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Co-Amplification of rRNA Genes with CAD Genes in N-(Phosphonacetyl)-L-Aspartate-Resistant Syrian Hamster Cells

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The amplified CAD genes in *N*-(phosphonacetyl)-L-aspartate (PALA)-resistant Syrian hamster cells are located in an expanded chromosomal region emanating from the site of the wild-type gene at the tip of the short arm of chromosome B-9. The terminus of B-9 in PALA-sensitive cells contains a cluster of rRNA genes (i.e., a nucleolus organizer, rDNA). We have used a molecular clone containing sequences complementary to Syrian hamster 28S rRNA to investigate whether rDNA is coamplified with CAD genes in the PALA-resistant mutants. In situ hybridization of this probe to metaphase chromosomes demonstrates that rDNA and CAD genes do coamplify in two independently isolated PALA-resistant mutants. The tight linkage of CAD and rDNA genes was demonstrated by their coordinate translocation from B-9 to the end of the long arm of chromosome C-11 in one mutant. Blot hybridization studies substantiate the in situ hybridization results. Both types of analysis indicate that only one or two rDNA genes, on the average, are coamplified per CAD gene. The data are consistent with the model that unequal exchanges between rDNA genes mediate the amplification of CAD genes in the Syrian hamster mutants that were analyzed.

Gene amplification occurs in organisms spanning the phylogenetic scale and provides a common mechanism for genome remodeling. For example, up to 3% of a bacterial population can harbor duplications of specific chromosomal regions (2). In yeasts, amplification within the ribosomal gene cluster occurs spontaneously with high frequency (29). Gene amplification in animal cells has been demonstrated to mediate drug resistance (see reference 29 for examples) and the phenotypic reversion of mutants with temperature-sensitive (14) or kinetically altered proteins (6).

The large size of most amplified units, 135 to approximately 3,000 kilobase pairs (5, 20, 21, 38), has made it difficult to analyze them in detail at the molecular level. However, one clue to a mechanism for CAD gene amplification was provided by our earlier studies which showed that CAD genes in two PALA-resistant mutants of baby hamster kidney cells (BHK-21/13 cells) are amplified from the site of the wild-type gene which is located adjacent to a nucleolus organizer on the short arm of chromosome B-9 (4, 38). (CAD is an acronym for the multifunctional protein which catalyzes the first three steps of uridine biosynthesis: C = carbamoylphosphate synthetase; A = aspartate transcarbamylase [ATCase]; D = dihydrooratase. PALA [N-phosphonacetyl-L-aspartate] is a transition-state analog inhibitor of ATCase.) Since rRNA genes (rDNA genes) are known to mediate the amplification of adjacent sequences in procaryotes (2, 13) and yeasts (29), and to amplify spontaneously in mammalian tissue culture cells (30), we have investigated whether rDNA genes coamplify with CAD genes in PALA-resistant cells.

(A preliminary account of this work has been published [26]).

MATERIALS AND METHODS

Cells and culture. The PALA-resistant cell lines B5-3 and B5-4 were isolated from BHK-21/13 cells by a multistep selection with increasing concentrations of PALA as described previously (36). The final PALA concentration used for their selection was 5 mM. The cell lines were maintained as described previously (15).

Isolation of clones containing ribosomal DNA sequences. The cosmid library that we used previously to isolate a functional CAD gene was used to obtain a clone containing rDNA sequences (25). The cosmid library (in *Escherichia coli* HB101) was spread on agar plates containing 10 μg of tetracycline per ml (approximately 1,000 to 2,000 colonies per 9-cm plate) and prepared for colony filter hybridization (11).

The probes used to detect colonies containing rDNA sequences were prepared as follows. Non-polyadenylate-containing [poly(A)$^-$] RNA (approximately 20 μg) from BHK-21/13 cells was fractionated on a methyl mercury agarose gel, and the 18S and 28S rRNA bands were excised and purified as described by Lemischka et al. (17). The probe was prepared from the purified 18S and 28S rRNA, using avian myeloblasto-

sis virus reverse transcriptase (RTase, kindly provided by J. Beard, Life Sciences, Inc.) and random oligodeoxynucleotide primers (32). Each 25-μl reaction contained 200 ng of RNA, 250 ng of random primer, 10 to 20 U of RTase, 50 mM Tris-hydrochloride (pH 8.3), 50 μCi of [^{32}P]dCTP (≥400 Ci/mmol), 40 mM KCl, 8 mM $MgCl_2$, 0.4 mM dithiothreitol, and 200 μM each of dGTP, dATP, and dTTP. The reactions were incubated at 42°C for 90 min and were terminated by adding 0.1 volume of 2% sodium dodecyl sulfate–200 mM EDTA (pH 8.0). The unincorporated [^{32}P]dCTP was removed from the reaction by filtration through a Bio-Gel P-60 column. The excluded fractions were pooled; sheared *E. coli* DNA and NaOH were added to final concentrations of 500 μg/ml and 0.6 M, respectively, and the solution was incubated at 50°C for 1 h to hydrolyze the RNA template.

