MOLECULAR AND CELLULAR BIOLOGY, Feb. 1983, p. 257-266
0270-7306/83/020257-10$02.00/0

Vol. 3, No. 2

Expression and Amplification of Engineered Mouse Dihydrofolate Reductase Minigenes

GRAY F. CROUSE,* ROBERT N. McEWAN, AND MARK L. PEARSON

Cancer Biology Program, National Cancer Institute-Frederick Cancer Research Facility, Frederick, Maryland 21701

Received 26 July 1982/Accepted 22 October 1982

We constructed mouse dihydrofolate reductase (DHFR) minigenes (*dhfr*) that had 1.5 kilobases of 5′ flanking sequences and contained either none or only one of the intervening sequences that are normally present in the coding region. They were ≤3.2 kilobases long, about one-tenth the size of the corresponding chromosomal gene. Both of these minigenes complemented the DHFR deficiency in Chinese hamster ovary *dhfr*$^{-/-}$ cells at a high frequency after DNA-mediated gene transfer. The level of DHFR enzyme in various transfected clones varied over a 10-fold range but never was as high as in wild-type Chinese hamster ovary cells. In addition, the level of DHFR in primary transfectants did not vary directly with the copy number of the minigene, which ranged from fewer than five to several hundred per genome. The minigenes could be amplified to a level of over 2,000 copies per genome upon selection in methotrexate, a specific inhibitor of DHFR. In one case, the amplified minigenes were present in a tandem array; in two other cases, a rearranged minigene plasmid and its flanking chromosomal DNA sequence were amplified. Thus, the mouse *dhfr* minigenes could be transcribed, expressed, and amplified in Chinese hamster ovary cells, although the efficiency of expression was generally low. The key step in the construction of these minigenes was the generation in vivo of λ phage recombinants by overlapping regions of homology between genomic and cDNA clones. The techniques used here for *dhfr* should be generally applicable to any gene, however large, and could be used to generate novel genes from members of multigene families.

The mouse dihydrofolate reductase (DHFR) gene (*dhfr*) is a "housekeeping" gene whose product, DHFR, is essential for amino acid and nucleotide biosynthesis in all eucaryotic cells. DHFR is present in relatively small amounts, typically representing <0.1% of the total cell protein, in contrast with most other well-studied mammalian genes, which generally code for proteins expressed at high levels in specialized cell types. The *dhfr* gene also encodes a surprisingly heterogeneous set of mature mRNAs, which exhibit heterogeneity at both their 5′ and 3′ ends (9, 25). In addition, the gene undergoes amplification in response to selective pressure, i.e., in the presence of methotrexate (MTX), a specific inhibitor of the DHFR enzyme (1, 24). Therefore, we were interested in studying the control mechanisms regulating the expression and amplification of this gene at the molecular level.

Transfection of cloned genes into eucaryotic cells is useful for analyzing the influence of DNA primary structure on transcription in vivo. For example, the transcription of various transfected globin genes (10, 15, 20, 32) has been studied and appears to be similar, if not identical, to that of normal globin genes found in the animal. Many transfected genes yield active protein products and, in addition, exhibit at least some aspects of normal regulation. Among these are the genes for herpes simplex virus thymidine kinase (31), mouse metallothionein (2), human β interferon (22), *Drosophila* heat shock (7), and rat $\alpha_{2\mu}$ globulin (17). However, most of these represent specialized genes present in only highly differentiated cell types and generally expressed at high levels in the fully induced state.

The ability to transfect various cell types under different physiological conditions with the cloned *dhfr* gene and with variants of the gene having modified primary structure would be useful for the study of the expression and regulation of the *dhfr* gene. Studies of the *dhfr* gene have been hampered by the enormous size of the gene; it is 31 kilobases (kb) long in the case of the mouse, even though the major mRNA species is only 1.6 kb, of which only 0.56 kb is required to code for the DHFR protein (9).

Although this gene size is not at the limit for present-generation cosmid cloning vehicles, it is sufficiently large to make in vitro manipulations of primary structure difficult. Therefore, to reduce the size of this gene for analysis of the factors regulating its transcription and amplification by using gene transfer techniques, we constructed a series of *dhfr* minigenes less than one-tenth the size of the original genomic structure. These *dhfr* minigenes contained 1.5 kb of 5′ flanking sequence, expressed DHFR, and were amplifiable under MTX selection pressure. In this report, we describe construction of the minigenes by use of a combination of in vitro and in vivo recombination techniques and their subsequent use in analyzing *dhfr* expression and amplification.

MATERIALS AND METHODS

Construction of minigenes. The general scheme for the construction of the minigenes is shown in Fig. 1. The phage λCh4ADHFR121 (9), which contains the 5′ flanking sequences and the first and second coding sequences of the *dhfr* gene, was digested with *Hin*dIII. The 3.4-kb fragment containing coding sequences I and II was cloned into the *Hin*dIII site of λ*rva* (3) to make λ*rva-dhfr*3.4. Plaques were plated on DP50 *supF* (18) and screened for an insert with the orientation shown in Fig. 1. The mouse *dhfr* cDNA plasmid pDHFR11 (5, 25) was digested with *Pst*I to excise the *dhfr* sequences, the ends were made blunt with T4 DNA polymerase, and then *Hin*dIII linkers were added. The cDNA was then cloned into the *Hin*dIII site of pBR322. The *dhfr* cDNA, flanked by *Hin*dIII sites, was cloned into the *Hin*dIII site of λ*rvb* (3). Phages with inserts in the *c*I gene were recognized by their clear plaque morphology.

Phage crosses to generate recombinant *dhfr* minigenes were done as follows. Clear λ*rvb*-DHFR11 plaques were respotted on a lawn of DP50 *supF* to make larger areas of confluent lysis of approximately 5 mm in diameter. These areas were suspended in 200 μl of TMG (10 mM Tris-hydrochloride [pH 7.5], 10 mM $MgCl_2$, 10 μg of gelatin per ml), treated with $CHCl_3$, and centrifuged to remove debris. LE392 (r^- m^+) bacteria (2×10^7) (11) were incubated with 10^8 λ*rva-dhfr*3.4 and 50 μl of a λ*rvb*-DHFR11 isolate in a total volume of 80 to 100 μl for 15 min at 37°C. The cells were then diluted to 2 ml in Luria broth and incubated for 90 min at 37°C with agitation and aeration. The phage lysate was treated with $CHCl_3$, and 10- and 100-μl portions were plated on S667 [*gal thi* Str^r *sup*$^+$ (P2) (λ)] indicator bacteria (3). When plated on S667 indicator bacteria, generally the entire 2 ml of phage lysate produced either <40 plaques (for phage without proper inserts) or 3×10^3 to 10×10^3 plaques. Plaques were picked and grown on plate stocks in DP50 *supF*, and DNA was made as described previously (9).

