MOLECULAR AND CELLULAR BIOLOGY, June 1988, p. 2316–2327
0270-7306/88/062316-12$02.00/0

Vol. 8, No. 6

Organization and Genesis of Dihydrofolate Reductase Amplicons in the Genome of a Methotrexate-Resistant Chinese Hamster Ovary Cell Line

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Received 4 November 1987/Accepted 2 March 1988

We have recently isolated overlapping recombinant cosmids that represent the equivalent of two complete dihydrofolate reductase (*dhfr*) amplicon types from the methotrexate-resistant Chinese hamster ovary (CHO) cell line CHOC 400. In the work described in this report, we used pulse-field gradient gel electrophoresis to analyze large *Sfi*I restriction fragments arising from the amplified *dhfr* domains. The junction between the 260-kilobase type I amplicons (which are arranged in head-to-tail configurations in the genome) has been localized, allowing the construction of a linear map of the parental *dhfr* locus. We also show that the 220-kilobase type II amplicons are arranged as inverted repeat structures in the CHOC 400 genome and arose from the type I sequence relatively early in the amplification process. Our data indicate that there are a number of minor amplicon types in the CHOC 400 cell line that were not detected in previous studies; however, the type II amplicons represent ca. 75% of all the amplicons in the CHOC 400 genome. Both the type I and type II amplicons are shown to be composed entirely of sequences that were present in the parental *dhfr* locus. Studies of less resistant cell lines show that initial amplicons can be larger than those observed in CHOC 400. Once established, a given amplicon type appears to be relatively stable throughout subsequent amplification steps. We also present a modification of an in-gel renaturation method that gives a relatively complete picture of the size and variability of amplicons in the genome.

There are numerous examples of aberrant DNA sequence amplification processes in organisms ranging from bacteria to mammals (see references 17, 31, and 33, for reviews). Amplification of mammalian genes in response to stepwise increases in selective agents has received particular attention in recent years. It is not known whether DNA sequence amplification in mammalian cells results from some form of rereplication process or whether it results from unequal sister chromatid exchange or other recombination mechanisms. However, it appears that virtually any gene for which there is a suitable selective agent can be amplified (17, 31, 33), and the copy number of the selected gene can be as high as 2,500 per cell in some cases (36). In murine cell lines, amplified genes such as the dihydrofolate reductase gene (*dhfr*) are usually contained in double minutes, which are small extrachromosomal elements that lack centromeres and that are rapidly lost when the cells are propagated in the absence of selection (see, e.g., reference 21). In Chinese and Syrian hamster cells, amplified genes such as *dhfr* and *cad* are usually found in one or more stable chromosomal locations (4, 24, 26, 35), which were first detected as abnormally or homogenously staining chromosome regions by Biedler and Spengler (4).

A rather surprising finding in mammalian systems is that the units of amplification (amplicons) are much larger than the gene whose product is selected: amplicons range in size from a few hundred to 10,000 kilobases (kb) in length (16, 23). This raises the question of why such a large amount of flanking DNA is coamplified with the selected gene. A second generalization that has emerged from studies in several laboratories is that the multiple amplicons within and among cell lines can be extremely variable in length and sequence arrangement. For example, Federspiel et al. cloned ca. 240 kb of DNA including and surrounding the amplified *dhfr* gene in a highly methotrexate (MTX)-resistant murine cell line containing double minutes (10); however, they were not able to clone a complete amplicon, because of multiple branch points that were encountered during the chromosomal walks outward from the *dhfr* gene, indicating the presence of amplicons of various sizes. The studies of Federspiel et al. also suggested that unrelated DNA sequences mapping at distant loci in the parental genome can become joined to the *dhfr* locus during the amplification process (10).

In other studies on cloned amplicon sequences, Stark and co-workers have drawn a similarly complex picture of the structure of amplicons in *N*-(phosphonacetyl)-L-aspartate (PALA)-resistant Syrian hamster cell lines, even within and among cell lines derived in a single selection step (2, 16, 37). In addition, Van der Bliek et al. (34) and de Bruijn et al. (9) have shown that amplified fragments encoding different overproduced mRNAs have different copy numbers in the same multidrug-resistant cell line. Since the genes encoding these mRNAs are apparently linked in the chromosome, their results suggest large differences in the sizes of amplicons in a single cell line.

In our own laboratory, we have been studying the amplification of the *dhfr* gene in methotrexate-resistant CHO cells and have developed a cell line (CHOC 400) that contains approximately 1,000 copies of the *dhfr* gene and flanking

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sequences. The amplified DNA is located at three positions on different chromosomes (one major and two minor sites), as determined by in situ hybridization with *dhfr*-specific probes (24). We have recently cloned overlapping recombinant cosmids representing the equivalent of two complete *dhfr* amplicon types from the CHOC 400 genome (23). The type I amplicons are 260 kb in length and appear to be arranged in head-to-tail configurations in the genome, although we have not previously identified the junction between the type I sequences that would allow us to formally demonstrate this proposal. The more prevalent type II amplicon in CHOC 400 cells is ca. 220 kb in length and represents a truncated version of the type I sequence, suggesting that it may have arisen by rearrangement of the type I amplicon at some point during the amplification process. On the basis of the presence of inverted repeat sequences at the boundaries of the type II sequence, we suggested that these amplicons might be arranged in head-to-head and tail-to-tail configurations in the genome, thereby forming giant palindromes (23).

During the chromosomal walking procedure that was used to clone the *dhfr* amplicons from the CHOC 400 genome, relatively few branch points were encountered that were not involved directly in forming either the type I or type II amplicons (23). Furthermore, when a collection of overlapping cosmids representing the 260-kb type I amplicon were used as probes on digests of DNA from six other independently isolated MTX-resistant Chinese hamster cell lines, we found that the entire 260-kb sequence appeared to be amplified to approximately the same extent as the *dhfr* gene itself in all but one of these cell lines (25; J. E. Looney, C. Ma, T.-H. Leu, W. F. Flintoff, W. B. Troutman, and J. L. Hamlin, manuscript in preparation). In total, these data suggested that the amplicons in MTX-resistant CHO cells are not as heterogenous as those observed in most of the other drug-resistant mammalian cell lines that have been examined in detail. Alternatively, it is possible that our methods of analysis partially masked amplicon heterogeneity in CHOC 400. For example, when using the overlapping cosmid clones we may have pieced together consensus maps of amplicons that predict the type I and type II amplicons, but these amplicons may not actually exist as such in the genome.

In the work described in the present report, we examined the organization of *dhfr* amplicons in the CHOC 400 genome by using the infrequently cutting restriction enzyme *Sfi*I in combination with pulse-field gradient gel electrophoresis to analyze large restriction fragments from the amplified *dhfr* domains. In addition, we examined the DNA sequence rearrangements that occurred in the series of increasingly resistant cell populations and clonal lines that gave rise to CHOC 400 to gain insight into the genesis of the type I and type II amplicons. Our results show that the type I and type II amplicons actually exist as such in the genome of CHOC 400 cells and arose relatively early in the amplification process. Furthermore, we have located the junction between the type I amplicons, which establishes the correct linear permutation of the map in the parental *dhfr* locus. Our data also indicate that there is heterogeneity among *dhfr* amplicons in the CHOC 400 genome that was not previously detected, but that the type I and type II structures together account for ca. 75 to 80% of all the amplicons in this cell line.

MATERIALS AND METHODS

Development of resistant cell lines and cell culture. All cell lines were grown in minimal essential medium supplemented with nonessential amino acids, 10% donor calf serum, and gentamicin sulfate. Cultures were maintained in an atmosphere of 8% CO_2 in a humidified chamber. Resistant CHO cell lines were selected in increasing concentrations of MTX by following a protocol in which 5×10^6 cells were exposed to a lethal dose of drug at each selection step (0.01 μg/ml for CHO cells). In the early stages of selection, the resistant cells obtained at each step were maintained as populations. Beginning at a level of 50 μg/ml, a single healthy clone was established and was subjected to a further increase in the MTX dose. By this procedure, resistant populations were isolated at 0.01, 0.05, 0.2, 1, and 5 μg of MTX per ml, and clones were obtained at 50 and 400 μg of MTX per ml. Cell populations are designated CHO/0.01, etc., and clones are designated as CHOC 50, etc. Drug-resistant cells were maintained in the appropriate MTX concentration at all times. Media, sera, and antibiotics were obtained from GIBCO Laboratories.

