Molecular cloning of Chinese hamster dihydrofolate reductase-specific cDNA and the identification of multiple dihydrofolate reductase mRNAs in antifolate-resistant Chinese hamster lung fibroblasts

John A. Lewis¹, David T. Kurtz² and Peter W. Melera¹

¹Sloan-Kettering Institute for Cancer Research, Walker Laboratory, Rye, NY 10580, and ²Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, NY 11724, USA

Received 5 January 1981

ABSTRACT

ds cDNA from the antifolate-resistant Chinese hamster lung fibroblast subline DC-3F/MQ19 was ligated to Eco RI and Sal I oligonucleotide linkers and cloned into Eco RI and Sal I digested pBR322. Transformed colonies containing dihydrofolate reductase (DHFR)-specific recombinant plasmid were identified by Grunstein Hogness assay using a Chinese hamster DHFR-specific cDNA probe. A recombinant plasmid, pDHFR6, containing a 650 bp DHFR insert was isolated and analyzed. This plasmid was used as a molecular probe in a Northern blot analysis of both cytoplasmic and polysomal DHFR, poly A+ mRNAs of the DC-3F/MQ19 subline, which overproduces a 20,000d DHFR 150-fold, and the DC-3F/A3 subline, which overproduces a 21,000d DHFR 170-fold. This analysis revealed the presence of three DHFR mRNA species of 1350, 2200, and 3300 nucleotides in both independently-derived cell lines. The relative abundance of each species however varied strikingly between the two cell lines.

INTRODUCTION

We have previously reported that under challenge by the antifolates, methotrexate or methasquin, Chinese hamster lung fibroblasts acquire resistance by an overproduction of the target enzyme dihydrofolate reductase (DHFR) mediated by proportional increases in DHFR mRNA transcribed from amplified DHFR structural gene loci (1,2). These results confirm earlier work of Chang and Littlefield (3) with resistant BHK cells, and work of Alt et al. (4,5) with resistant sarcoma 180 cells. Unique to the Chinese hamster lung fibroblast system is the observation of Shanske et al. (6) that of 15 independently-derived resistant sublines, 4 overproduce a 20,000d DHFR characteristic of the parental sensitive DC-3F control line, whereas 11 others
overproduce a DHFR variant of 21,000 molecular weight. This molecular weight difference is also characteristic of the DHFR protein translated in vitro in a rabbit reticulocyte system primed with resistant cell RNA, and, therefore, is inherent in the nucleotide sequence of the respective DHFR mRNA (1). We are interested in the molecular genetic basis of this DHFR protein variant phenomenon and as a first step in its analysis have cloned DHFR ds cDNA sequences into the vector pBR322. We report here the details of this cloning. The use of these cloned DHFR sequences as hybridization probe in Northern blot analysis reveals an unexpected multiplicity of cytoplasmic DHFR poly A+ RNA in various resistant cell lines and, intriguingly, a variation between cell lines in the relative abundance of each of these poly A+ species.

MATERIALS AND METHODS

Poly A+ RNA purification, cDNA and ds cDNA synthesis. Poly A+ mRNA from the DHFR overproducing DC-3F/MQ19 cell line was selected by oligo(dT)-cellulose chromatography from cytoplasmic RNA obtained from polysomal pellets (1). DHFR mRNA was partially purified by sucrose density gradient sedimentation (2), and fractions corresponding to the 14S region of the gradient were pooled, concentrated by ethanol precipitation and used as template for cDNA synthesis by AMV reverse transcriptase (provided kindly by Dr. J. Beard) under reaction conditions as described by Alt (4) and Melera (2). Second strand synthesis with E. coli Pol I and terminal hairpin digestion with S1 nuclease were conducted as described by Harpold et al. (7).