Colony filters were hybridized with 5×10^5 cpm of random primed 18S and 28S probe per filter. The hybridization was carried out at 68°C for 12 to 16 h in a solution consisting of 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M $NaPO_4$ [pH 7.7], 1 mM EDTA), 2× Denhardt reagent (8), 200 μg of sheared *E. coli* DNA per ml, and 0.1% sodium dodecyl sulfate. The filters were removed and washed batch-wise (greater than 25 ml of washing agent per filter) in the following solutions: 1× SSPE–0.1% sodium dodecyl sulfate at 25°C for 15 min, then 0.1× SSPE–0.1% sodium dodecyl sulfate at 50°C for 30 min. Colonies containing sequences which hybridized with both 18S and 28S rRNA probes were localized by autoradiography, isolated from the master plate, and purified by two successive rounds of single colony isolation. A colony isolated from the last purification was then grown into mass culture, and the plasmid DNA was isolated (7).

Restriction digestion and blot hybridization. Samples of genomic DNA were prepared as described previously (36). When quantitative comparisons were performed, the following procedure was adopted to insure digestion of equal amounts of DNA for each sample. DNA samples were diluted to a concentration of approximately 100 μg/ml based on the initial measurements of optical density at 260 nm. Aliquots of the samples were then analyzed by the diphenylamine method to determine the actual DNA concentration (23). A second aliquot of each sample was then taken so that each restriction digestion contained ~5 μg of DNA (except where indicated). DNA samples were digested with two successive additions of two- to fourfold excesses of restriction enzymes for a total of 4 h to insure complete digestion. Different enzyme lots produced equivalent results. Hybridizations were performed as described previously with 1×10^6 to 2×10^6 cpm of nick translated probes (1×10^8 to 2×10^8 cpm/μg) (24) or reverse transcribed probes per ml.

Gel electrophoresis and blotting of DNA to nitrocellulose (18, 28; Schleicher & Schuell Co., BA 83) were carried out by the following modifications of the procedure of Wahl et al. (37). The total depurination time in 0.25 M HCl was shortened to 10 min. Complete transfer from the 0.7% agarose gels used for all restriction analyses was achieved after 1 to 2 h of blotting. The transfer buffer was 1 M ammonium acetate instead of 20× SSPE. (Ammonium acetate affords the same transfer efficiency as 20× SSPE, but it does not leave behind the salt residue often encountered when SSPE or SSC is used.) Transfer of RNA samples to nitrocellulose (BA 83) was performed as described by Thomas (33).

In situ hybridization. In situ hybridization to fixed metaphase chromosomes of the indicated cell lines was performed as described by Wahl et al. (38), except that the isotope used for nick translation was [^{3}H]dTTP (100 Ci/mmol, New England Nuclear Corp.). Probes made with [^{3}H]dTTP were found to give the same signal-to-noise ratios as those made with [^{125}I]dCTP but were far more stable. In all cases, hybridization reactions contained 2.5×10^5 cpm of nick translated probe (0.5×10^8 to 1.0×10^8 cpm/μg) in a volume of 20 to 25 μl as described previously (38). Exposures were for 1 week at −70°C.

RESULTS

Isolation of rDNA-specific probes. The initial assay for rDNA and CAD gene coamplification was in situ hybridization of [^{3}H]rDNA probes to spreads of metaphase chromosomes isolated from PALA-sensitive and PALA-resistant cell lines. Since this procedure employs nonstringent washing conditions to preserve chromosome morphology, it was first necessary to insure that the rDNA probe used would not hybridize to CAD sequences under equivalent conditions. It was also necessary to isolate a probe devoid of the middle repetitive elements which are interspersed in mammalian ribosomal repeat units and which are dispersed throughout the genome (3, 12). A probe fulfilling these requirements was isolated in the following way. A cosmid (rcos11a) containing the largest ribosomal sequence insert was digested with *Eco*RI, and the resulting restriction fragments were fractionated on an agarose gel and purified (35). The fragments were nick translated (24) and hybridized to *Eco*RI-digested DNA isolated from PALA-sensitive BHK-21/13 cells. Four of the five Syrian hamster *Eco*RI fragments in rcos11a contained interspersed repeated sequences since they produced a continuum of hybridization to the *Eco*RI-digested BHK-21/13 DNA, and one of the fragments displayed a discrete band of hybridization (an example of hybridization with this probe to genomic DNA is shown in Fig. 3). This 7.1-kilobase (kb) fragment was subcloned into the *Eco*RI site of the vector MUA3 (19) for use in further experiments. The subclone containing this fragment is referred to as p11a-2.