DNA preparation, restriction digestion, and Southern blotting. For analysis of small restriction fragments less than 20 kb in length, genomic DNA was purified by standard procedures (15) and was incubated with the appropriate restriction enzyme in the buffer recommended by the supplier (Bethesda Research Laboratories, Inc.). Digests were separated on agarose gels, were transferred to GeneScreen Plus (New England Nuclear Corp.) by an alkaline blotting procedure (27), and were hybridized with probes as previously described (23). Probes were isolated from recombinant cosmids from the amplified *dhfr* domain in CHOC 400 cells (23). An appropriate digest of a cosmid was separated on low-melting-point agarose (Bethesda Research Laboratories), and the fragment of interest was excised and labeled with [^{32}P]dCTP (New England Nuclear Corp.) by the random primer method (11).

Genomic DNA was separated on pulse-field gradient gel electrophoresis essentially as outlined in reference 14. Approximately 10^5 cells were embedded in a 20-μl low-melting-point agarose plug (0.5%) formed in 3/32-in. (0.24-cm) Tygon tubing (Norton Plastics). Each plug (ca. 0.5 cm in length) was removed from the tubing and processed and digested with *Sfi*I as described previously (14). The plugs were then inserted into the wells of a 0.8% standard agarose gel (7.5 by 8.5 by 0.6 cm) formed in a GeneLine pulsed-field gradient (PFG) electrophoresis apparatus (Beckman Instruments, Inc.). Gels were run in 10 mM Tris-acetate–0.5 mM EDTA (pH 8.2) at 15°C and were pulsed for either 15 or 30 s at 150 mA (see legend to Fig. 3). The digests were transferred to GeneScreen Plus and hybridized with appropriate probes as described above.

In-gel renaturation. For *Eco*RI digests, the in-gel renaturation procedure was performed exactly as described by Roninson et al. (29, 30). Digests were end labeled with [^{32}P]dCTP by using T4 polymerase (29) and were separated on 0.8% standard agarose gels prior to two cycles of denaturation, renaturation, and S1 nuclease treatments. For *Sfi*I digests, DNA was labeled intrinsically by incubating cells for one cell cycle with 1 μCi of [^{3}H]thymidine per ml (80 Ci/mmol; New England Nuclear Corp.). Approximately 1.5×10^5 cells were then embedded in an agarose plug (see above), and the samples were processed and digested with *Sfi*I as described above. After electrophoresis, the gel was subjected to one cycle of a modified in-gel renaturation procedure (29) comprising the following steps: (i) two 30-min incubations in 0.5 M NaOH–0.6 M NaCl; (ii) five 15-min incubations with 50% formamide–3 × SSPE until the wash attained a pH of 8.0 (1 × SSPE is 10 mM sodium phosphate

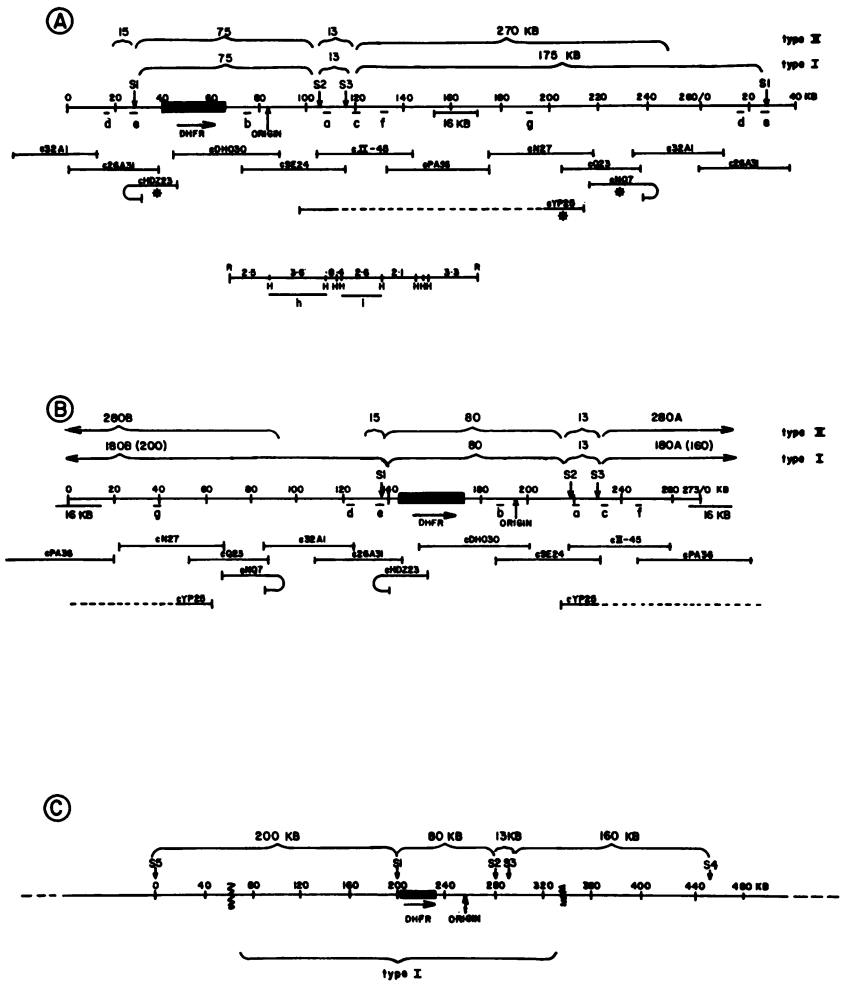

FIG. 1. Linear arrangement of overlapping recombinant cosmids from the CHOC 400 *dhfr* amplicons. (A) The predicted 260-kb type I amplicon is represented by a circularly permuted map that has been broken arbitrarily in cosmid c32A1, with the 5′ end of c26A31 placed at zero. The predicted 220-kb type II amplicon maps between the two inverted sequences depicted in cHDZ23 and cNQ7 (*). cYP25 is a cosmid that joins sequences in cSE24 and cN27 and appears to represent a 90-kb deletion (– – – –). The *dhfr* gene is shown on the linear scale (▬), and the origin of replication is also shown (19). *Sfi*I sites are indicated as S1, S2, etc., above the scale, as are the predicted *Sfi*I fragments from the type I and type II amplicons. Note that the *Sfi*I sites S3 and S1 in cosmids cII-45 and c26A31 define a ca. 175-kb fragment in the type I amplicon; in the type II amplicon, this fragment would be ca. 270 kb, since it would extend rightward from S3 to the palindrome in cNQ7 and back to S3. The positions of probes referred to in the text (a to g) are shown below the scale, as is the 16-kb *Eco*RI junction fragment from cosmid cPA36. A *Hin*dIII map of this fragment is shown at the bottom. (B) The correct order of recombinant cosmids is shown as their sequences would occur in the CHO genome. The linear scale begins and ends at zero, which represents the type I junction in cPA36. Note that the correct sizes are shown for the various *Sfi*I fragments discussed in the text (determined in pulse-field gradient gels), and the scale has been adjusted to show that the type I amplicon is 273 kb in length. Note also that the 180- and 280-kb fragments that characterize the type I and type II amplicons, respectively, are broken into two parts (A and B) by the type I junction. The 3′ portion derives from the 160-kb parental fragment, and the 5′ portion derives from the 200-kb parental fragment (compare with panel C). (C) The correct linear permutation of the *Sfi*I map in the parental CHO genome is shown, which covers 453 kb of the *dhfr* locus. The position of the ends of the type I amplicon are indicated by the wavy vertical lines on the linear scale.

[pH 6.0], 0.18 M NaCl, and 1 mM EDTA); (iii) a 20-h incubation in 50% formamide–3 × SSPE; (iv) four 20-min incubations in S1 buffer (50 mM sodium acetate [pH 4.6], 0.2 M NaCl, 1 mM $ZnSO_4$); (v) digestion with 120 U of S1 nuclease (Bethesda Research Laboratories) per ml for 2.25 h; and (vi) four 30-min incubations with 3 × SSPE–0.1% sodium dodecyl sulfate. All of the above steps took place in a volume of 80 ml at 37°C. After being rinsed with two changes of water, the digests were transferred to GeneScreen Plus by the alkaline blotting procedure (27), and the transferred digests were sprayed with En^{3}Hance (New England Nuclear Corp.) prior to autoradiography on Kodak X-Omat film with the aid of an intensifying screen.

