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Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair region that includes the dihydrofolate reductase gene

(restriction fragments/drug resistance/homogeneously staining region/tetrahydrofolate dehydrogenase)

JEFFREY D. MILBRANDT, NICHOLAS H. HEINTZ, W. CARLTON WHITE, STEVEN M. ROTHMAN, AND JOYCE L. HAMLIN*

Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia 22908

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ABSTRACT **For the eventual purpose of isolating and studying a single animal cell replicon, we have developed a methotrexate-resistant Chinese hamster ovary cell line that has amplified an early-replicating DNA sequence approximately 500 times; this sequence includes the gene coding for dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3). DHFR composes 30% of the cytoplasmic protein in this cell line, and DHFR mRNA represents 25% of the message translatable *in vitro*. After digestion of genomic DNA from resistant cells with restriction enzymes, a unique set of highly repetitive restriction fragments can be visualized on agarose gels by ethidium bromide staining. These bands are not present in digests of parental DNA. We estimate the total length of the unit repeated sequence to be 135 ± 15 kilobase pairs. Regardless of the restriction enzyme utilized, a subset of these repetitive fragments hybridizes to radioactive DHFR cDNA. The homogeneously staining regions on mitotic chromosomes in which these amplified sequences are located are shown to be early-replicating, as are the highly repeated restriction fragments themselves. These data suggest that an early replicon can be isolated from this region, and that this entire, normally unique, genomic segment can be cloned and mapped with respect to origins of DNA synthesis and promoters for transcription, as well as other genetic features of interest.**

In order to study control of DNA synthesis in complex eukaryotic systems, one would like to examine a single replicon whose time of synthesis within the S period is known. Certain cell lines contain amplified sequences that appear to replicate synchronously with respect to one another and therefore behave as if they were composed of one or a few replicon types (1, 2). These sequences are found in a variety of methotrexate (MTX)-resistant mouse and hamster cell lines, and arise through the amplification of a large segment which includes the gene for dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) (3–6) as well as unknown other genetic material. Several hundred copies of the amplified sequence are arranged in tandem on one or a few chromosome arms, and constitute homogeneously staining regions (HSRs) on mitotic chromosomes when subjected to G- and C-banding protocols (1, 3, 4, 6). It has been shown that the HSRs in Chinese hamster lines begin to synthesize DNA over their length synchronously at many loci at the very beginning of the S period; in one of these lines, replication of the HSR is completed by the third hour of a 7-hr S period (2). These data suggest that each amplified unit may contain one or a few replicon types that respond to an initiation signal in the early S period.

For the purpose of defining the molecular properties of an animal cell replicon, we have developed a MTX-resistant Chinese hamster ovary (CHO) cell line with 700–1000 copies of an early-replicating sequence. As a result, the amplified nucleotide sequence can be viewed as a unique set of restriction endonuclease fragments on ethidium bromide-stained gels against the background smear of genomic DNA. Thus, this unusual amplification process will allow an examination of the molecular organization and replication of a large, normally unique genetic segment that would otherwise be difficult to study.

MATERIALS AND METHODS

Selection of MTX-Resistant CHO Cells. CHO cells were obtained originally from D. F. Peterson (Los Alamos, NM). Cells were selected for increasing resistance to MTX essentially as described by Nunberg *et al.* (3), except that cells were maintained in Eagle's minimal essential medium supplemented with nonessential amino acids, 10% *non*dialyzed donor calf serum, and 8% CO_2. Drug concentrations were 1, 10, 20, 40, 80, 400, and 800 nM, 2, 10, 100, and, finally, 800 μM; cells remained at each level for approximately 2 weeks. Cells resistant to 800 μM MTX (400 μg/ml) were cloned, and the resulting lines were maintained at this drug concentration at all times. They will be referred to as CHOC 400s.

