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Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line

(*in situ* hybridization/gene amplification)

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ABSTRACT **Methotrexate-resistant Chinese hamster ovary cells selected for high resistance by progressive increments of methotrexate in the culture medium have levels of dihydrofolate reductase (tetrahydrofolate dehydrogenase, 7,8-dihydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) 200 times that of sensitive cells and a corresponding increase in the number of copies of the dihydrofolate reductase gene. The resistant cells contain an expanded region on the second chromosome (homogeneously staining region) that is not present in sensitive cells. *In situ* hybridization of DNA complementary to dihydrofolate reductase mRNA shows that the dihydrofolate reductase genes are specifically localized to the homogeneously staining region of this chromosome in the resistant cells.**

We have reported that methotrexate (MTX) resistance in several murine cell lines associated with increased levels of dihydrofolate reductase (tetrahydrofolate dehydrogenase, 7,8-dihydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) (DHFR, H_2folate reductase) results from increased rates of synthesis of the reductase (1) and a corresponding increase in the gene copy number (2). In some cell lines the amplified genes are lost when the cells are grown in the absence of MTX, whereas in other cell lines the amplified genes are stable. More recently we have found that amplification of H_2folate reductase genes occurs in a number of MTX-resistant cell lines derived from both murine and hamster origins, which have stable karyotypes or are highly aneuploid (3). As part of our studies on the mechanism of the amplification, loss, and stabilization of the H_2folate reductase genes, it is important to determine the chromosomal localization of these amplified genes.

Indirect evidence suggested that there is a specific localization of the H_2folate reductase genes. Biedler and Spengler (4) have reported that MTX resistance and elevated reductase levels are associated with a specific chromosomal abnormality in a Chinese hamster lung cell line. In these resistant cells there exists an expanded chromosomal region which displays no banding pattern when stained by the trypsin–Giemsa method, a so-called "homogeneously staining region" (HSR). These workers also reported that in this cell line, in which MTX resistance is unstable, there is a diminution in the average size of the HSR upon loss of MTX resistance (5).

We have studied a resistant Chinese hamster ovary (CHO) cell line with H_2folate reductase levels and gene copy number approximately 150 times that of sensitive cells. The amplified genes in this cell line are stable, and there exists a characteristic HSR on the second chromosome that is not present in sensitive cells. *In situ* hybridization of DNA complementary to mRNA of murine H_2folate reductase indicates that the reductase genes are specifically localized to the HSR region.

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MATERIALS AND METHODS

Isolation of a MTX-Resistant Variant CHO Clone. Five million unmutagenized CHO-K1 cells were exposed to increasing concentrations of MTX in Ham's F12 medium (6) lacking glycine, hypoxanthine, and thymidine, and supplemented with 10% (vol/vol) dialyzed fetal calf serum. At each concentration, cells were cultured until the majority of the culture was growing well; then an inoculum of 5×10^6 cells was used to initiate the next step. The concentrations of MTX used were 0.01, 0.03, 0.05, 0.07, 0.1, 0.4, 0.8, 1.0, 4.0, 10, 50, and 200 μM. A colony was picked from the final mass culture, and recloned in 200 μM MTX. This clone, designated MK42, was routinely maintained in medium containing 200 μM MTX.

Enzyme Assay. H_2folate reductase activity was measured at room temperature in sonic extracts (7) essentially as described by Frearson *et al.* (8) except that the mixture was preincubated for 10 min prior to initiating the reaction with 0.12 mM dihydrofolate. Cultures were grown for at least 1 week in MTX-free medium before they were harvested for assays. Protein was measured by the Lowry method, with bovine serum albumin as a standard.

