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Single-Copy and Amplified CAD Genes in Syrian Hamster Chromosomes Localized by a Highly Sensitive Method for In Situ Hybridization

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Syrian hamster cells resistant to *N*-(phosphonacetyl)-L-aspartate (PALA), a specific inhibitor of the aspartate transcarbamylase activity of the multifunctional protein CAD, overproduce this protein as a result of amplification of the CAD gene. We have used a sensitive in situ hybridization technique to localize CAD genes in spreads of metaphase chromosomes from several independent PALA-resistant lines and from wild-type PALA-sensitive cells. The amplified genes were always found within chromosomes, usually in an expanded region of the short arm of chromosome B9. In wild-type cells, the CAD gene was also on the short arm of chromosome B9. In one mutant line, 90 to 100 CAD genes were found within an expanded B9 chromosome and 10 to 15 more were near the distal end of one arm of several different chromosomes. Another line contained most of the genes in a telomeric chromosome or large chromosome fragment. The amplified genes were in chromosomal regions that were stained in a banded pattern by trypsin-Giemsa. A few double minute chromosomes were observed in a very small fraction of the total spreads examined. The in situ hybridizations were performed in the presence of 10% dextran sulfate 500, which increases the signal by as much as 100-fold. Using recombinant DNA plasmids nick-translated with [^{125}I]dCTP to high specific radioactivity, 10 CAD genes in a single chromosomal region were revealed after 1 week of autoradiographic exposure, and the position of the unique gene could be seen after 1 month.

Enzyme overproduction is a common mechanism by which many kinds of mammalian cells resist a broad range of selective agents. (For a summary of 14 examples with references, see R. A. Padgett, G. M. Wahl, O. Brison, and G. R. Stark, *in The Third Cleveland Symposium on Macromolecules: Recombinant DNA*, in press.) In four cases, the availability of cloned cDNA probes has allowed the molecular basis of the increases in enzyme levels to be examined. Cells resistant to PALA, methotrexate (MTX), or cadmium are now known to contain more copies of the genes encoding the target proteins CAD (34), dihydrofolate reductase (DHFR) (2), or metallothionine I (3), respectively. In contrast, canavanine-resistant cells contain highly elevated levels of arginosuccinate synthetase and the corresponding mRNA without an increase in the number of genes (30; T.-S. Su, H.-G. Bock, A. L. Beaudet, and W. E. O'Brien, Fed. Proc. **40**:1224, 1981).

The frequency of drug resistance due to gene amplification is not increased substantially by mutagens (14) and is much higher than the spontaneous frequency of point mutations that create drug-resistant forms of the target protein (1, 33) or affect drug transport (10). For resistance to PALA, gene amplification is the only mechanism that has been observed until now. It is reasonable to expect that gene amplification will be a common mechanism of resistance to specific and potent enzyme inhibitors such as PALA or MTX, and it may even be important in cases of resistance to other types of drugs. However, in different systems the genetic and cytogenetic details of gene amplification may be different. For example, the stability of amplified genes varies widely: amplified CAD genes are not lost from PALA-resistant Syrian hamster cells grown without PALA (14), whereas amplified DHFR genes are lost rapidly from some resistant mouse cell lines grown without MTX (13) but not from some MTX-resistant mouse L5178Y cells (9), Chinese hamster ovary cells (20), or baby hamster kidney (BHK) cells (18). Interestingly, in BHK cells selected sequentially to resist PALA and then MTX, the amplified CAD genes are significantly more stable than the amplified DHFR genes (A. Karlin, R. A. Padgett, and G. M. Stark, unpublished data). Resist-

ance to cadmium can be either stable or unstable (3; K. Mayo and R. Palmiter, personal communication).

The cytogenetic bases of stable and unstable MTX resistance have been examined. Stably amplified DHFR genes in Chinese hamster ovary or mouse L5178Y cells are in an expanded portion of a single chromosome, termed a homogeneously staining region since it does not contain bands after treatment with trypsin-Giemsa (9, 20). In contrast, the unstable DHFR genes in mouse S180 cells are found in acentromeric chromosomal material called double minute chromosomes (13). The rapid loss of DHFR genes from unstable MTX-resistant cells grown in the absence of drug results from disproportionate segregation of double minute chromosomes at mitosis and selection of those cells with fewer copies due to faster growth (13, 13a).

We have used a highly sensitive method for in situ hybridization to determine the chromosomal location of amplified CAD genes in four independently derived PALA-resistant mutants. The method has also allowed us to determine the location of the CAD gene in wild-type cells. Significant differences are revealed between the cytogenetic consequences of CAD and DHFR gene amplification.

MATERIALS AND METHODS

Cells and cell culture. Cells were grown as described previously (31). B5-3 and B5-4, BHK clones previously selected with PALA in steps to resist a final concentration of 5 mM, have 56 and 51 times the parental number of CAD genes (34). BH3-1A and BH3-2A were selected recently in similar fashion to resist 1.25 mM PALA.

Chromosome preparation. Confluent cells in 100-mm plates were released with trypsin 1 day before preparing chromosome spreads, and the culture was divided equally into two T150 flasks (Falcon) containing Dulbecco modified Eagle medium plus 10% fetal calf serum. After 12 to 16 h, colcemid dissolved in medium plus serum was added to a final concentration of 0.1 μg/ml. The flasks were incubated at 37°C for 1.25 h and then tapped vigorously to dislodge mitotic cells. The supernatant suspension was separated, and the cells were pelleted from it by centrifugation at 1,000 × *g* for 2 min. All but 0.5 ml of the supernatant solution was removed, the cells were resuspended gently, and 10 ml of 0.075 M KCl was added dropwise at 25°C with gentle agitation to keep the cells suspended. After 12.5 min, the cells were pelleted by centrifugation, all but 0.5 ml of the supernatant solution was removed, and the cells were resuspended gently. Fixative (methanol-glacial acetic acid, 3:1 [vol/vol]) was added dropwise at 4°C to a final volume of 5 ml. After 5 min, the cells were pelleted, and the fixation process was repeated three more times. Cell suspensions were dropped or streaked onto microscope slides (cleaned in 95% ethanol) to form metaphase spreads. The cell density was chosen to minimize the chances of chromosome contamination from adjacent spreads.