Analysis of Cytoplasmic Proteins and mRNA. Exponentially growing cells were trypsinized and treated on ice for 5 min with 10 mM Tris·HCl, pH 7.6/10 mM NaCl/1.5 mM $MgCl_2$/1% Nonidet P-40, and nuclei were pelleted at 600 × *g* for 2 min. The cytoplasmic fraction was either electrophoresed on NaDodSO$_4$/10% polyacrylamide (7) or was subjected to two cycles of oligo(dT)-cellulose chromatography (8). The ethanol-precipitated messenger fraction was dissolved and was used as the source of mRNA in the rabbit reticulocyte *in vitro* translation system (9) with [^{35}S]methionine as the labeled amino acid. Reaction mixtures were electrophoresed as above and were visualized by fluorography (10).

Preparation of Animal Cell DNA, Restriction Endonuclease Digestion, Blotting, and Hybridization. The recombinant DNA experiments reported herein were performed according to current National Institutes of Health guidelines. DNA was purified by standard procedures, and was digested with restriction enzymes in the buffers suggested by the supplier (Bethesda Research Laboratories, Rockville, MD). Restriction digests were electrophoresed in agarose and were transferred to diazobenzyloxymethyl (DBM)-paper (Schleicher & Schuell) by the

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Abbreviations: DHFR, dihydrofolate reductase; HSR, homogeneously staining region; MTX, methotrexate; DBM, diazobenzyloxymethyl; CHO, Chinese hamster ovary; kb, kilobase pair(s).

* To whom reprint requests should be addressed.

method of Wahl *et al.* (11), except that excess diazonium groups were neutralized with 0.1% phloroglucinol and 1% glycine for 10 min. The DNA on the filters was hybridized with radioactive murine DHFR cDNA from plasmid pBR322 clone DHFR 21, obtained from Robert Schimke at Stanford University (12). DNA probes were nick-translated (13) to a specific activity of 5×10^7 dpm/μg with [^{32}P]dCTP (New England Nuclear), and after denaturation, 2×10^7 dpm was hybridized with each filter for 16 hr at 42°C. Results of hybridizations were detected by exposing Kodak XR-5 film to the dried blot at −70°C.

Chromosome Banding, *in Situ* Hybridization, and Retroactive Labeling (14). Fixed mitotic chromosomes were G-banded by the procedure of Seabright (15). For determinations of the replication pattern of the HSRs, replicate cultures in exponential growth were labeled for 10 min with 10 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]thymidine per ml of culture medium (3 Ci/mmol), label was removed, and cells were returned to fresh medium. At hourly intervals thereafter, Colcemid at 0.1 μg/ml was added to one plate for 60 min. Mitotic cells were harvested and spread on microslides, and the slides were coated with emulsion as described (2). In CHOC 400, the S period lasts for 10 hr and G2 for 1 hr. Labeled mitotic cells harvested 6 and 11 hr after the radioactive pulse were therefore in mid- and early S period, respectively.

In situ hybridizations were performed essentially by the method of Pardue and Gall (16). A recombinant plasmid (pDHFR-He) containing an 8-kilobase pair CHO DHFR genomic fragment was nick-translated with [^{3}H]dTTP (10^7 dpm/μg) and 1×10^4 dpm was hybridized to fixed mitotic cells for 48 hr. Slides were dipped in Kodak NTB2 emulsion and were exposed for 10–20 days.

All tissue culture media, serum, and antibiotics were obtained from GIBCO. MTX was obtained from the National Cancer Institute.

RESULTS

Overproduction of DHFR and Its mRNA in CHOC 400. When the cytoplasmic fraction of the MTX-resistant CHOC 400s is compared on a NaDodSO$_4$/acrylamide gel to that of the sensitive, parental CHO cells, it is apparent that a 21,000-dalton polypeptide that comigrates with legitimate DHFR is overproduced in the resistant cells (Fig. 1*A*). Densitometric scans indicate that in rapidly growing cultures this band can represent as much as 25–30% of the cytoplasmic protein. Fig. 1*B* demonstrates that overproduction of DHFR in CHOC 400 can be attributed to the overproduction of the mRNA for DHFR; 25% of the total CHOC 400 mRNA translated in the rabbit reticulocyte lysate system codes for DHFR. We have noticed that the observed levels of both DHFR and DHFR mRNA are lower in resting cultures, possibly because this protein may be made during a relatively brief interval prior to the S period (17).

