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Properties of Single-Step Mutants of Syrian Hamster Cell Lines Resistant to N-(Phosphonacetyl)-L-Aspartate

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Eleven independent lines of Syrian hamster cells were selected by using very low levels of *N*-(phosphonacetyl)-L-aspartate (PALA), an inhibitor of aspartate transcarbamylase. The protocol employed insured that each resistant cell arose during one of the last divisions before selection was applied. Cells of each mutant line contained an amplification of the structural gene for CAD, a trifunctional protein which includes aspartate transcarbamylase and two other enzymes of UMP biosynthesis. Strikingly, despite the minimal selection employed, the degree of amplification of the CAD gene was 6 to 10 times the normal diploid number in all 11 cases. In situ hybridization indicated that the amplified CAD genes were almost always present at a single chromosomal site in each line. Therefore, one of the two alleles was amplified 11- to 19-fold. The rates at which cells became resistant to PALA, determined by fluctuation analysis, were 100 times less dependent on drug concentration than were the frequencies of resistant cells in steady-state populations. The relatively shallow dependence of this rate upon PALA concentration is consistent with our independent observation that most events gave rise to a similar degree of amplification. In six of six cell lines examined, the levels of CAD mRNA and aspartate transcarbamylase activity were elevated two- to fourfold. These lines were resistant to PALA concentrations 20- to 80-fold higher than the ones used for selection. The organization of amplified DNA was examined by hybridizing Southern blots with cloned DNA fragments containing amplified sequences, previously isolated from two cell lines resistant to high levels of PALA. A contiguous region of DNA approximately 44 kilobases long which included the CAD gene was amplified in five of five single-step mutants examined. Outside this region, these mutants shared amplified sequences with only one of the two highly resistant lines.

Gene amplification is a major mechanism through which mammalian cells (2, 12, 21) and microorganisms (9) achieve resistance to a variety of anti-proliferative agents. Amplification also plays a role in normal development when there is need for very rapid synthesis of RNA, i.e., rRNAs in *Xenopus laevis* oocytes (5) and mRNAs for chorion proteins in *Drosophila melanogaster* follicle cells (19). Although the mechanisms differ greatly in each of these systems, detailed analyses of each may reveal common features.

N-(Phosphonacetyl)-L-aspartate (PALA) potently and specifically inhibits the aspartate transcarbamylase activity of the trifunctional protein CAD, which catalyzes the first steps of UMP biosynthesis (7, 20). When challenged with PALA in culture, resistant Syrian hamster cells can be selected which have elevated levels of CAD protein (13), of the corresponding mRNA (16), and of the gene coding for CAD (21). By analyzing the organization of the amplified DNA, we hope to understand more about the mechanism of amplification in this system.

In the accompanying paper (1), we show that the arrangement of amplified DNA is complex in mutant cell lines selected in several steps for resistance to very high concentrations of PALA. These lines have experienced several independent amplification events, and it is likely that new novel joints, through which amplified regions are linked, were formed during each event. Ardeshir et al. (1) also show that two different regions of DNA may be amplified to different extents in the same mutant cell line and that different mutant cell lines may have amplified some regions of DNA which are entirely different. There is a strong suggestion that extensive rearrangement or movement of DNA accompanies amplification. In view of these complica-

tions for the multiple step mutants, we have decided to examine the structure of amplified DNA in simpler mutants which have undergone only a single step of selection, since their amplified DNA may be much more amenable to detailed analysis. We now describe a selection scheme designed to isolate single-step mutants and an initial characterization of their properties.

MATERIALS AND METHODS

Cells, cell culture, and selections. All cells were grown as described previously (20). A clone designated WT-BHK-1 was derived from BHK-21/13 cells (21) and was used to select six of the single-step mutants. The concentration of PALA which allowed a half-maximal increase in the number of WT-BHK-1 cells in 72 h was 2.5 μM. This analysis was performed as described by Kempe et al. (13). Since a very large number of colonies was obtained at 2.5 μM PALA, selections were performed at 5, 10, and 20 μM. Colonies of resistant cells were picked after 20 days in PALA and expanded into populations by growth for about 2 months more in the continued presence of the drug.

The parental population of BHK-21/13 cells (WT-BHK-1) and the six PALA-resistant mutant lines derived from it were found to be infected with mycoplasma at a low level, using an assay based on staining in the cytoplasm with the DNA-specific fluorescent dye Hoechst 33258. To be sure that the infection played no role in the selection or properties of resistant cells, a mycoplasma-free line was derived from WT-BHK-1 by growing the cells for 48 h at 41°C, 48 h at 37°C, and 48 h at 41°C, followed by cloning of single cells (10; F. Calvo Riera, unpublished data). A mycoplasma-free clone, WT-BHK-2, was used for selecting the five additional single-step mutants. WT-BHK-2 and the resistant lines derived from it have remained free of mycoplasma contamination. The concentration of PALA which allowed a half-maximal increase in the number of WT-BHK-2 cells in 72 h was 6 μM. Again, selection at this concentration gave a very large number of colonies, so selections were performed at 12, 16, 20, and 40 μM PALA. Colonies of resistant cells were picked after approximately 3 weeks and grown to ca. 2×10^7 cells in the presence of PALA before the cells were frozen. For assays of gene copy number and in situ hybridizations, frozen cells were thawed and grown for a short time in the absence of PALA.