The lack of hybridization of p11a-2 with CAD sequences and its specificity for rRNA sequences are shown in Fig. 1. Figure 1a shows the results of hybridizing p11a-2 with *Eco*RI-digested DNA isolated from a cosmid which contains a functional CAD gene as well as approximately 10 to 15 kb of flanking cellular sequences (cCAD6 [25]). The single prominent band of hybridization is to the vector which is excised from the insert sequences by *Eco*RI digestion (compare in Fig. 1a the tracks labeled

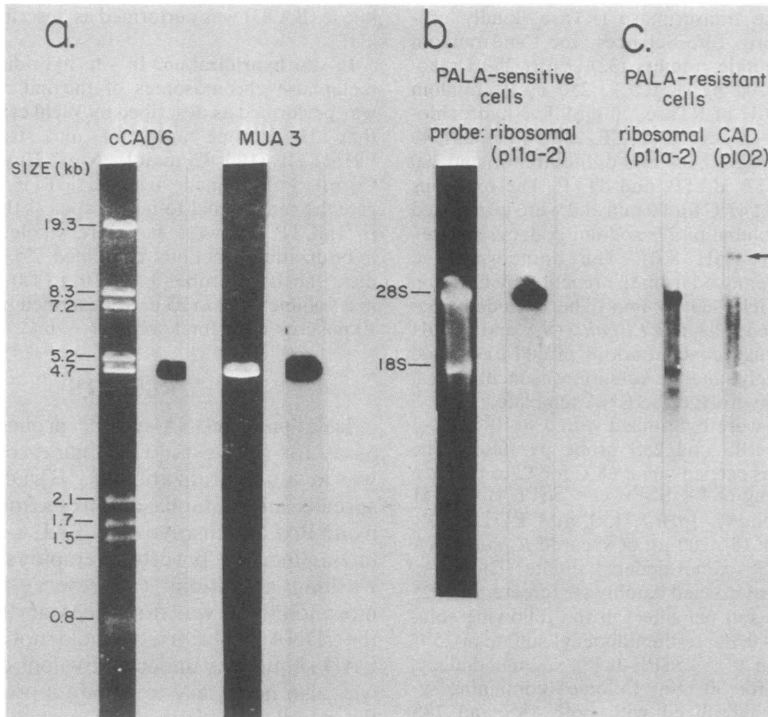

FIG. 1. Specificity of plasmid p11a-2 for rRNA sequences. Plasmid p11-2 was hybridized with the following nucleic acid samples: (a) 5 μg of *Eco*RI-digested cCAD6 (a cosmid which contains a functional CAD gene and 10 to 15 kb of flanking cellular sequences) or 1 μg of *Eco*RI-digested MUA3 (the vector used to construct cCAD6) DNA; (b) 10 μg of poly(A)$^+$ RNA isolated from PALA-sensitive BHK-21/13 cells; (c) 1 μg of poly(A)$^+$ RNA isolated from mutant 165-28 (this mutant contains 100 to 200 times the level of CAD mRNA in BHK-21/13 cells). The leftmost columns in (a) and (b) show the ethidium bromide-stained gels. The last lane shows the hybridization pattern of the CAD-specific probe p102 to the same blot as that used for hybridizations in the adjacent lane after removal of the P11a-2 hybridization signal. The arrow in the last lane designates the position of migration of CAD mRNA. We were able to use the poly(A)$^+$ fraction from an oligodeoxythymidylate-cellulose column to detect rRNA or CAD mRNA since some 18S and 28S rRNA elutes with poly(A)$^+$ RNA after oligodeoxythymidylate-cellulose chromatography under our conditions. The smear of hybridization below the 28S rRNA and CAD mRNA reflects degradation of these large RNA species.

cCAD6 and MUA3). Figures 1b and c show the result of hybridizing p11a-2 to size-fractionated, poly(A)$^+$ RNA isolated from PALA-sensitive BHK-21/13 cells of PALA-resistant 165-28 cells which overproduce CAD mRNA by 100- to 200-fold (36). Probe p11a-2 hybridizes strongly with 28S rRNA, weakly with 18S rRNA, and does not hybridize with CAD mRNA (compare in Fig. 1c hybridization of the same RNA blot with p11a-2 and the CAD probe p102). These results demonstrate that p11a-2 provides a probe which is specific for rRNA sequences and which is incapable of hybridizing with CAD genomic or CAD mRNA sequences under nonstringent washing conditions. A comparison of these results (and other restriction and DNA blotting analyses not shown) with the map of the Syrian hamster rDNA repeat unit indicates that fragment 11a-2 contains sequences corresponding to the 3′ end of the 18S rRNA gene, the internal transcribed spacer, and almost the entire 28S rRNA gene (see reference 34 and Fig. 3a).

Co-amplification of CAD genes and ribosomal sequences in PALA-resistant BHK cells. Previous experiments have shown that most and probably all of the 100 amplified CAD genes in cell line B5-4 (36) are localized to an extension of the short arm of one homolog of chromosome B9 (referred to as B9p$^+$ [38]). The B9p$^+$ chromosome is an obvious cytogenetic marker since it is the largest submetacentric chromosome in cell line B5-4. To determine whether ribosomal sequences are amplified in B5-4 cells, metaphase chromosomes from this line were hybridized with the rRNA-specific probe p11a-2. Significant hybridization is clearly visible over the entire length of the expanded short arm of chromosome B9 (Fig. 2a). Hybridization is also