Number of DHFR Gene Copies in CHOC 400. In order to determine whether the CHOC 400s contained amplified DHFR genes that could account for the overproduction of DHFR mRNA, by analogy to MTX-resistant cell lines studied in other laboratories (3, 5, 18), total genomic DNA from the sensitive parent and from the resistant CHOC 400s was digested to completion with *Eco*RI restriction endonuclease, CHOC 400 DNA was serially diluted, and the digestion products were separated according to size on agarose gels. The separated products were transferred to DBM-paper and were hybridized with radioactive murine DHFR cDNA probe, in order to detect fragments of DNA containing complementary genomic DHFR sequences. This dilution experiment was performed in order that an equivalent radioactive signal could be obtained on the same blot from the parental CHO DNA (arbitrary value of 1 for the DHFR gene copy number) and from resistant DNA (relative

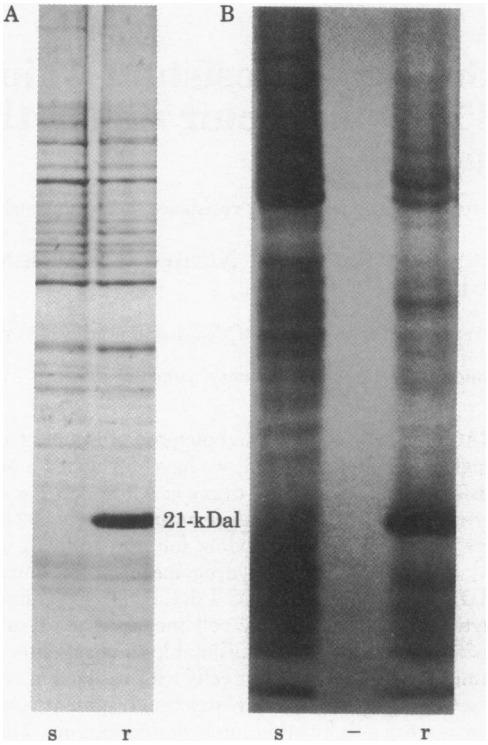

FIG. 1. Overproduction of DHFR and its mRNA in CHOC 400. (*A*) Cytoplasmic fractions from sensitive parental CHO cells (s) and from resistant CHOC 400 cells (r) separated on NaDodSO$_4$/10% polyacrylamide gels and stained with Coomassie brilliant blue. Note the presence in the CHOC 400 cytoplasm of large amounts of a 21,000-dalton (21-kDal) polypeptide that is absent in the parent. No differences were detected in the nuclear protein fractions (not shown). (*B*) Polypeptide products of mRNA from CHO (s) and from the CHOC 400 (r) cells translated in the rabbit reticulocyte lysate system and separated on 10% polyacrylamide. A control lysate without added mRNA (−) was included. Note that the CHO lane was loaded with twice as much translation mixture as the CHOC 400 lane, in order to demonstrate more clearly the absence of a significant band of 21,000-dalton polypeptide in the sensitive CHO lane. The figure shown is a fluorogram of the gel (10). The anode is at the bottom.

copy number expected to be greater than 100). From the experiment presented in Fig. 2 we estimate that the gene copy number is slightly less than 500 in the CHOC 400s relative to the sensitive parental CHO line; i.e., the radioactive signal obtained from 0.02 μg of CHOC 400 DNA was slightly less than that obtained from 10 μg of CHO DNA. Because CHO cells apparently contain two copies of the DHFR gene (19), the copy number of this gene per diploid nucleus must therefore be in the range of 700–1000 in CHOC 400.

