The organization of the dihydrofolate reductase gene of baby hamster kidney fibroblasts

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Received 3 September 1981

ABSTRACT

Using cloned DNA complementary to mouse dihydrofolate reductase (DHFR) mRNA, the organization of the hamster DHFR gene has been determined in two baby hamster kidney (BHK) cell lines, A5 and B1. A5 cells are highly methotrexate-resistant, containing 200-fold more copies of the DHFR gene than do the parental B1 cells. The DHFR gene has the same organization in A5 and B1 cells, suggesting that it has not been altered by the amplification process. The BHK DHFR gene spans a maximum of 10.7 kb and contains at least three introns. Thus the BHK DHFR gene is much smaller than the mouse DHFR gene, which has a minimum size of 42 kb and at least five introns. This striking size difference is probably due to much smaller introns in the BHK DHFR gene.

INTRODUCTION

Dihydrofolate reductase (DHFR), the initial enzyme in folate metabolism, is inhibited by the folate analog, methotrexate (MTX), at concentrations as low as 10^{-9} M (1). In cultured mammalian cells, three classes of mutants resistant to MTX have been isolated. Mutant cells possessing an altered form of DHFR with decreased affinity for MTX and mutants defective in MTX transport have a low level of MTX-resistance, about 10- to 50-fold greater than the parental cells (2,3). A third class of mutants with a high level of MTX-resistance, at least 1000-fold greater than the parental cells, possess high DHFR activity (100-fold or more above the parental cells) (4,5). These mutant cells contain an increased number of DHFR genes which results in a proportionate increase in DHFR synthesis (6,7).

We have studied the organization of the genomic DHFR gene in two lines of baby hamster kidney (BHK) cells, A5 and B1. A5

is a spontaneous MTX-resistant line derived by exposing the parental B1 cells to small step-wise increases in MTX concentration (8). Tryptic fingerprint analysis of DHFR purified from A5 and B1 cells demonstrated that the DHFR amino acid sequence is identical in both cell lines (9). A5 is 10,000-fold more resistant to MTX than the parental B1 line and possesses 140-fold more DHFR due to increased synthesis (9). The A5 genome encodes for 200-fold more copies of the DHFR gene than does the B1 genome (7).

We have analyzed the structure of the BHK DHFR gene with cloned DNA complementary (cDNA) to mouse DHFR mRNA (10). The mouse DHFR probe hybridizes strongly to BHK sequences under stringent conditions, suggesting that the DHFR gene sequences are highly conserved, as expected for two closely related species. However, following restriction endonuclease digestion of mouse or BHK DNA, the DNA fragment sizes hybridizing to the mouse probe are completely different. Since most of the cleavage sites in both mouse and BHK DNA are in the DHFR introns rather than the coding sequences (11, Schoner and Littlefield, this paper), they must have diverged considerably. Furthermore, the coding sequences for the mouse DHFR gene span a minimum of 42 kb (11), while the DHFR coding sequences in BHK DNA span a maximum of approximately 10.7 kb. Therefore, the BHK DHFR introns are smaller than those of the mouse, and there may be fewer of them.

MATERIALS AND METHODS

Preparation of plasmid DNA. The recombinant DNA plasmid pDHFR11 (10) was amplified as described by Norgard et al. (12). Plasmid DNA was extracted from cells as described by Clewell and Helinski (13) and purified by ethidium bromide-cesium chloride density centrifugation (14).

Preparation, digestion and electrophoresis of BHK DNA. BHK cells (5) were grown in Eagle's minimal essential medium (Gibco) supplemented with 10% fetal calf serum until nearly confluent. Cells were washed once with calcium- magnesium-free phosphate buffered saline (Gibco) and harvested by trypsinization. The DNA extracted from cells as described by Kunkel et al. (15) was typically >50kb. A sample of each DNA batch was routinely

analyzed by electrophoresis in a 1% agarose gel to check for degradation. All restriction endonucleases were purchased from Bethesda Research Labs and were used with the suggested buffers. DNA was incubated with 2 enzyme units/ μ g DNA for 18 h. The digested DNA was analyzed by electrophoresis in 0.8% agarose gels at 2V/cm for 18 h in a buffer of 40mM Tris-acetate (pH 7.9), 5 mM sodium acetate and 1mM EDTA.