In situ hybridization. After treatment with RNase and denaturation (6), the slides were exposed to acetic anhydride to reduce the background often observed with iodinated probes (12). The hybridization mixture for each slide contained, in a total volume of 20 to 25 μl, 10% sodium dextran sulfate 500 (DS 500; Pharmacia), 50% formamide, 2× standard saline citrate (SSC) (pH 7.0), 1× Denhardt reagent (Denhardt reagent [7] contains 0.02% [wt/vol] each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll; M_r = 400,000), 1 mM iododeoxycytidine (Sigma), 100 μg of denatured herring sperm DNA per ml, cleaved to an average size of 500 nucleotides, and 2.5 × 10^5 cpm of probe (specific activity, 0.5 × 10^8 to 1.0 × 10^8 cpm/μg). The probe was prepared by nick translating (25) the DNA of p102, a plasmid containing 8.6 kb of genomic DNA devoid of repetitive sequences, from within the CAD gene (21, 22). Each 20 μl of reaction mixture contained 0.1 to 0.2 μg of DNA and 20 μCi of [^{125}I]dCTP (New England Nuclear Corp.; more than 1,000 Ci/mmol), and the reaction was performed as described by Wahl et al. (34). The hybridization mixture was deposited onto slides and covered with a 24-by-50-mm cover slip, and the edges were sealed with Carter's rubber cement. Slides were placed in a plastic box above a layer of water, the top was sealed, and the whole was incubated for 14 to 24 h at 39 to 42°C. The cover slips were then removed, up to 18 slides were placed in a Wheaton dish slide holder, and the unhybridized probe was removed by sequential washing for 3 min each in the following solutions: 2× SSC (pH 7.0), 1 liter at 25°C; 2× SSC (pH 7.0), 500 ml at 25°C; 50% formamide–2× SSC (pH 7.0), twice with 200 ml at 39°C; 2× SSC (pH 7.0), eight times with 200 ml at 39°C. The slides were immersed sequentially in 70%, 80%, and 95% ethanol for 2 min each, dried with a stream of air, and dipped in 0.1% gelatin–0.01% chromalum subbing solution (11) and air-dried. The dipping step was necessary to prevent the emulsion from moving on the slide during subsequent procedures. In total darkness, the slides were dipped once in Kodak NTB-2 liquid emulsion diluted 1:1 with water, dried in a light-tight air box designed by T. C. Hsu (personal communication) at 25°C for 1 to 3 h, and placed in light-tight boxes with desiccant at 4°C for 4 days to 1 month. The slides were developed and stained as described by Harper and Saunders (11a). Chromosomes were observed under oil immersion with bright-field or phase-contrast optics with a Zeiss photomicroscope equipped with a 64× objective. To be sure that the collection of metaphase spreads scored maximized the detection of all chromosomes with specific hybridization, only the following criteria were used to reject spreads: (i) high backgrounds, (ii) more than two overlapping chromosomes, or (iii) fewer than 36 chromosomes (to minimize the chances of analyzing specimens that had lost chromosomes during spreading). Our use of DS 500 in the hybridization reaction is based upon M. Wolfner's results with *Drosophila* polytene chromosomes (Ph.D. thesis, Stanford University, Stanford, Calif., 1980) and our previous demonstration of its effect on hybridizations to DNA or RNA immobilized on paper (35). Harper and Saunders (11a) have also performed in situ hybridizations in the presence of DS 500.

Chromosome banding. Chromosomes were banded by a modification of the Giemsa-trypsin procedure (28). Slides were dipped for 45 s to 2 min in trypsin (Difco; 1:250) diluted 1:1,000 in Gurr buffer (pH 7.2;

Bio/medical Specialties, Santa Monica, Calif.), rinsed in distilled water, and stained for 1.5 min in 4% Gurr Giemsa made with Gurr buffer, pH 6.8 (Bio/medical Specialties).

RESULTS

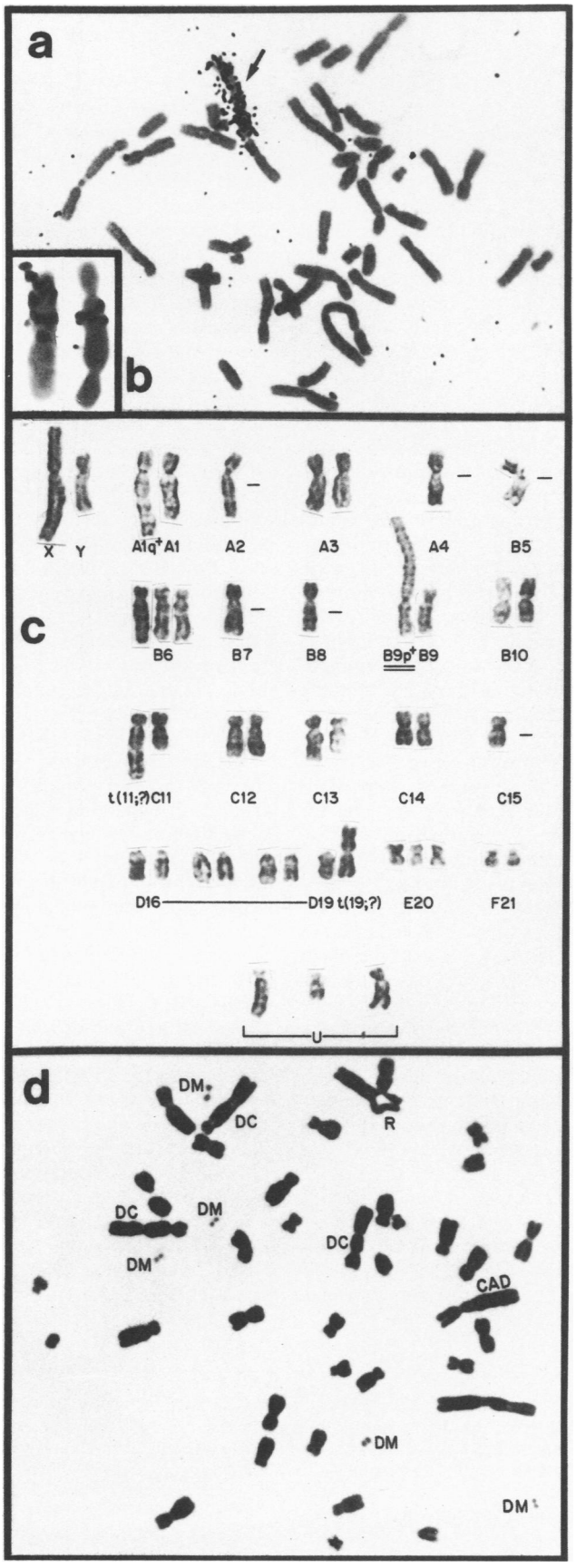
Effect of DS 500 on hybridization of nick-translated probes to metaphase chromosomes. When nick-translated probes are hybridized to DNA immobilized on nitrocellulose or diazobenzyloxymethyl-paper, 10% (wt/vol) DS 500 can increase the signal obtained by as much as 100-fold (35). As a logical extension of this observation, we investigated the effect of hybridizing metaphase chromosomes from PALA-resistant B5-3 cells which contain approximately 110 CAD genes (34) with different probe concentrations in the absence or presence of 10% DS 500 (Table 1). The data of Table 1 and Fig. 2a show that, in the presence of DS 500, hybridization can be detected over both a large and a small chromosome by using 6.25×10^4 cpm (about 40 ng/ml) of an iodinated probe having 8.6 kilobases (kb) of homologous DNA devoid of repetitive sequences. In the absence of DS 500, an equivalent amount of probe gave no hybridization at the minor hybridization site in the small chromosome and a signal barely above background at the major site. The background was equal in the presence or absence of DS 500. Since the extent of hybridization at the major site is reproducibly 7 to 10 times the extent at the minor site, and since line B5-3 contains about 110 CAD genes, we estimate that the minor site contains 10 to 15 CAD genes. Therefore, the use of DS 500 has enabled us to detect 86 to 130 kb of homologous sequence after a 1-week exposure using 6.25×10^4 cpm of an iodinated probe. It is difficult to estimate accurately the magnitude of enhancement by DS 500 since we observed so little hybridization in its absence. The data of Table 1 show that the number of grains over the major site of hybridization was about 20 times higher in the presence of DS 500 than in its absence, when the same amounts of probe were used. The effect of DS 500 is greatest at low concentrations of probe, where the degree of enhancement can be as large as 100-fold (35; unpublished data). Variability in the amount of probe hybridized is typical for in situ hybridizations performed under the conditions stated above in the presence or absence of DS 500, and we have not been successful in making the hybridizations more uniform. The extent of hybridization in the experiment described in Table 1 was less than usual. In other experiments, we have observed as many as 50 to 60 grains over the major site of hybridization after a 1-week exposure (see Fig. 1a and 2a) and have seen the minor site after as little as 3 to 5 days of exposure.