Visualization of Restriction Fragments Arising from the Amplified Sequence. When restriction digests of CHOC 400 genomic DNA were separated on agarose gels, 18–26 discrete bands appeared against the background smear of unique fragments observed by ethidium bromide staining (Fig. 3). These bands were reproducible for a given enzyme, and the position and number of the bands varied with the enzyme used to digest the DNA (compare the *Eco*RI, *Xba* I, and *Hin*dIII digests of CHOC 400 DNA). These highly repetitive bands are *not* observed in the sensitive parental DNA (right lane, Fig. 3), or in digests of MTX-resistant CHO cells with levels of MTX resistance lower than approximately 100 μM (not shown). In order to determine whether some of these fragments contain the coding sequences for the DHFR gene, three different enzyme digests of CHOC 400 genomic DNA were separated on agarose, blotted onto DBM-paper, and hybridized to ^{32}P-labeled DHFR

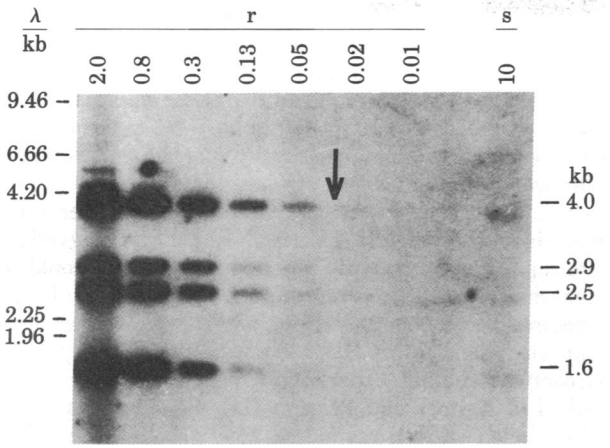

FIG. 2. Number of DHFR gene copies in CHOC 400. DNA from CHOC 400 and from the parental CHO cells was digested to completion with *Eco*RI, and the number of micrograms of each DNA sample indicated at the top was loaded into the wells of a 0.7% agarose gel. After electrophoretic separation of fragments, transfer to DBM-filters, and hybridization with DHFR [^{32}P]cDNA, the filters were washed at 45°C and autoradiographed. Note that fragments coding for DHFR sequences in the parental CHO digest (labeled s, 10 μg of DNA) are barely detectable; they correspond to the signal recorded for 0.02–0.05 μg of CHOC 400 DNA (arrow). This estimate was made from the x-ray autoradiograph itself, which did not photograph well. Note also that there are small amounts of partial digestion products detectable at the two highest CHOC 400 levels, and that this portion of the gel does not include the 23-kb *Hin*dIII λ DNA restriction fragment; the positions of other λ DNA fragments are indicated on the left.

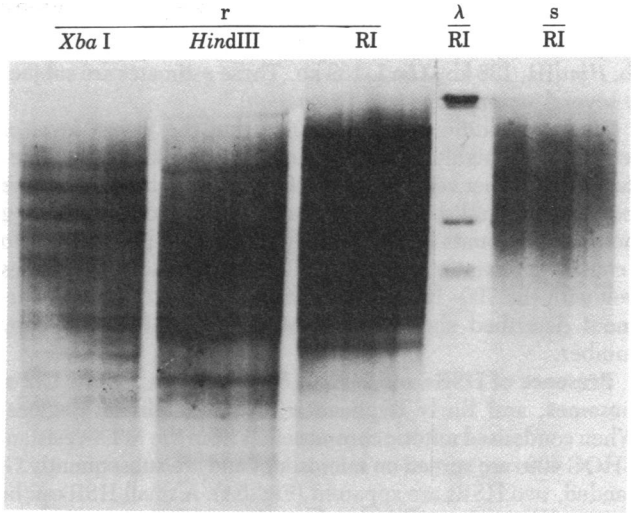

FIG. 3. Restriction patterns of CHOC 400 DNA. CHO and CHOC 400 genomic DNAs were digested to completion with various restriction enzymes. Digests were separated on 0.85% agarose gels and were stained with ethidium bromide. The figure shown is a high-contrast negative of the gel. CHOC 400 DNA (r) was digested with *Xba* I, *Hin*dIII, or *Eco*RI (RI). λ DNA size markers are 49 kb (uncut), 21.8, 7.5, 5.9, 5.4, 4.8, and 3.4 kb (not visible in this photograph).