Insert preparation, recombinant construction, cell transformation and colony identification. All experiments were carried out under the revised NIH Guidelines for Recombinant DNA Research, February, 1980. A fuller description of the cloning technique to be described below is detailed in Kurtz and Nicodemus (8). To ensure that the S1-treated ds cDNA was flush
ended before linker addition, 400 ng were suspended in 40 μl of a buffer
(66 mM Tris-HCl, pH 7.4, 7 mM MgCl2, 10 mM spermidine, 1 mM ATP, and 1 mM
β-mercaptoethanol) containing 200 μM dXTPs and 5 units of *E. coli* poly-
merase I (Klenow fragment). Incubation was carried out for 30 min at 37°C,
at which time 500 ng each of Eco RI and Sal I linkers solubilized in the
same buffer, and 10 units of T4 polynucleotide kinase were added. The
final reaction volume was 60 μl. Incubation was continued for another 30
min at 37°C and terminated by heating to 65°C for 7 min. The kinased
linkers and ds cDNA were ligated by addition of 10 units T4 DNA ligase and
10 units RNA ligase followed by incubation for 12 hr at 4°C. The volume
of the incubation mixture was then expanded to 300 μl and simultaneously
adjusted to Eco RI buffer conditions. Fifty units each of Eco RI and Sal I
restriction enzymes were added and the linker containing ds cDNA digested
for 5 hr at 37°C. The reaction mixture was then heated to 65°C for 5 min,
extracted with phenol and chromatographed over Sephadex G-150. Eco RI and
Sal I linker containing ds cDNA was pooled from the void volume and col-
lected by ethanol precipitation.

The use of the heterogeneous linker mixture to mediate recombination of
insert and plasmid was selected to preclude self-religation of the pBR322
cloning vector during the annealing procedure described below. To inform
the choice of linker specificity, approximately 40 ng ds cDNA to DC-3F/MQ19
poly A+ RNA gradient enriched for DHFR mRNA was digested with either Eco RI,
Bam HI, Hind III or Sal I, and analyzed by gel electrophoresis and Southern
blot transfer with a Chinese hamster DHFR-specific cDNA probe. This
analysis demonstrated only a Bam HI restriction site within the DHFR ds
cDNA. Eco RI and Sal I linkers were therefore selected and used in combi-
nation.

pBR322 was prepared for recombination by simultaneous digestion with 20
units each Eco RI, Bam HI, Hind III and Sal I, followed by phenol-chloroform
extraction and ethanol precipitation. Since Bam HI and Hind III both cut within the fragment of pBR322 generated by Eco RI and Sal I, this step mini-
mized the possibility of plasmid fragment religation, thereby favoring the ligation of ds cDNA with the major 3700 bp Eco/Sal ended pBR322 fragment. Approximately 2 µg of enzyme-digested pBR322 were recombined with 200 ng linker containing ds cDNA in 2.0 ml total volume of 66 mM Tris-HCl 7 mM MgCl₂, 10 mM spermidine, 1 mM β-mercaptoethanol 1 mM ATP and incubated at 4°C for 24 hours with 10 units T₄ DNA ligase. Ligation products were phenol-
extracted and concentrated by ethanol precipitation. Approximately 1 µg recombinant DNA was recovered by centrifugation, resuspended in 10 µl ligation buffer as above, and exposed to 100 µl of transformation competent cells of E. coli strain MM 294 prepared as described by Dagert and Ehrlich (9). Transformant colonies were identified by growth on LB agar plates con-
taining 150 µg/ml ampicillin.

Colonies were transferred and grown on nitrocellulose filters to a diameter of 1 mm and processed according to Grunstein and Hogness (10). Five filters were hybridized in 12.5 ml of a solution of 4x SSC, 2.5 mM EDTA, containing 25 µg/ml denatured salmon sperm DNA, and 2 ng/ml DHFR cDNA probe, specific activity 2 x 10⁸ [³²P] cpm/µg. Plasmid DNA was purified by the techniques of Guerry et al. (11) or Clarke and Carbon (12).