Cells and DNA transfection. Chinese hamster ovary (CHO) DUK XB11, a nonreverting *dhfr*$^{-/-}$ cell line (29), was used as a recipient in transfection experiments. Cells were maintained in Eagle minimum essential medium plus 10% fetal calf serum supplemented with 10^{-4} M hypoxanthine, 1.7×10^{-5} M thymidine, 3×10^{-4} M proline, and 10^{-4} M glycine. Transfectants of DUK XB11 cells with DHFR activity (Dhfr$^+$) were selected in minimum essential medium plus 10% dialyzed fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) plus proline. DUK XB11 cells were transfected with calcium phosphate precipitates of DNA as described by Corsaro and Pearson (8). When carrier DNA was used, 0.3 μg of supercoiled plasmid DNA was added to 20 μg of mouse embryo DNA per 100-mm dish containing 10^6 cells. Transfections without carrier included 20 μg of supercoiled plasmid DNA per dish.

DNA analysis. DNA and RNA were isolated from the cells by a modification of the technique of Chirgwin et al. (6). Cells were harvested and lysed by gentle homogenization in a solution of 4 M guanidinium thiocyanate, 0.5% Sarkosyl, 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol. The homogenate was layered over a block gradient of 1 to 1.5 ml of 5.7 M CsCl in 0.1 M EDTA (pH 7.0) and 1 to 1.5 ml of 4.5 M CsCl in 0.1 M EDTA (pH 7.0) in a Beckman SW50.1 tube. The homogenate was centrifuged at 35,000 rpm for 17 h or longer at 20°C. The guanididium thiocyanate solution was removed, and the DNA was taken from the 4.5 M CsCl layer and dialyzed against TE (10 mM Tris-hydrochloride, 1 mM EDTA [pH 8.0]). The remainder of the solution was drained, and the RNA pellet was dissolved in water, extracted with 4 volumes of 4:1 $CHCl_3$:*n*-butanol, and precipitated with ethanol. DNA was quantitated by a fluorescence assay with Hoechst no. 33258 dye (Sigma Chemical Co., St. Louis, Mo.) (4).

The copy number of the DNA in the various clones was generally determined by a dot blot hybridization, essentially as described by Kafatos et al. (16). A 2.5-μg sample of DNA in 100 μl of TE was diluted to 0.3 to 0.4 N in NaOH, incubated for 10 min at room temperature, and neutralized by the addition of an equal volume of 2 M ammonium acetate, and the solution was kept on ice. Various amounts of *Hin*dIII-restricted p*dhfr*3.2 were used as standards. The DNA was slowly filtered through a nitrocellulose filter (HAWP; Millipore Corp., Bedford, Mass.) prewetted with 1 M ammonium acetate, dried under a heat lamp, and baked for 1 h at 80°C in a vacuum oven. Restriction analyses, Southern transfers, and hybridizations were done as previously described (9). Some of the filters were probed with nick-translated mDHFR11, the *dhfr* cDNA fragment from pDHFR11 (5, 25) cloned into M13mp8 (a cloning vector developed by J. Messing, University of Minnesota, St. Paul, Minn., and obtained from Bethesda Research Laboratories, Bethesda, Md.).

DHFR assays. Cells were grown to 75% confluency (approximately 10^7 cells) and then were harvested by trypsinization, washed in phosphate-buffered saline, and suspended in 0.5 ml of 50 mM potassium phosphate (pH 7.2). The cells were sonicated with three 10-s bursts of a Branson sonifier (microtip probe, lowest power setting). The extract was centrifuged at 35,000 rpm for 45 min in a Beckman type 50 rotor. The supernatant was quick-frozen in liquid nitrogen and stored at −20°C. MTX binding to DHFR was determined as described by Haber et al. (13). DHFR activity was measured by reduction of [^{3}H]dihydrofolate (13, 14). Before determination of DHFR activity of cells selected for MTX resistance, the cells were grown for 1 week in the absence of MTX. Protein was quantitated by the method of Lowry et al. (19).

RESULTS

Construction of *dhfr* minigenes. Although the mouse DHFR protein is coded for by only 558 nucleotides (21), the gene itself is 31 kb long and includes intervening sequences of up to 16.5 kb long (9). To study structural features of the gene that affect its expression, we needed to construct smaller derivatives that would still be functional. We assumed at the outset that most of the intervening sequences could be deleted without blocking expression of DHFR. To achieve precise deletion of most of the intervening sequences, we devised the scheme shown in Fig. 1. λ*rva* and λ*rvb* are phage vectors developed by Carroll et al. for studies on genetic recombination (3). They were constructed with no internal homologies between selectable phage markers conferring the Spi and Imm phenotypes; any recombination between these two markers requires homologies between the inserted sequences. Therefore, a 3.4-kb *Hin*dIII genomic fragment containing coding sequences I and II from λCh4ADHFR121 (9) was cloned into λ*rva*, and the 1.5-kb *dhfr* insert from pDHFR11 (5, 25) was cloned into λ*rvb* with *Hin*dIII linkers. λ*rva-dhfr*3.4 with the desired orientation of the genomic *Hin*dIII fragment was chosen. Rather than screen the λ*rvb*-DHFR11 phages for inserts with the desired orientation, the λ*rva-dhfr*3.4 clone was crossed with different λ*rvb*-DHFR11 phages as described above. Twelve phages from these crosses were picked, and their DNA was analyzed by restriction with *Hin*dIII (Fig. 2). All 12 phages exhibited the pattern expected from the phage cross; 11 were the result of recombination between coding sequence I and the cDNA, and 1 was the result of recombination between coding sequence II and the cDNA. The structures were verified by further restriction analysis (results not shown).