Measurement of Rate of H_2folate Reductase Synthesis. The relative rate of enzyme synthesis was determined by direct immunoprecipitation of enzyme protein from extracts of pulse-labeled cells in early logarithmic phase under conditions of antibody excess, as described by Alt *et al.* (1). Preparation of rabbit gamma globulin against murine H_2folate reductase has been described (1). The crossreactivity of the antibody against murine H_2folate reductase with Chinese hamster H_2folate reductase was demonstrated by its ability to quantitatively remove reductase activity from MTX-resistant CHO MK42 cell extracts after immunoprecipitation. In a control experiment, gamma globulin against ovalbumin did not affect the activity in the supernatant. Immunoprecipitable radioactivity is expressed as a percent of the trichloroacetic acid-precipitable radioactivity of total soluble protein.

Preparation of [^{3}H]cDNA. [^{3}H]cDNA specific for H_2folate reductase mRNA sequences was prepared as described by Alt *et al.* (2).

Preparation of DNA. Cell pellets were resuspended in 9–19 vol of 10 mM Tris·HCl, pH 8.0/10 mM EDTA/10 mM NaCl/0.5% sodium dodecyl sulfate lysis buffer and disrupted vigorously with a Dounce homogenizer to reduce viscosity. Proteinase K (EM Labs) was added to 50 μg/ml and the lysate was incubated overnight at 37°C. After addition of sodium dodecyl

Abbreviations: C_0t, initial concentration of DNA (mol of nucleotide/liter) × time (sec); MTX, methotrexate; H_2folate reductase, dihydrofolate reductase (DHFR); HSR, homogeneously staining region; CHO, Chinese hamster ovary.

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sulfate to 1% and solid $NaClO_4$ to 0.6 M, the lysate was shaken at room temperature for 30 min and the DNA was further sheared by passage through a 27-gauge needle several times. The lysate was extracted twice with an equal volume of phenol/$CHCl_3$ (1:1 vol/vol) (saturated with lysis buffer) and the DNA was precipitated by addition of 2 vol of EtOH. After centrifugation the DNA was redissolved in 10 mM Tris·HCl, pH 7.4/2 mM EDTA/10 mM NaCl and sheared to approximately 400 base pairs by passage through a French pressure cell (Aminco) at 20,000 lb./inch^2 (1 lb./inch^2 = 6.89 × 10^3 Pa). RNA was hydrolyzed and the DNA denatured by bringing the sample to 0.3 M NaOH and incubating at 37°C for 20 hr. The DNA was cooled and neutralized by addition of an equimolar amount of HCl, buffered with Tris·HCl (pH 7.4) to 50 mM, and precipitated by addition of 2 vol of EtOH. After centrifugation, the DNA was redissolved in the above Tris/EDTA/NaCl buffer and used for C_0t analyses. (C_0t is initial concentration of DNA in mol of nucleotide/liter multiplied by time in sec.)

C_0t Analysis to Measure Relative Number of Copies of H_2folate Reductase Gene. C_0t analysis was carried out as described by Alt *et al.* (2). Reaction mixtures contained 25 mM Tris·HCl (pH 7.4), 300 mM NaCl, 1 mM EDTA, 50 pg of [^{3}H]cDNA (500 cpm), and 200 μg of CHO MK42 DNA or 600 μg of CHO K1 DNA and were incubated at 68°C for various times to achieve the appropriate C_0t values. Single- and double-stranded DNA was fractionated by hydroxylapatite chromatography as described by Alt *et al.* (2). At a C_0t of 0 (assayed immediately after the DNA was denatured), 3.2% of the [^{3}H]cDNA was retained on hydroxylapatite and eluted with the double-stranded portion of DNA. This value has been subtracted in calculations of the percent double-stranded DNA. The percent double-stranded DNA was determined by dividing the cpm in the double-stranded fraction by the cpm recovered in the single- plus the double-stranded fractions.

Chromosome Analysis. Metaphase chromosome spreads were prepared from Colcemid-treated cultures by air drying and were Giemsa banded by a modification of the method of Seabright (9). In most cases, slides were allowed to age from several days to several weeks to improve subsequent banding.