Localization of amplified CAD genes in independent PALA-resistant BHK mutants. The four PALA-resistant BHK lines used for these studies were isolated in two separate experiments. Mutants B5-4 and B5-3, cloned from a single population of PALA-resistant BHK 21/13 cells, were shown previously to contain approximately 100 and 110 CAD genes, respectively (34). Mutants BH3-1A and BH3-2A were cloned from two new, independent populations of PALA-resistant BHK 21/13 cells and were studied soon after isolation. A typical result for cell line B5-4 is shown in Fig. 1a. Accumulation of grains was observed consistently over the long arm of a single large chromosome which is not present in the wild-type BHK 21/13 cells (see below). All 32 metaphase spreads examined showed substantial hybridization to the large marker chromosome. Of these, 28 showed hybridization to this chromosome only, 1 spread contained two such chromosomes, and the 3 others had, in addition, low levels of hybridization to different

TABLE 1. Effect of DS 500 on in situ hybridization[a]

Amt of probe (cpm × 10^4)	Without DS 500				With DS 500			
	Avg no. of grains over chromosomes		% of spreads with grains over chromosomes		Avg no. of grains over chromosomes		% of spreads with grains over chromosome	
	Major site	Minor site	Major site	Minor site	Major site	Minor site	Major site	Minor site
1.25	0	0	0	0	6.4 (0–9) ± 2.9	0.8 (0–3) ± 1.0	85	45
6.25	0.8 (0–3) ± 1.1	0	41	0	13.7 (10–18) ± 2.8	2.5 (2–3) ± 0.5	100	100
25	0.8 (0–3) ± 1.1	0	57	0	15.5 (8–38) ± 7.5	2 (0–5) ± 1.9	100	64

[a] Metaphase spreads of mutant B5-3 were prepared and hybridized with the amounts of probe indicated in the absence or presence of DS 500. The slides were washed, coated with subbing solution and emulsion, and exposed for 7 days to detect hybridization to CAD genes located in the long chromosome (major site) or short chromosome (minor site). The slides were developed and stained with Giemsa, and the grains were counted. At least 10 metaphase spreads were counted for each data point. The numbers in parentheses represent the range of grains counted ± standard deviation.


a
b
c
X
Y
A1q+A1
A2
A3
A4
B5
B6
B7
B8
B9p+B9
B10
t(11;?)C11
C12
C13
C14
C15
D16
D19 t(19;?)
E20
F21
U
d
DM
DC
R
DC
DM
DM
DC
CAD
DM
DM

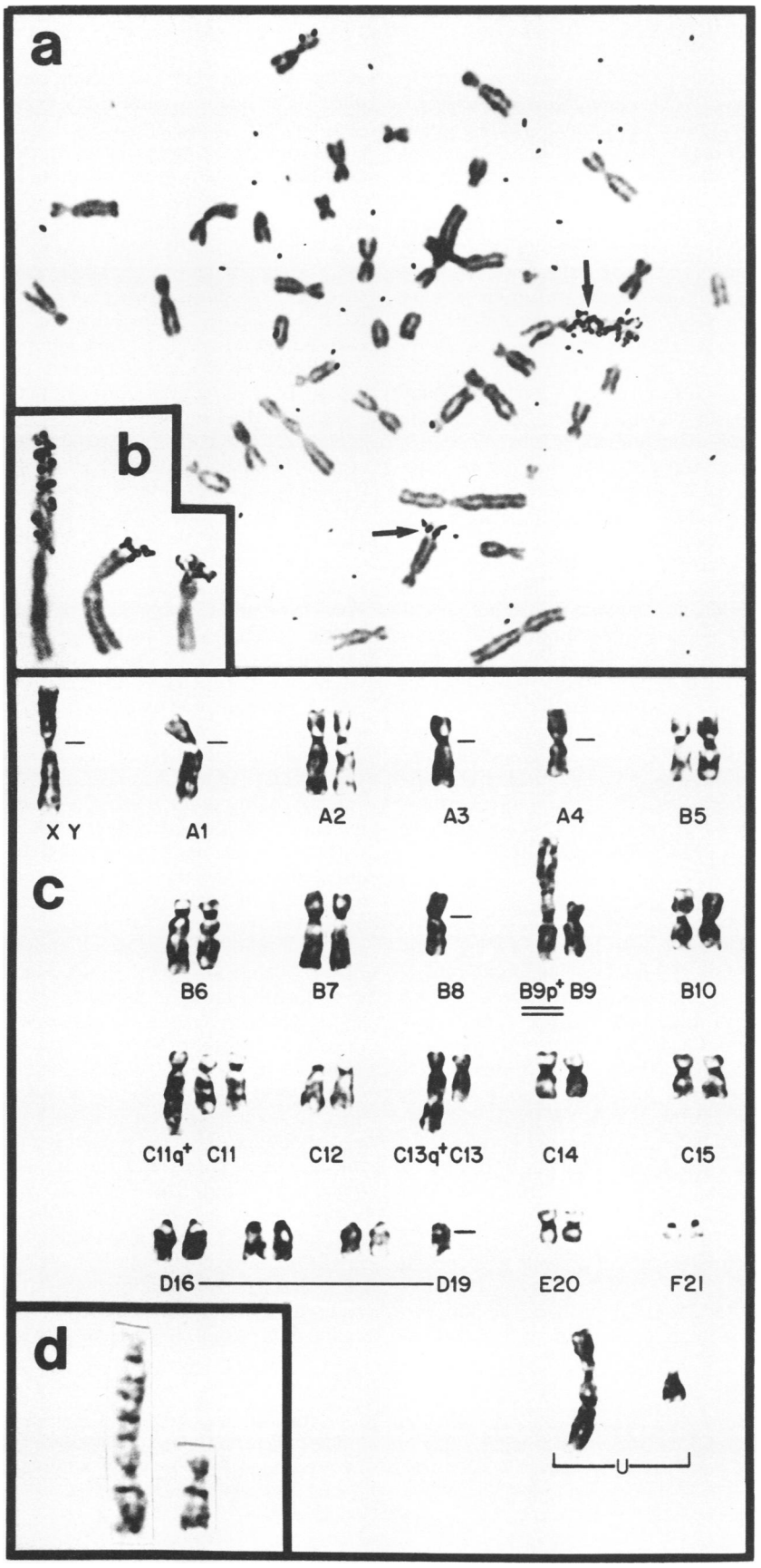
chromosomes (Fig. 1b). The cells used in this experiment were derived from a stock frozen soon after isolation. Equivalent results were obtained with cells grown for about 60 generations without PALA. A karyotype of one B5-4 cell is shown in Fig. 1c. In general, the PALA-resistant mutants contained many chromosomal rearrangements not present in wild-type cells, and these rearrangements varied from cell to cell in a population. However, one marker chromosome seen consistently in B5-4 cells resembles chromosomes B9 and is designated B9p$^+$ in Fig. 1c. (The nomenclature of Syrian hamster chromosomes is that of Lehman et al. [15]). In the metaphase spreads of B5-4, the short arm of chromosome B9 appears to have become elongated and has acquired many G bands not present in the B9 chromosomes of the wild-type cells. Other anomalies apparent in the metaphase spread shown in Fig. 1d were six double minute chromosomes (DM), several dicentric chromosomes (DC), and an undefined chromosome exchange (R). Double minute chromosomes have been seen in only 1 other metaphase spread of B5-4 out of more than 50 examined. They were not seen in any of the other mutants described below, although chromosomal rearrangements similar to those shown in Fig. 1d were observed with high frequency.