Transfection of Dhfr⁻ CHO cells by *dhfr* minigenes. The *dhfr* minigenes from the recombinant phages were then cloned into the *Hin*dIII site of pBR327, a derivative of pBR322 with a 1,089-base pair deletion (27). Plasmids were screened to obtain minigenes in the same orientation, with and without intervening sequence I, as illustrated in Fig. 3. The plasmids were identical with the exception of the intervening sequence which was missing in p*dhfr*2.9. The plasmids were then transferred into Dhfr⁻ CHO cells by a standard calcium phosphate DNA transfer, with or without carrier mouse embryo DNA. Dhfr⁺ cells were selected by growth in the absence of hypoxanthine, thymidine, and glycine. The transfection frequencies with carrier DNA and either plasmid were $>10^{-4}$ Dhfr⁺ transfectants per recipient cell. Transfections with 20 μg of plasmid DNA and no carrier DNA were similarly efficient. No colonies were seen when transfections were done with the pBR327 plasmid and carrier DNA in place of the *dhfr* minigenes.

Analysis of transfectants. Clones arising from

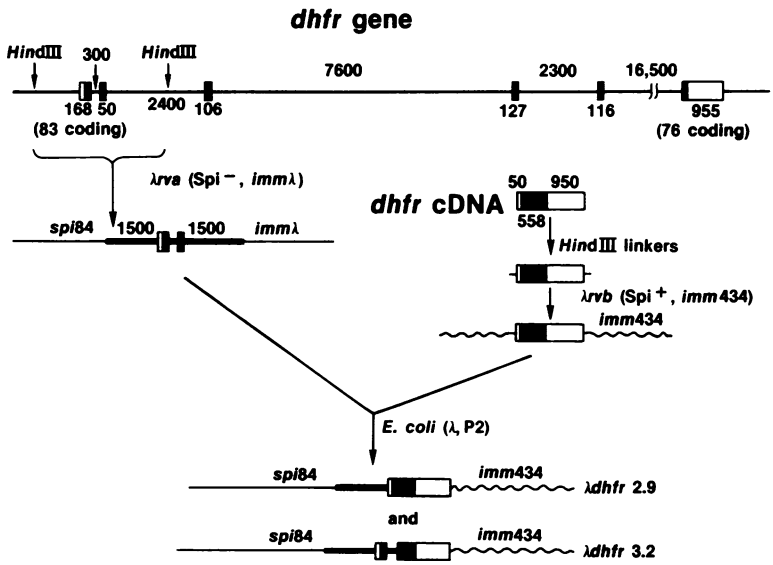

FIG. 1. Construction of *dhfr* minigenes. The top line illustrates the mouse *dhfr* gene drawn to scale (with the exception of the 16,500-base pair intervening sequence) (9). The boxes represent the sequences found in the mRNA as determined from the cDNA clones pDHFR26 and pDHFR11 (5). The black boxes represent coding sequences, and the open boxes represent the 5′ and 3′ untranslated regions. Underneath each box is the number of nucleotides as determined by DNA sequencing of the region (9). For details of the construction, see the text.

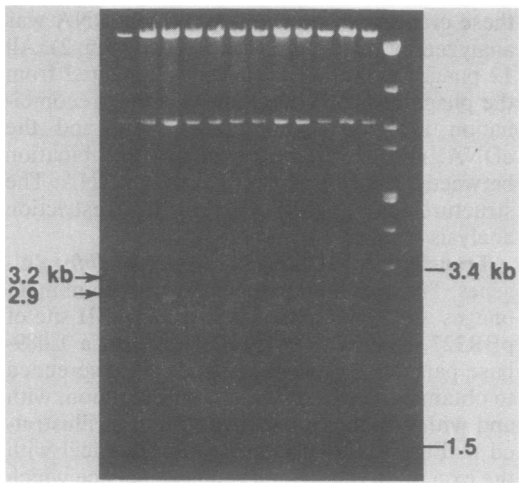

FIG. 2. *Hin*dIII digest of recombinant phages selected in S667 and grown in DP50 *supF*. The last lane on the right is a *Hin*dIII-*Eco*RI digest of λ. The lines marked at 1.5 and 3.4 kb represent the position where the inserts in the parent phages would have run. The recombinant inserts, with and without the 300-base pair intervening sequence, are shown at 3.2 and 2.9 kb, respectively.

transfections with each minigene were further analyzed for *dhfr* gene content and DHFR protein (Table 1). The copy number of the *dhfr* minigene ranged from less than five to more than several hundred, and the amount of DHFR protein varied by more than a factor of 10. The DHFR level was not directly proportional to *dhfr* gene copy number. Also, the clones that

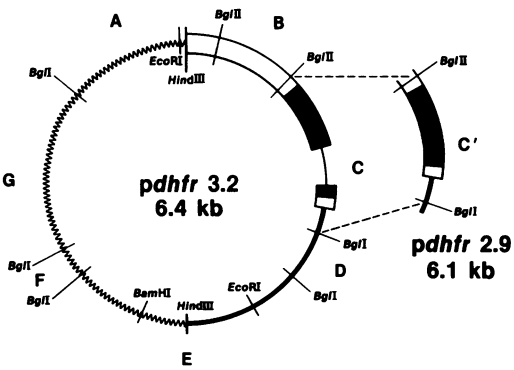

FIG. 3. Structure of the two minigenes cloned into pBR327. The plasmid sequences are indicated by a wavy line, the 5′ flanking region of the *dhfr* gene by a solid line, and the cDNA sequences by a black box for coding sequence and an open box for untranslated regions. The letters (A through G) refer to the fragments generated by a digestion with *Bgl*I and *Bgl*II. p*dhfr*2.9 is identical to p*dhfr*3.2, with the exception of the intervening sequence.