***In Situ* Hybridizations.** Metaphase chromosome spreads were prepared by the method of Deaven and Peterson (10) from exponentially growing cultures blocked for 60 min with 0.06 μg of Colcemid per ml. These slides were prepared for *in situ* hybridization by a modification (11) of the method of Pardue and Gall (12). Hybridizations were carried out in 15 μl of 0.3 M NaCl/0.03 M sodium citrate containing 1 mg of denatured chicken DNA per 0.1 mg of poly(rA) (Miles) per ml, and approximately 1.2 × 10^4 cpm of H_2folate reductase [^{3}H]cDNA. The hybridization mixture was placed on the slide, covered with a 22-mm^2 coverslip, and incubated at 65°C for 36 hr in a moist chamber enclosed within a plastic bag. After hybridization, the slides were washed extensively in 0.3 M NaCl/0.03 M sodium citrate at 60°C and finally dehydrated in 70% EtOH followed by 95% EtOH, and air dried. Autoradiography, using Kodak NTB-2 liquid emulsion, and Giemsa staining were performed as described by Pardue and Gall (12). Photographs were taken with a Zeiss microscope using Kodak high contrast copy film at ×500 magnification and after 6 months of exposure.

RESULTS

Biochemical Characterization of MTX-Resistant CHO Cell Line. Clone MK42 is approximately 50,000 times more resistant to MTX than the parental CHO K1 cells (Table 1). This resistance is accompanied by a 200-fold increase in the specific activity of H_2folate reductase. The reductase activity of the MK42 cells is as sensitive to MTX as that of the K1 cells, with 50% inhibition by 2 mM MTX (data not shown). The increase in activity is accounted for by an increase in the relative rate of reductase synthesis determined by immunoprecipitation of the enzyme from extracts of pulse-labeled cells. Drug resistance and high enzyme level in MK42 is a stable phenotype: no significant decrease in either trait has occurred after growth for approximately 75 cell doublings in medium containing no MTX. This is in contrast to certain murine cell lines where there is a 50% reduction in enzyme content within 15–20 cell doublings of growth in the absence of MTX (2, 3).

Table 1. Comparison of MTX-sensitive and MTX-resistant cell lines

Cell line	D_{50}, M	H_2folate reductase: Specific activity	H_2folate reductase: Relative rate of synthesis	H_2folate reductase: Relative gene copies
CHO K1	10^{-8}	3.2	<0.2	1
MK42	5×10^{-4}	770	4.1	150

D_{50} is the concentration of MTX required to reduce relative plating efficiency to 50%. Enzyme specific activity is nmol/min per mg of soluble protein. The relative rate of enzyme synthesis is expressed as a percent of trichloroacetic acid-precipitable soluble protein. Values below 0.2% are below the resolution of this assay. The relative number of gene copies is calculated from the data in Fig. 1.

We have previously shown that the increased synthesis of H_2folate reductase protein in several murine MTX-resistant cell lines is due to a corresponding increase in the number of copies of the reductase gene (2, 3). Fig. 1 shows a kinetic analysis of the relative number of reductase genes in the MK42 and K1 cells, using labeled DNA complementary to mRNA for murine H_2folate reductase mRNA (2). Hybridization of this cDNA occurs to an extent of 25–35% and the $C_0t_{1/2}$ value is shifted to the left when MK42 DNA is compared to K1 DNA. In addition, the hybridization with K1 DNA has a $C_0t_{1/2}$ essentially the same as that of the unique sequence portion of the total genome. The acceleration in the rate of hybridization corresponds to a 150-fold increase in the number of reductase gene copies in MK42 relative to K1 and indicates that the increased enzyme content and rate of synthesis of H_2folate reductase in the MK42 cell line result from a corresponding increase in gene copy number. We ascribe the lack of complete hybridization under the conditions used to a lack of complete sequence homology between murine and hamster H_2folate reductase DNA se-