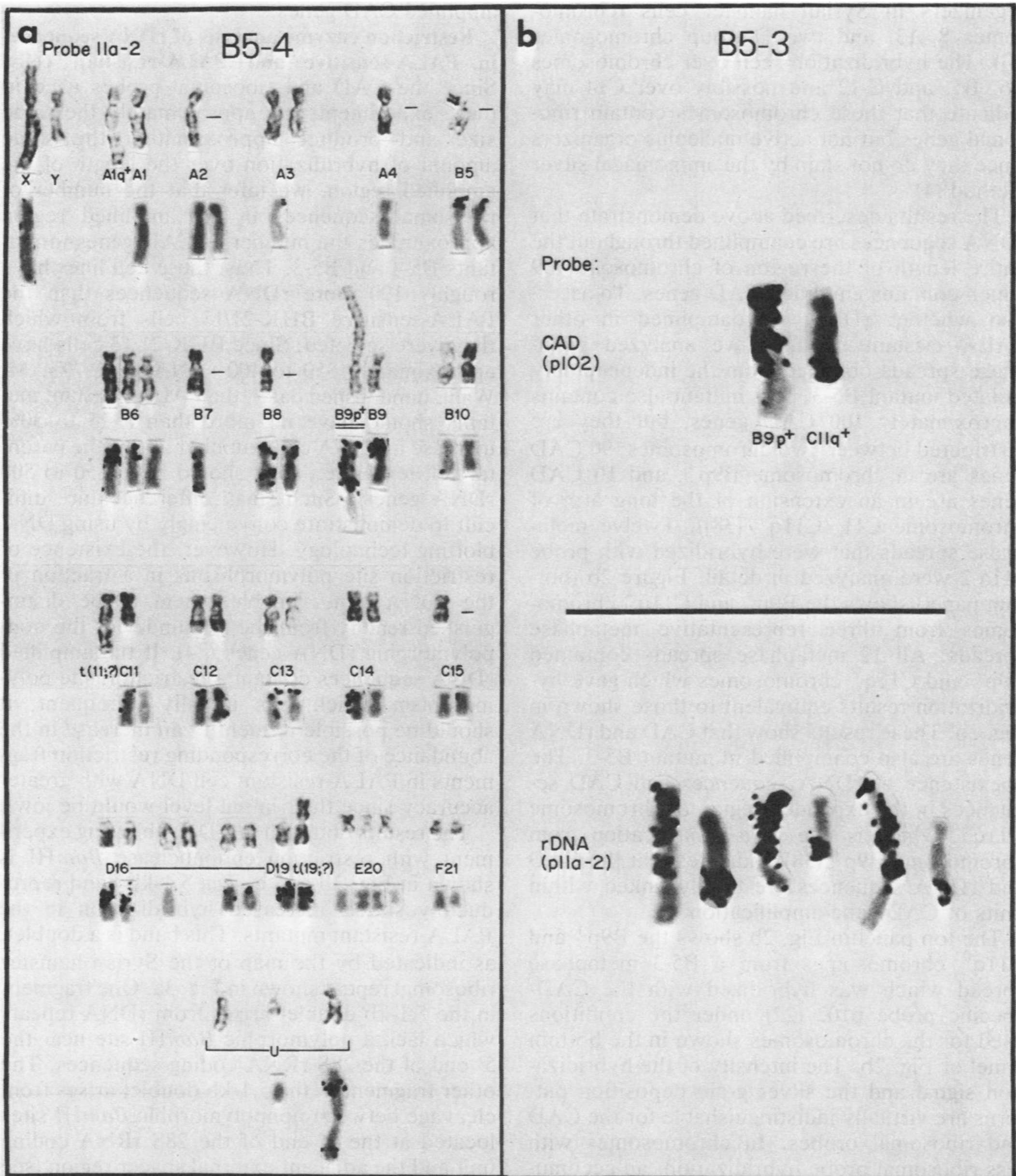

FIG. 2. In situ hybridization of ribosomal (p11a-2) and CAD (p102)-specific probes to metaphase chromosomes of PALA-resistant mutants B5-4 and B5-3. (a) Metaphase spreads were prepared from cell line B5-4 and were either banded by the trypsin-Giemsa method (upper row) or hybridized with ^{3}H-labeled p11a-2 (lower row) prepared by nick translation as described in the text. This cell line has a number of chromosome rearrangements, as we reported previously (38). (b) The upper panel shows the pattern of hybridization of the ^{3}H-labeled CAD-specific probe p102 to the chromosomes containing amplified CAD genes in the PALA-resistant mutant B5-3, and the lower panel shows the pattern of hybridization with probe p11a-2 to chromosomes isolated from the same mutant. In all panels, representative hybridization patterns were chosen from the 12 spreads examined for each experiment. "U" in panel a indicates unidentified rearranged chromosomes.

apparent over the normal B9 homolog and over the telomeric regions of the short arms of other chromosomes known to contain active nucleolus organizers in Syrian hamster cells (chromosomes 8, 13, and two D-group chromosomes [4]). The hybridization seen over chromosomes B6, B5, and C-12 and possibly over C14 may indicate that these chromosomes contain ribosomal genes but not active nucleolus organizers since they do not stain by the ammoniacal silver method (4).