TABLE 1. *dhfr* minigene copy number and DHFR enzyme levels in various p*dhfr* transfected clones[a]

Clone	*dhfr* copy no. per genome[b]	DHFR assay[c]	
		^{3}H-labeled MTX binding (U/mg) ± SD	Enzyme assay (U/mg)
p*dhfr*3.2 DNA			
32S1	5–10	0.16 ± 0.02 (5)[d]	
32S2	450	1.2 ± 0.3 (5)	1.0
32S3	<5	1.1 ± 0.2 (5); 0.52 ± 0.11 (6)	
32S4	10	1.1 ± 0.12 (4)	
32S5	5	0.19 ± 0.03 (3)	
32S6	10–20	0.26 ± 0.03 (6)	
32S7	5–10	0.81 ± 0.12 (2); 0.24 ± 0.03 (5)	
32CF1	<5	0.13 ± 0.03 (14)	
32CF2	<5	0.07 ± 0.02 (7)	
32CF3	<5	0.08 ± 0.03 (9)	
32CF4	<5	0.26 ± 0.06 (11)	0.29
p*dhfr*2.9 DNA			
29S2	<5	0.24 ± 0.01 (3)	
29S3	<5	0.29 ± 0.06 (9)	
29S4	300	0.14 ± 0.01 (4)	0.09
29S6	270	0.16 ± 0.01 (5)	0.13
29CF1	<5	0.08 ± 0.02 (6)	
29CF2	<5	0.29 ± 0.03 (5); 0.11 ± 0.01 (3)	
29CF3	<5	0.08 ± 0.02 (12)	
29CF4	<5	0.12 ± 0.03 (7)	0.09
CHO-K1 (parent)		1.7 ± 0.26 (8)	2.3
CHO DUK XB11		<0.01	

[a] The clones with a CF in their designation were obtained by transfection without carrier DNA; all other clones were obtained with carrier.

[b] The copy number per genome was determined by DNA dot blot analysis as described in the text with p*dhfr*3.2 as a standard.

[c] The standard for both the MTX-binding and DHFR enzyme assays was an extract from mouse liver; 1 mg of this extract bound 35,000 cpm of ^{3}H-labeled MTX and contained 26.1 ± 1.1 U of DHFR per mg (1 U = the production of 1 nmol of tetrahydrofolate from dihydrofolate in 15 min at 37°C). In several cases, extracts were made from a clone at different times. With three exceptions, extracts from the same clone contained similar amounts of DHFR: for clones 32S3, 32S7, and 29CF2, extracts made from cells cultured for longer times showed a significant loss of DHFR (two assay values are shown for these clones).

[d] Numbers in parentheses indicate the number of determinations made.

arose from the transfection without carrier DNA appeared to have fewer copies of the minigene and low DHFR activities.

Minigene amplification. To test whether the transferred *dhfr* minigenes could be amplified

under conditions known to favor amplification of the chromosomal *dhfr* gene, three transfectant cell lines, 32S3, 32CF4, and 29CF4, were grown in increasing concentrations of MTX. Cells were taken at various concentrations of MTX and analyzed as described above; the results are shown in Table 2. 32S3 cells grown in 0.1 μM MTX had approximately 2,000 copies of the gene, approximately a 1,000-fold amplification, but the level of DHFR was only 20-fold above that of the parent 32S3 cell line. Cells selected from this preparation grew poorly in 0.5 or 1.0 μM MTX, and, as is evident from Table 2, they contained approximately the same levels of gene and enzyme as 32S3 cells grown in 0.1 μM MTX.

After 32S3M.1a were grown for approximately 5 months in 0.1 μM MTX, six subclones were examined for the *Hin*dIII restriction patterns of their amplified *dhfr* minigenes. As can be seen in Fig. 4, these patterns were identical; the predominant species was at 3.2 kb, identical with the original *Hin*dIII *dhfr* fragment in p*dhfr*3.2, and the background pattern of other *dhfr* fragments was the same in different subclones. A digest of one of these clones with *Bam*HI, which cuts once in p*dhfr*3.2, revealed that only one of many bands had the expected size of 6.4 kb. This band represented a much smaller proportion of the total hybridization than one would predict on the basis of the pattern of hybridization for the *Hin*dIII-digested DNA, indicating that there are many different sites of integration of the *dhfr* sequences in this transfectant.

The DNA of one of the 32S3M.1a clones was also digested with a combination of *Bgl*I and *Bgl*II and a bidirectional transfer was probed with either mDHFR11 or pBR327. The result (Fig. 5) indicates that all of the bands seen in p*dhfr*3.2 were also present in the clones.

Cells at various stages of selection with MTX in the clones 32CF4 and 29CF4 were also analyzed to determine the state of their transferred *dhfr* minigenes in a similar fashion. Figure 6 shows the result of digesting the 32CF4 DNAs with *Bam*HI and the 29CF4 DNAs with *Hin*dIII and probing with mDHFR11. The pattern of restriction remained the same at each stage of selection, although it was different from the expected p*dhfr*3.2 and p*dhfr*2.9 patterns. The amount of the minigene increased, as would be predicted on the basis of the dot blot hybridization. In contrast to the results with the 32S3 clone, the level of DHFR showed a more reason-

TABLE 2. *dhfr* minigene copy number and DHFR protein in amplified clones selected for MTX resistance[a]

Clone	Selected in MTX (μM)	*dhfr* copy no. per genome	DHFR assay	
			^{3}H-labeled MTX binding (U/mg) ± SD	Enzyme assay (U/mg)
32S3 (parent)		<5	1.1 ± 0.18 (6)[b]	
M.1a	0.1	2,200	14 ± 2.7 (10)	16
M.1b	0.1	2,300	14 ± 2.2 (7)	
M.1c	0.1	1,800	11 ± 1.5 (4)	
M.1aM.5	0.5	1,900	14 ± 1.3 (3)	
M.1aM1	1.0	2,000	16 ± 0.9 (3)	14
M1	1.0	1,300	21 ± 4.3 (8)	22
32CF4 (parent)		<5	0.26 ± 0.06 (11)	0.29
M.01	0.01	35	6.3 ± 0.4 (5)	
M.05	0.05	120	9.8 ± 0.7 (5); 21 ± 2.1 (3)	
M.1	0.1	170	35 ± 4.9 (5)	
M.3	0.3	340	30 ± 1.0 (3)	35
29CF4 (parent)		<5	0.12 ± 0.03 (7)	0.09
M.01	0.01	30	1.9 ± 0.2 (3)	2.0
M.05	0.05	90	6.3 ± 1.6 (6)	
M.1	0.1	200	4.7 ± 0.4 (4)	
M.3	0.3	370	3.8 ± 0.3 (3)	8.0

[a] Clones selected stepwise for resistance to the MTX concentration shown were assayed for copy number and DHFR as described in footnotes *b* and *c*, respectively, to Table 1. For the 29CF4 and 32CF4 clones, populations of cells were subjected to selection in increasing concentrations of MTX, and cells at each stage of the selection were analyzed. The 32S3 clone was placed into 0.1 μM MTX, and three clones that grew up, M.1a, M.1b, and M.1c, were picked. The M.1a clone was then selected in 0.5 μM MTX to yield the M.1aM.5 population, and these cells were then selected in 1 μM MTX to yield the M.1aM1 population. The M1 population was selected in two similar steps from a population of cells that grew in 0.1 μM MTX.

