RI and *Dpn*I (lanes b and d) and fractionated on a 0.7% gel. pUC13 was added to the samples to be digested with *Dpn*I. The gel was blotted to nitrocellulose and hybridized with nick-translated pUC13. *Eco*RI cleaves pLT-1 into two bands of approximately 5 and 4 kbp which contain hybridizing plasmid sequences. The complementary vector sequences in cCAD1 are contained in a 4.7-kbp *Eco*RI band (40). The low-molecular-weight (<2 kbp) bands in lanes b and d are cleavage products of the added pUC13. (b) Cells were transfected with pLT-1 as described above, and plasmid DNA was isolated after 1 (lanes a to c) or 2 (lanes d to f) days by alkaline lysis. pUC13 was added to all samples before digestion. Lanes a and d were digested with *Eco*RI alone, lanes b and e were digested with *Eco*RI and *Dpn*I, and lanes c and f were digested with *Eco*RI and *Mbo*I. The gel was transferred to nitrocellulose, and the blot was probed with nick-translated pUC13. The intensely hybridizing band at 2.7 kbp is linear pUC13. The *Dpn*I cleavage products of pUC13 were run off the gel.

transfected with cCAD1 appeared to be *Dpn*I sensitive (compare lanes d and c [the latter is a control which contains cloned cCAD1 digested with *Eco*RI alone]).

Since pLT-1 contains a viral origin and a modified T antigen gene, it is conceivable that this plasmid could replicate many times per cell cycle in the CHO cells used for these analyses. It could be argued that it is because of such runaway replication that its replication can be detected and that a molecule which replicates only once or a few times per cycle could not be detected in such an assay. To investigate this issue, the replication of pLT-1 was analyzed over a 2-day period, and the total number of pLT-1 sequences recovered and the extent of replication were compared. Lanes a and d, Fig. 7b, show the *Eco*RI digestion products on, respectively, days 1 and 2 after transfection. The total amount of pLT-1 DNA recovered was not dramatically different, indicating that many rounds of replication did not take place. That replication did occur was demonstrated by conversion of most or all of the pLT-1 molecules from *Dpn*I sensitivity on day 1 (lane b) to approximately 50% of the molecules being *Dpn*I resistant on day 2 (lane e). Similarly, whereas most of the pLT-1 sequences were resistant to *Mbo*I on day 1 (*Mbo*I only cleaves unmethylated DNA; lane c), a substantial proportion of the pLT-1 DNA was converted to an *Mbo*I-sensitive form (i.e., unmethylated; lane f) by day 2 in this experiment. These data show that pLT-1 replicated once or at most a few times, on the average, in these CHO cells. This result further strengthens the conclusion that cCAD1 is incapable of even a single round of replication under these conditions and therefore does not contain a functional origin of replication.

DISCUSSION

To identify and tag regions which engender high-frequency gene amplification, we introduced functional genomic clones of the Syrian hamster CAD gene into random chromosomal locations in CAD-deficient CHO cells (49). An analysis of 25 independent transformants (49; P. Gaudray, J. Emery, and G. Wahl, unpublished data) has revealed one clone, T5, which amplifies the donated sequences with a frequency 100- to 1,000-fold higher than any of the other transformants or wild-type CHO and Syrian hamster cell lines. Recently, we have used a different vector system and found 2 such "hyperamplifiable" transformants among 47 that were analyzed (J. Ruiz and G. M. Wahl, manuscript in preparation). In addition, Glanville (20) has reported that 1 of 40 mouse cells transformed with the v-*src* gene demonstrated high-frequency amplification of this gene. These results strongly suggest that hyperamplifiable sites are relatively common in rodent genomes.

The gel migration characteristics, isolation by alkaline lysis, and agarose trapping experiments we report provide compelling evidence that some T5 subclones (e.g., T5C5 and others not shown) produce CAD episomes which are covalently closed circular moducules with a size of approximately 250 kbp. Restriction analysis showed that the CAD episome contains all of the sequences present in the CAD cosmid that was used in the original transfection and at least three novel fragments not present in the CAD cosmid (49; J. M. Clement, P. Gaudray, B. Windle, and G. M. Wahl, unpublished data). These novel fragments are unique to T5 and its derivatives and provide a molecular fingerprint of the CAD sequences in T5 cells. We have cloned and analyzed two of these junctions, previously referred to as J1 and J2 (49), and have shown that they are formed, at least in part, by rearrangements within the donated CAD sequences. It is as yet unclear whether the novel fragments we have identified thus far also contain host genomic sequences. By comparing the hybridization intensity of these novel fragments with the corresponding unrearranged fragments in the remaining CAD sequences, we have determined that the CAD episome contains one J1 or J2 sequence for five CAD genes. This is the same ratio as was found in the original T5 transformant and in PALA-resistant T5 clones in which the donated genes are amplified chromosomally. These restriction analyses show that the CAD episome and the chromosomally amplified CAD genes share common ancestoral sequences, and the process of episome formation did not produce other large-scale rearrangements.

The slow rate of loss of the CAD episome (60 to 70% loss over 25 to 30 cell doublings) from C5R500 cells grown in the absence of selection provided the first indication that the episome could not only replicate but also segregate with reasonable efficiency. The kinetics of incorporation of BrdU into the CAD episome, ribosomal RNA genes, and the chromosomally amplified CAD sequences in another T5 clone (data not shown) were indistinguishable within the limits of sensitivity of our measurements, indicating that the CAD episome replicates semiconservatively and approximately once per cell cycle. These results also show that most, if not all, of the CAD episomes in each cell replicate in each cycle and that few, if any, replicate more than once in a single S phase. Although the data do not address whether the episome replicates at a specified time in each cell cycle, they indicate that the general controls which result in one round of DNA replication per S phase probably operate in this molecule. Since in vivo replication assays showed that cCAD1 cannot replicate, we infer that autonomous, regulated replication of the CAD episome derives from the presence of a functional DNA replication origin which is not derived from the CAD sequences. It seems likely that this putative DNA replication origin was derived from CHO DNA and became associated with the CAD sequences as a consequence of episome formation (see below). However, we cannot rule out the less likely possibility that a functional replication origin was created by one or more of the DNA rearrangements which are unique to the CAD genes in T5. Current experiments are directed toward isolation and characterization of the sequence which enables the CAD episome to replicate autonomously.