The results described above demonstrate that rDNA sequences are coamplified throughout the entire length of the region of chromosome B9 which contains amplified CAD genes. To ascertain whether rDNA is coamplified in other PALA-resistant mutants, we analyzed metaphase spreads obtained from the independently isolated mutant B5-3. This mutant also contains approximately 100 CAD genes, but they are distributed between two chromosomes; 90 CAD genes are in chromosome B9p$^+$, and 10 CAD genes are in an extension of the long arm of chromosome C11 (C11q$^+$ [38]). Twelve metaphase spreads that were hybridized with probe p11a-2 were analyzed in detail. Figure 2b (bottom panel) shows the B9p$^+$ and C11q$^+$ chromosomes from three representative metaphase spreads. All 12 metaphase spreads contained B9p$^+$ and C11q$^+$ chromosomes which gave hybridization results equivalent to those shown in Fig. 2b. These results show that CAD and rDNA genes are also coamplified in mutant B5-3. The coexistence of rDNA sequences and CAD sequences in the expanded region of chromosome C11q$^+$, which is due to a translocation from chromosome B9p$^+$ (38), indicates that the CAD and rDNA sequences are tightly linked within units of CAD gene amplification.

The top panel in Fig. 2b shows the B9p$^+$ and C11q$^+$ chromosomes from a B5-3 metaphase spread which was hybridized with the CAD-specific probe p102 (22) under the conditions used for the chromosomes shown in the bottom panel of Fig. 2b. The intensity of the hybridization signal and the silver grain deposition patterns are virtually indistinguishable for the CAD and ribosomal probes. In chromosomes with less ribosomal probe hybridization, an accumulation of grains is often visible in the region which was formerly the terminus of the B9 short arm (e.g., see B9p$^+$ in Fig. 2a). This observation indicates that in the homolog with amplified CAD genes, some of the rDNA genes of the nucleolus organizer are apparently still present in their normal location at the terminus of the short arm of chromosome B9. Dense grain clusters indicating local accumulation of many genes have not been observed at either the distal end of B9p$^+$ or along the length of the amplified region. Rather, the grain distribution pattern indicates that rDNA sequences are dispersed uniformly throughout the region containing the amplified CAD genes.

Restriction enzyme analysis of rDNA sequences in PALA-sensitive and PALA-resistant cells. Since the CAD and ribosomal probes used in these experiments are approximately the same size and produce approximately the same amount of hybridization over the length of the amplified region, we infer that the number of ribosomal sequences in the amplified region approximates the number of CAD genes in mutants B5-4 and B5-3. Thus, these cell lines have roughly 100 more rDNA sequences than the PALA-sensitive BHK-21/13 cells from which they were selected. Since BHK-21/13 cells have approximately 350 to 400 rDNA genes (G. M. Wahl, unpublished data), the PALA-resistant mutants should have no more than a 25 to 30% increase in rDNA copy number above the parental cell level (i.e., they should have 450 to 500 rDNA genes). Such small differences are difficult to demonstrate convincingly by using DNA blotting technology. However, the existence of restriction site polymorphisms in a fraction of the rDNA genes enables them to be distinguished readily from the remainder of the nonpolymorphic rDNA genes (34). If the amplified rDNA sequences contain a restriction site polymorphism which was initially infrequent, it should be possible to identify an increase in the abundance of the corresponding restriction fragments in PALA-resistant cell DNA with greater accuracy since their initial level would be low.

The results obtained in a DNA blotting experiment with restriction endonuclease *Bam*HI is shown in Fig. 3b and c. The 5.1-kb band reproducibly shows increased hybridization in the PALA-resistant mutants. This band is a doublet, as indicated by the map of the Syrian hamster ribosomal repeat shown in Fig. 3a. One fragment in the 5.1-kb doublet arises from rDNA repeats which lack a polymorphic *Bam*HI site near the 5′ end of the 28S rRNA coding sequences. The other fragment in the 5.1-kb doublet arises from cleavage between nonpolymorphic *Bam*HI sites located at the 3′ end of the 28S rRNA coding unit and the adjacent external spacer region (see Fig. 3a). *Bam*HI cleavage of those repeats which contain the polymorphic *Bam*HI site near the 5′ end of the 28S rRNA coding sequence generates fragments approximately 1.4 and 3.7 kb long. Another *Bam*HI fragment approximately 1.3 kb long is generated by cleavage between the remaining two *Bam*HI sites within the 28S rRNA coding sequence (see Fig. 3a). Probe 11a-2 hybridizes with all of these fragments since it spans sequences from the 3′ end of the 18S rRNA gene to the 3′ end of the 28S rRNA gene. Note that