[b] Numbers in parentheses indicate the number of determinations made.

able correlation with the *dhfr* copy number. In both cases, there was approximately a 100-fold amplification of both the *dhfr* genes and the DHFR protein.

Digestion and hybridization of 32CF4M.3 and 29CF4M.3 with *Bgl*I and *Bgl*II, also shown in Fig. 5, revealed that in both clones the fragment containing the coding sequences of the gene was intact, but that both had undergone rearrangement in the 3′ untranslated region of the gene. A pBR327 probe showed that 32CF4M.3 did not contain fragments A and G but did exhibit an additional band. The *Hin*dIII site in fragment A was missing, indicating that the rearrangement involved the 3′ untranslated region of the *dhfr* minigene as well as pBR327 sequences (results not shown). Surprisingly, no pBR327 sequences were detectable in 29CF4M.3, a result that has been observed in several independent experiments.

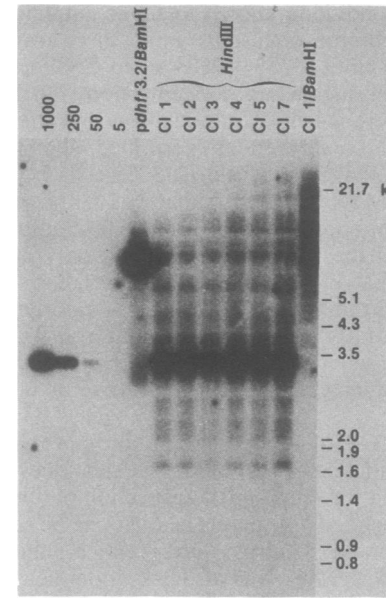

FIG. 4. Minigene organization in subclones (Cl 1 through 7) of the amplified line 32S3M.1a. 32S3M.1a was subcloned after approximately 5 months of growth in 0.1 μM MTX. DNA from various subclones was restricted and electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose paper, and probed with mDHFR11, the *dhfr* cDNA cloned into M13. *Hin*dIII-digested p*dhfr*3.2, representing 5, 50, 250, or 1,000 copies per genome, was run as a copy number and size standard. *Bam*HI digests of p*dhfr*3.2 and one of the subclones, Cl 1, were also run. The numbers at the right of the gel indicate the positions of the *Hin*dIII-*Eco*RI fragments of λ DNA.

DISCUSSION

***dhfr* minigene construction.** The bacteriophage λ derivatives, λ*rva* and λ*rvb*, allowed us to create *dhfr* minigenes by homologous recombination in *E. coli*. In an analogous fashion, we could reintroduce intervening sequences other than the first one in the *dhfr* gene back into the minigene, regardless of what restriction sites were available in the coding sequence of the gene. Only the largest intervening sequence, because of its size, required some splicing in vitro after the phage cross. One limitation of this method at present is that the vectors λ*rva* and λ*rvb* are only suitable for cloning *Hin*dIII fragments. We are now in the process of constructing new vectors which will allow this method to be extended to inserts bounded by a wide array of restriction enzyme sites.

Although we used this technique for making recombinants between phages carrying regions that are completely homologous, recombination between similar, but not identical, members of multigene families showing partial homology could also occur, yielding new hybrid genes, without requiring conveniently located restriction sites. This could be useful in the construction of synthetic hybrids with novel biological properties, such as hybrid interferon genes, for example, since there is extensive homology among various leukocyte interferon genes (12).

Minigene expression. Minigenes both with and without the first intervening sequence were clearly expressed in CHO DUK XB11 cells at levels high enough to allow the cells to grow under selection for the Dhfr$^+$ phenotype. Even though most of the clones had more than one copy of the minigene per genome, none of the clones produced as much DHFR protein as the parent CHO-K1 cells, and only two clones produced levels comparable to that found in mouse liver. The reasons for this low level of expression are not known. The minigenes, as presently reconstituted, may be lacking some important element needed for efficient transcription or translation.

Not only were low levels of DHFR produced, in general, but they were also variable from clone to clone. In many instances, the yield of enzyme appeared to bear little relation to the number of gene copies present in the cell. This fact is not too surprising because not all copies of the minigene present in a transfectant will necessarily be expressed or even be intact in the coding region. In addition, the chromosomal location of the transfected genes probably can also influence their level of expression. Therefore, it is difficult to determine whether there are any intrinsic differences in expression between transfectants carrying p*dhfr*3.2 and those carrying p*dhfr*2.9. Any such differences caused by the absence of the intervening sequence in p*dhfr*2.9