Fluctuation analyses. Fluctuation analysis experiments were performed according to Luria and Delbrück (15). One thousand WT-BHK-2 cells were seeded onto 20 10-cm plates for each concentration of PALA. The cells were allowed to grow for approximately 10 days until confluent (ca. 10^7 cells per plate). A suspension of cells from each plate was prepared with trypsin and EDTA, and 2×10^5 cells from each sample were seeded onto fresh 10-cm plates. The cells were allowed to attach for 4 h before PALA was added. For the steady-state experiments, 1×10^3 to 4×10^5 cells from a mass culture were challenged with PALA in the same manner. The medium was changed once, after 5 days. After approximately 10 days, the colonies (ca. 0.5 mm in diameter) were stained with 0.1% bromphenol blue in methanol and counted. The mean, variance, and mutation rate based on the mean were calculated according to Lea and Coulson (14).

Nucleic acids. It was important to prepare RNA from actively growing, subconfluent cells, since we have observed that the level of CAD mRNA is decreased substantially in confluent cells (W. S. L. Liao and G. R. Stark, unpublished data). Total RNA was extracted from subconfluent cells after lysis with guanidinium thiocyanate (6). CAD mRNA was quantitated by a modification (P. C. Brown and W. S. L. Liao, unpublished data) of the dot hybridization procedure of Kafatos et al. (11). Amounts of CAD mRNA and of dihydrofolate reductase mRNA (as an internal standard) were estimated by scanning autoradiograms with a densitometer. Similar results were obtained when the RNAs were separated by electrophoresis in an agarose gel before transfer and hybridization. High-molecular-weight DNA was prepared by the method of Brison et al. (4). DNA samples were treated twice with RNase and proteinase K and extracted twice with phenol to remove RNA and protein contaminants before their concentrations were determined by measuring the absorbancy. DNA digested with *Eco*RI endonuclease (kindly provided by J. Carlson) and glyoxal-treated RNA were fractionated by electrophoresis in agarose gels and stained with ethidium bromide (21). The intensities of the stained bands were compared to insure that equal amounts had been loaded on each track. DNA and RNA were transferred to diazotized *o*-aminophenylthioether paper (18). Probe DNAs were labeled with ^{32}P to a specific activity of 1×10^8 cpm/μg or higher (17). The hybridization conditions described by Brison et al. (4) were used. Amounts of DNA were compared by matching bands of equal intensity, obtained by varying the times of exposure (Table 1) or by determining the intensity of each band by using a densitometer (Table 2). In each case, preflashed film was used, and care was taken to use the linear range of the film.

Aspartate transcarbamylase assays. [^{14}C]carbamyl phosphate (10.4 mCi/mmol, New England Nuclear Corp.) was diluted to a specific activity of 0.5 mCi/mmol as described by Davies et al. (8). We modified the assay of Kempe et al. (13). Cells from subconfluent plates were washed in TD buffer (25 mM Tris-chloride [pH 7.4]–137 mM NaCl–5 mM KCl–0.6 mM sodium phosphate) and detached in 0.1 mM EDTA in TD buffer. After brief centrifugation at low speed, the cell pellet was suspended in ice-cold 50 mM Tris-chloride (pH 7.5)–30% dimethyl sulfoxide–5% glycerol, using a final volume of 1 ml per 10-cm plate. The cells were disrupted twice by sonication at low power for 20 s each at 4°C. The homogenate was centrifuged at $12{,}000 \times g$ for 30 min at 4°C, and samples of the supernatant solution were stored at −80°C. Assays were done in 1 ml of 20 mM Tris-hydrochloride (pH 8)–15 mM potassium aspartate. Samples were preincubated at 37°C for approximately 2 min before the reaction was started by adding 0.015 μCi of [^{14}C]carbamyl phosphate, 0.5 mCi/mmol. After 15 min at 37°C, 200 μl of 50% acetic acid was added, and unreacted [^{14}C]carbamyl phosphate was removed as $^{14}CO_2$ at 100°C in a hood. The protein concentration in each cell extract was determined by the method of Bradford (3).