Analysis of the CAD episome in various T5 clones over a 1-year period has shown that the episome has a minimum size of approximately 250 kbp. We have never observed CAD episomes smaller than this size in any T5 clone, even though circular molecules as small as 5 kbp can be resolved in the gel system we used. This is somewhat unexpected since the parental CAD-deficient CHO cells can recombine exogenously introduced molecules with high efficiency (41), and the head-to-tail tandem array of CAD genes in the episome should provide an excellent substrate for homologous intramolecular recombination. It is unlikely that the size of the CAD episome reflects the minimum size required for efficient extrachromosomal replication in eucaryotic cells since Garvey and Santi (19) and Rassoulzadegan et al. (39) have reported that covalently closed circular molecules ranging from 3 to approximately 80 kbp can replicate extrachromosomally and, in some cases, approximately once per cell cycle. Rather, it may be that the 250-kbp size minimum reflects the lower size limit for molecules to be segregated with acceptable efficiency in CHO cells.

The data presented above demonstrate that the CAD episome is an autonomously replicating circular molecule. Minute chromosomes, which are frequently associated with gene amplification in mouse and human cells (for reviews see references 6 and 13), also replicate autonomously (1, 23), are probably circular, but are at least 1,000 to 5,000 kbp (13, 24). Thus, the CAD episome is structurally and functionally similar to these larger extrachromosomal elements. The presence of extrachromosomally amplified sequences in T5 is unusual since hamster cells generally amplify sequences within chromosomes, although there are examples of extrachromosomal amplification in these cells (27, 29). It has been postulated that amplification in hamster cell lines is mediated by production of extrachromosomal elements, followed by their rapid and presumably site-specific integration into the chromosome (8). Our data suggest one reason why amplification via extrachromosomal elements has rarely been seen in hamster cells. The initially produced circular molecules may be submicroscopic as they are in some T5 subclones. Furthermore, it is not posssible to isolate such molecules by standard procedures such as Hirt lysis (26) or SDS lysis followed immediately by CsCl-EtBr equilibrium gradient centrifugation. Interestingly, we have observed that there is a tendency for the episome to increase in size upon passage of the cells or selection for resistance to higher PALA concentrations and that some of the cells in highly resistant T5 clones do contain minute chromosomes (Carroll et al., manuscript in preparation). These observations suggest a novel mechanism, different from those previously described (3, 4), for the formation of minute chromosomes from submicroscopic circular precursors. In addition, we have found that the CAD episome does integrate infrequently into apparently random chromosomal locations and that amplification can be achieved from such sites (Carroll, manuscript in preparation).

The presence of the CAD episome can be accounted for by two scenarios; either the molecule has always existed extrachromosomally or it was excised from the chromosome. We believe that the CAD sequences from which the episome arose were initially integrated into a single chromosomal site in T5. This conclusion is based upon the general finding that DNA sequences introduced by the calcium phosphate coprecipitation technique have always been shown to be intregrated into cellular chromosomes (38, 40, 42, 49). In addition, six independently derived PALA-resistant T5 mutants, obtained within weeks after isolation of the parental T5 clone, all contain an expansion of the donated CAD genes at the same chromosomal location (49; Fig. 1; unpublished data). Since, as described above, the CAD episome and the chromosomally amplified CAD sequences share a common molecular precursor, it seems most likely that this precursor was a unit of five CAD genes integrated into one chromosomal site.

The results presented here address the mechanism of formation of the CAD episome. As mentioned above, when T5 cells containing the episome were labeled for approximately one generation with BrdU, most CAD episomes were labeled in only one strand. The few CAD episomes sometimes observed to be labeled in both strands were quantitatively the same as the fraction of chromosomal sequences labeled in both strands, indicating that a small fraction of cells underwent more than one S phase during the labeling period. These results are inconsistent with models proposing that a chromosomal copy generates CAD episomes by multiple rounds of DNA synthesis within one S phase (43). In addition, we have isolated T5 clones which have lost the episome after passage under nonselective conditions and which have also lost chromosomally localized copies of the donated CAD genes (Carroll et al., manuscript in preparation). Both the BrdU labeling patterns and the isolation of cell lines which have lost all of the donated CAD sequences are consistent with the conclusion that an excision event deleted the chromosomal copies of the donated CAD gene and generated the autonomously replicating CAD episome. It has also been reported that in HeLa cells, formation of a small extrachromosomal molecule is associated with a chromosomal deletion (28, 30). Chromosomal deletions are also known to occur preferentially in some CHO chromosomes (1).

Our use of gene transfer to identify chromosomal regions which engender high-frequency amplification has given new

insight into mechanisms for gene amplification. We originally postulated that some amplification hot spots might represent DNA replication origins since perturbation of regulated replication may be involved as an early step of gene amplification (43, 48a). It is intriguing in this regard that we detected the donated CAD sequences as semiconservatively replicating autonomous elements in some subclones of one hyperamplifiable transformant. It is tempting to speculate that the CAD episome was formed by excision of the donated sequences along with a nearby replication origin or that an origin was created by the insertion event itself. We are now in a position to identify the origin functionally and characterize it at the molecular level.

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