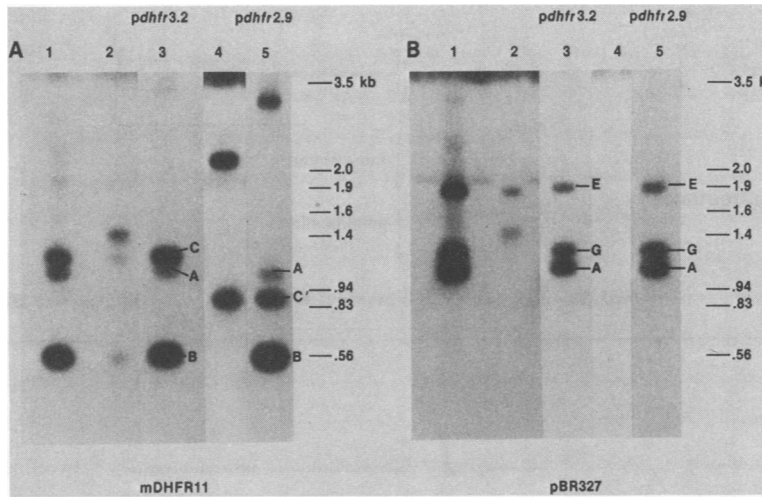

FIG. 5. *Bgl*I-*Bgl*II restriction pattern of amplified *dhfr* minigenes in different cell lines. Five micrograms of DNA from the amplified clones was digested with *Bgl*I and *Bgl*II and electrophoresed on a 0.8% agarose gel, and a bidirectional transfer was performed as described by Smith and Summers (26). One of the filters (A) was probed with nick-translated mDHFR11, and the other (B) was probed with nick-translated pBR327. Lane 1 is 32S3M.1a subclone 7; lane 2 is 32CF4M.3, and lane 4 is 29CF4M.3. p*dhfr*3.2 (lane 3) and p*dhfr*2.9 (lane 5) were also digested with *Bgl*I and *Bgl*II and electrophoresed on the same gel to serve as markers. The size markers are those described in the legend to Fig. 4. The A, B, C and C', E, and G fragments of the plasmids p*dhfr*3.2 and p*dhfr*2.9 shown here are labeled in Fig. 3.

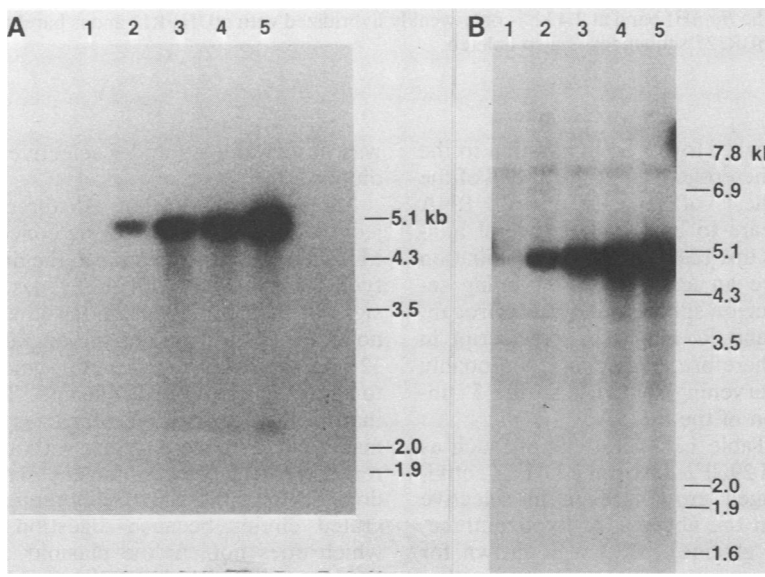

FIG. 6. Amplification of minigenes in 32CF4 and 29CF4 clones selected in increasing concentrations of MTX. (A) Ten micrograms each of 32CF4 (lane 1), 32CF4M.01 (lane 2), 32CF4M.05 (lane 3), 32CF4M.1 (lane 4), and 32CF4M.3 (lane 5) DNAs were digested with *Bam*HI and electrophoresed on a 0.8% agarose gel, blotted onto nitrocellulose, and hybridized with nick-translated mDHFR11. (B) Ten micrograms each of 29CF4 (lane 1), 29CF4M.01 (lane 2), 29CF4M.05 (lane 3), 29CF4M.1 (lane 4), and 29CF4M.3 (lane 5) DNAs were digested with *Hin*dIII, electrophoresed on a 0.8% agarose gel, blotted onto nitrocellulose and hybridized with nick-translated mDHFR11. The size standards are based on a *Hin*dIII-*Eco*RI digest of λ and an *Eco*RI digest of λCh4A.

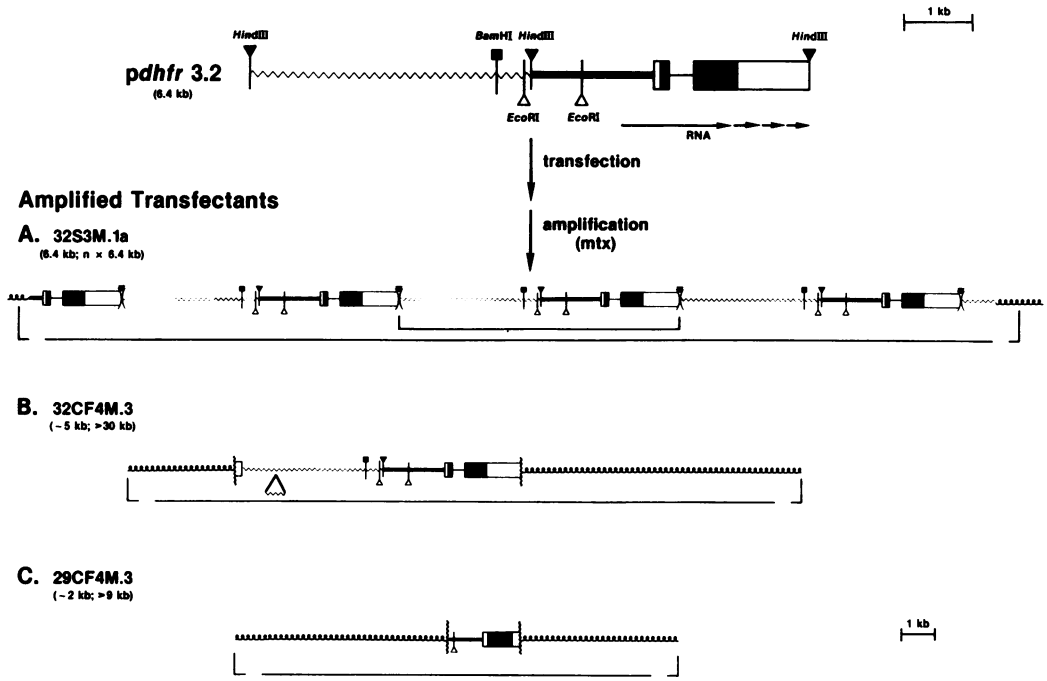

FIG. 7. Proposed structures for the amplified *dhfr* minigenes. p*dhfr*3.2 is shown at the top with the location of some restriction enzyme sites. The structure of the minigenes in 32S3M.1a (A), 32CF4M.3 (B), and 29CF4M.3 (C) are shown at half the scale for p*dhfr*3.2. Estimates of the sizes of the amplified minigenes (6.4 kb in A, ~5 kb in B, and ~2 kb in C) and a minimum size for the amplified unit are also shown. The curly line represents genomic sequences that are not part of the minigene. The amplified unit in 32S3M.1a is complex, having variable size with variable ends. *Hin*dIII and *Bam*HI digests of 32CF4M.3 both show two bands that hybridize with both mDHFR11 and pBR327, suggesting that a part of the 3′ end of the *dhfr* minigene is separated from the main part of the minigene (the *Bam*HI band at 2.4 kb is only weakly hybridized with mDHFR11 and is barely visible in Fig. 6A). Part of the pBR327 sequences is also deleted.