Independent confirmation of the identity of DHFR cloned sequences was accomplished by selection of poly A⁺ RNA complementary to recombinant plasmid essentially as described by Smith et al. (13) and Stark and Williams (14). 500 µg of recombinant plasmid was bound to a 4 cm x 4 cm DBM filter and hybridized with 60 µg poly A⁺ RNA from the resistant DC-3F/MQ19 cell line for 19 hours at 37°C under the buffer conditions of Smith et al. (13). RNA bound specifically in hybrid form was eluted (14) and translated in vitro in a rabbit reticulocyte system modified from Pelham and Jackson (15) by Melera et al. (1).
RNA Transfer. RNAs for Northern blot analysis were denatured and electrophoresed through formaldehyde gels as described by Lehrach et al. (16). RNA gel transfers to DMB filters were conducted according to Alwine et al. (17). Filters were hybridized for 18 hours at 42°C in 5 ml of a solution of 4x SSC, 50% formamide, 2.5 mM EDTA, containing 25 ng/ml pDHFR6 nick-translated (18) to a specific activity of $2.0 \times 10^8$ [$^{32}$P] cpmpg.

RESULTS

Molecular cloning. Analysis by Southern transfers (19) using a kinetically purified Chinese hamster DHFR-specific cDNA as probe demonstrated the presence of DHFR ds cDNAs of approximately 500-700 base pairs in size among the ds cDNA population obtained from poly A+ RNA enriched for DHFR mRNA by sucrose density gradient sedimentation. These DHFR ds cDNAs were smaller than expected for full length transcripts of a 14S DHFR mRNA. Although we are ultimately interested in obtaining fully copy ds cDNA for sequencing purposes, we proceeded with a molecular cloning of these less than full copy ds cDNAs to obtain probes for ongoing studies of DHFR gene isolation.

Transformation frequencies using the linker technique outlined here were approximately $1.2 \times 10^4$ transformants per µg ds cDNA. 300 of approximately 1200 colonies transformed to ampicillin resistance were replated for screening with a DHFR-specific cDNA probe. A single recombinant colony, designated pDHFR6, was recovered on the basis of its strong hybridization signal. Its plasmid was isolated, nick-translated and used as a probe in a dot blot analysis of poly A+ mRNA from control drug-sensitive DC-3F cells and drug-resistant DC-3F/QM19 cells. The results, not shown, suggested that pDHFR6 was indeed a DHFR recombinant based on its intense reactivity with resistant but not sensitive cell RNA.

Although its DHFR specificity was strongly presumed on the basis of its identification by Grunstein Hogness assay with a DHFR-specific cDNA and by
its reactivity under dot blot conditions with RNA from drug-resistant but not drug-sensitive cells, we used the method of positive mRNA selection by plasmid linked covalently to DBM filters in conjunction with in vitro translation to establish the identity of the pDHFR6 insert as DHFR-specific. 500 μg pDHFR6 was sonicated, bound to DBM filters and hybridized with 60 μg poly A+ RNA from the DC-3F/MQ19 cell line for 18 hours at 42°C. Filters were washed stringently and the RNA not bound was pooled and collected by ethanol precipitation. RNA bound to the filter was eluted at 65°C in 70% formamide and precipitated in the presence of DC-3F control cell RNA. Both RNAs were resuspended and translated in vitro in a rabbit reticulocyte system. An SDS polyacrylamide gel analysis of the translation products of both RNA fractions is presented in Figure 1. Filter-bound RNA translated in the presence of control cell RNA contained an intense band at a molecular weight of 20,000d, as expected for DC-3F/MQ19 DHFR. No such band was present in the DC-3F control cell translation products. A faint band of DHFR was evident in the non-hybridizing RNA fraction, suggesting that the DBM filters failed to bind all the DHFR mRNA present in the 60 μg sample, but also demonstrating that the large majority of the DHFR mRNA had been removed by specific hybridization.

Having determined its DHFR specificity, the insert of pDHFR6 was removed by digestion with Eco RI and Sal I and purified by agarose gel electrophoresis and electroelution. The insert, which migrated as a 650 bp species, was nick-translated and used to screen the remaining 900 ampicillin-resistant colonies. From this screening, 3 additional recombinants whose inserts ranged from 400-500 base pairs were recovered. Since pDHFR6 contained the largest insert among the four DHFR recombinant plasmids identified, it was used in subsequent Northern blot analysis. It has been preliminarily mapped for use in DHFR gene isolation studies (Fig. 2).