cDNA (Fig. 4). It can be seen that a subset of the repeated restriction fragments visible in each digest appears to hybridize to the ^{32}P-labeled DHFR cDNA (compare lanes a to b, c to d, and e to f). Thus, we conclude that these bands correspond to restriction fragments arising from digestion of a larger amplified region in these MTX-resistant cells, only a fraction of which corresponds to coding sequences for DHFR. Note that the second largest DHFR band in the *Eco*RI digest in Fig. 2 (2.9 kb) is barely detectable in the *Eco*RI digest in Fig. 4. The murine cDNA probe apparently has the least homology to this fragment; thus, the more stringent washing conditions utilized in the experiment in Fig. 4 (60°C) have eluted the probe from this fragment.

In the experiment represented in Fig. 4, we have compared the sizes of the highly repeated bands in each of the three digests to a standard restriction digest of λ phage DNA. Our estimates for the position and number of fragments in each digest are shown in the figure. The results of this experiment suggest that

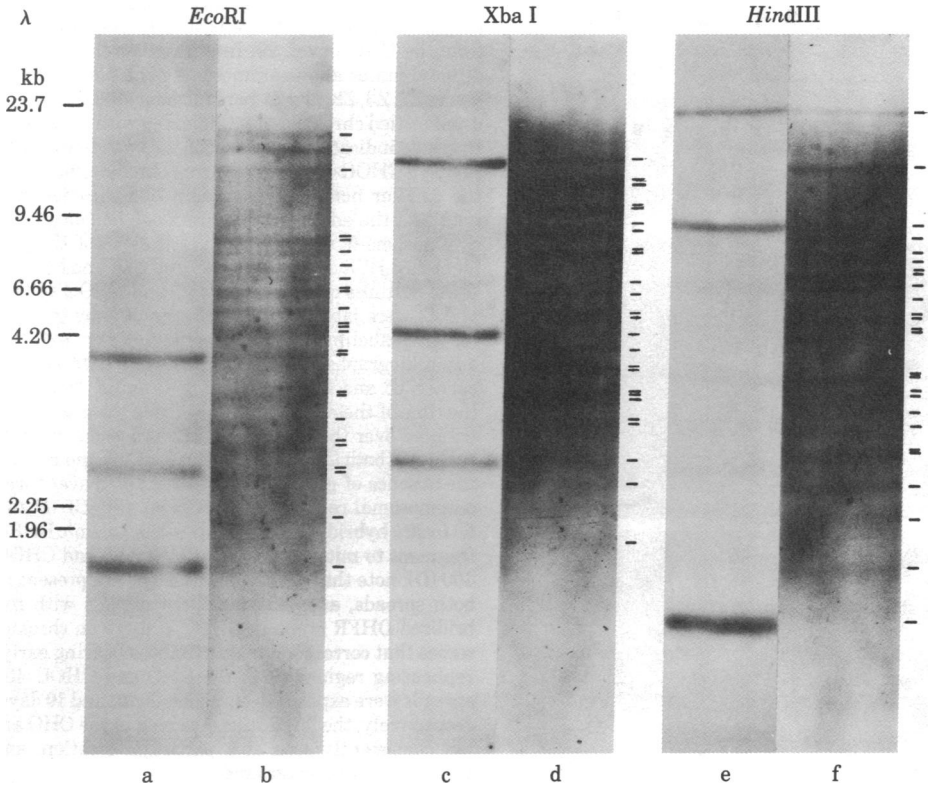

FIG. 4. Hybridization of DHFR cDNA to a subset of the highly repeated restriction fragments in CHOC 400. CHOC 400 genomic DNA was digested with *Eco*RI, *Xba* I, or *Hin*dIII, and the digests were separated on 0.85% agarose gels. Gels were stained with ethidium bromide and photographed, and a high-contrast negative was made from the positive (lanes b, d, and f). The gel was blotted onto DBM-paper, which was then hybridized with DHFR [^{32}P]cDNA (lanes a, c, and e) and washed at 60°C. The approximate positions in kb of markers from a *Hin*dIII digest of λ DNA are shown, and our estimates for the number and positions of fragments in each digest are indicated to the right of each group. Note also that the smallest fragments in lanes b, d, and f do not show up well on the high-contrast negative, but their positions were detected on the original photograph.

the sum of the repetitive fragment lengths is approximately 135 kb, with the following individual determinations; *Eco*RI, 132 kb; *Hind*III, 136 kb; *Xba* I, 139 kb. These estimates are subject to several assumptions (see *Discussion*).