Karyotyping and in situ hybridization. CAD genes were localized on chromosomes by in situ hybridiza-

tion as described by Wahl et al. (22), with minor modifications. To reduce cytoplasmic contamination, cell suspensions were allowed to soak in fixative for 45 min before the final centrifugation and spreading. The probe used was pCAD 142, a 6.5-kilobase (kb) CAD cDNA clone (K. Shigesada and G. R. Stark, unpublished data) labeled by nick translation (17) to a specific activity of 2×10^7 to 4×10^7 cpm/μg with [^{3}H]dTTP (100 Ci/mmol, Amersham Corp.). Pretreatment with acetic anhydride was unnecessary, and unlabeled iododeoxycytidine was omitted from the hybridization. The number of washes in 2× SSC was reduced to two, followed by two washes in 2× SSC containing 0.1% sodium pyrophosphate (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate). Results were obtained from 40 spreads which had significant labeling (more than three grains in one place) and low backgrounds. Spreads were not used if they had grains on overlapping chromosomes or if more than four chromosomes were overlapping. Chromosomes were banded by a combination of the trypsin-Giemsa and Wright procedures. Fresh spreads were left at room temperature for 10 to 15 days and then were pretreated for 15 to 45 s with trypsin, prepared as by Wahl et al. (22), followed by treatment with Wright stain for 1.5 min.

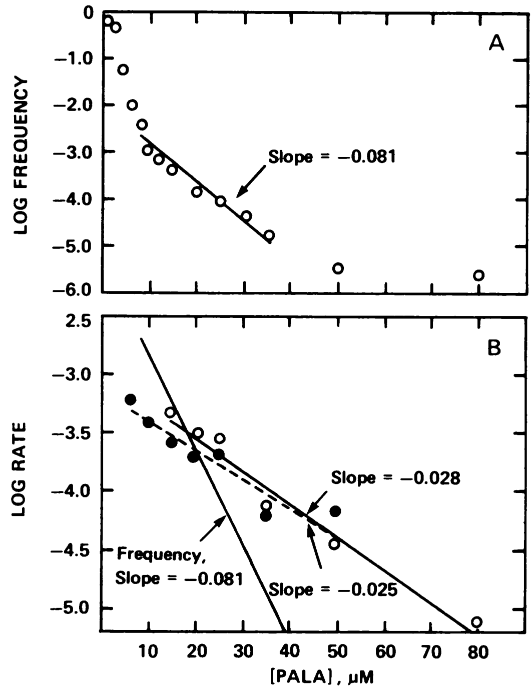


FIG. 1. Frequencies and rates of resistance as a function of PALA concentration. The points were fitted to straight lines by linear regression. The straight-line portion of the curve in panel A is reproduced in panel B. In panel B, two independent experiments are shown with different symbols.

RESULTS

Isolation of "single-step" mutants. Our intention was to require a cell to amplify the CAD gene minimally to achieve resistance. Mutants which arose only one or a few cell divisions before PALA was added were selected by using a scheme based on the Luria-Delbrück fluctuation analysis (15). Thirty samples of 1×10^3 WT-BHK-1 cells each were grown in nonselective medium for 10 generations. The cells in each plate were dispersed with trypsin, and a portion of each suspension corresponding to 1×10^5 cells was placed onto a fresh plate. PALA was added at 5, 10, or 20 μM, 10 plates at each concentration. When concentrations of PALA lower than 2.5 μM were used, the cells grew to confluence. After 20 days of selective growth, colonies were isolated only from those plates which contained no more than four colonies per plate. If a resistant cell were present in an original sample of 1×10^3 cells, or if it arose early during nonselective growth, the many mutant cells present after 10 generations would have given rise to many colonies upon dispersal and subsequent selection. Seven of the 30 samples represented such a situation: 4 at 5 μM PALA, 2 at 10 μM PALA, and 1 at 20 μM PALA. However, if resistance arose just before PALA was applied, only a few colonies would be present after dispersal and selection. Six independent PALA-resistant mutants were obtained from plates with four or fewer colonies: two at 5 μM PALA (SS5-1 and SS5-3), three at 10 μM PALA (SS10-1, SS10-2, and SS10-4), and one at 20 μM PALA (SS20-2). The remaining plates had no resistant colonies. Selection of five more independent mutants from WT-BHK-2 was done similarly, but with a higher range of PALA concentrations (12, 16, 20, and 40 μM) to take account of the fact that WT-BHK-2 is less sensitive to PALA than is WT-BHK-1.

Rates and frequencies of resistance as a function of PALA concentration. Experiments on resistance as a function of PALA concentration were done with WT-BHK-2. The fraction of cells resistant to PALA (frequency) varied from approximately 10^{-1} at 4 μM PALA to nearly 10^{-6} at 80 μM PALA (Fig. 1A). The data were not corrected for plating efficiency, 63% in this experiment. At 6 μM, the concentration of PALA which allowed a half-maximal increase in the number of WT-BHK-2 cells in 72 h, the frequency was 10^{-2}. It is not clear that amplification was responsible for resistance at PALA concentrations as low as 6 μM, but every resistant mutant tested that was obtained at 12 μM PALA or more contained amplified CAD genes. Note that the data between 10 and 35 μM fit a straight line in a semilog plot. A similar experiment performed with WT-BHK-1 also gave a straight line with a very similar slope but displaced toward lower PALA concentrations, as

TABLE 1. Relative amounts of CAD genes, CAD mRNA, and aspartate transcarbamylase activity in PALA-resistant cells derived from WT-BHK-1 (also shown are the concentrations of PALA which reduced the growth rates by one-half during 72 h)