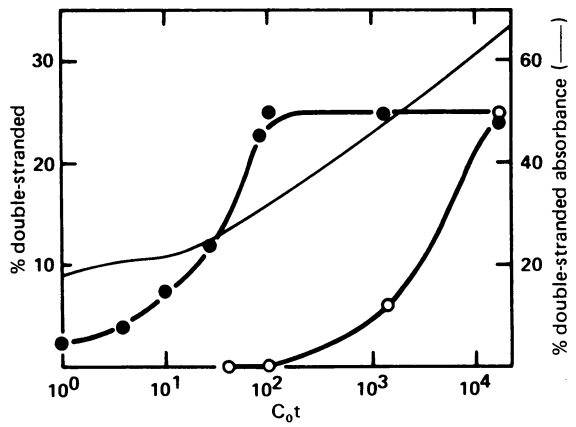

FIG. 1. Hybridization of cDNA of murine H_2folate reductase to DNA of MTX-sensitive and -resistant CHO cells. ○, MTX-sensitive CHO K1 DNA; ●, MTX-resistant CHO MK42 DNA. The thin line indicates the renaturation kinetics of the total hamster DNA.

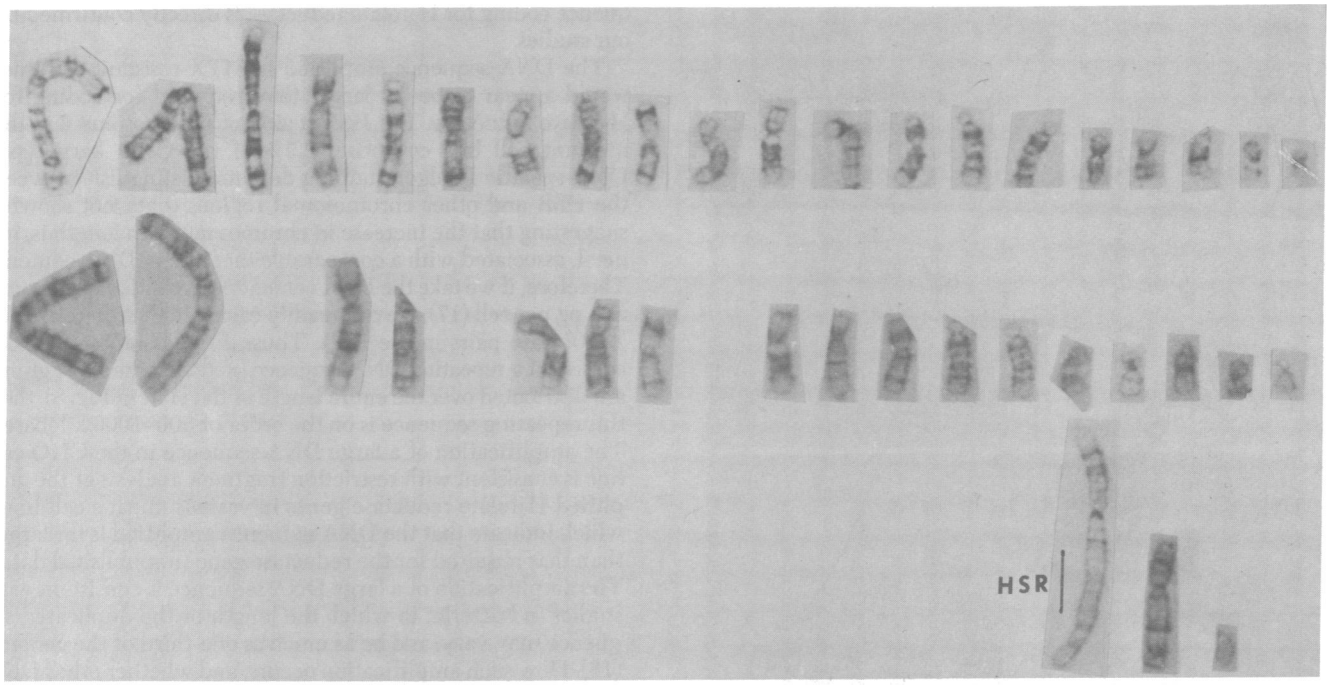

FIG. 2. Chromosome analysis of MTX-sensitive (*Top*) and MTX-resistant (*Middle* and *Bottom*) CHO cells. The *bottom* row shows the chromosomal abnormalities in the MTX-resistant cell line.