RESULTS

Preliminary map of the *dhfr* amplicons in CHOC 400. Figure 1A shows an ordered series of overlapping cosmid clones isolated from the amplified *dhfr* locus in the CHOC 400 cell line (23). With the exception of cHDZ23, cNQ7, and cYP25 (marked with asterisks in the figure), the cosmids in this array represent the 260-kb type I amplicon, which has a circularly permuted map (23). The amplicons of this type were therefore suggested to be arranged head-to-tail in the genome. In previous studies, we were not able to identify the junction between the type I amplicons (23), and the circular map was therefore arbitrarily linearized beginning with the 5′

end of cosmid c26A31 at the zero position in the map (as in Fig. 1A). Note that the map repeats itself again 260 kb downstream with cosmid c26A31.

The 220-kb region mapping between the hairpins indicated in cosmids cNQ7 and cHDZ23 (Fig. 1A, asterisks) defined the type II amplicon. Since cNQ7 and cHDZ23 were each shown to contain palindromes, we suggested that the 220-kb type II amplicons are arrayed head-to-head and tail-to-tail in the genome, with the center of the palindromes in cHDZ23 and cNQ7 representing the joints between amplicons.

The 20 kb of DNA sequence mapping to the left of the cosmid cHDZ23 and the 20 kb extending from the right of cNQ7 to the 260/0-kb junction appeared to be present in only ca. 15% of the amplicons in the CHOC 400 genome, on the basis of the results of quantitative hybridization studies (23). Thus, the type I amplicon, as well as any other larger types that include this 40-kb sequence, represents a minority of the amplicons in this cell line. The type II sequence was suggested to represent ca. 80% of the amplicons in CHOC 400 cells, on the basis of hybridization studies with diagnostic type II junction fragments (23).

A third minor arrangement was defined by cosmid cYP25, which represents a union between sequences in cSE24 and cN27. This arrangement (Fig. 1A) appeared to represent a deletion of 80 to 90 kb in a region common to both the type I and type II amplicons (represented by a dashed line in Fig. 1A, cYP25). We could therefore not determine in which of the two amplicon types this deletion occurs.

Mapping the amplicons in genomic DNA with *Sfi*I. Since the three amplicon types described above were predicted from rather complicated mapping studies of a large number of overlapping cosmids (only a few of which are shown in Fig. 1), it was important to confirm that the suggested type I and type II structures actually exist in the CHOC 400 genome. We therefore mapped the amplicons in CHOC 400 with the infrequently cutting enzyme, *Sfi*I (*Sfi*I sites are denoted S1, S2, etc., in Fig. 1). This enzyme predicts the presence of three diagnostic *Sfi*I fragments from the circularly permuted type I amplicon (75, 13, and 175 kb in length) and four fragments from the palindromic type II amplicon (75, 13, 270, and 15 kb) (Fig. 1A, map). Note that the 75- and 13-kb fragments are common to both the type I and type II amplicons. However, the 175-kb *Sfi*I fragment predicted from the map of the type I sequence would become a 270-kb palindromic fragment in the type II amplicon that extends 3′ from *Sfi*I site S3 to the turnaround in cNQ7 and back to S3. Note also that the 15-kb *Sfi*I fragment is unique to the type II amplicon and extends 7.5 kb upstream from S1 to the turnaround in cHDZ23 and back to S1.

It was also necessary to localize the position of the junction between the head-to-tail type I amplicons in CHOC 400 to establish the correct linear permutation of this 260-kb sequence in the parental *dhfr* locus. As shown below, a new restriction fragment (P′K′) is formed when a fragment from one end of an amplicon (P) is joined to a fragment from the beginning of the next amplicon (K). The new restriction fragment P′K′ will usually be a different length than either of the parental-sized fragments that contributed to its formation, but P′K′ should hybridize with both of the parental fragments P and K in Southern blotting experiments on restriction digests of parental DNA.

abcdefghijKLMNOPqrstuvwxyz (parental arrangement)

abcdefghijKLMNOP′K′LMNOPqrstuvwxyz
(after amplification)

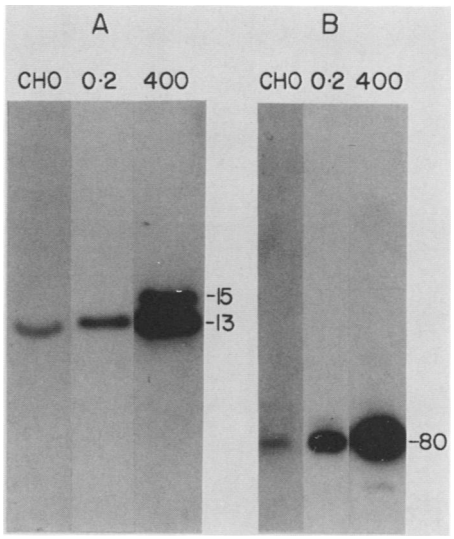

FIG. 2. Parental-sized 13- and 80-kb *Sfi*I fragments in the CHOC 400 genome (A). Genomic DNAs from CHO, CHO/0.2, and CHOC 400 cells were digested with *Sfi*I, and the digests were separated on a 0.7% agarose gel, transferred to GeneScreen Plus, and probed with a 1.1-kb *Kpn*I fragment (probe a) isolated from cSE24. (B) CHO, CHO/0.2, and CHOC 400 cells were embedded in 0.5% low-melting-point agarose plugs, and the plugs were processed, digested with *Sfi*I, and subjected to PFG electrophoresis, as described in Materials and Methods. After being transferred to GeneScreen Plus, the digests were hybridized with a 1.75-kb *Eco*RI fragment isolated from cSE24 (probe b). In both panels A and B, transfers were exposed to X-ray film for 120 and 18 h, to illuminate the CHO and drug-resistant DNA samples, respectively.

For the type I amplicon, the junction could be located in any one of the 50 to 60 *Eco*RI or *Hin*dIII fragments derived from this 260-kb sequence, each of which would have to be examined individually for the properties of a junction fragment. However, the use of the infrequently cutting enzyme *Sfi*I to localize the junction greatly simplifies this problem, since the junction must be located in one of only three *Sfi*I restriction fragments arising from the type I amplicon, if our predicted map of this amplicon is correct (i.e., the 13-, 75-, or 175-kb fragment in Fig. 1A). Therefore, if an *Sfi*I digest is hybridized with probes from either end of the junction-containing fragment, each probe will recognize a different-sized fragment in parental CHO DNA, but both probes will hybridize with the same-sized restriction fragment in CHOC 400 DNA. Once this fragment is identified, the exact position of the junction within the *Sfi*I fragment can be narrowed down by the successive use of hybridization probes that approach the center of the fragment.

We therefore used *Sfi*I, in combination with PFG and standard gel electrophoresis and Southern blotting, to analyze genomic DNA isolated from CHOC 400, from CHO, and from CHO/0.2 (a much less resistant cell population in the series that gave rise to CHOC 400 and that contains 30 to 40 copies of the *dhfr* gene). The positions of the hybridization probes (a to i) used to detect these *Sfi*I fragments are shown below the linear scales in Fig. 1A and B.