Cells	Concn of PALA for selection (μM)	Concn of PALA for half-maximal growth (μM)	Relative amt of:		
			CAD DNA[a]	CAD mRNA[b]	ATCase[c]
WT-BHK-1		2.5	1	1	1.0
SS5-1	5	30	6	1.4	1.9 ± 0.2
SS5-3	5	110	8	3.6	2.3 ± 0 4
SS10-1	10	190	8	3.8	3.1 ± 0.2
SS10-2	10	52	7	ND	2.9 ± 0.2
SS10-4	10	62	8	2.8	3.0 ± 0.2
SS20-2	20	200	10	4.3	3.3 ± 0.5

[a] Values are based on relative intensities of bands in DNA blot hybridization experiments, estimated by matching different exposures.
[b] Values are based on relative intensities of bands in RNA blot hybridization experiments, estimated with a densitometer. ND, Not determined.
[c] Average of three experiments, normalized to WT-BHK-1.

expected from the greater sensitivity of WT-BHK-1 to the drug (data not shown; thanks to Anna Karlin for performing the experiment).

The number of resistant colonies formed per cell per generation (rate of amplification), determined according to Luria and Delbrück (15) and Lea and Coulson (14), was also a function of PALA concentration (Fig. 1B). The results of two independent experiments, plotted separately, were quite similar. The rate had a much shallower dependence on PALA concentrations than did the frequency. The slopes of the semilog plots of rate and frequency differed by a factor of approximately 3, meaning that an increase in PALA concentration which caused the rate to decrease by 10-fold caused the frequency to decrease by 1,000-fold. The significance of these data is discussed below. The fluctuation data used to calculate the rates (14, 15) were also used to calculate a ratio of the variance to the mean at each concentration of drug. The average of 10 such ratios was 30 ± 7 (standard deviation). Three values (6, 5, 53) which were more than two standard deviations from the average were excluded. Clearly, the ratio of the variance to the mean was not 1, indicating that the mutations arose spontaneously (15), in agreement with our previous conclusion for SV28 cells at a single concentration of PALA (13). The ratio did not depend upon the concentration of PALA.

Levels of CAD DNA, mRNA, and enzyme activity in single-step mutants. To determine the relative CAD gene copy number in the mutant cell lines, genomic DNA was prepared from each, as well as from the two parental WT-BHK lines. The DNAs were digested with *Eco*RI, fractionated by gel electrophoresis, transferred to paper, and probed with ^{32}P-labeled CAD cDNA. As shown in Fig. 2 of Ardeshir et al. (1), the relative intensities of hybridization indicated that the gene copy number was increased 6- to 10-fold in the six single-step mutants derived from WT-BHK-1. The results are summarized in Table 1. A similar experiment was performed with each of the mutants derived from WT-BHK-2 (Table 2).

The mRNA levels of the six single-step lines derived from WT-BHK-1 were increased by two- to fourfold, and the aspartate transcarbamylase levels were increased by two- to threefold relative to WT-BHK-1 (Table 1). Thus, the increases in mRNA levels and enzyme activities were smaller than the increases in gene copy number. Note also that all of the mutants listed in Table 1 resisted concentrations of PALA far higher than those used for selection.

Localization of amplified CAD genes by in situ hybridization. Both parental WT-BHK cells were heteroploid, and we could not assign specific chromosomal alterations to the drug-resistant mutants. None of the resistant cells had

TABLE 2. Relative amounts of CAD genes in PALA-resistant cells derived from WT-BHK-2[a]

Cells	Concn of PALA for selection (μM)	CAD DNA[b]
WT-BHK-2		1
SS12-1	12	6
SS16-1	16	10
SS20-3	20	6
SS20-4	20	8
SS40-1	40	10

[a] The concentration of PALA which reduced the growth rate of WT-BHK-2 by one-half during 72 h was 6 μM.
[b] Values are based on relative intensities of bands in DNA blot hybridization experiments, estimated with a densitometer.

double-minute chromosomes. The CAD gene has been localized by in situ hybridization to the short arm of chromosome B9 in wild-type BHK-21/13 cells (22). Several lines selected in multiple steps to high resistance had grains over most of the long arm of an abnormally long chromosome (22). An example for the line B5-3 is shown in Fig. 2A. For all of the mutants derived from WT-BHK-1, grains were usually found in one location, but occasionally two were seen. In five of the six lines, the grains were over an extremely long abnormal chromosome. Cell line SS5-1 was approximately tetraploid, and approximately 80% of the spreads showed labeling along a long metacentric chromosome (Fig. 2C). A few spreads had additional grains over a small telocentric chromosome or had lost the long chromosome and gained two extra copies of the CAD-containing telocentric chromosome (Fig. 2D). SS5-3 had a highly variable karyotype, with 55 to 80 chromosomes, but consistent labeling was seen on the long arm of an acrocentric chromosome similar to B8, B9, or C11 (Fig. 2B). All of the SS10 lines were pseudodiploid and carried the amplified CAD genes near a secondary constriction in the long arm of one abnormally long chromosome (Fig. 2E, G, H). Most cells of these lines had several abnormal, elongated chromosomes, as did WT-BHK-1. Occasionally there were cells with only one such abnormal chromosome; the banding pattern of a typical example of this chromosome, which probably carries the amplification, is shown in Fig. 2F. The banding does not bear much resemblance to that of B9, and neither the pattern nor the position of the grains is consistent with a simple elongation of the short arm of B9. In 10% of the spreads of SS10-2, a small metacentric chromosome was also labeled, and 25% of the spreads had a second, telocentric site instead (data not shown). SS20-2 showed two alternative labeling patterns. Half were very similar to those of the SS10 lines (Fig. 2I), and the rest had an even longer chromosome with labeling on each arm, possibly derived by fusion of two chromosomes containing amplified CAD genes (Fig. 2J).