**RNA Analysis.** Northern blot analysis of poly A+ RNA from the drug-resistant subline DC-3F/MQ19 (a 20,000d DHFR overproducer) and DC-3F/A3 (a
Fig. 1. In vitro translation of mRNA hybridized by pDHFR 6. 500 µg pDHFR 6 was bound to DBM filter paper and hybridized with 60 µg of poly A+ RNA from polysomal pellets of DC-3F/MQ19 cells. RNA not hybridized by the plasmid was pooled, precipitated and translated. RNA hybridized, i.e., selected, by the plasmid was eluted from it with 70% formamide at 65°C, mixed with parental drug-sensitive DC-3F mRNA as carrier, precipitated from ethanol and translated. Lane A. Translation products of DC-3F/MQ19 mRNA; Lane B. Translation products of DC-3F/MQ19 mRNA containing DC-3F/MQ19 mRNA positively selected by pDHFR 6; Lane C. Translation products of DC-3F/MQ19 mRNA not selected by pDHFR 6. Protein X is a product of endogenous synthesis by the rabbit reticulocyte system. It varies in amount from lysate to lysate and is refractory to micrococcal nuclease treatment.

21,000d DHFR overproducer) revealed the presence of 3 molecular weight species of RNA which reacted with pDHFR6 (Fig. 3). Molecular size estimates in formaldehyde gels, using both Physarum polycephalum 26S and 19S and Chinese
Fig. 2. Restriction map of recombinant insert pDHFR 6. The terminal Eco RI and Sal I sites result from the linker construction used. The overall length of the insert is approximately 650 nucleotides.

Hamster 28S and 18S rRNAs as standards, indicated molecules of 1350, 2200 and 3300 nucleotides in length. Remarkably, the relative abundance of each poly A⁺ DHFR RNA species can be seen to vary between the DC-3F/MQ19 and DC-3F/A3 cell lines. A quantitative analysis of this variation by X-ray film scanning with a Joyce-Loebl densitometer indicates that the DHFR poly A⁺ RNAs of DC-3F/MQ19 are distributed with 55-60% occurring as the 1350 base species, 25-30% as the 2200 base pair species, with the remainder as the 3300 base species. By contrast, DC-3F/A3 DHFR poly A⁺ RNA species are distributed with 50-55% as the 3300 base species, 20-25% as the 2200 base species, with the remainder as the 1350 bp species (data not shown). These results have been consistently obtained and are largely invariant, not only between repeated analyses of individual RNA preparations, but between various preparations of poly A⁺ RNA as well.

Since nonpolysomal cytoplasmic ribonucleoprotein particles would have been expected as a contaminant element of the polysomal pellets used for the isolation of poly A⁺ RNA, experiments were conducted to establish directly that the three DHFR poly A⁺ RNA species detected by Northern blotting were engaged as polysomes and, hence, presumably available for in vivo translation. Poly A⁺ mRNA was isolated by oligo(dT) chromatography from polysomes isolated on 10-50% sucrose gradients as described by Melera et al. (1). The
Fig. 3. Northern blot analysis of DC-3F/MQ19 and DC-3F/A3 mRNA. Two μg of mRNA from DC-3F/MQ19 and DC-3F/A3 were denatured in 50% deionized formamide at 85°C for 5 min in the presence of 2.2 M formaldehyde, and electrophoresed for 18 hr at 50 volts through a 1.5% horizontal agarose gel containing 2.2 M formaldehyde. RNA was denatured in situ (Alwine et al., 1977) and blotted under gravity to DBM filters. Preparation of the filters for probing with nick-translated pDHFR 6 was as described in Materials and Methods. Lane A, DC-3F/A3 mRNA; Lane B, DC-3F/MQ19 mRNA; Lane C, 20 μg DC-3F mRNA.