quences. We have previously shown that reductase cDNA, which hybridizes to approximately 85% with murine DNA and with a melting temperature (t_m) the same as that of total murine DNA, hybridizes to an extent of only 25–30% with human DNA under the same conditions and that such DNA·cDNA hybrids have a t_m approximately 10°C less than murine DNA·cDNA hybrids (2). The t_m of the hamster DNA·cDNA hybrids is reduced 9–11°C from that of hamster DNA itself (data not shown). Hence, we can conclude that the lack of complete hybridization results from considerable mismatching between murine and hamster H_2folate reductase sequences.[1]

Chromosome Analysis. Karyotype analysis reveals the presence of a distinctive chromosome anomaly in the MK42 cells. The single identifiable chromosome 2 has undergone an increase in length of approximately 40% due to the addition of a HSR to the long arm (Fig. 2). Such new chromosomal regions associated with MTX resistance were first described by Biedler and Spengler (4) and called homogeneously staining because of their lack of trypsin–Giemsa bands. These authors, studying an entirely different line of Chinese hamster cells, also found that the HSR in MTX-resistant cells was often associated with chromosome 2. As can be seen in Fig. 2, there is at least one other difference in the MK42 karyotype; most likely a translocation involving the sixth and tenth chromosomes (deleted from the second row of Fig. 2 and shown in row 3).

***In Situ* Hybridization.** In view of the fact that individual chromosomes can be easily identified in the CHO cells, we undertook to localize the amplified H_2folate reductase genes in the MK42 cells by *in situ* hybridization. Fig. 3 shows representative chromosome spreads from MTX-resistant (Fig. 3 *A–C*) and sensitive (Fig. 3*D*) cells. The resistant cells contain a large submetacentric chromosome not present in sensitive cells. This chromosome is easily identified as the chromosome containing the HSR in the middle of the long arm on trypsin–Giemsa banding (Fig. 2). It is to this region of the chromosome that hybridization with the H_2folate reductase cDNA has occurred. Silver grains were counted in 10 chromosome spreads of resistant cells and the number of grains over chromosome 2 (mean 7.9, SD 2.2) were compared with the sum of grains over all other chromosomes (mean 3.0, SD 2.2). A Student's *t*-test analysis confirmed a highly significant accumulation of grains over chromosome 2 ($P < 0.001$). Such an analysis obscures the fact (Fig. 3) that essentially all of the grains are localized entirely over the middle of the long arm of chromosome 2; i.e., the HSR. Although in these studies the number of grains present in the chromosome spreads of sensitive cells was far greater (Fig. 3*D*), they were distributed throughout the slide unrelated to any specific chromosome. We therefore conclude that the HSR in the middle of the long arm of chromosome 2 in MTX-resistant MK42 cells contains most of the amplified H_2folate reductase genes.

DISCUSSION

We have previously reported that the H_2folate reductase genes are amplified in MTX-resistant cultured murine cells and that the amplified genes can be in a stable or unstable state (2, 3). We have proposed various mechanisms for this process (15). More recently, using a fluorescein conjugate of MTX and the fluorescence-activated cell sorter to analyze the reductase content of individual cells in a population (16), we have concluded that in the unstable state the amplified genes are distributed unequally among daughter cells, and that the cells with low numbers of gene copies multiply more rapidly, eventually resulting in the dominance of the cell population with cells with