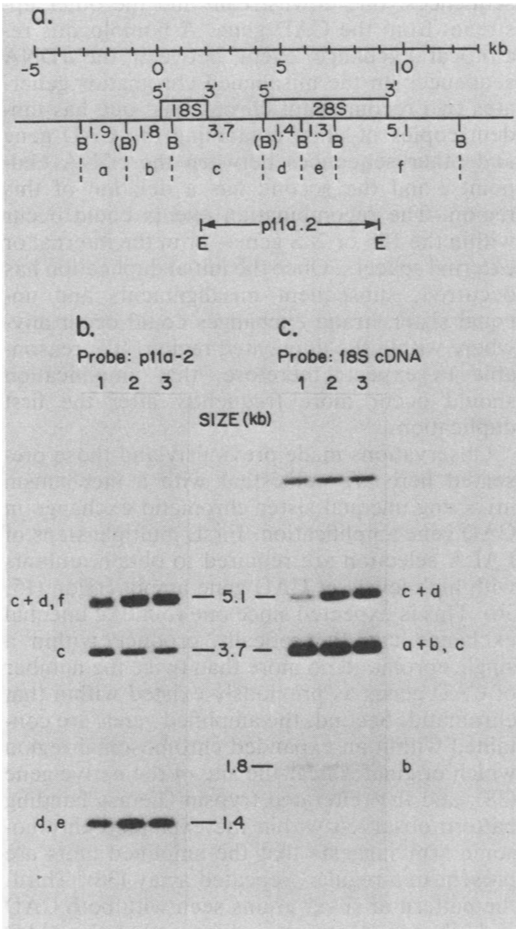

FIG. 3. Hybridization of rRNA-specific probes with *Bam*HI-digested DNA from PALA-sensitive and PALA-resistant cells. (a) Map of the *Bam*HI sites (indicated by a to f) in the ribosomal repeat of Syrian hamster cells indicating the sizes (in kb) of the fragments relevant to these studies (the map was adapted from Triezenberg et al. [34] with permission from W. Folk) and the positions of the polymorphic sites (B). The position of the *Eco*RI fragment contained in plasmid p11a-2 is indicated. (b) DNA (5 μg) from PALA-sensitive BHK-21/13 cells (lane 1) and from PALA-resistant mutants B5-3 and B5-4 (lanes 2 and 3, respectively) was digested with *Bam*HI, fractionated on a 0.7% agarose gel, transferred to nitrocellulose filters, and hybridized with the indicated probes as described in the text. The autoradiographic exposure was for 2 h at −70°C with a Lightning-Plus screen. (c) The blot shown in panel b was washed, reexposed to XR-5 film for 24 h to insure that no signal remained, and then rehybridized under the same conditions with a probe made by reverse transcription of gel-purified 18S rRNA in the presence of random oliodeoxynucleotide primers as described in the text. The exposure was for 6 h at −70°C. The letters a to f in panels b and c represent the corresponding *Bam*HI fragments indicated in panel a.

the 3.7-kb band in the BHK-21/13 DNA is at least as intense as it is in the B5-3 and B5-4 DNA samples (compare, in Fig. 3b, lane 1 with lanes 2 and 3). This internal control establishes that at least as much DNA was transferred and hybridized in all samples. Thus, differences in hybridization intensities must reflect differences in the abundance of the indicated sequences in the mutant cell DNA.

Although the hybridization results with probe p11a-2 consistently demonstrated small increases in the abundance of the 5.1-kb band in the mutant DNA, the true extent of the increase is obscured by the hybridization to the nonpolymorphic sequence of the same size which comes from the 3′ end of the 28S rRNA gene. The contribution of this sequence to the hybridization signal can be eliminated by using a probe which is specific for the polymorphic region. Since the genomic probe derived from the polymorphic region contains repetitive elements, a probe was prepared from gel-purified 18S rRNA. The pattern of hybridization of this probe with the same blot used in Fig. 3b is shown in Fig. 3c. Two differences are readily apparent. First, there is a fivefold increase in the abundance of the 5.1-kb band in the PALA-resistant cell DNA (determined by densitometry). Second, the 5.1-kb band is more discrete than that shown in Fig. 3b, substantiating the idea that this region contains two fragments with rDNA sequences. These results show that some rDNA genes with the indicated *Bam*HI polymorphism are amplified in the two PALA-resistant mutants studied.

A densitometric analysis revealed that the polymorphic *Bam*HI site is absent in 10% of the rDNA repeats of the wild-type BHK-21/13 cells used in these experiments (the ratio of the intensities of the 3.7- and 5.1-kb bands is 9.3:1). This estimate agrees well with that made by Triezenberg et al. (34). Since there are 350 to 400 rDNA genes in the PALA-sensitive cells, the blotting analysis indicates that the number of rDNA genes without the *Bam*HI polymorphic site increases from 35 to 40 in the BHK-21/13 cells to 175 to 200 in mutants B5-3 and B5-4. The net increase of approximately 150 rDNA genes arrived at by blotting analysis is thus in agreement with the estimate made by in situ hybridization.

DISCUSSION

We have used two independent methods, in situ hybridization and DNA restriction analysis, to determine whether rDNA sequences are coamplified with CAD genes in PALA-resistant mutants of Syrian hamster BHK-21/13 cells. This analysis was undertaken because our previous studies demonstrated that the wild-type

CAD gene is adjacent to a nucleolus organizer and that the amplified genes extend outward from a site near the wild-type gene (38). The results of both the in situ hybridization and restriction analyses reported in this paper demonstrate that rDNA genes do coamplify with CAD genes in the two mutants that were analyzed. The coamplified rDNA and CAD genes are inferred to be tightly linked since both genes translocate together to the tip of another chromosome in one of the mutants that was studied (mutant B5-3). We estimate that an average of one or two rDNA genes coamplify per CAD gene in the mutants studied.