resultant mRNA was electrophoresed, blotted and hybridized as described above. Results (not shown) were essentially identical to those obtained with poly A+ RNA obtained from polysomal pellets, providing evidence that the multiple RNA species were organized polysomally and presumably therefore represented messenger RNA species actively engaged in translation. To establish whether the phenomenon of multiple poly A+ DHFR mRNA species is consequential to the process of DHFR gene amplification documented to have occurred in these two cell lines (2), cytoplasmic poly A+ RNA was prepared from the drug-sensitive DC-3F control cell line, known to produce the 20,000d DHFR (6), and analyzed by Northern blot techniques. The results
of such an analysis shown in Figure 3c reveal the presence of three RNA species reacting with pDHFR6 probe, distributed with the 1350 nucleotide species as the predominant form, similar to the pattern observed for DHFR poly A⁺ RNA from the DC-3F/MQ19 cell line.

DISCUSSION

The successful cloning of DHFR ds cDNA sequences into the plasmid pBR322 has permitted the study of the DC-3F/MQ19 and DC-3F/A3 protein variant phenomenon to be extended to the level of DHFR gene expression and revealed an unexpected multiplicity of poly A⁺ DHFR mRNA in both lines. This multiplicity has been confirmed under the denaturing conditions afforded by 10 mM methylmercury hydroxide (20), which has verified the 1350, 2200, and 3300 nucleotide molecular size estimates obtained from formaldehyde gels. The interpretation that pDHFR6 encodes nucleotide sequences shared by DHFR mRNA with other Chinese hamster mRNA is made untenable by the results of the positive selection experiment (Fig. 1), which clearly showed DHFR mRNA to be essentially the only DC-3F/MQ19 mRNA selected by the plasmid. Of equal importance the methylmercury gels have confirmed the distributional differences found between the DHFR mRNAs of sublines DC-3F/MQ19 and DC-3F/A3.

Preliminary data of Melera et al. have indicated that the correlation of relative DHFR transcript abundance with DHFR protein molecular weight is a consistent observation among seven other independently-derived antifolate-resistant sublines. The molecular basis of this correlation - whether both RNA species abundance and DHFR molecular weight are governed by a unique nucleotide sequence mutation - is presently unknown. Experiments are currently in progress to establish whether the multiple DHFR mRNAs reported here are the products of multiple functionally discrete DHFR transcription units or whether they arise through an ambiguity in the options presumably available for transcription initiation or termination or transcript processing.
Recent data from Setzer et al. (21) studying the transcriptional products of the murine DHFR locus in both control and resistant S-180 cells describe a complex pattern of 4 DHFR mRNAs species. These multiple mRNAs have been shown to arise through options in transcription termination at the 3' terminus of the murine DHFR locus. Similar options may be characteristic of the 3' terminus of the Chinese hamster DHFR locus as well. The importance of multiple DHFR mRNAs to gene expression at the DHFR gene locus of both mouse and Chinese hamster remains to be established.

The molecular basis for the variation in the relative abundance of the 3 DHFR cytoplasmic poly A+ mRNA species observed between the independently-derived resistant DC-3F/MQ19 and DC-3F/A3 Chinese hamster lung fibroblast lines is currently under investigation. Although the poly A+ DHFR mRNA pattern of the DC-3F/MQ19 cell line is characteristic of the sensitive DC-3F control cell line, the pattern in the DC-3F/A3 cell line clearly is not. It remains possible that the variation arises either through infidelities in the process of DHFR gene amplification, or, alternatively, from the amplification and activation of a normally repressed DHFR gene in the Chinese hamster cell genome which generates DHFR RNA species in a manner distinct from the normally active DHFR gene. Studies of the DHFR genes of the amplified DC-3F/MQ19 and DC-3F/A3 cell lines by Southern blotting may distinguish between these alternatives.

ACKNOWLEDGEMENTS

We thank Dr. J. L. Biedler for supplying cell lines and Dr. M. Wigler for helpful discussions. The excellent technical assistance of Ms. C. A. Hession is gratefully acknowledged. This work was supported in part by grants from the National Cancer Institute to the Sloan-Kettering Institute for Cancer Research and to P.W.M. The data reported here has been submitted
in partial fulfillment for the Ph.D. degree by J.A.L.

†To whom requests for reprints should be sent.

REFERENCES