Analysis of 26 metaphase spreads of line B5-3 revealed a situation different from the one for B5-4. A typical example is shown in Fig. 2a. Most of the hybridization is localized to a chromosome similar to the one seen in line B5-4 (24 of 26 spreads). However, in addition, a minor site of hybridization was also observed in 20 of 26 spreads over an expanded region at the distal end of the long arm of a different chromosome. The same observation was made both in cells grown for more than 60 generations after isolation and in freshly thawed cells frozen soon after isolation. As described above, the minor hybridization site contains about 10 to 15 CAD genes. Hybridization only to a single chromosome similar to the one that predominates in B5-4 was seen in 4 of 26 spreads, and hybridization to additional chromosomes was seen in 2 of 26 spreads. Figure 2b shows the other chromosomes that hybridize with the CAD-specific probe, and a karyotype of line B5-3 is shown in Fig. 2c. A chromosome similar to the abnormally long B9 chromosome, with many G bands, was seen consistently, as was another chromosome with an elongated long arm and a banding pattern similar to that of C11. A better picture of the B9 chromosomes from another spread of cell line B5-3 is shown in Fig. 2d. Other abnormal chromosomes were also seen occasionally.

Since mutants B5-3 and B5-4 were grown without PALA for varying lengths of time after selection, it was possible that unstable genetic elements containing amplified CAD genes could have been lost, especially since Kaufman et al. (13) had shown previously that amplified DHFR genes can be located in double minute chromosomes. Lines BH3-1A and BH3-2A were cloned from independent populations of cells selected in three consecutive steps to resist a final concentration of 1.25 mM PALA and grown to mass populations in the presence of PALA. Representative results are shown in Fig. 3. Line BH3-2A showed hybridization consistently over a single large chromosome very much like the one seen in cell lines B5-4 and B5-3 (Fig. 3a). In contrast, line BH3-1A showed significant hybridization only over a large structure resembling either a telomeric chromosome or a chromosome fragment (Fig. 3b).

Localization of the CAD gene in wild-type BHK cells. Since most of the amplified CAD genes in three of the four mutants examined were localized to an expanded region of the short arm of chromosome B9, we tested the possibility that the CAD gene might be located on the short arm of this chromosome in the PALA-sensitive parental cells. Metaphase spreads from wild-type cells were hybridized as described above, but the slides were exposed for 1 month. The results with mutant cells indicated that one gene copy yields about one grain per week, so we expected to see an average of four grains over the wild-type chromosome containing a single CAD gene after 1 month. Figure 4 shows four metaphase spreads from the 16 that were examined. Two of these (Fig. 4a and b) were chosen because there was significant hybridization over each of two chromosomes which appear to be homologs, as judged by arm length ratios. In the remaining two there was significant hybridization over only one chromosome. In each of these four cases, hybridization was over the same chromosome and the grains were near the short arms.

FIG. 1. Localization of amplified CAD genes in the PALA-resistant mutant B5-4. Metaphase spreads were prepared and hybridized with 2.5×10^5 cpm of probe in the presence of DS 500. The exposure was for 1 week at 4°C. (a) Metaphase spread of B5-4. (b) Marker chromosomes exhibiting significant hybridization. (c) Karyotype prepared by trypsin-Giemsa banding (arranged and numbered according to Lehman et al. [15]). (d) Multiple chromosome anomalies in a single metaphase spread. DM, double minute chromosomes; DC, dicentric region; R, rearrangement of unknown origin; CAD, chromosome with amplified CAD genes.


a
b
c
d
X Y
A1
A2
A3
A4
B5
B6
B7
B8
B9p+ B9
B10
C11q+ C11
C12
C13q+C13
C14
C15
D16
D19
E20
F21

Of approximately 750 chromosomes in 16 spreads, 52 had two or more grains over a single region and accounted for a total accumulation of 174 grains. Of these, 106 grains (61%) were near the short arms of 22 chromosomes which resembled B9 on the basis of arm length ratios, even though the short arms of these chromosomes represent only about 0.5% of the total length of all chromosomes. To provide a more stringent criterion, only chromosomes with four or more grains over a single region were scored. The number of positive regions was reduced to 16 (93 grains), and 13 (77 grains, 83%) were over the short arms of chromosomes having the same size and ratio of arm length as B9. Since the data were obtained using unbanded chromosomes, we cannot say with certainty that the chromosomes with most of the grains were B9, although their sizes and arm length ratios suggest this conclusion strongly.