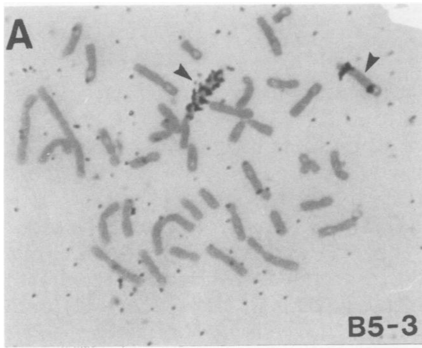
Three of the five PALA-resistant lines derived from WT-BHK-2 (Table 2) were also examined by in situ hybridization (data not shown). Again, in all three cases, grains were usually found over limited regions of a single arm of a single chromosome. SS12-1 was very similar to the SS10 lines just discussed in that the labeled chromosome was extremely long and abnormal. In SS16-1 and SS20-4, the grains were localized over one arm of a smaller metacentric or acrocentric chromosome, respectively.

Organization of amplified sequences in the single-step mutants. Amplified DNA fragments isolated from 165-28 and B5-4, mutant cell lines resistant to high concentrations of PALA, were used to probe blots of *Eco*RI-digested DNA from five of the six mutants derived from WT-BHK-1, to determine whether any of the same sequences had been amplified. Cell line 165-28, derived from SV28, has a 200-fold amplification of the CAD gene. B5-4, derived from the same BHK-21/13 cells as WT-BHK-1, has a 50-fold amplification. Approximately 44 kb of contiguous DNA including the CAD gene was amplified in all five of the single-step lines examined. Cloned amplified sequences from 165-28 outside this 44-kb region were not amplified in any of these five cell lines: see Table 2 of Ardeshir et al. (1). However, all of the cloned amplified sequences from B5-4 were amplified in four of the five mutants. SS5-1 was exceptional in that only a few such sequences from B5-4 were also amplified in this line. For example, clone 11-200 (group L, Table 2, Ardeshir et al. [1]), an amplified fragment from B5-4, was used to probe a blot prepared from the DNAs of the single-step mutants (Fig. 3). This fragment was not amplified in SS5-1, although it was in the other four mutants. These data also illustrate that, even at this early stage of gene amplification, the organization of amplified sequences in the single-step lines was not necessarily simple. Fragment 11-200 was amplified approximately 30-fold in SS5-3, which had an eightfold amplification of the CAD gene. A different extent of amplification of two fragments within the same cell line is inconsistent with an array of identical units in which each fragment is amplified to the same degree.

Novel fragments, which do not exist in the genome of the parental cell line, are generated by joining two parental sequences during amplification. For example, the 4.3-kb novel fragment from phage 9-900 (group H, Table 2, Ardeshir et al. [1]) appeared only in the DNA of 165-28 (Fig. 4). It was homologous to two wild-type fragments of 20 and 6.3 kb, which were present at single-copy levels in the DNAs of all of the single-step mutants. None of the novel fragments isolated from 165-28 or B5-4 were present in any of the single-step mutants analyzed. They were homologous instead to wild-type fragments, some of which were amplified. No novel fragments were detected in any of the single-step cell lines by homology with any amplified fragment derived from 165-28 or B5-4, novel or not.