Figure 2 shows the results obtained when probes derived from the predicted 13- and 75-kb fragments were hybridized to *Sfi*I digests of genomic DNA from the three cell lines. Probe a (Fig. 1A) illuminates a 13-kb *Sfi*I fragment in the genomic DNA of the parental CHO cell line, in the CHO/0.2 cell population, and in CHOC 400 (Fig. 2A). Thus, in the

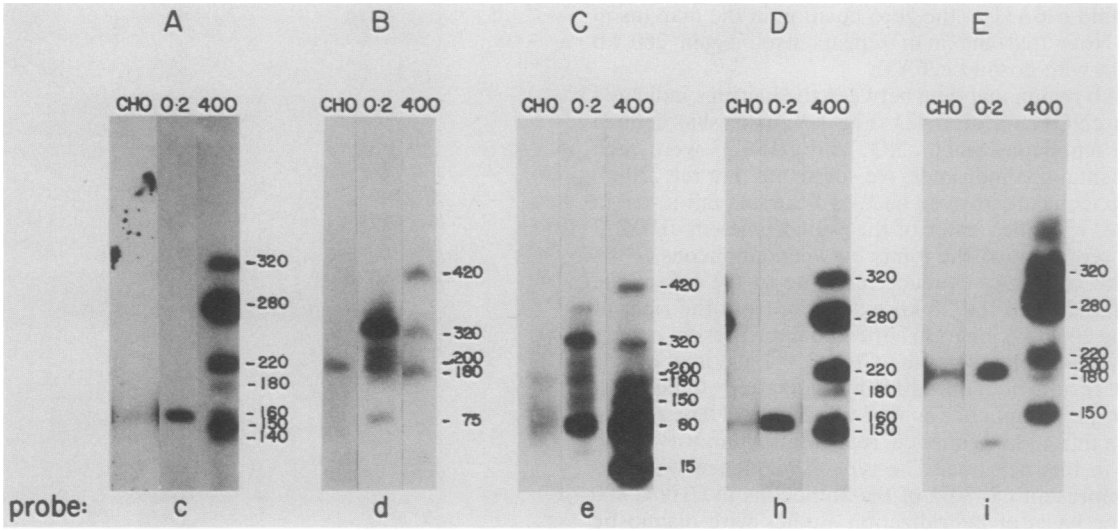

FIG. 3. Location of the type I junction in the 180-kb *Sfi*I fragment. *Sfi*I digests were prepared and separated on PFG gels. A 15-s pulse time was used for the gels in panels A, D, and E, and a 30-s pulse was used for the gels in panels B and C. The DNA in the gels was transferred to GeneScreen Plus as in Fig. 2. Individual transfers were hybridized with the following probes (Fig. 1A): a 0.8-kb *Sfi*I-*Eco*RI fragment isolated from cII-45 (probe c) (panel A); a 6.3-kb *Eco*RI fragment isolated from c26A31 (probe d) (panel B); a 6.7-kb *Eco*RI fragment containing the *Sfi*I site in cHDZ23 (probe e) (panel C); the 3.6-kb *Hin*dIII fragment isolated from the 16-kb *Eco*RI fragment contained in cPA36 (probe h) (panel D); the 2.6-kb *Hin*dIII fragment isolated from the 16-kb *Eco*RI fragment in cPA36 (panel E). In each case, the transfers were exposed to film for 120 and 18 h to bring out the detail in the parental CHO and the drug-resistant samples, respectively. Fragment sizes were determined by comparison with a ligated ladder of bacteriophage lambda DNA in a separate well (see Fig. 7).

amplified cell lines, this predicted fragment is present in the genome and is parental sized (with the exception of a minor 15-kb variant in CHOC 400). The 13-kb *Sfi*I fragment therefore does not appear to contain the junction between the type I amplicons. The 15-kb variant fragment in CHOC 400 represents ca. 10% of the total hybridization signal (determined from lower film exposures). We have not cloned this variant and cannot presently explain its origin.

When probe b was hybridized to *Sfi*I digests separated by PFG electrophoresis (Fig. 2B), an 80-kb fragment was detected in all three cell lines (sizes were determined by comparison with a ladder of ligated lambda DNA; see Fig. 7). Thus, this 80-kb band, which must correspond to the predicted 75-kb fragment, is also parental sized in the amplified cell lines and does not contain the junction between the type I amplicons. A minor 40-kb cross-reacting fragment of unknown origin was observed in CHOC 400 and in CHO/0.2 (Fig. 2B), as well as in two other amplified cell lines (data not shown). Very minor fragments of approximately 100 and 140 kb in length were also detected in the CHO/0.2 and CHOC 400 digests, respectively, and were not studied further.

The hybridization results were considerably more complicated when probes for the predicted 175-kb (type I) and 270-kb (type II) *Sfi*I fragments were used (Fig. 1A). The hybridization patterns obtained with probe c from cosmid cII-45 are shown in Fig. 3A. This probe detects a minor 180-kb fragment in CHOC 400 DNA, which is close to the 175 kb predicted from the map of the type I amplicon. Probe c also hybridizes intensely to a 280-kb fragment; this fragment undoubtedly corresponds to the predicted 270-kb palindromic fragment from the type II amplicon that extends between S3 and cNQ7, since it is not detected by probes that map to the 3′ side of the turnaround in cNQ7 (see Fig. 1A, map, and results with probe d below).

Thus, we conclude that both the predicted type I and type II structures actually do exist in the genome of CHOC 400 cells. However, the sizes of these two amplicons are closer to 273 kb (13 + 80 + 180 kb) and 240 kb (13 + 80 + 280/2 + 15/2 kb), respectively (as opposed to the original estimates of 260 and 220 kb, which did not include restriction fragments less than 1 kb in length [23]). However, several other variant fragments were detected in the CHOC 400 genome by probe c that are not so easily explained (Fig. 3A). These include rather prominent 150-, 220-, and 320-kb bands and a minor band at 140 kb. The possible origin of these fragments will be addressed in the discussion.

A minor band at 160 kb was also detected in CHOC 400 with probe c; this fragment corresponds to the only detectable band in parental CHO cells and to the major band in the CHO/0.2 cell population (Fig. 3A). If the 180-kb fragment in CHOC 400 contains the junction between the type I amplicons, then the 160-kb band could represent one of the two parental *Sfi*I fragments that were joined together. If this is the case, then a minor fraction of the amplicons in CHOC 400 and almost all of the amplicons in CHO/0.2 are probably larger than the type I amplicon, since they contain the 160-kb *Sfi*I fragment in its entirety.

To confirm that the 180-kb fragment contains the junction between the type I amplicons, we also hybridized *Sfi*I digests with probe d from the right end of this fragment (Fig. 1A, map). This probe should detect the 180-kb fragment, but should not detect the prevalent 280-kb palindromic *Sfi*I fragment present in the type II amplicons, since probe d maps to the right of the turnaround in cNQ7 (Fig. 1A, map). Figure 3B shows the results of this analysis. Again, the 180-kb type I fragment, in addition to bands at 420, 320, and approximately 75 kb, is observed in CHOC 400. With the exception of the 180-kb band, we are unsure of the origin of these fragments. Note that, together, the bands detected by this probe in CHOC 400 DNA represent less than ca. 10% of the amplicons in this cell line, since the probe does not detect the major 280-kb type II *Sfi*I fragment, nor the 220-, 160-, 150-, or 140-kb fragments detected by probe c in Fig.

3A. The 75-kb band (but not the 420-, 320-, or 180-kb bands) is observed in the CHO/0.2 population, which also displays a prominent band at ca. 330 kb and faint bands at 250 and 200 kb.

Most importantly, the only fragment detected by probe d in the parental CHO digest is 200 kb in length (Fig. 3B). This result, in combination with the data presented in Fig. 3A, shows that the 180-kb *Sfi*I fragment from the type I amplicon represents the union of two parental-sized fragments of 160 and 200 kb in length. The 180-kb *Sfi*I fragment therefore contains the junction between the type I amplicons.

Figure 3C shows the hybridization results obtained when an *Eco*RI fragment that spans the *Sfi*I site in the cosmid cHDZ23 (probe e) was hybridized to the *Sfi*I digests. In the CHOC 400 digest, this probe should detect the 80-kb fragment (common to the type I and type II amplicons), the 180-kb junction fragment unique to the type I sequence (defined by *Sfi*I sites S3 and S1), and the 15-kb palindromic fragment mapping on the 5′ end of cHDZ23 in the type II amplicon (defined by S1 [Fig. 1A]). As predicted, all of these fragments are observed in CHOC 400 DNA, as are the variants at 150, 320, and 420 kb that were detected by probes c and d in Fig. 3A and B. In the CHO/0.2 digest, 80- and 330-kb bands are detected, in addition to minor bands at 200, 150, and ca. 370 kb.