A typical karyotype of PALA-sensitive BHK 21/13 cells is shown in Fig. 5. The cells were aneuploid but the chromosomal morphology still reflected that of secondary cultures of Syrian hamster embryo cells (8, 24). Although dicentric chromosomes and other markers were observed occasionally, the karyotype of the parental cells was remarkably constant with respect to chromosome number (47 ± 1, from 25 spreads) and chromosome morphology. The marker chromosomes and occasional double minute chromosomes observed in the PALA-resistant mutants were not seen in the wild-type cells.

DISCUSSION

Method of hybridization in situ. DS 500 accelerates by about 100-fold the apparent rate of hybridization of nick-translated probes to DNA immobilized in fixed metaphase chromosomes. Wolfner (Ph.D. thesis) and Harper and Saunders (11a) have also used DS 500 in hybridizations to fixed chromosomes and have also seen large enhancements of the signals. With an exposure time of 1 week and 2.5×10^5 cpm of probe DNA having a specific activity of 0.5×10^8 to 1×10^8 cpm/μg, we can easily localize 10 copies of a DNA sequence 8.6 kb long. For comparison, Robins et al. (26), working in the absence of DS 500, used 1.5×10^7 cpm of a probe at a specific activity of 10^9 cpm/μg to detect 39 kb of homologous DNA after a 3-day exposure. The improvement in apparent rate afforded by DS 500, together with the very low backgrounds observed, allows the localization of single-copy sequences even in metaphase spreads of Syrian hamster cells, which have more than 40 chromosomes. In addition to centromere position and relative size, other morphological features of chromosomes, especially banding patterns, might be used in conjunction with autoradiography to provide unambiguous mapping information in complex situations. However, up to now we have not succeeded in doing the banding and autoradiography on the same spreads.

Location of the amplified and normal CAD genes. In two of the four mutant cell lines examined (B5-3 and B5-4), most of the amplified genes are found within expanded chromosome arms which exhibit bands after staining with trypsin-Giemsa. Preliminary results indicate that this will also be true for a third mutant, BH3-2A. This result contrasts with the finding that chromosomally amplified DHFR genes are localized within a region devoid of G bands (4, 9, 20). Therefore, amplified genes may be found either within banded or unbanded (homogeneously staining) regions of chromosomes.

The karyotypes of the two independent PALA-resistant lines we have studied in detail are much more anomalous than the karyotype of the wild-type, PALA-sensitive cells. A single abnormally long chromosome, seen in all spreads of mutants B5-4, B5-3, and BH3-2A, hybridizes to cloned genomic CAD sequences within an expanded region probably formed by elongation of the short arm of chromosome B9. Since one normal B9 chromosome was also present in these cells, we conclude that amplification within both homologs is rare. In mutant B5-3, 90 to 100 copies of the amplified genes were located in the expanded B9 chromosome, and the remaining 10 to 15 copies were found at the distal end of the long arm of another chromosome. A few spreads of B5-3 exhibited significant hybridization at the distal portions of one arm of several different chromosomes. A secondary constriction was often located between the amplified genes and the adjacent chromosome arm in both the major and minor sites of hybridization. A constriction at the major site in chromosome B9 was also noted in spreads of line B5-4. Such constrictions have been correlated with the presence of nucleolus organizers (17) and with sites of translocations (16). It is interesting to note that, in rat hepatoma lines contain-

FIG. 2. Localization of amplified CAD genes in the PALA-resistant mutant B5-3. Metaphase spreads were analyzed as described in the legend of Fig. 1. (a) In situ hybridization to metaphase chromosomes of mutant B5-3. Regions of significant hybridization are indicated by arrows. (b) Other marker chromosomes exhibiting significant hybridization. (c) Karyotype of Giemsa-banded chromosomes. (d) B9 chromosomes taken from another metaphase spread of B5-3.

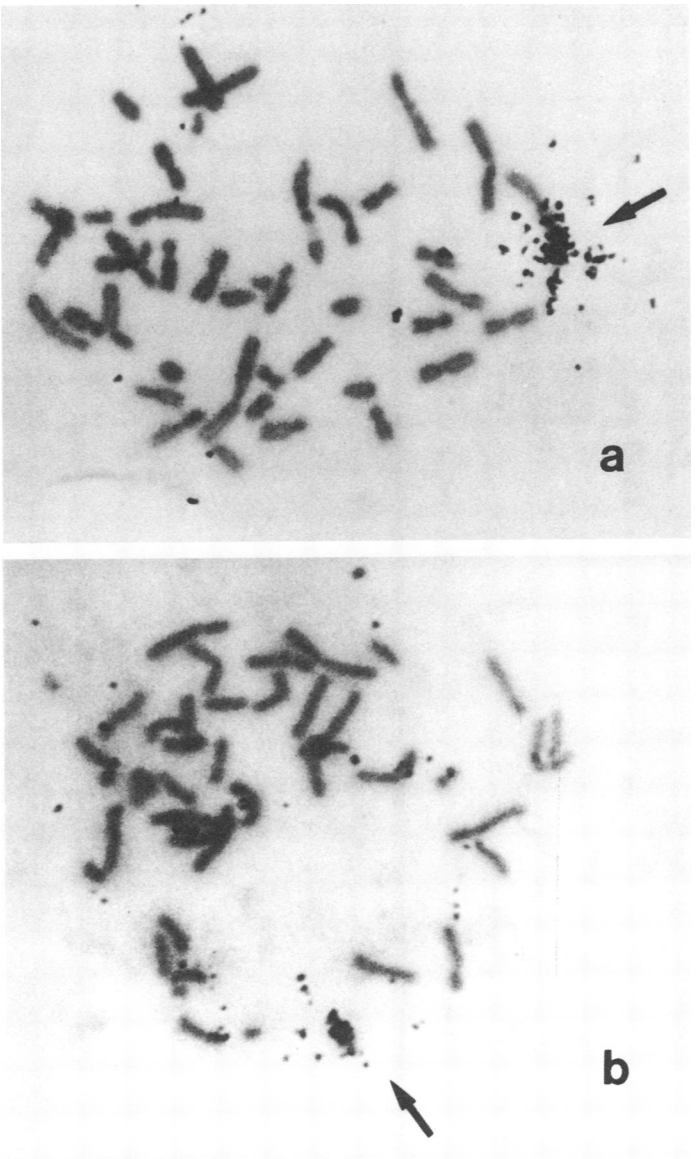


FIG. 3. Localization of the amplified CAD genes in PALA-resistant mutants BH3-1A and BH3-2A. Metaphase spreads were prepared and hybridized as described in Fig. 1. Those shown are typical for cell lines BH3-2A (a) and BH3-1A (b).

ing amplified rRNA genes, the expanded chromosomal region has an interspersed banding pattern in which the unstained portions probably represent secondary constrictions known to be sites of nucleolus organizers (19, 32).