We have additionally determined that the lowest level of detection of a highly repeated sequence in ethidium bromide-stained gels is between 700 and 1400 copies relative to a unique sequence (unpublished observations). This was done by adding increasing amounts of a restriction digest of λ phage DNA to a constant amount of a digest of CHO DNA. This range agrees well with the 700–1000 figure obtained in the dilution experiment described above for estimation of DHFR gene copy number.

Presence of HSRs and DHFR Sequences on Mitotic Chromosomes, and Early Replication of the Amplified Regions. When condensed mitotic chromosomes from the MTX-resistant CHOC 400s are spread on microslides and are subsequently G-banded, two HSRs are apparent (Fig. 5*A*). A small HSR can be detected on an elongated arm of one chromosome 1. In addition, there appears in CHOC 400 an entire mid-sized chromosome of unknown origin that contains approximately 80% nonbanding chromatin. Two examples of this chromosome are shown in Fig. 5*A*: the fifth from the left appears to be somewhat banded, although unusual in appearance relative to the other chromosomes; the chromosome farthest to the right in the CHOC 400 karyotype was taken from a second cell in which the nonbanded character is more apparent. This chromosome can be either the fifth or the sixth largest in the karyotype, depending upon the spread.

In situ hybridization experiments with a radiolabeled CHO DHFR genomic fragment indicated the presence of a total of three sites of DHFR amplification, one of which we are unable to detect in G-banded preparations of CHOC 400 (Fig. 5*D*). However, this chromosome appears to be the fifth or sixth largest in the CHOC 400, and therefore could be chromosome Z4. We have estimated the combined size of the amplified regions in several spreads of CHOC 400 to be 7–8% of the total condensed genomic chromosome length from photographs of *in situ* hybridizations.

Our interest in establishing these cell lines arose because we determined that similar HSRs in Chinese hamster lung cell lines were replicated in the early S period (2), and thus could represent the amplification of one or a few replicon types. In order to determine whether the HSRs, and, hence, the sequences composing them in the CHOC 400s were also early-replicating, retroactive labeling experiments were performed by the method of Bostock and Prescott (14). Replicate plates were pulse-labeled with [^{3}H]thymidine, label was chased with carrier thymidine, and each culture was collected at mitosis with Colcemid at intervals thereafter. Cells were spread on microslides and were autoradiographed. Grains appeared over the chromosome regions synthesized at the time of the radioactive pulse. Fig. 5 *B* and *C* shows mitotic spreads from CHOC 400s that were pulsed in early and in mid S. It is evident from these photographs that most chromosome regions (including the HSRs) begin to incorporate [^{3}H]thymidine in very early S (Fig. 5*B*); midway through the 10-hr S period, three chromosomal regions have ceased replication entirely in CHOC 400 (Fig. 5*C*); no such regions are detected in the parental CHO (Fig. 5*F*); these early-replicating regions in CHOC 400 correspond to the amplified sequences detected by *in situ* hybridization, judging by their location on the first-, fifth-, and sixth-largest chromosomes. In agreement with these studies is the observation that radioactive DNA that is prepared from CHOC 400 cells