Preparation of probe DNA fragments. Purified pDHFR11 DNA was digested with restriction endonucleases to yield specific probes. PstI digestion yielded two DNA fragments, pBR322 and the DHFR gene, separable by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining and UV illumination. The 1.53 kb fragment containing the DHFR gene was cut out of the gel, eluted by diffusion, purified on a DE52 column and concentrated by ethanol precipitation as described by Smith (16). All DNA fragments resulting from subsequent digestion of the 1.53 kb fragment were separated on 5% or 6% polyacrylamide gels at 12V/cm for 4 h in a buffer of 200 mM glycine-NaOH (pH 8.5) with an acrylamide/bis acrylamide ratio of 20:1. The fragments were cut out of the gel, eluted by diffusion and purified as described by Smith (16).

Nick translation of probe DNA. Aqueous α - 32 P dATP and α - 32 P dCTP (Amersham, >400 Ci/mmol) were incorporated into probe DNA as described by Rigby et al. (17). The amount of DNase I required for maximum incorporation of 32 P-labeled dATP and dCTP was determined for each lot of E. coli polymerase I (Bethesda Research Labs). Typically, incorporation was $>1 \times 10^8$ cpm/ μ gDNA.

DNA transfer and hybridization. DNA was transferred from 0.8% agarose gels to nitrocellulose filters (Millipore) as described by Southern (18) except that 20X SSC (1X SSC is 150 mM NaCl, 15 mM trisodium citrate) was used as the transfer medium. Prehybridization and hybridization of the filters was done in sealed plastic bags essentially as described by Scott et al. (19). Prehybridization was at 37°C in 50% formamide, 3X SSC, 0.1% SDS, 1mM EDTA, 10 mM HEPES (pH 7.5), 1 mM sodium pyrophosphate, 0.1 mg/ml salmon sperm DNA, 0.2% bovine serum albumin, 0.2% Ficoll and 0.2% polyvinylpyrrolidone (20). After 24 h the

bags were drained and refilled with prehybridization solution containing 10% dextran sulfate (Pharmacia) (21) and $0.5\text{--}1.0 \times 10^6$ cpm/ml of probe DNA. The bags were rotated for 18-24 h at 37°C . The filters were washed three times in 2X SSC and 0.5% SDS for 20 min/wash at room temperature, four times in 0.1X SSC and 0.5% SDS for 30 min/wash at 45°C and five times in 0.1X SSC for 5 min/wash at room temperature. The filters were dried and autoradiographed at -70°C with X-Omat R Kodak X-ray film with screens.

RESULTS AND DISCUSSION

Comparison of DHFR gene organization in A5 and B1. Fig. 1 shows the fragments obtained after digestion of A5 or B1 DNA by several restriction endonucleases and hybridization to the cloned 1530-bp mouse cDNA probe (Fig. 2). In each case, digestion of A5 or B1 DNA by a restriction endonuclease yielded

Figure 1. Autoradiogram of A5 and B1 DNA digested with restriction endonucleases, transferred to nitrocellulose paper and hybridized with the 1530-bp mouse cDNA. Lanes 1-5 A5 DNA, lanes 6-10 B1 DNA. Lanes 1 and 6 digested with *Hpa*I, 2 and 7 *Bgl*II, 3 and 8 *Xba*I, 4 and 9 *Eco*RI and 5 and 10 *Hind*III. *Hind*III digested lambda DNA served as the molecular weight markers, expressed in kb.

Figure 2. Cloned cDNA to mouse DHFR mRNA. The thick line represents the sequence coding for DHFR. The cDNA contains approximately 60 bp 5' to the AUG start codon for DHFR, and at least 900 bp of DNA 3' to the sequence coding for DHFR. The mouse cDNA was digested by restriction endonucleases to give the fragments shown for use as probes in Southern hybridizations of BHK DNA.

the same DNA fragments. Thus, the step-wise amplification of the DHFR gene has not grossly altered the arrangement of the DHFR coding sequence or introns.