Since the amplified CAD genes were found in the expanded short arm of chromosome B9 in several independent mutant cell lines, it was possible that the single copy of the CAD gene resided in the corresponding region of this chromosome in wild-type cells. In fact, the only significant hybridization in wild-type cells is localized to the short arms of a B-group chromosome with a ratio of arm lengths the same as that of B9. Since chromosome B9 also contains a nucleolus organizer (5), we have investigated whether rRNA genes are amplified in concert with CAD genes. (Recent evidence to be presented elsewhere shows that ribosomal genes are co-amplified with CAD genes in cell lines

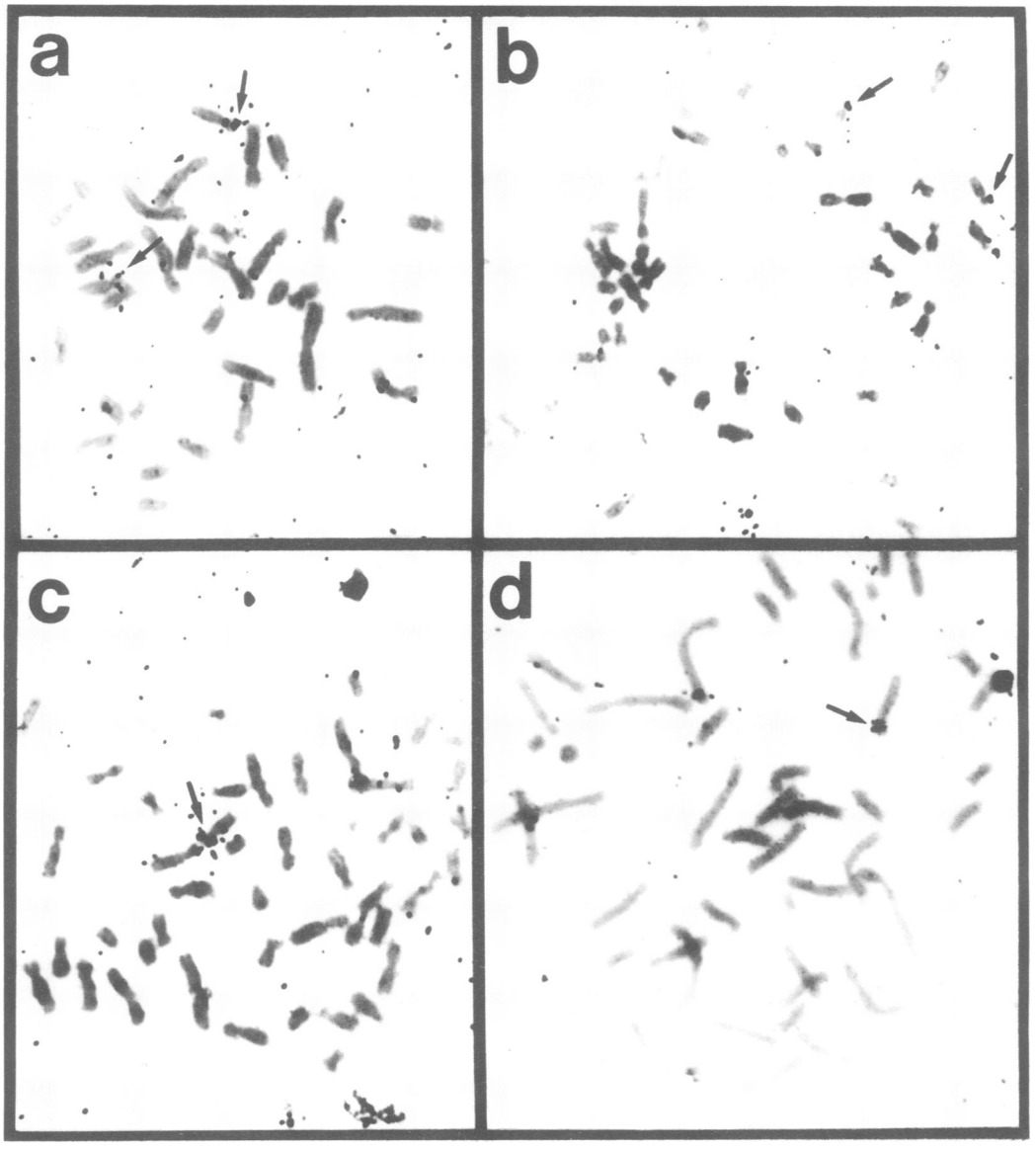


FIG. 4. Localization of CAD genes in wild-type BHK cells. Metaphase spreads were prepared and hybridized as described in Fig. 1. Those shown were derived from a single experiment in which the exposure was for one month at 4°C. In (a) and (b), two homologous chromosomes exhibit hybridization over their short arms, and in (c) and (d) a single chromosome is labeled over the short arm (see arrows).

B5-3 and B5-4 [L. Vitto, J. Rubnitz, and G. M. Wahl, manuscript in preparation].)

Chromosomal abnormalities in PALA-resistant cell lines. PALA-sensitive wild-type cells have very few chromosomal rearrangements, whereas each of the PALA-resistant lines examined has many. An extreme example is shown in Fig. 1d, where a single cell contains, in addition to the abnormal chromosome carrying the amplified CAD genes, several dicentric chromosomes, a rearrangement of unknown origin, and double minute chromosomes. Only 2 of 50 spreads of this cell line contained double minute chromosomes, and none were observed in spreads of the other mutants or the wild-type cells. The types of chromosomal rearrangements in the mutant cells are reminiscent of abnormalities often seen in the karyotypes of patients with Fanconi's anemia (27), a recessive hereditary disorder associated with failure to repair damaged DNA. It is conceivable that the PALA-resistant mutants represent a subpopulation of