Fine mapping of the junction between the type I amplicons. To localize the position of the type I junction more precisely, we used several other small probes isolated from the region mapping between probes c and d (Fig. 1A). We found that probe f hybridized to the 160-kb parental *Sfi*I fragment, whereas probe g hybridized to the 200-kb parental fragment (Fig. 1A; hybridization data not shown). This result suggested that the junction lay somewhere in the neighborhood of cosmid cPA36. In previous studies, we detected a large (>16-kb) *Eco*RI fragment in cPA36 and in the CHOC 400 genome that is not present as such in parental CHO cells (23). Instead, this fragment hybridized to two smaller fragments in CHO DNA that were approximately 10 and 9 kb in length. However, since we could not detect a difference in *Hin*dIII digests of CHOC 400 DNA in this region when it was probed with cPA36, we assumed that the large *Eco*RI fragment resulted from the loss of an *Eco*RI site early in the course of amplification (23). More extensive restriction analysis of this region shows that, in fact, the junction between the type I amplicons in CHOC 400 is located approximately in the center of the 16-kb *Eco*RI fragment contained in cPA36. The *Hin*dIII map of this fragment is shown at the bottom of Fig. 1A. When the 3.6- and 2.6-kb *Hin*dIII subfragments (probes h and i) were used as probes on *Sfi*I digests of CHO DNA, they hybridized to the parental 160- and 200-kb *Sfi*I fragments, respectively (Fig. 3D and E). Thus, the junction between the type I amplicons is contained in one of the two small *Hin*dIII fragments mapping between probes h and i (Fig. 1A). The two parental *Hin*dIII fragments that formed the junction must also be very small to explain why we detected no differences between parental and CHOC 400 *Hin*dIII digests when they were probed with cosmid cPA36 in previous studies (23). Note that the hybridization patterns of probes h and i on *Sfi*I digests of CHOC 400 DNA were almost identical to each other and to those observed with probe c (compare Fig. 3D and E with Fig. 3A). This result is expected, because both probes h and i lie in a region common to the 180-kb type I and 280-kb type II *Sfi*I fragments, as does probe c.

The localization of the junction between the type I amplicons in cPA36 establishes the correct permutation of the overlapping cosmid series that represents the type I sequence (shown in Fig. 1B). Note that the corrected sizes of the *Sfi*I fragments were used in this figure and that the linear scale has been adjusted to indicate that the type I and type II amplicons are ca. 273 and 240 kb, respectively. The order of the four *Sfi*I fragments in the parental CHO genome is 200, 80, 13, and 160 kb, reading from left to right (Fig. 1C). An interesting outcome of the revised map shown in Fig. 1B is that neither the head-to-head nor the tail-to-tail junction between the type II amplicons coincides with the type I junction mapping in cPA36. Our studies also indicate that the type I and type II amplicons arose exclusively from the *dhfr* locus and do not contain unrelated sequences that were introduced into the amplicons by illegitimate recombination events.

Origin and stability of junction fragments. To determine when, during the amplification process, the type I and type II amplicons arose, we used the junction fragments between these amplicons as probes on *Eco*RI digests of genomic DNA isolated from the series of cells that gave rise to CHOC 400. This series includes the parental CHO cells, the cell populations CHO/0.01, CHO/0.2, CHO/1, and CHO/5, and the clonal lines CHOC 50 and CHOC 400 (see Materials and Methods for the development of cell lines). The origins of probes for the type I and type II junctions are shown in Fig. 1A and in the map of the junction regions (Fig. 4).

When the 16-kb *Eco*RI type I junction fragment from cPA36 (Fig. 1A) was used to probe *Eco*RI digests of the series of increasingly resistant cell lines, the junction was detected first in the CHO/1 cell population (which is estimated to contain ca. 100 copies of the *dhfr* gene; Fig. 5A). The 16-kb junction fragment represents approximately 50% of the hybridization signal in CHO/1.0 DNA; the remaining 50% derives from the parental 10- and 9-kb *Eco*RI fragments. Interestingly, in the more resistant CHO/5 population, which contains ca. 300 copies of the *dhfr* gene per cell, the type I junction is present, but appears to represent a very minor species, suggesting that the cell type carrying the type I sequence arrangement was at a growth disadvantage and was overtaken by other cell lines with larger amplicons during this selection step. However, we apparently isolated the minor cell type containing the type I sequence again when we cloned CHOC 50, since the 16-kb type I junction fragment represents the prevalent hybridizing species in this cell line and in CHOC 400 (Fig. 5A). The type I amplicon therefore apparently persisted during amplification from less than 100 to more than 1,000 copies of the *dhfr* gene per cell. The asterisks in the figure represent cross-reacting (possibly middle repetitive) elements that apparently occur elsewhere in amplified DNA, since they are detected in all of the resistant cell lines after long film exposures (not shown) and increase with the degree of amplification. The arrowhead indicates a variant cross-reacting fragment of unknown origin that occurs only in the CHOC 50 and CHOC 400 samples.

The results obtained with the type II junction fragments are shown in Fig. 5B and C, and a map of the junction regions is shown in Fig. 4. The 4.2-kb *Eco*RI fragment containing the head-to-head junction in cHDZ23 (Fig. 4, fragment T/U) hybridizes only to parental-sized fragments in CHO cells and in the moderately resistant cell populations CHO/0.01 and CHO/0.2 (Fig. 5B). The 4.2-kb head-to-head junction fragment can first be detected as a minor variant in the CHO/1 cell population, appears only faintly in the CHO/5 population, and then becomes a very prominent band in the first clonal cell line, CHOC 50. The two parental 4.9- and

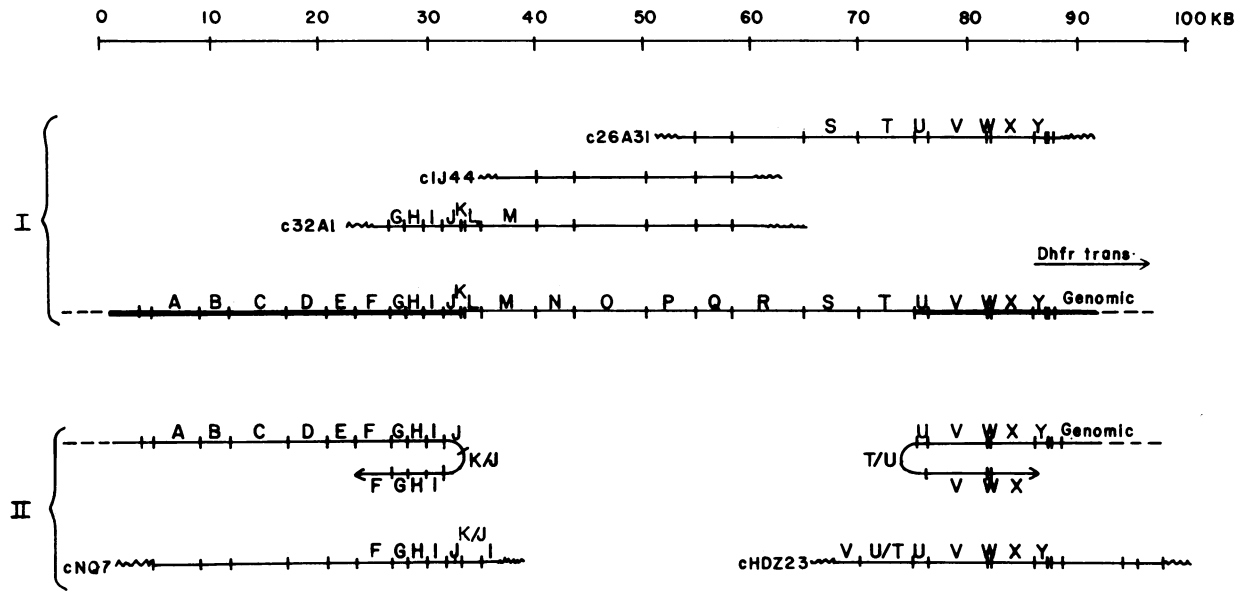

FIG. 4. *Eco*RI map of the region encompassing the two palindromic junctions in the type II amplicons. The map of the type I amplicon in this region is shown, with the sequences common to the type II amplicon highlighted with a bold line. The cosmids used to determine the type I map are shown above. The map of the type II amplicon indicates the stucture of the two palindromic regions defining the head-to-head (cHDZ23) and tail-to-tail (cNQ7) junctions. Note that one of the two fragments forming the K/J junction (J) is contained in both the type I and II amplicons, as is fragment U from the T/U junction.