DISCUSSION

An accompanying paper by Ardeshir et al. (1) illustrates the complex organization of amplified sequences in mutant cell lines resistant to high concentrations of PALA. Because these lines were selected in several steps, they presumably have undergone several independent events,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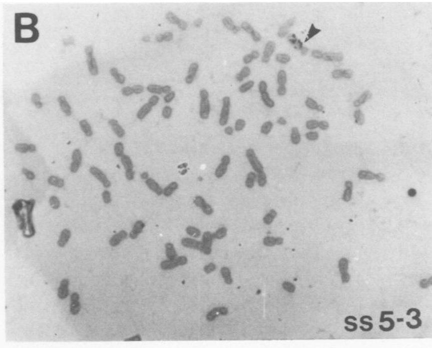
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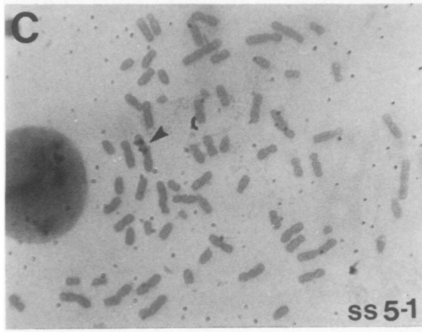
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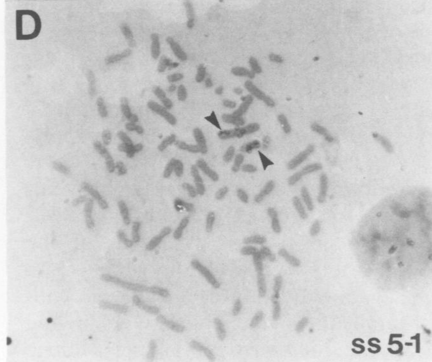
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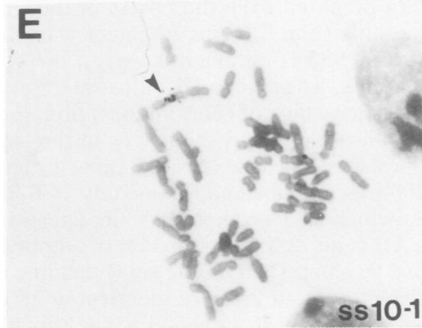
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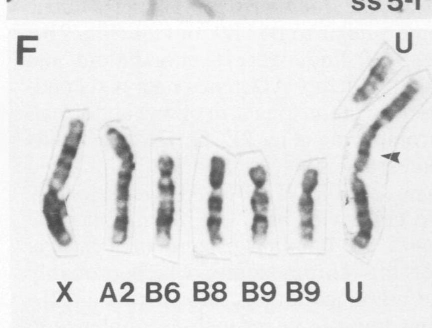
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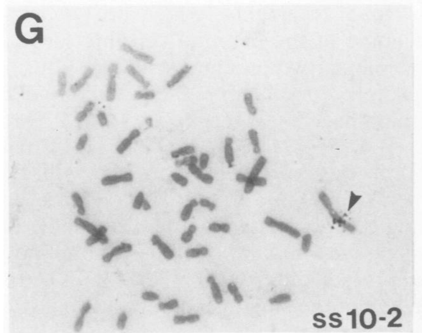
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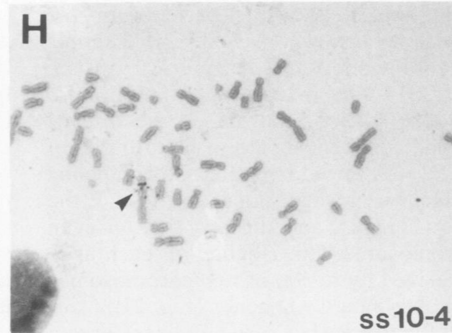
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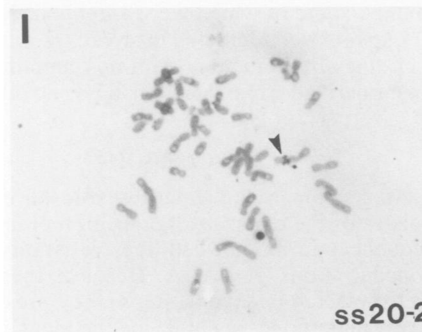
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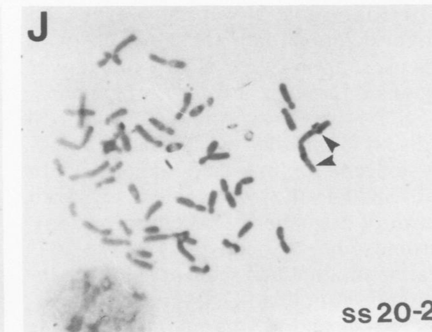
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each of which contributed to the complex arrangement of the amplified DNA. It will be necessary to study single-step mutants to understand the structure of amplified DNA in more detail. In selecting such mutants, we wanted to use as little PALA as possible, to determine the extent to which the CAD gene was amplified under minimal selective pressure, and we wanted to be sure that the amplification events were recent. The scheme used, based on the principle of the Luria-Delbrück fluctuation analysis (15), insured that each mutation arose one to three generations before selection was applied.

We were surprised to find a 6- to 10-fold amplification of the CAD gene in each of the 11 single-step lines examined. Since amplification usually occurred on only one chromosome, the gene copy number at that locus must have changed from 1 to 11 to 19. It may be that the primary event of amplification leads to such an increase in copy number most of the time. If so, this result would not be consistent with the predictions of amplification by a simple unequal sister chromatid exchange, which would give an increase of only twofold in a single cell division.