Observations in diverse organisms indicate that unequal reciprocal recombination between ribosomal sequences can be a frequent event which results in gene duplication. For example, Anderson and Roth (2) have found that the highest frequency of spontaneous duplications in *Salmonella typhimurium* occurs in the *purD-purH* region, which is bounded by three rDNA cistrons. Duplications in this region are harbored by as much as 3% of the population. Hill and co-workers (13) demonstrated directly that unequal exchange between adjacent rRNA cistrons mediates the duplication of the *glyT-purD* region in *E. coli*. In mitotically dividing yeast cells, *leu* genes inserted into the rDNA repeat unit duplicate spontaneously with high frequency by unequal crossing over between flanking rDNA genes (29). Magnification of rDNA genes in *Drosophila melanogaster* bobbed mutants apparently takes place by unequal mitotic exchange as well (31). The variability in the number of rDNA repeats on different human chromosomes within the same individual and between individuals has been interpreted in terms of frequent duplication and deletion of rDNA repeating units (16). Unequal sister chromatid exchange has been used to explain the observation that nucleolus organizers in human chromosomes often contain multiple copies of a limited set of the known polymorphic rDNA genes (i.e., all of the known polymorphisms have never been observed on a single chromosome [16]). Spontaneous, intrachromosomal expansion of rDNA repeats has also been reported in rat hepatoma cells (30), indicating that mitotic mammalian cells can amplify rDNA genes. The mechanism for rDNA amplification in the hepatoma cells has not been elucidated.

In light of the results described above, the coamplification of rDNA and CAD genes raises the possibility that the rDNA sequences near the CAD gene also mediate its amplification by unequal sister chromatid exchange. A simplified view of this model (see Fig. 4) is that an occasional misalignment of sister chromatids during mitosis results in the pairing of two rDNA sequences, one downstream and the other upstream from the CAD gene. A homologous reciprocal exchange event between the rDNA sequences on the misaligned chromatids generates two recombinant chromatids; one has tandem copies of units containing the CAD gene and other sequences between the rDNA endpoints, and the second has a deletion of this region. The recombination events could occur within the 18S or 28S genes, or in the internal or external spacers. Once the initial duplication has occurred, subsequent misalignments and unequal sister strand exchanges could occur anywhere within the duplicated region. It is reasonable to expect, therefore, that amplification should occur more frequently after the first duplication.

Observations made previously and those presented here are consistent with a mechanism involving unequal sister chromatid exchange in CAD gene amplification. First, multiple steps of PALA selection are required to obtain mutants with high levels of CAD gene amplification (15, 36). This is expected since one round of unequal exchange can theoretically produce within a single chromatid no more than twice the number of CAD genes as previously existed within that chromatid. Second, the amplified genes are contained within an expanded chromosomal region which originates near the site of the native gene (38), and the reiterated trypsin-Giemsa banding pattern observed within the expanded chromosome arm suggests that the amplified units are present in a regular, repeated array (38). Third, the pattern of silver grains seen with both CAD and ribosomal probes indicates that the CAD and ribosomal genes are evenly distributed along the length of the amplified region and are not present in large numbers in one or a few discrete regions. Although it has been found that some units of amplification are heterogeneous with regard to sequence composition (F. Ardeshir, O. Breson, J. Zieg, and G. Stark, personal communication), such heterogeneity may reflect the result of rearrangements which have occurred during the multiple rounds of selection and the hundreds of generations the cells have experienced before their analysis rather than reflecting the initial composition of each amplified unit.

Although the data and examples presented above make a strong case for the direct involvement of rDNA genes in CAD gene amplification, the observed stability of the amplified CAD genes (15) is unexpected if the proposed unequal exchange process is freely reversible. In fact, cells which have lost CAD genes are expected eventually to overtake the population in the absence of selection since such cells generally have a slight growth advantage (15). Thus, it is possible that either (i) the generation and remov-