Comparison of BHK and mouse DHFR gene organization. Since the BHK DHFR has a molecular weight of 23,000 daltons (8) approximately 600 nucleotides should be required to code for it. The size and number of fragments found after digestion of A5 and B1 DNA suggest that the BHK DHFR gene contains several introns (Fig. 1). A similar analysis of the mouse DHFR gene demonstrated that the 564 nucleotides of the coding sequence span at least 42 kb of DNA due to at least five introns (11). Comparison of the BHK and mouse DNA fragments generated by restriction endonuclease digestion leads to two observations (Table I): 1) the fragment sizes generated by a restriction endonuclease are very different, and 2) the fragment size is generally smaller in the BHK digests. The amino acid sequence of DHFR in two species as closely related as mouse and hamster are probably very similar; for example, the amino acid sequences of mouse and bovine DHFR are approximately 85% homologous, while mouse and chicken DHFR are approximately 75% homologous (22). Since we expect the coding sequence and size of the BHK and mouse DHFR genes to be highly homologous, the most likely explanation for the observed difference in the size and number of DNA fragments is that most of the restriction sites are in introns and the introns have diverged markedly.

TABLE I
BHK and mouse DNA fragments hybridizing
to 1530-bp mouse cDNA probe^a

Hamster	Mouse ^b	Hamster	Mouse	Hamster ^c	Mouse
<u>HindIII</u>		<u>EcoRI</u>		<u>PstI</u>	
4.8	14	14	17	11	3.9
2.4	3.7	3.4	16	7.3	2.8
2.2	3.2	2.8	6.2	2.9	2.0
	3.2	1.4	5.8	2.7	1.6
	1.8		3.5	1.7	1.6
					1.2
Hamster					
	<u>BglII</u>	<u>XbaI</u>	<u>HpaI</u>		
	13	13	8.9		
	9.8	8.4	1.2		
	5.9	7.1			
	2.4	1.5			

^aDNA fragment sizes in kb.

^bmouse DNA fragment sizes are from Nunberg et al. (11).

^cdata not shown.

Organization of the BHK DHFR gene. To map the BHK DHFR gene, the 1530-bp mouse DHFR cDNA was digested with BglII to produce 3 fragments: a 710-bp 5'-fragment which contains all of the coding sequences; a 520-bp middle fragment and a 300-bp 3'-fragment, both of which contain only 3'-non coding sequences (Fig. 2). When hybridized to BHK DNA digested with various restriction endonucleases, the 520-bp and the 300-bp probes both hybridized to only one fragment. Thus, in HindIII digests the 2.2-kb fragment contains all of the 3'-non coding sequences of the mRNA, while in BglII digests these sequences are found on a 13-kb fragment (Fig. 3). The strong hybridization between these

Figure 3. Autoradiogram of restriction endonuclease-digested A5 DNA hybridized to *Bgl*II fragments of mouse DHFR cDNA. BHK DNA in lanes 1, 3 and 5 digested with *Bgl*II; BHK DNA in lanes 2, 4 and 6 digested with *Hind*III. DNA in lanes 1 and 2 hybridized to 710-bp probe; lanes 3 and 4 hybridized to 500-bp probe; lanes 5 and 6 hybridized to 300-bp probe. *Hind*III digested lambda DNA served as the molecular weight markers, expressed in kb.

two fragments and BHK DNA fragments demonstrates that the mouse sequence in the 3'-non coding region of the DHFR mRNA is highly conserved in BHK DNA.

Detailed analysis of the globin genes from several organisms has demonstrated that the DNA sequences complementary to the 3'-non coding region of the mRNA diverge faster than the coding sequence (23). Therefore, the 520-bp and 300-bp probes from the 3'-non coding region of the mouse cDNA should have the least homology to the BHK DHFR gene. The strong and specific hybridization between these probes and BHK DNA indicates that the mouse DHFR cDNA can be used successfully to probe for BHK DHFR sequences.

To order the fragments containing coding sequences, the 710-bp fragment was digested with *Hae*III to yield: a 140-bp 5'-fragment, a 310-bp middle fragment and a 260-bp 3'-fragment (Fig. 2). Hybridization of these probes to digested BHK DNA allowed ordering of the coding sequence fragments (Fig. 4).