The results shown in Fig. 1 bear on this issue strongly. The slopes of the lines in Fig. 1A and B are related to the relative numbers of cells resistant to different concentrations of PALA. Consider one limiting case as an example: if each amplification event were to give an increase of exactly 10-fold in the number of CAD genes, if each amplified gene were expressed at the same level, and if that level of expression always gave the same level of resistance to PALA, then the rate of amplification shown in Fig. 1B would show no dependence on PALA concentration at all until some limiting concentration had been reached. None of these approximations are exactly true, but the relatively shallow slope of the rate curve does mean that the degree of amplification is similar in most independent events. Amplifications of 6-fold or more are probably somewhat more frequent than amplifications of 10-fold or more (see Tables 1 and 2).

The levels of aspartate transcarbamylase activity, and of the corresponding mRNA, were elevated only 2- to 4-fold in cells containing a 6- to 10-fold increase in gene copy number. We do not understand the basis of this difference between relative gene copy number and relative expression, which has been noted before (21). The resistant cell lines obtained from WT-BHK-1 were 20- to 80-fold less sensitive to PALA than were the parental cells, despite the fact that the specific aspartate transcarbamylase activity increased by only 2- to 3-fold (Table 1). This disproportionate increase in resistance, noted previously by Kempe et al. (13), is probably due in large part to the fact that CAD is a trifunc-

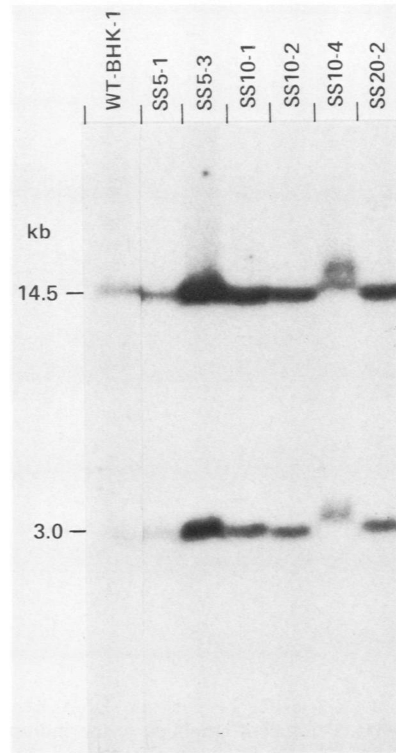

FIG. 3. Hybridization of 11-200, a fragment amplified in the multiple-step mutant B5-4 (1), to genomic DNAs from WT-BHK-1 and the six single-step mutants derived from it. Ten micrograms of *Eco*RI-digested DNA was run in each lane. The DNA of SS10-4 was not digested completely. Sizes of the bands (in kb) are shown on the left. Repetitive sequences were partially removed from the probe as described by Ardeshir et al. (1).

FIG. 2. In situ hybridization of PALA-resistant BHK lines with a CAD cDNA probe. Examples are shown of each of the six single-step lines derived from WT-BHK-1, together with B5-3, a multiple-step mutant with an approximately 50-fold amplification of CAD (22). Also shown (panel F) are selected banded chromosomes from SS10-1, including an abnormally long chromosome and another unidentified telocentric chromosome with similar bands, both marked "U." Arrows indicate sites of significant hybridization on the chromosomes and the corresponding position on the banded elongated chromosome from SS10-1. The exposure times were 1 week for B5-3 and 3 weeks for all other cell lines.

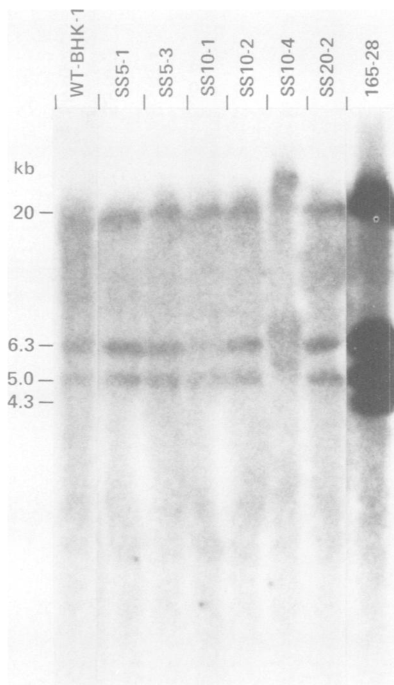

FIG. 4. A transfer of genomic DNA hybridized with 9-900, a fragment amplified in the multiple-step mutant 165-28 (1). Each lane contained 10 μg of *Eco*RI-digested DNA. The DNA of SS10-4 was not digested completely. Sizes of the bands (in kb) are shown on the left. Repetitive sequences were partially removed from the probe as described by Ardeshir et al. (1).

tional enzyme. The higher levels of carbamyl phosphate synthetase activity also present in the resistant cells provide higher concentrations of carbamyl phosphate, which competes with PALA. Note from Fig. 1A that 6 μM, the concentration of PALA giving half-maximal growth of WT-BHK-2, was barely selective. The corresponding value for clone 20-2, 200 μM, would also be expected to be barely selective, and yet this clone was selected at 20 μM. Thus, the increase in resistance was approximately 10-fold larger than minimally required.