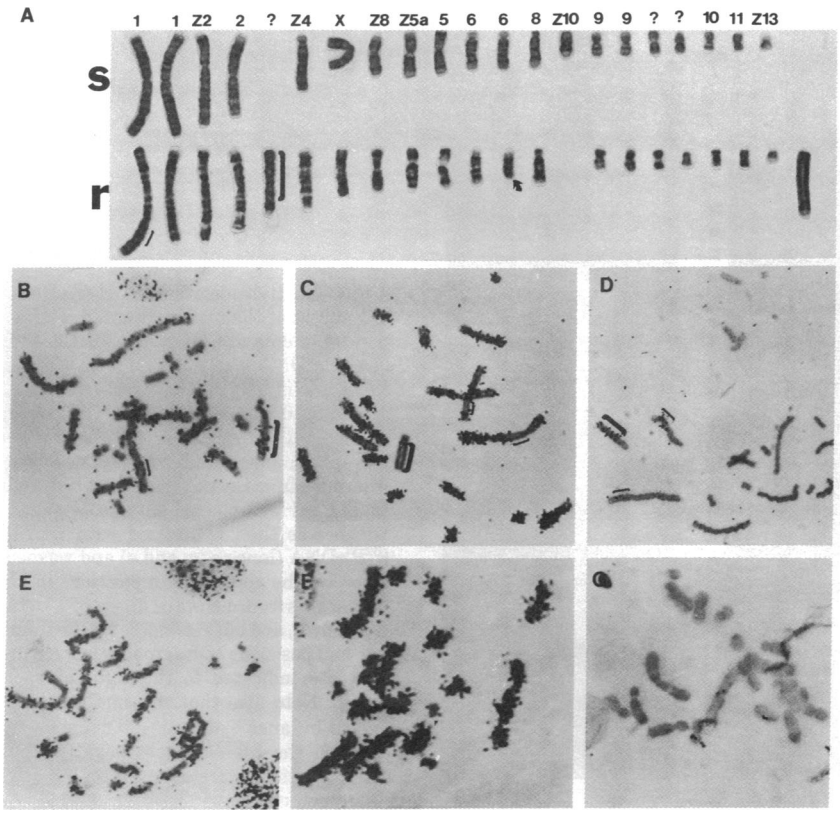

FIG. 5. Early-replicating HSRs in CHOC 400. (*A*) Mitotic cells from logarithmically growing cultures of CHO and CHOC 400 were fixed on a microslide and were G-banded. CHO chromosomes are arranged by decreasing size, and are designated as far as possible as in ref. 20. Note that the parental cell line is an aneuploid and rearranged variant of normal Chinese hamster cells, and differs somewhat from the CHO in ref. 20. In particular: there is additional material on chromosome Z2; a normal 5 replaces Z7; Z3, Z9, and Z12 are missing, and two small unidentified chromosomes (indicated with ?) appear. Brackets indicate the position of the two identifiable HSRs in CHOC 400: HSR-bearing chromosome 1 and the smaller nonbanding unidentified chromosome; note that the end is missing from one homologue of chromosome 6, which resembles the end of the unidentified HSR chromosome. (*B* and *C*, *E* and *F*) Replicate cultures of CHO (*E* and *F*) and CHOC 400 (*F* and *C*) were labeled with [^{3}H]thymidine by the retroactive labeling protocol described in the text; the autoradiographs shown represent cells that were in early S (*B* and *E*) and in mid-S (*C* and *F*) period at the time of the radioactive pulse; note the grains distributed over the length of all chromosome arms in early S in both CHOC 400 and in CHO (*B* and *E*), and the absence of grains by the fifth hr of S over three chromosomal regions only in CHOC 400 (*C*). (*D* and *G*) *In situ* hybridization of ^{3}H-labeled genomic DHFR fragment to mitotic spreads of CHO (*G*) and CHOC 400 (*D*); note that all 20 chromosomes are present in both spreads, and that the three regions with hybridized DHFR probe in CHOC 400 are on chromosomes that correspond in size to those bearing early-replicating regions in *C*; the CHO and CHOC 400 spreads were exposed to emulsion for 20 and 10 days, respectively; the few grains observed in the CHO are not consistently seen at a particular location, and they represent background.

labeled with [^{14}C]thymidine in early S is enriched for the amplified restriction fragments (unpublished data).

DISCUSSION

We have developed a CHO cell line that is extremely resistant to the antifolate drug methotrexate. We have shown this cell line to contain 700–1000 copies of the DHFR gene, which results in the increased production of DHFR mRNA and a concomitant increase in the target enzyme, DHFR. The CHOC 400s described here have three chromosomal regions that contain amplified DHFR sequences. Two of these are demonstrable HSRs. All three chromosome regions are shown to be early-replicating in retroactive labeling experiments.