[1] There are two potential reasons for the lack of complete hybridization due to sequence mismatching. Mismatching results in a lower rate of hybridization of the cDNA to genomic DNA relative to the rate of hybridization of the two genomic DNA strands, effectively removing genomic single-stranded DNA from the reaction (13). Another potential reason is that the mRNA of murine H_2folate reductase is about 1500 nucleotides in length, with the approximately 600 coding nucleotides at the far 5′ end (14). If the noncoding 3′ sequences have diverged between mouse and hamster more than the coding sequences, and knowing that the mass of the cDNA is more representative of the 3′ than the 5′ end of the mRNA, then it would follow that not all of the cDNA would be sufficiently homologous to the hamster genomic DNA to be detected as a DNA·cDNA hybrid.

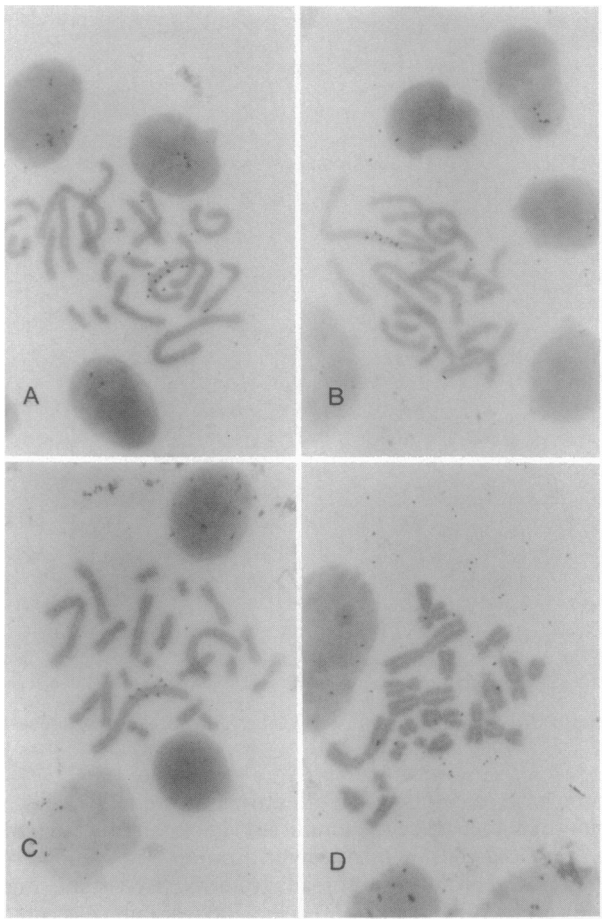

FIG. 3. *In situ* hybridization of H_2folate reductase cDNA to chromosomes of MTX-resistant (*A–C*) and MTX-sensitive (*D*) CHO cells.

low numbers of gene copies (3). As a part of our studies on the mechanism(s) of amplification, fixation, and loss of genes, it is important to determine if the genes are localized to specific chromosomes. In the studies reported herein we have used CHO cells whose karyotype is identifiable and relatively stable. MTX resistance in the CHO MK42 line is stable when cells are grown in the absence of MTX. The H_2folate reductase genes are amplified approximately 150 times relative to sensitive cells, an amplification that corresponds to the increase in reductase levels.

In the resistant cells there is a readily identifiable expansion on the single identifiable chromosome 2, which is not present in sensitive cells (Fig. 2). Such expanded regions have been denoted as "homogeneously staining regions," based on the lack of trypsin–Giemsa banding patterns (5). *In situ* hybridization of reductase cDNA clearly shows that the reductase genes are distributed along this HSR. Although we cannot entirely exclude the possibility that a few of the reductase genes are not present in this HSR, Urlaub and Chasin (unpublished data) have shown, in chromosome segregation experiments using intraspecific hybrid cells, that MTX resistance (high H_2folate reductase levels) is associated with the chromosome 2 containing the HSR. Biedler and Spengler (5) have also noted HSRs in MTX-resistant Chinese hamster lung cell lines, which are also associated with chromosome 2. Thus, their original suggestion that the HSR is associated with amplification of a DNA sequence coding for H_2folate reductase is directly confirmed by our studies.