Amplifications of the CAD gene in WT-BHK-2 occurred often, from ca. 4×10^{-4} to ca. 4×10^{-5} events per cell per generation over the 16 to 40 μM range of PALA concentrations used for selection. It is surprising that the rate of amplification to 11 or more copies per chromosomal site was so high. Unselected amplifications or other rearrangements of the DNA may have occurred during growth of each original resistant cell to a population, but these should be rare and would be expected to contribute minimally to the analyses performed. More seriously, a primary resistant cell might have amplified the CAD gene enough to allow survival but not growth at an optimum rate. As the population derived from this cell increased in the continued presence of PALA at the original concentration, it would become more and more likely for a second independent amplification to occur in one cell, further increasing the gene copy number and thus permitting that cell to grow more rapidly. Continued growth in PALA would eventually allow such a cell to become dominant in the population. This scenario is possible for the six single-step mutants derived from WT-BHK-1, since each population of resistant cells was grown for approximately 2 months in the continued presence of PALA, at the original concentration. However, the five single-step mutants derived from WT-BHK-2 were not grown in PALA for very long, and they were very similar to the six mutants from WT-BHK-1 in extent of gene amplification and patterns of in situ hybridization. Each resistant cell derived from WT-BHK-2 was grown to only ca. 2×10^7 cells in PALA, and without PALA thereafter. It is unlikely that a second amplification of the CAD gene could have arisen in one cell and become dominant in such a small population even once, and highly unlikely that it would have happened in five of five independent populations. A secondary mutant would have had to arise with a rate of about 1×10^{-3} mutation per cell per generation to be present at the 1,000-cell stage and would also have had to grow approximately twice as fast in PALA as the remaining cells to become dominant by the 2×10^7-cell stage. The rates of mutation at the PALA concentrations employed were substantially smaller than 1×10^{-3} (Fig. 1B).

The chromosomal alterations associated with resistance are difficult to interpret fully in the BHK cells. The single copy of the CAD gene has been localized previously to the short arm of Syrian hamster chromosome B9 (22). However, in the single-step experiments, the parental and mutant lines were heteroploid, and the B9 chromosomes were often difficult to identify. In situ hybridizations revealed that the extra copies of the CAD gene were always located in chromosomes, usually at a single site. No double-minute chromosomes were observed. We can also say, very approximately, that no more DNA was coamplified per CAD gene than was found for four more highly resistant cell lines by Wahl et al. (22) and that the degree of amplification did correlate very approximately with the number of grains in in situ hybridizations. In six of the nine single-step mutants analyzed, in situ hybridization was observed within a chromosome arm much longer than the short arm of chromosome B9, but hybridization was evident over only a relatively small region of this arm

(Fig. 2). It is difficult to imagine how such structures could have been formed within the short arm of B9 without a translocation, but we cannot say whether such an event might have happened before, during, or after amplification. The results of in situ hybridizations also indicated heterogeneity in the chromosomal location of the CAD gene within some of the cloned lines.

Cloned DNAs from the highly resistant cell lines 165-28 and B5-4 were used to probe genomic blots prepared from the DNA of six single-step mutants, to determine whether the sequences coamplified with the CAD gene in 165-28 or B5-4 were also amplified in any of these mutants. The data from these experiments, summarized in Table 2 of the accompanying paper (1), are discussed briefly here. Fragments totaling 44 kb contiguous with and including the CAD gene in the DNA of wild-type cells were amplified to the same extent as the CAD gene itself in all six of the single-step mutants analyzed. The other cloned amplified sequences from 165-28 (approximately 100 kb) were not amplified in any of the six mutants. Most of the other sequences from B5-4 (approximately 68 kb) were amplified in most of the single-step lines, but only a few were amplified in SS5-1. B5-4 and the single-step mutants were derived from the same parental line, BHK-21/13. As shown in the accompanying paper (1), two other related cell lines, BH2-1 and BH3-1A, derived from BHK-21/13 in several steps of selection, also did not share amplification of any of the sequences from B5-4 outside the region of the CAD gene. Thus, mutants derived from a common parent often shared amplified sequences, but there were several substantial exceptions. DNA prepared from SS5-1 after several weeks of growth in PALA contained a novel *Eco*RI fragment longer than 20 kb and homologous to CAD cDNA (Fig. 2 in reference 1). This fragment was absent from earlier DNA preparations, suggesting that the SS5-1 line underwent a second event. In another line, SS5-3, different fragments were found to be amplified to different extents. In a simple tandem array, all amplified sequences should be present at the same abundance, arguing that the arrangement of amplified DNA is more complex in this line.

No novel fragments were seen in the single-step cell lines by homology with any cloned fragment amplified in either 165-28 or B5-4. In further work, we hope to be able to clone fragments containing novel joints from the mutant cell lines shown in Table 2. Hybridization of such fragments to Southern blots of DNA from each mutant and the WT-BHK-2 parental line should provide evidence at a molecular level pertaining to the nature of DNA rearrangements associated with amplification. Comparison of the DNA sequence at a novel joint with the sequences of the two wild-type fragments which came together to form the joint will also be informative.

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