1.9-kb fragments (fragments T and U) persist throughout the amplification process, although the 4.9-kb band (T) characteristic of the type I amplicon does not increase in CHOC 50 or CHOC 400, whereas the 1.9-kb fragment (U) does (since it is contained in both the type I and II amplicons [Fig. 4]). A similar result was obtained with the type II junction fragment isolated from cNQ7 (Fig. 4, fragment K/J). This junction fragment hybridizes to the two parental fragments at 0.5 and 2.5 kb (fragments K and J) at low levels of resistance, but also hybridizes to its 1.0-kb counterpart K/J in CHO/1 DNA (Fig. 5C). A signal at this position can barely be detected in CHO/5 cells, but then appears again as a prominent band in the CHOC 50 and CHOC 400 clones. In Fig. 5B and C the asterisks indicate cross-reacting species that are seen in all cell lines, and the arrowhead in Fig. 5B indicates a variant observed only in the CHOC 400 cell line.

Heterogeneity of amplicon structures in different CHOC/5 clones. The rather abrupt appearance of both the type I and

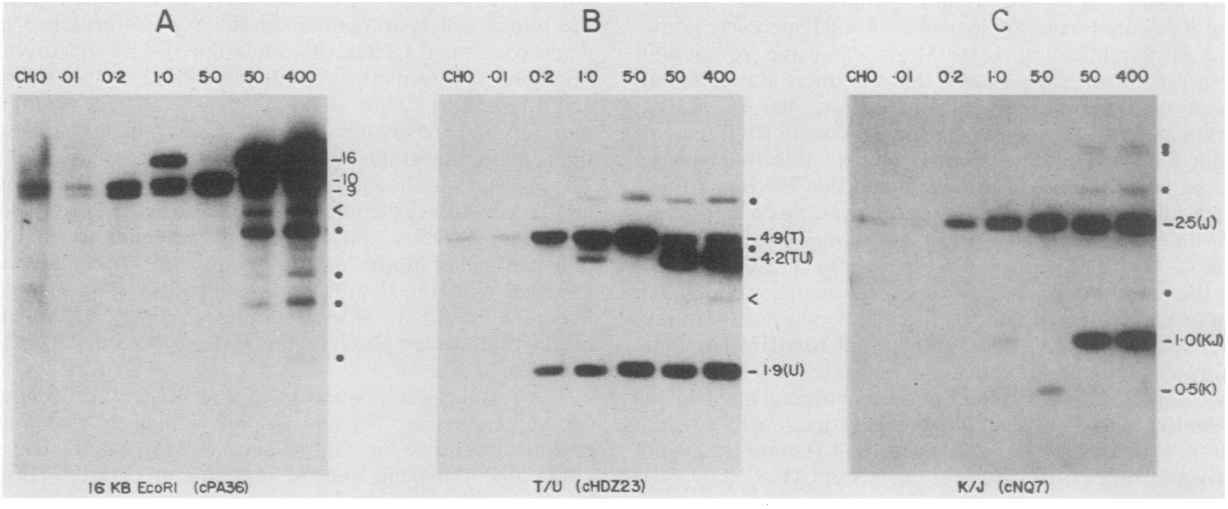

FIG. 5. Origin of the type I and type II junctions during the development of CHOC 400. (A) Genomic DNA was purified from the indicated cell lines and was digested with *Eco*RI. CHO DNA (7 μg) and DNA (1 μg) from each of the resistant cell lines were separated on 0.8% agarose. After being transferred to GeneScreen Plus, the transferred DNA was hybridized with the 16-kb *Eco*RI junction fragment isolated from cPA36. Symbols: *, cross-reacting fragments common to all cell lines; <, a variant fragment unique to CHOC 50 and CHOC 400 (B) *Eco*RI digests identical to those described in panel A above were probed with the 4.2-kb *Eco*RI junction fragment (T/U) derived from cHDZ23 (Fig. 4). Symbols: *, cross-hybridizing species common to all cell lines; <, a variant found in CHOC 400 only. (C) *Eco*RI digests were probed with the 1.0-kb *Eco*RI junction fragment (K/J) from cNQ7 (Fig. 4).

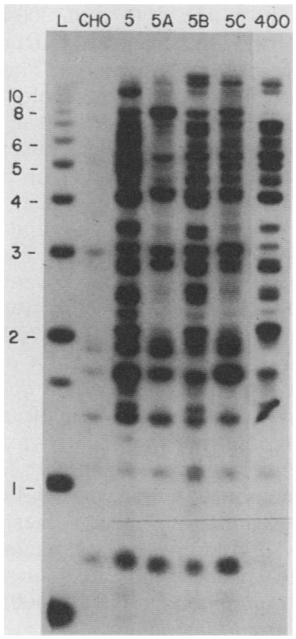

FIG. 6. In-gel renaturation analysis of individual clones from the CHO/5 population. *Eco*RI digests of DNA were isolated from CHO and CHOC 400 cells, from the CHO/5 population, and from three clones isolated from the CHO/5 population (clones 5A, 5B, and 5C). After the DNA had been end labeled with [^{32}P]dCTP (29) and separated on a 1.0% agarose gel, the gel was subjected to the in-gel renaturation procedure described by Roninson (29). A 1-kb end-labeled ladder (Bethesda Research Laboratories) was included as a standard.

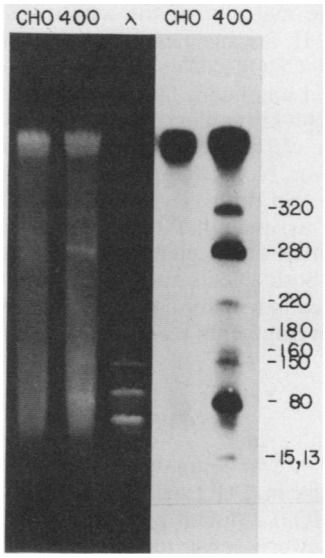

FIG. 7. In-gel renaturation analysis of *Sfi*I digests of CHO and CHOC 400 DNA. CHO and CHOC 400 cells were labeled with [^{3}H]thymidine in vivo for one cell cycle, and genomic DNA was processed for *Sfi*I digestion as described in Materials and Methods. The digests were separated on a 0.8% agarose gel by PFG electrophoresis (150 mA, 15-s pulse, 16 h), along with a ligated ladder of lambda phage DNA (14), and the gel was then subjected to one cycle of the modified in-gel renaturation procedure as described in Materials and Methods. Digests were transferred to GeneScreen Plus, and the transferred DNA was sprayed with En^{3}Hance and placed next to X-ray film with an intensifying screen. The right panel shows the resulting autoradiogram, and the left panel shows the ethidium bromide-stained agarose gel after electrophoresis. The smallest lambda fragment is the 48.5-kb monomer.

type II amplicon junctions in the CHO/1 cell population, their virtual disappearance in the CHO/5 population, and their reappearance in the CHOC 50 cell line suggested that the CHO/5 population from which CHOC 50 was cloned was heterogeneous and that the less resistant progenitor of CHOC 50 in this population was a very minor species. To test this hypothesis, we cloned several cell lines from the CHO/5 population and analyzed the amplified DNA in each clone by the in-gel renaturation technique developed by Roninson (29). In this method, end-labeled restriction digests of genomic DNA are electrophoretically separated on an agarose gel and the DNA in the gel is subjected to denaturation, renaturation, and S1 nuclease digestion. By this method, single-copy background sequences that do not efficiently reanneal are removed and only repeated sequences are visualized when the gel is subsequently exposed to X-ray film. The method therefore does not require hybridization probes to detect amplified sequences.

The results of this analysis on CHOC 400, on the original CHO/5 population, and on three CHOC 5 clones chosen at random are shown in Fig. 6. Parental CHO DNA is also included to allow correction for repetitive elements unrelated to *dhfr* gene amplification per se (e.g., mitochondrial DNA and rDNA). Although the patterns of amplified *Eco*RI fragments in each cell line are relatively complex and contain many doublets and triplets, it can be seen that each of the three clones (Fig. 6, clones 5A, 5B, and 5C) manifests a different pattern of amplified *Eco*RI fragments. In at least one cell line, the prevalent amplicons appear to be quite small (the CHOC 5A clone). It is also clear that each pattern differs from that observed in CHOC 400, which itself does not differ from CHOC 50 by this criterion (data not shown). We therefore conclude that the CHO/5 population is heterogeneous and that the clone that gave rise to CHOC 50 probably represented a minor species in the CHO/5 population.