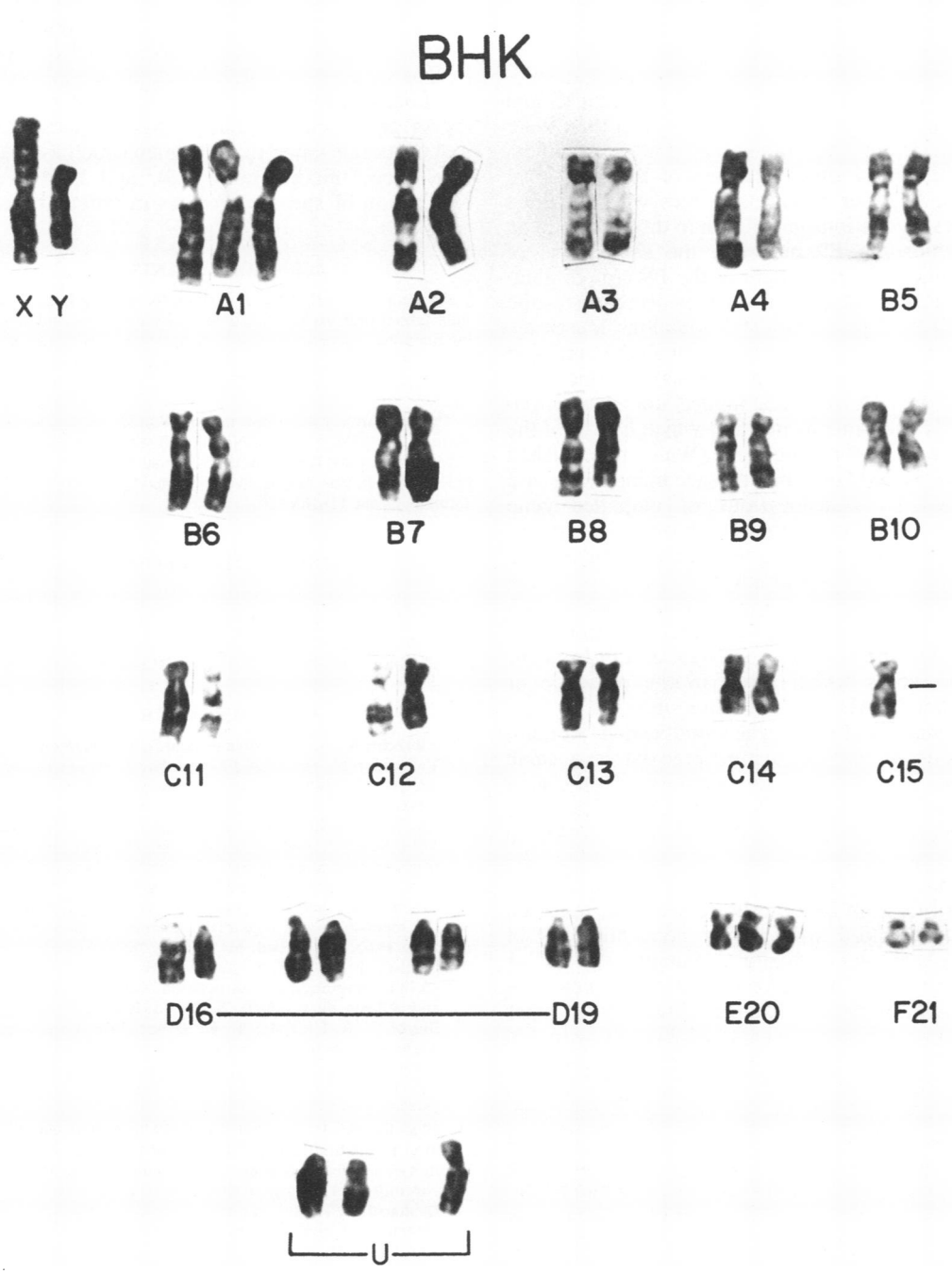


FIG. 5. Giemsa-banded karyotype of the wild-type BHK cells described in Fig. 4.

cells whose chromosomes rearrange more frequently because of an undefined genetic defect. Such a lesion might even be necessary for gene amplification to occur at high frequency.

Size of the amplified region. Amplified regions account for approximately 2% of the chromosome length in mutants B5-3 and B5-4. Since the haploid DNA content of hamster cells is about 3 $\times 10^6$ kb and since each of these mutants has 100 to 110 CAD genes, we calculate that the average

amount of DNA amplified is approximately 500 kb per copy of the CAD gene, comparable to estimates for the size of the region coamplified with the DHFR gene. We know that the transcriptional unit for CAD is 25 kb long (22) and that an active CAD gene is not longer than 40 kb (25a). Therefore, the average unit of amplification is many times the size of the functional gene. In other mutant cell lines with amplified genes at locations in addition to the major site on chromosome B9 but with the same level of amplification, the length of the B9 arm containing most of the amplified genes decreases as the number of genes at other locations increases. Furthermore, the total length of all the amplified regions remains constant, so that the amount of additional DNA per amplified unit is approximately the same as in B5-4, which has all of the genes on chromosome B9 (Wahl, unpublished data). It is likely that the secondary sites are caused by translocations of amplified gene copies from the parental chromosome rather than by new amplifications. Since we did not see any evidence that amplified CAD genes were translocated to the short arm of a second B9 chromosome, we conclude that recombination between the two B9 homologs is less frequent than events which result in the presence of amplified CAD genes at other sites.

Dynamics of CAD gene amplification. Amplifications of varying degree preexist in a small subset of cells within the population (14). Use of a particular concentration of PALA leads to selection of those cells with enough CAD to carry out UMP synthesis in the presence of the inhibitor (14; Padgett and Stark, unpublished data). Growth of the cells so selected, followed by reselection with higher concentrations of PALA, leads eventually to mutants with 300 or more CAD genes. The rate at which new mutants are generated is rapid (about 2×10^{-5} per cell per generation in C13/SV28 cells with 100 μM PALA), and a steady state is achieved not because the mutants are intrinsically unstable but rather because they tend to grow appreciably more slowly than wild-type cells (14). The mutants studied here were observed many generations after the initial events in amplification, so our findings that the amplified genes are localized on chromosomes and that many rearrangements have taken place may not be true for cells early in the process. It is important to investigate the nature of the initial events and to study the structure of the very large region of DNA that appears to be coamplified with the CAD gene. Experiments in progress are designed to examine the arrangement of amplified sequences, the nature of the novel joints between them, and the frequency with which each subregion is amplified in independent events. Other experiments will investigate the role of the sequences flanking a gene that can be amplified in determining the size, stability, and cytogenetic characteristics of the amplification. We hope eventually to obtain a more detailed understanding of gene amplification in higher eucaryotes and of its relevance to phenomena such as drug resistance, tumorigenesis (23), and high-level expression of specific proteins in differentiated cells (29).

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ADDENDUM IN PROOF

We have found recently that DNA nick-translated with [^{3}H]dTTP to a specific activity of approximately 10^8 cpm/μg works as well for in situ hybridization as the iodinated probes described in this paper.