Figure 4. Autoradiograms of restriction endonuclease-digested A5 DNA hybridized to HaeIII fragments of the 710-bp coding region fragment of mouse DHFR cDNA. A5 DNA in lanes 1, 3 and 5 digested with BglII, A5 DNA in lanes 2, 4 and 6 digested with HindIII. DNA in lanes 1 and 2 hybridized to the 190-bp fragment, lanes 3 and 4 hybridized to the 310-bp fragment, and lanes 5 and 6 hybridized to the 260-bp fragment. HindIII digested lambda DNA served as the molecular weight markers, expressed in kb.

Fig. 5 is a map of the DHFR gene which indicates the number and order of the DNA fragments obtained after digestion with HpaI, HindIII and BglII.

Size of the BHK DHFR gene. HpaI digests of BHK DNA yield two fragments, 1.2 kb and 8.9 kb, when hybridized with the 1530-bp mouse cDNA probe. The 140-bp probe hybridizes with only the 1.2-kb HpaI fragment (Fig. 6). Thus the 1.2-kb fragment contains only the very 5'-end of the DHFR gene, while the 8.9-kb fragment contains all the sequences 3' to the 140-bp probe. By determining the distance between the 1.2-kb and 8.9-kb HpaI fragments, the size of the genomic BHK DHFR gene was determined. Two enzymes, HindIII and BglII, generate fragments that span the 1.2-kb and

Figure 5. Organization of the BHK DHFR gene. The figure shows the restriction endonuclease used to map the BHK DHFR gene, the sizes of the BHK DNA fragments containing DHFR sequences resulting from the digestions, the arrangement of the DNA fragments in the DHFR gene and the mouse cDNA fragments hybridizing to each BHK DNA fragment.

8.9-kb HpaI fragments. HindIII digestion results in three fragments: 1) a 4.8-kb fragment hybridizing to the 140-bp and 310-bp probes; 2) a 2.4-kb fragment hybridizing to the 260-bp probe; 3) a 2.2-kb fragment hybridizing to the 3'-non coding sequences (Figs. 1, 4). A HindIII-HpaI double digest yields fragments of 2.4-kb and 2.2-kb as before, but the 4.8-kb fragment disappears and is replaced by a 1.2-kb and a 3.1-kb fragment (Figs. 6,7). The 1.2-kb fragment hybridizes to only the 140-bp probe, while the 3.1-kb fragment hybridizes to only the 310-bp probe. Therefore, the 4.8-kb HindIII fragment must span the 1.2-kb and 8.9-kb HpaI fragments (Fig. 4). Similarly, BglII digests yield a 5.9-kb fragment that hybridizes to the 140-bp and 310-bp probes. In BglII-HindIII double digests, the 5.9-kb fragment disappears and the two new fragments appear at 1.2 kb and 4.1 kb (Fig. 5). The 1.2-kb fragment hybridizes to only the 140-bp probe and the 4.1-kb fragment hybridizes to only the 310-bp probe (Fig. 6). Therefore, the 5.9-kb BglII fragment must also span the 1.2-kb and 8.-kb HpaI fragments (Fig. 4).

Figure 6. Autoradiograms of double restriction endonuclease digests of A5 DNA hybridized to HaeIII fragments of the 710-bp coding region fragment of mouse DHFR cDNA. A5 DNA in lane 1 digested with HpaI, A5 DNA in lanes, 2 4 and 6 digested with BglII and HpaI, A5 DNA in lanes 3, 5 and 7 digested with HindIII and HpaI. DNA in lanes 1, 2 and 3 hybridized with the 140-bp fragment, lanes 4 and 5 hybridized with the 260-bp fragment. HindIII digested lambda DNA served as the molecular weight markers, expressed in kb.