appear to be small. However, in addition to the transcriptional heterogeneity at the 3′ end of the gene, the synthesis of some (if not all) RNA molecules appears to be initiated several hundred base pairs upstream of the AUG initiation codon and have an additional intervening sequence in this region spliced out (M. McGrogan, C. Simonsen, and R. Schimke, manuscript in preparation). Therefore, even p*dhfr*2.9 probably contains an intervening sequence in the 5′ untranslated region of the mRNA.

As noted in Table 1, several clones, such as 32S3, 32S7, and 29CF2, lost some DHFR activity after prolonged growth, even in selective medium (i.e., in the absence of hypoxanthine, thymidine, and glycine). 32S3 cells grown for 142 days in the presence of hypoxanthine, thymidine, and glycine lost about 75% of their DHFR activity. 29S3 and 32CF1 cells grown for 84 days in the absence of selection lost about 50% of their DHFR activity (results not shown). Thus, some DHFR activity was retained in the absence of selection, although loss of DHFR was observed even under selective growth conditions.

Minigene amplification. All three clones subject to selection in increasing concentrations of MTX developed resistance to the drug by amplifying their resident *dhfr* minigenes. There were significant differences among the three lines nonetheless. A digest of the amplified genes in 32S3M.1a yielded a series of fragments identical to those of the original plasmid. This suggests that the transferred *dhfr* minigenes are arranged mainly in a tandem array without significant rearrangement of the minigene sequences. We do not believe these are freely replicating unintegrated circles because digestion with *Kpn*I, which does not cut the plasmid, left the *dhfr* sequences in high-molecular-weight DNA (results not shown). Also, a *Bam*HI digestion yielded many fragments both larger and smaller than linear molecules of p*dhfr*3.2. Because a corresponding heterogeneity was not observed in the combined *Bgl*I-*Bgl*II digestion, we presume that the structure of these genes consisted of several

copies of p*dhfr*3.2 arranged in a perfect tandem array with random ends joined to chromosomal DNA. A proposed structure for the amplified gene in 32S3M.1a is shown in Fig. 7.

We noted that the level of DHFR increased only 15- to 20-fold, even though the minigene was amplified approximately 1,000-fold in this line. This appears to be a general result, in that clones 32S2, 29S6, and 29S4 (Table 1), which also have high *dhfr* copy numbers arranged in tandem arrays (results not shown), were also expressed inefficiently. This limited evidence seems to suggest that most *dhfr* minigenes, arranged in tandem arrays, either are not expressed or are expressed poorly relative to other configurations of the genes. A similar result has been reported in a clone containing a tandem array of pSV2-*neo* (28). Therefore, we feel that the low levels of expression in the tandem arrays were not attributable solely to an absence of important 3′ sequences, although this conclusion remains to be tested. The amplified genes also appeared to be relatively stable; 32S3M.1a retained over 75% of its DHFR activity after growth for 1 month in the absence of MTX (results not shown).

In contrast to the amplified 32S3 line, 32CF4M.3 and 29CF4M.3, selected after transfection in the absence of carrier DNA, underwent substantial alterations (Fig. 7). The rearrangements observed in the 29CF4M.3 cells could also be detected in the original 29CF4 clone; however, the restriction pattern of the minigene in the 32CF4 clone differed substantially from the pattern observed in the amplified derivatives (results not shown). A rearrangement apparently occurred in the 32CF4 line in the very early stages of amplification, and the rearranged minigene was then subsequently faithfully amplified, in a manner similar to the altered minigene in 29CF4. Both underwent rearrangement in the 3′ untranslated region of the minigene and the adjacent plasmid sequences. In fact, 29CF4M.3 appeared to have lost all of the pBR327 sequences. The reason for the loss of these sequences is unclear. The 5′ flanking regions of the *dhfr* gene appeared to be intact as far upstream as the *Eco*RI site, about 1 kb from the start codon (results not shown). The fact that these minigenes both underwent rearrangement in the 3′-untranslated region may indicate that the structure of this region is not optimal for expression. Although three minor *dhfr* mRNAs had 3′ ends within this region and the fourth major species had an end coterminal with the end of this minigene (25), genomic sequences downstream from the polyadenylation sites may be important for proper termination and polyadenylation and thus efficient expression. We are currently testing this hypothesis by adding 3′ genomic sequences to the minigene in a manner analogous to the method in Fig. 1.

The fact that a minigene such as 29CF4M.3, which contains only a portion of the 3′-untranslated region, can be amplified and that a *dhfr* cDNA joined to eucaryotic promoters with no 5′ genomic sequences can also be amplified (23) suggests that there are no special sequences responsible for directing the process of amplification. This conclusion is consistent with the idea that gene amplification is a process which occurs normally at a low frequency, as has already been suggested (24). The amplified region clearly extends well beyond the minigene itself, for in the case of the amplified 32CF4 and 29CF4 clones, the flanking genomic CHO DNA was also amplified. Digestion of the 32CF4 amplified lines revealed that two *Hin*dIII *dhfr* bands >10 kb and an *Eco*RI *dhfr* band >20 kb were amplified, and digestion of the 29CF4 amplified line with *Bam*HI showed a single amplified *dhfr* band of ~9 kb (results not shown). These data indicate that the amplified region in 32CF4M.3 was >30 kb and that the amplified region in 29CF4M.3 was >9 kb. Unlike the amplified minigene in 32S3M.1a, there appeared to be only one structure for the amplified minigenes in 32CF4M.3 and 29CF4M.3, although there may be heterogeneity in the amplified flanking DNA beyond the regions indicated in Fig. 7. Thus, the *dhfr* minigenes can be used to selectively amplify linked DNA in a manner analogous to that previously demonstrated with the intact *dhfr* gene (30).