The CHOC 400 cell line that we have described in this report is unique in at least two important ways amongst other MTX-resistant cell lines containing amplified DHFR genes. First, CHOC 400s apparently contain many more gene copies than do similar resistant cell lines (3, 6), with the result that an amplified sequence containing this gene in CHOC 400 gives rise to a discrete set of restriction fragments that can be detected after agarose gel electrophoresis and staining with ethidium bromide (Figs. 3 and 4). Other of our own cell lines with lower levels of resistance, as well as cell lines developed by others, appear not to have amplified this sequence to such a degree. Second, the amplified sequence (of which the DHFR gene is probably only a part) appears to be approximately 135 ± 15 kb long, as opposed to the 500–3000 kb estimated for other amplified sequences in murine (4, 6) and hamster (3) lines. The 135-kb estimate assumes that we are able to detect all the restriction fragments arising from the amplified sequence and that we can distinguish doublets and triplets from single fragments by the relative ethidium bromide staining in each band. Indeed, we probably do not detect some of the smallest fragments, and, as a consequence, slightly underestimate the combined length of the amplified region. Undiscernible doublets with large molecular weights (i.e., those that would affect the total estimate the most) would be unlikely to arise in the digests with *every* restriction enzyme utilized, and we therefore expect that our largest estimate for the length of the entire amplified segment (139 kb, *Xba* I digest) is a reasonable one.

The data presented here do not indicate whether a precise sequence (for example, *ABCDEFG*, in which *D* is the DHFR gene) is amplified, or whether several slightly different sequences are amplified that bracket *D*, but that include more or less of the flanking sequences (e.g., *BCDE*, *ABCDE*, *CDEFG*, etc.). The bands we are able to detect on gels would then arise only from region *CDE*, which is the consensus sequence in each repeated unit. Restriction fragments arising from regions *A*, *B*, *F*, and *G* would be underrepresented in gels, and would therefore escape detection. We are presently not able to distinguish between a precise and an imprecise amplification process, but the first of these models predicts that 135 kb repeated approximately 1000 times (a total of 1.4×10^5 kb) would represent 3% of the Chinese hamster genome (5×10^6 kb). This figure compares reasonably well with our estimate that 7–8% of the condensed mitotic chromosome length is contained in amplified regions in the CHOC 400 (as judged from *in situ* hybridization data). Nunberg *et al.* (3) have estimated that 3.5% of the condensed mitotic chromosome length in another MTX-resistant CHO cell contains approximately 150 copies of an amplified sequence. Assuming that condensed chromosome length is proportional to the DNA contained within any chromosomal region, they calculated that the amplified sequence must be 500–1000 kb long. It is not clear whether the amplified sequences in these two cell lines are intrinsically different, or whether the methods used to estimate the size of the amplified sequence would give such different results. We have also obtained evidence that all the highly repeated fragments we observe on gels are amplified to the same degree when a cell becomes resistant to a higher level of MTX, and, furthermore, that new highly repeated fragments do not appear during this process (unpublished). Thus, our data are consistent with a relatively precise amplification process in this cell line.

It was somewhat surprising to find that one of the three chromosome regions that bind a specific genomic DHFR fragment in *in situ* hybridization studies was not detectable as an HSR by G-banding. This could mean that amplified sequences are interspersed with unrelated sequences that are packaged differently in chromatin and therefore obscure the usual nonbanding characteristic of the amplified sequences. If this were true, the unrelated sequences must also be early-replicating, as indicated in the retroactive labeling experiments.

Because the amplified regions are early-replicating and can be viewed as a set of restriction fragments on gels, we are in a unique position to study the synthesis of a defined genetic segment. It should be possible to determine which of these restriction fragments contains an origin (or origins) of DNA synthesis. Furthermore, it should also be possible to clone the entire amplified segment, which will allow the isolation of an origin of DNA synthesis, as well as detailed structural and functional mapping of this extensive nucleotide sequence.

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