The DNA sequence amplified in MTX-resistant cell lines would appear to be far larger than required for coding for H_2folate reductase. The HSR region of chromosome 2 in the resistant cell line comprises 3.5% of the CHO karyotype. DNA-specific Feulgen staining does not distinguish between the HSR and other chromosomal regions (data not shown), suggesting that the increase in chromosome arm length is, indeed, associated with a comparable increase in DNA content. Therefore, if we take the DNA content per mammalian genome as 5 pg per cell (17), we can readily calculate that there are 1.6×10^8 base pairs in the HSR. Thus, if we assume that HSR consists of a repeating DNA sequence (note that the silver grains are distributed over the entire length of the HSR in Fig. 3), then this repeating sequence is on the order of 500–1000 kilobases. The amplification of a large DNA sequence in the CHO cell line is consistent with restriction fragment analysis of the amplified H_2folate reductase genes in various murine cell lines, which indicate that the DNA sequence amplified is far larger than that required for the reductase gene (unpublished data). The amplification of a large DNA sequence is consistent with studies in bacteria, in which the length of the duplicated sequence may vary, and be as much as one-third of the genome (18). How such amplification occurs, and whether other DNA sequences in the amplified unit are expressed, are the subjects of current studies.

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1. Alt, F. W., Kellems, R. E. & Schimke, R. T. (1976) *J. Biol. Chem.* **251**, 3063–3074.
2. Alt, F. W., Kellems, R. E., Bertino, J. R. & Schimke, R. T. (1978) *J. Biol. Chem.* **253**, 1357–1370.
3. Schimke, R. T., Kaufman, R. J., Nunberg, J. H. & Dana, S. L. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, in press.
4. Biedler, J. L. & Spengler, B. A. (1976) *Science* **191**, 185–187.
5. Biedler, J. L. & Spengler, B. A. (1976) *J. Cell Biol.* **70**, 117a.
6. Ham, R. G. (1965) *Proc. Natl. Acad. Sci. USA* **53**, 288–293.
7. Chasin, L. A. (1974) *Cell* **2**, 37–41.
8. Frearson, P. M., Kit, S. & Dubbs, D. R. (1966) *Cancer Res.* **26**, 1653–1660.
9. Seabright, M. (1971) *Lancet* ii, 971–972.
10. Deaven, L. L. & Peterson, D. F. (1974) in *Methods in Cell Biology*, ed. Prescott, D. M. (Academic, New York), Vol. 8, pp. 179–204.
11. Bonner, J. J. & Pardue, M. L. (1976) *Chromosoma* **58**, 87–99.
12. Pardue, M. L. & Gall, J. G. (1975) in *Methods in Cell Biology*, ed. Prescott, D. M. (Academic, New York), Vol. 10, pp. 1–16.
13. Marsh, J. L. & McCarthy, B. J. (1974) *Biochemistry* **13**, 3382–3388.
14. Chang, A. C. Y., Nunberg, J. H., Kaufman, R. J., Erlich, H., Schimke, R. T. & Cohen, S. N. (1978) *Nature (London)* **275**, 617–624.
15. Schimke, R. T., Alt, F. W., Kellems, R. E., Kaufman, R. J. & Bertino, J. R. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 649–657.
16. Kaufman, R. J., Bertino, J. R. & Schimke, R. T. (1978) *J. Biol. Chem.* **253**, 5852–5860.
17. Fasman, G. D., ed. (1976) *Handbook of Biochemistry and Molecular Biology Nucleic Acids* (CRC, Cleveland, OH), Vol. 2, 3rd Ed., pp. 302–306.
18. Anderson, R. P. & Roth, J. R. (1977) *Annu. Rev. Microbiol.* **31**, 473–505.