As drawn in Fig. 5, the genomic DHFR gene spans a maximum of approximately 10.1 kb. In Fig. 5, 0.5 kb of the 4.8-kb HindIII fragment and 0.6-kb of the 5.9-kb BglII fragment are drawn lying 5' to the 1.2-kb HpaI fragment. However, we cannot exclude the possibility that these fragments lie between the 1.2-kb and 8.9-kb HpaI fragments. Internal fragments would occur if two HpaI sites separated by about 0.6 existed between the 1.2-kb and 8.9-kb HpaI fragments. If these two small fragments contained only intron sequences, we would not detect them with the mouse cDNA probe. An internal 0.6-kb fragment would increase the maximum size of the DHFR gene to 10.7 kb. In the HindIII-HpaI and BglII-HpaI double digests, each of the four probes used (140 bp, 310 bp, 260 bp, and 3'-non coding) hybridizes

Figure 7. Autoradiograms of single and double restriction endonuclease digests of A5 DNA hybridized to the 1530-bp mouse DHFR cDNA. A5 DNA digested with: lane 1, HindIII; lane 2, HindIII and HpaI; lane 3, BglII; lane 4, BglII and HpaI. The DNA in all four lanes was hybridized to the 1530-bp mouse DHFR cDNA. HindIII digested lambda DNA served as the molecular weight markers.

to a single fragment. Thus, each probe appears to represent a block of DHFR gene sequence that is separated from the others by an intron. Therefore, the BHK DHFR gene contains at least three introns.

Limitations to mapping. There are limitations to gene mapping by the technique of genomic DNA digestion followed by hybridization to specific fragments of a cDNA probe. A small DNA fragment may not bind to nitrocellulose paper, and be missed. A small intron may be missed because it lacks a restriction site for the enzymes employed. However, our data is consistent with a maximum gene size of 10.7 kb and at least three introns.

The 9.8-kb BglII fragment that hybridizes faintly to the 1530-bp and 310-bp probes cannot be assigned a position within the genomic BHK DHFR gene consistent with our data (Figs. 1,6).

This DNA fragment is not cleaved in BglII-HpaI double digests and although it is 9.8 kb, only the 310-bp probe hybridizes to it. Since the 9.8-kb fragment occurs in BglII digests of A5 and B1 DNA (Fig. 1), its appearance is not related to amplification of the DHFR gene. There are several possible explanations for the anomaly. First, the BHK cell line was established from outbred one-day old hamsters (24). The DHFR genes on the homologous chromosomes may be heterogeneous with regard to size and restriction sites in introns and we may have detected such a difference with BglII digests. Second, a pseudogene may be associated with the functional DHFR gene. A pseudogene may contain substitutions or deletions leading to a BglII-fragment not found in the functional gene. Third, a portion of the DHFR gene that hybridizes to the 310-bp probe may have transposed to a new site an unknown distance from the DHFR gene, but still within the repeating unit of the amplification. Childs et al. (25) have found portions of the tandemly repetitive histone gene cluster and ribosomal gene cluster in sea urchin and D. melanogaster at new sites away from the gene cluster. We cannot presently distinguish between these possibilities to rigorously determine if the 9.8-kb fragment is a part of the DHFR gene. However, all of our data, with the exception of the 9.8-kb BglII fragment, are consistent with 10.7 kb as the maximum size of the DHFR gene and the organization presented in Fig. 5.

BHK and mouse DHFR introns. The DHFR of A5 and B1 cells is identical in amino acid sequence (9) and digestion of A5 or B1 DNA with each restriction endonuclease we used has yielded the same size fragment. Therefore, the gene amplification process that occurred in A5 has not detectably altered the DHFR coding sequence or the introns.

All portions of the mouse DHFR cDNA, including the 3'-non coding region, hybridize strongly to BHK DNA, suggesting that the mouse DHFR sequences transcribed into cytoplasmic mRNA are highly conserved in the BHK genome. However, the different sizes and numbers of fragments obtained after restriction endonuclease digestion of BHK and mouse DNA suggest that the introns are very different. The minimum size of the mouse DHFR

gene is 42 kb (11), while we find that the maximum size of the BHK DHFR gene is 10.7 kb. This size difference must be due to the presence of much larger introns in the mouse DHFR gene and is further evidence that introns evolve rapidly between species.

ACKNOWLEDGEMENTS

We thank Drs. Stanley Wolf and John Phillips for helpful discussions, Dr. Robert Schimke for the pDHFR11 plasmid, and Mrs. Deborah K. Kuriga for skillful secretarial assistance in the manuscript preparation. This work was supported by grant CA 16754 from the NCI and by institutional funds made available by the Mellon Foundation.

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