In-gel renaturation analysis of *Sfi*I digests of CHOC 400 DNA. After completion of the in-gel renaturation studies, it occurred to us that a modification of this method might be used to give a more simple and complete picture of amplified sequences in genomic DNA. We therefore adapted the in-gel renaturation method to *Sfi*I digests separated on pulse-field gradient gels. In addition, instead of end-labeling the *Sfi*I genomic digests with ^{32}P, we labeled the DNA intrinsically for one cell cycle with [^{3}H]thymidine. Tritium labeling in vivo has the advantage of being extremely reliable, and samples can be useful for extended periods because of the long half-life of the isotope. After one cycle of the denaturation, renaturation, and S1 nuclease steps (instead of the usual two [29]), *Sfi*I digests were transferred to GeneScreen Plus, and the transfer was sprayed with En^{3}Hance. The resulting autoradiogram of labeled *Sfi*I fragments from the CHOC 400 genome is shown in Fig. 7.

Because of the large size of *Sfi*I fragments, the pattern is extremely simple relative to the *Eco*RI patterns shown in Fig. 6. All of the major amplified fragments (280, 80, and 15/13 kb), as well as all of the minor ones (320, 220, 180, 160, 150, and 140 kb), that were detected in the CHOC 400 genome in hybridization studies were detected by the in-gel renaturation method. The only variant not detected in this experiment was the 420-kb band, which was buried in the large band at the top of the gel; this band was not resolved at the pulse time used in this experiment (15 s). These data

confirm that the majority of the amplicons in CHOC 400 have the type II arrangement. A minor fraction of the amplicons in the CHOC 400 genome appear to be larger than the 273-kb type I amplicon, but most contain junctions that lie within the 200-kb and/or the 160-kb parental *Sfi*I fragments (since we were able to detect them with probes from these fragments). Note that there is a large amount of radioactive DNA in the wells of both the CHO and CHOC 400 lanes. We assume that this material represents long tracts of repetitive DNA elements that were not digested with *Sfi*I, since both digests appear to be complete and since material this size is usually not observed when *Sfi*I digests are probed with fragments from the *dhfr* domain (e.g., Fig. 3).

DISCUSSION

In this study, we have analyzed the *Sfi*I fragments from the *dhfr* domains in CHO cells and in two MTX-resistant derivatives, CHO/0.2 (a cell population) and CHOC 400 (a clone). Digests were separated by pulse-field gradient gel electrophoresis and were analyzed with hybridization probes from the cloned type I and type II CHOC 400 *dhfr* amplicons. Our results support the previous suggestion that the type II sequence is a major amplicon type, accounting for ca. 75% of all of the amplicons in CHOC 400 (23). The alternating head-to-head and tail-to-tail configuration of the type II amplicons was confirmed by demonstrating the presence of the predicted 280- and 15-kb *Sfi*I fragments that result from this palindromic arrangement. Our data support and extend the results of Ford and Fried, who used a snap-back hybridization procedure to detect large inverted repeat sequences in the amplicons of PALA-resistant Syrian hamster cells, as well as in human tumor cell lines containing amplified *myc* genes (13).

The type I amplicon is detected in the CHOC 400 genome in head-to-tail arrays, as predicted, although it appears to represent less than 2 to 3% of the amplicons in this cell line. We previously estimated that the type I amplicons might represent as many as 15% of all of the amplicons in CHOC 400, since sequences mapping beyond the boundaries of the type II amplicon (e.g., those represented by the cosmid c32A1) are present in ca. 15% of the amplicons in this cell line (23). However, we now know that there are several other amplicon types larger than the type I amplicon that also include these sequences, accounting for this discrepancy. Our inability to detect these larger amplicon types in previous studies arises from the fact that their junction fragments probably lie beyond the boundaries of the cloned type I sequence and therefore would not have been detected in our walking procedure or when cosmids from the type I and II amplicons were used as probes on genomic digests of CHOC 400 DNA.

Since the interamplicon junction between the type I sequences in cosmid cPA36 has been localized, the correct permutation of the 260-kb map of the *dhfr* locus can now be drawn (Fig. 1B). In addition, a 453-kb *Sfi*I map of the parental CHO *dhfr* locus can be constructed, since we have established the order of *Sfi*I fragments to be 200, 80, 13, and 160 kb, reading from left to right (Fig. 1C).

The fact that we detected several minor variants in this study that were not predicted from our previous mapping data on cosmids suggests that an analysis of large restriction fragments gives a more complete picture of the true variability among amplicon structures. For example, a 320-kb *Sfi*I fragment in the CHOC 400 genome was observed to react with probes for both the 160- and 200-kb parental *Sfi*I fragments. Thus, the 320-kb fragment must represent a union between these two parental fragments. The resulting amplicon could therefore be circularly permuted, as is the type I amplicon, and would be ca. 413 kb (80 + 13 + 320 kb) in length. In addition, a 220-kb band was observed that reacted only with probes c, h, and i, but not with probes d and e; a minor 420-kb band was also detected with probes d and e, but not with any other probes (Fig. 1A and 3). It is conceivable that the 220-kb *Sfi*I fragment represents a truncated palindromic version of the 160-kb parental fragment, by analogy to the 280-kb palindromic *Sfi*I fragment present in the type II amplicon. However, the 420-kb *Sfi*I fragment cannot be explained simply on the basis of the known *Sfi*I fragments in this region of the genome and therefore must arise from a more complicated amplicon arrangement. For example, it is possible that the 420-kb variant represents a joint between the *dhfr* domain and another locus unrelated to *dhfr*. This phenomenon was suggested by Federspiel et al. to occur in MTX-resistant murine cell lines that display extremely complex *dhfr* amplicon arrangements (10). However, our data clearly show that the sequences forming the type I and type II amplicons in CHOC 400 cells are derived exclusively from the *dhfr* domain.

Our previous studies also suggested that there was at least one other amplicon type in CHOC 400 in which sequences from cSE24 and cN27 were joined (Fig. 1A, cYP25 variant) (23). The rearranged map in Fig. 1B suggests that this amplicon might be a truncated version of the type I sequence that is only 150 to 160 kb in length. However, we did not detect the predicted 90-kb *Sfi*I fragment characteristic of this arrangement with any of the probes used. The cYP25 variant must therefore occur in one of the other minor variant *Sfi*I fragments detected in these studies.

Studies of the less resistant cell population, CHO/0.2, show that most of the amplicons in this population are larger than the type I and type II sequences found in CHOC 400 (Fig. 3). The parental 160-kb *Sfi*I fragment is prevalent in this cell population, indicating that most of the amplicons extend beyond the left end of the 160-kb *Sfi*I fragment in CHO/0.2 cells (Fig. 1C). A prevalent 330-kb *Sfi*I fragment is detected in the CHO/0.2 population with a probe for the 200-kb *Sfi*I fragment (Fig. 3C), indicating that most of the amplicons in the CHO/0.2 population may be greater than ca. 583 kb (330 + 80 + 13 + 160 kb) in length. This result suggests that amplification of the *dhfr* gene in CHO cells cannot simply be explained by the overreplication of just the parental *dhfr* replicon, since replicons are unlikely to be 583 kb in length (18). However, our data do not rule out the possibility that overreplication occurred from an origin of replication near the *dhfr* gene, but that the forks read through into adjacent replicons before the extra DNA sequences were recombined into the genome.