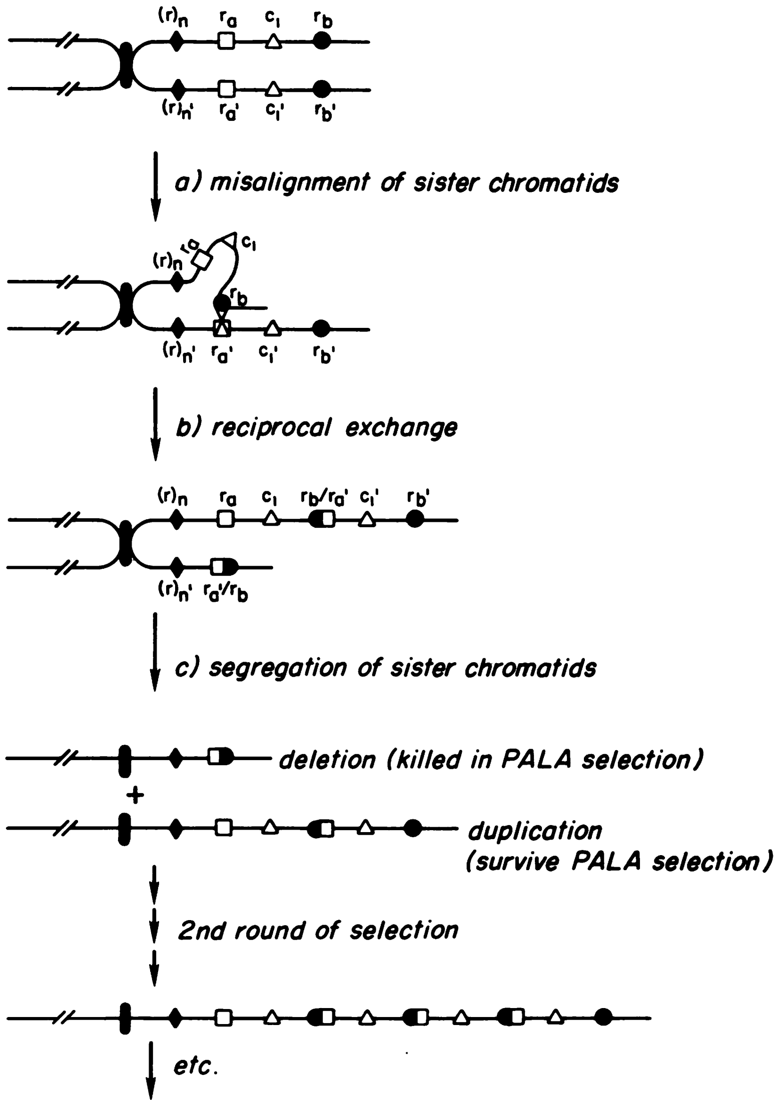

FIG. 4. Model for the amplification of CAD genes by unequal exchanges between rDNA sequences on sister chromatids during mitosis. The symbols are as follows: $(r)_n$ (◆) is a cluster of rDNA genes in the nucleolus organizer; r_a (□) and r_b (●) are ribosomal sequences which flank the CAD gene (C_1[△]). Symbols with a "prime" have the same meaning as described above, but they indicate that the sequences are on the sister chromatid. The model is presented with a cluster of ribosomal genes centromere-proximal to the CAD gene to be consistent with the in situ hybridization results presented in Fig. 2. Note that either r_a or r_b (or both) should contain the *Bam*HI polymorphism described in the text and shown in Fig. 3. One of these sequences may not be a part of the nucleolus organizer as described in the text. The proposed unequal exchanges could take place within the 18S or 28S rDNA genes, or in the internal or external spacers. The symbol ●□ indicates that a novel joint was formed by recombination between rDNA genes on sister chromatids. Subsequent rounds of unequal exchanges can generate chromatids with odd or even numbers of amplified CAD genes, depending on where the unequal exchange occurs.

al of amplified sequences by unequal exchanges occur at different rates, or (ii) the simple unequal exchange model described above is not entirely correct. One point that is noteworthy in this regard is that in contrast to some tandem gene arrays in *E. coli* (1), cloned DNA introduced as tandem repeats in mammalian cell chromosomes can be very stable (9; G. M. Wahl, unpublished data).

One observation which remains puzzling is that units of CAD gene amplification are 500 to 1,000 kilobase pairs long. In the context of the unequal exchange model, it is conceivable that this distance reflects the separation between two independent ribosomal sequences which flank the CAD gene. It is interesting in this regard that rDNA genes 250 kilobase pairs apart have been shown to mediate duplications in bacteria (13). Alternatively, the proposed unequal exchanges may take place between an rDNA gene associated with a cluster of rDNA genes (e.g., a nucleolus organizer) and another rDNA sequence located elsewhere on the B9 chromosome (3).

The data we have presented are consistent with, but do not prove, the hypothesis that unequal sister chromatid exchange mediated by rDNA genes leads to CAD gene amplification in the two mutants analyzed. Other PALA-resistant mutants which have suffered rearrangements of sequences near the CAD gene may require other sequences or different mechanisms for amplifying their CAD genes. Proof for the unequal exchange model will require finding ribosomal sequences at the ends of the amplification units and demonstrating that such terminal rDNA sequences have the novel joints which are expected to be generated by an unequal exchange event (see reference 1 and Fig. 4). In the absence of such information, it is important to point out that our data are also consistent with the idea that one or a few rDNA genes are coamplified as passengers with CAD genes, like the majority of the other sequences within each large amplification unit. It is equally important to emphasize that all of the data concerning CAD gene amplification, when taken together, are not easily reconciled with alternative models which involve only saltatory or disproportionate replication, or extrachromosomal sequence amplification (see reference 26).

Our results emphasize the potential importance of unequal sister chromatid exchange as one mechanism for modulating gene copy number in mammalian cells. The generation of triplicated α-globin loci in humans (10) and of recombinant chromosomes with adjacent T-locus alleles in mice (27) has also been interpreted in terms of unequal exchange models. Since repetitive sequences of similar homology are abundant in eucaryotic genomes, there are many potential sites in addition to the rDNA sequences reported here where sister chromatid exchanges could occur. Hence, there are likely to be many chromosomal regions in which unequal exchange between homologous or nearly homologous sequences may be an essential part of the amplification process.

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LITERATURE CITED

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