ACKNOWLEDGMENTS

We thank Larry Chasin for the DUK XB11 cells and Peter Southern and Mike McGrogan for communicating results before publication. We thank Steve Beverley for protocols for the dihydrofolate reductase assays. Methotrexate was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. We thank Hilda Marusiodis for excellent assistance in the preparation of this manuscript.

These studies were supported by the National Cancer Institute under contract no. N01-C0-75380 with Litton Bionetics, Inc.

LITERATURE CITED

1. **Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke.** 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. **253**:1357–1370.
2. **Brinster, R. L., H. Y. Chen, R. Warren, A. Sarthy, and R. D. Palmiter.** 1982. Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. Nature (London) **296**:39–42.
3. **Carroll, D., R. S. Ajioka, and C. Georgopoulos.** 1980. Bacteriophage lambda cloning vehicles for studies of genetic recombination. Gene **10**:261–271.
4. **Cesarone, C. F., C. Bolognesi, and L. Santi.** 1979. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. Anal. Biochem. **100**:188–197.
5. **Chang, A. C. Y., J. H. Nunberg, R. J. Kaufman, H. A.**

Erlich, R. T. Schimke, and S. N. Cohen. 1978. Phenotypic expression in *E. coli* of a DNA sequence coding for mouse dihydrofolate reductase. Nature (London) 275:617–624.
6. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 18:5294–5299.
7. Corces, V., A. Pellicer, R. Axel, and M. Meselson. 1981. Integration, transcription, and control of a *Drosophila* heat shock gene in mouse cells. Proc. Natl. Acad. Sci. U.S.A. 78:7038–7042.
8. Corsaro, C. M., and M. L. Pearson. 1981. Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. Somatic Cell Genet. 7:603–616.
9. Crouse, G. F., C. C. Simonsen, R. N. McEwan, and R. T. Schimke. 1982. Structure of amplified normal and variant dihydrofolate reductase genes in mouse sarcoma S180 cells. J. Biol. Chem. 257:7887–7897.
10. Dierks, P., A. van Ooyen, N. Mantel, and C. Weissmann. 1981. DNA sequences preceding the rabbit β-globin gene are required for formation in mouse L cells of β-globin RNA with the correct 5′ terminus. Proc. Natl. Acad. Sci. U.S.A. 78:1411–1415.
11. Enquist, L. W., M. J. Madden, P. Schiop-Stansly, and G. F. Vande Woude. 1979. Cloning of herpes simplex type 1 DNA fragments in a bacteriophage lambda vector. Science 203:541–544.
12. Goeddel, D. V., D. W. Leung, T. J. Dull, M. Gross, R. M. Lawn, R. McCandliss, P. H. Seeburg, A. Ullrich, E. Yelverton, and P. W. Gray. 1981. The structure of eight distinct cloned human leukocyte interferon cDNAs. Nature (London) 290:20–26.
13. Haber, D. A., S. M. Beverley, M. L. Kiely, and R. T. Schimke. 1981. Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J. Biol. Chem. 256:9501–9510.
14. Hayman, R., R. McGready, and M. B. Van der Weyden. 1978. A rapid radiometric assay for dihydrofolate reductase. Anal. Biochem. 87:460–465.
15. Hsiung, N., R. S. Roginski, P. Henthorn, O. Smithies, R. Kucherlapati, and A. I. Skoultchi. 1982. Introduction and expression of a fetal human globin gene in mouse fibroblasts. Mol. Cell. Biol. 2:401–411.
16. Kafatos, F. C., C. W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. 7:1541–1552.
17. Kurtz, D. T. 1981. Hormonal inducibility of rat $\alpha_{2\mu}$ globulin genes in transfected mouse cells. Nature (London) 291:629–631.
18. Leder, P., D. Tiemeier, and L. Enquist. 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the λgtWES system. Science 196:175–177.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
20. Mantei, N., W. Boll, and C. Weissman. 1979. Rabbit β-globin mRNA production in mouse L cells transformed with cloned rabbit β-globin chromosomal DNA. Nature (London) 281:40–46.
21. Nunberg, J. H., R. J. Kaufman, A. C. Y. Chang, S. N. Cohen, and R. T. Schimke. 1980. Structure and genomic organization of the mouse dihydrofolate reductase gene. Cell 19:355–364.
22. Ohno, S., and T. Taniguchi. 1982. Inducer-responsive expression of the cloned human interferon β1 gene introduced into cultured mouse cells. Nucleic Acids Res. 10:967–977.
23. Ringold, G., B. Dieckmann, and F. Lee. 1981. Co-expression and amplification of dihydrofolate reductase cDNA and the *Escherichia coli* XGPRT gene in Chinese hamster ovary cells. J. Mol. Appl. Genet. 1:165–175.
24. Schimke, R. T., R. J. Kaufman, F. W. Alt, and R. E. Kellems. 1978. Gene amplification and drug resistance in cultured murine cells. Science 202:1051–1055.
25. Setzer, D. R., M. McGrogan, J. H. Nunberg, and R. T. Schimke. 1980. Size heterogeneity in the 3′ end of dihydrofolate reductase messenger RNAs in mouse cells. Cell 22:361–370.
26. Smith, G. E., and M. D. Summers. 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. 109:123–129.
27. Soberon, X., L. Covarrubias, and F. Bolivar. 1980. Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene 9:287–305.
28. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1:327–341.
29. Urlaub, G., and L. A. Chasin. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. U.S.A. 77:4216–4220.
30. Wigler, M., M. Perucho, D. Kurtz, S. Dana, A. Pellicer, R. Axel, and S. Silverstein. 1980. Transformation of mammalian cells with an amplifiable dominant-acting gene. Proc. Natl. Acad. Sci. U.S.A. 77:3567–3570.
31. Wilkie, N. M., J. B. Clements, W. Boll, N. Mantei, D. Lonsdale, and C. Weissmann. 1979. Hybrid plasmids containing an active thymidine kinase gene of herpes simplex virus 1. Nucleic Acids Res. 7:859–877.
32. Wold, B., M. Wigler, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Introduction and expression of a rabbit β-globin gene in mouse fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 76:5684–5688.