Whatever the mechanisms responsible for amplification of the *dhfr* domain in CHO cells, there appear to be fewer variations in either initial amplification events or subsequent amplifications to high copy number than for several other systems that have been examined in detail. For example, Federspiel et al. detected marked heterogeneity in amplicon structure in a single MTX-resistant murine cell line and among different resistant cell lines (most of which carry the amplified *dhfr* genes on double-minute chromosomes) (10). Junction fragments defining multiple amplicon types were found during their chromosomal walking steps, and some of these were very close to the gene. Some junctions were observed to change with the time of propogation, even when

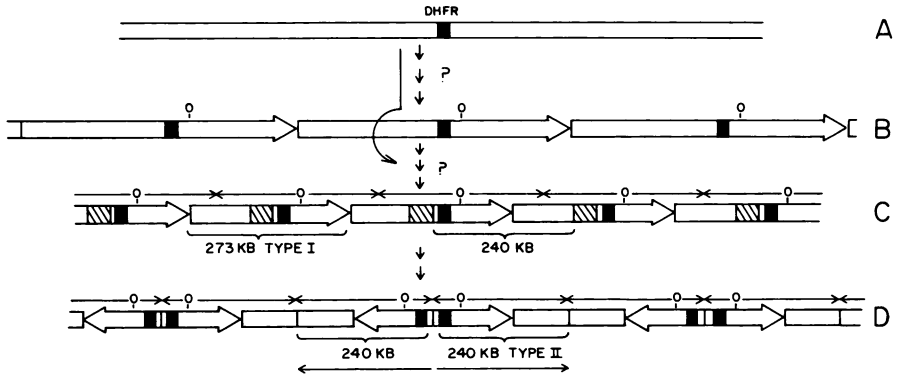

FIG. 8. Formation of the type I and type II amplicons. The schematic diagram indicates the possible rearrangements that led to the formation of the type I and type II amplicons in the CHOC 400 genome (line D), starting from the parental arrangement depicted in line A. The *dhfr* gene is indicated (■). A large amplicon is depicted in an initial step (line B), which was trimmed to the smaller type I amplicon (line C). A deletion of ca. 40 kb then occurred (▨), coincident with or followed by the amplification of a new unit defined by the head-to-head and tail-to-tail junctions found in the type II amplicon (line D). It is also possible that the 273-kb type I amplicon represents an initial amplicon type that persisted through multiple amplification steps to the CHOC 400 cell line. The location of the origin of replication (19) in the type I and type II amplicons is indicated above the respective maps, and termini are defined by the position at which the forks from adjacent origins meet.

the drug level was not increased, and, in some cases, DNA sequences unrelated to the *dhfr* locus per se were found to be covalently linked to the *dhfr* amplicons. Stark and co-workers have also detected significant heterogeneity among amplicons in different PALA-resistant Syrian hamster cell lines, even in single-step mutants (2, 37). The amplicons in these cell lines are maintained in stable chromosomal arrays (as opposed to double minutes), suggesting that the heterogeneity observed in the murine systems cited above cannot be attributed solely to the unstable nature of double minutes (21).

Interestingly, we recently found that the amplicons in two other independently isolated MTX-resistant Chinese hamster cell lines (A3 and MK42) (4, 26) are considerably larger and more uniform in structure than those observed in CHOC 400 cells (Looney et al., in preparation). In both of these cell lines, there appears to be only one site of *dhfr* amplification by the criterion of in situ hybridization with *dhfr*-specific probes (4, 21), whereas in CHOC 400 there are three sites (chromosomes 1, Z4, and an unidentified marker [24]). In in situ hybridization studies on the series of resistant cell lines that gave rise to CHOC 400, the first detectable amplicons were located on chromosome 1 (unpublished results), as opposed to the native sites on chromosomes 2 and Z2 (28). An initial amplification event may therefore have involved chromosomal breakage and translocation, as suggested by Flintoff et al. (12). Alternatively, an initial overreplicated *dhfr* domain may have had an extrachromosomal existence, followed by reintegration into chromosome 1, as suggested by Schimke (31), Biedler (3), and Carroll et al. (5). With further amplifications in the CHO/1 cell line, extra *dhfr* genes can also be detected on the Z4 chromosome. In the CHO/5 population, a new marker chromosome appears in many of the spreads, which becomes the major site of amplification in the CHOC 50 and CHOC 400 clones (24; J. L. Hamlin, unpublished observations). It is possible that each of these translocation-breakage events resulted in truncations of the initial amplicon to finally yield the predominant 240-kb type II sequence observed in CHOC 400.

Hybridization analyses of the characteristic type I and type II junction fragments in the present report show that once established (at or before the CHO/1 population), the junctions defining these amplicons are not subjected to significant further rearrangement (Fig. 5). Furthermore, although a few other minor variant fragments were detected by individual probes for the type I and type II junctions, our results do not suggest that the parental sequences that were joined to form the type I or type II junctions represent hot spots for the joining of amplicons. This result agrees with results of studies in other laboratories on murine *dhfr* amplicons and on the *cad* amplicons in PALA-resistant Syrian hamster cells (2, 10, 37). In contrast, Hyrien and co-workers have detected a hot spot for recombination of the adenylate deaminase amplicons into the genome in coformycin-resistant Chinese hamster cells (8, 20). A 2.6-kb clone representing this region contained multiple *Alu*-like sequences, as well as several long imperfect palindromes, each of which could mediate recombination processes in this system.

Since the 16-kb *Eco*RI junction fragment characteristic of the minor type I amplicon has roughly the same copy number in the CHOC 400 genome as the prevalent type II junction fragments have, it is possible to conclude that the type II amplicon must contain the type I junction. It follows that the type II amplicon must have been derived from the larger type I sequence. Furthermore, the type II palindromic junction fragments do not coincide with the type I junction, and thus they define a new amplified unit. A diagram of the possible rearrangements that gave rise to the type I and type II amplicons is shown in Fig. 8. An initial unit that may have been larger than the 273-kb type I sequence was amplified and was then trimmed to the 273-kb version sometime before the isolation of the CHO/1 population. An internal deletion of ca. 40 kb then occurred, followed by or in concert with the formation of the 240-kb unit of amplification defined by the two palindromic type II junction fragments. It is formally possible, of course, that the 273-kb type I unit of amplification represents an initial amplicon type that is too rare in the CHO/0.01 population to be detected, but which persisted during multiple amplifications and then gave rise to the type II arrangement that is prevalent in CHOC 400 cells. Since we did not clone resistant cells at each stage of amplification, we cannot distinguish between these possibilities.

Clearly, very complex rearrangements produced the type II amplicons, and it is not obvious whether they involved recombination alone or overreplication processes coupled

with recombination. However, the arrangements that we have deduced for the type I and type II amplicons have interesting consequences. Neither the origin of DNA replication in this domain (19) or the *dhfr* gene is located in the center of either amplicon type (Fig. 8). Furthermore, the two replication forks that emanate from the origin in the prevalent type II amplicon apparently have to travel very different distances to their respective termini (which presumably coincide with the head-to-head and tail-to-tail junctions in this amplicon). It would be of interest to know whether any of these junctions corresponds to a natural terminus region used by the parental, single-copy *dhfr* replicon.

The modified in-gel renaturation procedure, in which large *Sfi*I fragments labeled intrinsically with [^{3}H]thymidine were separated by PFG electrophoresis, confirmed the hybridization studies on amplified *Sfi*I fragments from the CHOC 400 genome, since it detected all of the amplified fragments that were detected with probes. We suggest that this adaptation may be an important addition to the standard in-gel procedure (29) during analysis of very large amplicons for which there are no (or few) cloned probes, e.g., the amplified sequences containing oncogenes in human tumors and tumor cell lines, and the amplicons of multidrug-resistant cells (1, 6, 7, 22, 32). In particular, comparisons among cell lines that have amplified the same gene will be facilitated, and estimates of the sizes of shared sequences may be more accurate, since the pattern of large fragments is so much simpler than the pattern generated by enzymes such as *Eco*RI and *Hin*dIII, which cut frequently, and most doublets and triplets are eliminated. We have not yet determined the lowest level of amplification that can be detected by this technique, but it should theoretically be the same as that observed by Roninson (29) in digests generated by enzymes that recognize six-membered sequences (15 to 30 copies per genome equivalent).

ACKNOWLEDGMENTS

We thank Igor Roninson for helpful discussions during the early stages of this work. We are grateful to Carlton White and Kevin Cox for dedicated and expert technical assistance.

This project was supported by research grants to J.L.H. from the National Institutes of Health and The American Cancer Society. J.E.L. was the recipient of a National Research Service Award, and J.L.H. was supported by an American Cancer Society Faculty Research Award.

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