LITERATURE CITED

1. **Albrecht, A. M., J. L. Biedler, and D. J. Hutchison.** 1972. Two different species of dihydrofolate reductase in mammalian cells differentially resistant to amethopterin and methasquin. Cancer Res. **32**:1539–1546.
2. **Alt, F., R. E. Kellems, J. R. Bertino, and R. T. Schimke.** 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. **253**:1357–1370.
3. **Beach, L. R., and R. D. Palmiter.** 1981. Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. Proc. Natl. Acad. Sci. U.S.A. **78**:2110–2114.
4. **Biedler, J. L., and B. A. Spengler.** 1976. Metaphase chromosome anomaly: association with drug resistance and cell-specific products. Science **191**:185–187.
5. **Bigger, T. R. L., and J. R. K. Savage.** 1976. Location of nucleolar organizing regions on the chromosomes of the Syrian hamster (Mesocricetus auratus) and the Djungarian hamster (Phodopus sungorus). Cytogenet. Cell Genet. **16**:495–504.
6. **Chandler, M. E., and J. J. Yunis.** 1978. A high resolution in in situ hybridization technique for the direct visualization of labeled G-banded early metaphase and prophase chromosomes. Cytogenet. Cell Genet. **22**:352–356.
7. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–651.
8. **DiPaolo, J. A., and N. C. Popescu.** 1979. Cytogenetics of Syrian hamster and its relationships to in vitro neoplastic transformation. Prog. Exp. Tumor Res. **24**:2–16.
9. **Dolnick, B. J., R. J. Berenson, J. R. Bertino, R. J. Kaufman, J. H. Nunberg, and R. T. Schimke.** 1979. Correlation of dihydrofolate reductase elevation with gene amplification in a homogeneously staining chromosomal region in L5178Y cells. J. Cell Biol. **83**:394–402.
10. **Flintoff, W. F., S. V. Davidson, and L. Siminovitch.** 1976. Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. Somat. Cell Genet. **2**:245–261.

11. **Gall, J. G., and M. L. Pardue.** 1971. Nucleic acid hybridization in cytological preparations. Methods Enzymol. **21**:470–480.

11a. **Harper, M. E., and G. F. Saunders.** 1981. Localization of single-copy DNA sequences on G-banded human chromosomes by *in situ* hybridization. Chromosoma **83**:431–439.

12. **Hayashi, S., I. C. Gillam, A. D. Delaney, and G. M. Tener.** 1978. Acetylation of chromosome squashes of Drosophila melanogaster decreases the background in autoradiographs from hybridization with [^{125}I]-labeled RNA. J. Histochem. Cytochem. **26**:677–679.

13. **Kaufman, R. J., P. C. Brown, and R. T. Schimke.** 1979. Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes. Proc. Natl. Acad. Sci. U.S.A. **76**:5669–5673.

13a. **Kaufman, R. J., P. C. Brown, and R. T. Schimke.** 1981. Loss and stabilization of amplified dihydrofolate reductase genes in mouse sarcoma S-180 cell line. Mol. Cell. Biol. **1**:1084–1093.

14. **Kempe, T. D., E. A. Swyryd, M. Bruist, and G. R. Stark.** 1976. Stable mutants of mammalian cells that overproduce the first three enzymes of pyrimidine nucleotide biosynthesis. Cell **9**:541–550.

15. **Lehman, J. M., I. Macpherson, and P. S. Moorehead.** 1963. Karyotype of the Syrian hamster. J. Natl. Cancer Inst. **31**:639–650.

16. **Lejeune, J., B. Dutrillaux, M.-O. Rethoré, M. Prieur, J. Couturier, S. Carpentier, and O. Raoul.** 1972. Analysis of 30 cases of translocation by the controlled heat denaturation, p. 191–200. *In* R. A. Pfeiffer (ed.), Modern aspects of cytogenetics: constitutive heterochromatin in man. F. K. Schattauer Verlag, Stuttgart.

17. **Lima-de-Faria, A.** 1972. The specific location of ribosomal genes in the eukaryotic chromosome, p. 39–44. *In* R. A. Pfeiffer (ed.), Modern aspects of cytogenetics: constitutive heterochromatin in man. F. K. Schattauer Verlag, Stuttgart.

18. **Littlefield, J. W.** 1969. Hybridization of hamster cells with high and low folate reductase activity. Proc. Natl. Acad. Sci. U.S.A. **62**:88–95.

19. **Miller, O. J., R. Tantravahi, D. A. Miller, L.-C. Yu, P. Szabo, and W. Prensky.** 1979. Marked increase in ribosomal RNA gene multiplicity in a rat hepatoma cell line. Chromosoma **71**:183–195.

20. **Nunberg, J. H., R. J. Kaufman, R. T. Schimke, G. Urlaub, and L. A. Chasin.** 1978. Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. Proc. Natl. Acad. Sci. U.S.A. **75**:5553–5556.

21. **Padgett, R. A., G. M. Wahl, and G. R. Stark.** 1982. Structure of the gene for CAD, the multifunctional protein that initiates UMP synthesis in Syrian hamster cells. Mol. Cell. Biol. **2**:293–301.

22. **Padgett, R. A., G. M. Wahl, and G. R. Stark.** 1982. Properties of dispersed, highly repeated DNA within and near the hamster CAD gene. Mol. Cell. Biol. **2**:302–307.

23. **Pall, M. L.** 1981. Gene-amplification model of carcinogenesis. Proc. Natl. Acad. Sci. U.S.A. **78**:2465–2468.

24. **Popescu, N. C., and J. A. DiPaolo.** 1972. Identification of Syrian hamster chromosomes by acetic-saline-Giemsa (ASG) and trypsin techniques. Cytogenetics **11**:500–507.

25. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.

25a. **Robert de Saint Vincent, B., S. Delbruck, W. Eckhart, J. Meinkoth, L. Vitto, and G. M. Wahl.** 1981. Cloning and reintroduction into animal cells of a functional CAD gene, a dominant amplifiable genetic marker. Cell **27**:267–277.

26. **Robins, D. M., S. Ripley, A. S. Henderson, and R. Axel.** 1981. Transforming DNA integrates into the host chromosome. Cell **23**:29–39.

27. **Schmid, W., and G. Fanconi.** 1978. Fragility and spiralization anomalies of the chromosomes in three cases, including fraternal twins, with Fanconi's anemia, type Estren-Dameschek. Cytogenet. Cell Genet. **20**:141–149.

28. **Seabright, M.** 1971. A rapid banding technique for human chromosomes. Lancet **ii**:971–972.

29. **Spradling, A. C., and A. P. Mahowald.** 1980. Amplification of genes for chorion proteins during oogenesis in Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A. **77**:1096–1100.

30. **Su, T.-S., A. L. Beaudet, and W. E. O'Brien.** 1981. Increased translatable mRNA for argininosuccinate synthetase in canavanine-resistant human cells. Biochemistry **20**:2956–2960.

31. **Swyryd, E. A., S. S. Seaver, and G. R. Stark.** 1974. N-(Phosphonacetyl)-L-aspartate, a potent transition state analog inhibitor of aspartate transcarbamylase, blocks proliferation of mammalian cells in culture. J. Biol. Chem. **249**:6945–6950.

32. **Tantravahi, U., R. V. Guntaka, B. F. Erlanger, and O. J. Miller.** 1981. Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methylcytosine. Proc. Natl. Acad. Sci. U.S.A. **78**:489–493.

33. **Urlaub, G., and L. A. Chasin.** 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. U.S.A. **77**:4216–4220.

34. **Wahl, G. M., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. **254**:8679–8689.

35. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.