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# 5′ Nucleotide Sequences Influence Serum-Modulated Expression of a Human Dihydrofolate Reductase Minigene

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**Human dihydrofolate reductase (DHFR) gene sequences were isolated from DHFR gene-amplified breast cancer cell line MCF-7. These genomic sequences plus human DHFR cDNA sequences were used to construct a DHFR minigene. Calcium phosphate-mediated transfer of minigene DHA into DHFR gene-deleted Chinese hamster ovary cells converted these cells to a DHFR$^+$ phenotype at a frequency of 0.12%. Minigene-transfected cells contained 20 to 30 minigene copies per cell and had DHFR enzyme levels similar to those of wild-type MCF-7 human cells (1.4 pmol/mg of protein). In contrast to gene-amplified MCF-7 cells, which contained multiple DHFR mRNA species (1.1, 1.6, 3.8, and 5.3 kilobases), only a single 3.8-kilobase DHFR mRNA was found in minigene-transfected cells. Previous studies on normal cells demonstrated modulation of DHFR levels by a variety of conditions which altered cell growth. When cell growth was induced in minigene-transfected cells by release from serum deprivation and DHFR levels were assayed at the time of maximum DNA synthesis, these levels were increased 2.4 to 3.7-fold. In contrast, the DHFR levels in cells transfected with a construct made from DHFR cDNA and viral promoter, intron, and termination sequences were unchanged. Minigene deletions were made and analyzed to determine the DHFR gene sequences responsible for regulation. Deletion of sequences upstream from 322 base pairs 5′ to the start of transcription or 90 base pairs downstream from the termination of translation (which removed most of the 3′ nontranslated region of the gene) did not alter the responsiveness of minigene-transfected cells to serum deprivation. However, when sequences between 322 and 113 base pairs 5′ to the start of transcription were deleted, serum-dependent expression in minigene-transfected cells was affected.**

Dihydrofolate reductase (DHFR) is an essential housekeeping enzyme that is necessary for the regeneration of reduced folate cofactors, which are required for the production of thymidylate, purines, and glycine (6). The availability of cell lines which contain amplified DHFR genes, as well as increased levels of DHFR mRNA and protein, has greatly facilitated studies on DHFR regulation. Although expression of the DHFR gene is necessary in all cells, previous studies in our laboratory and other laboratories have shown that cellular DHFR levels are responsive to a variety of modulators. Among these are estrogen, cAMP, viral infection, and methotrexate, as well as serum deprivation, amino acid deprivation, and contact inhibition (9, 10, 15, 24, 29, 32, 45, 51, 53). Some of the changes in DHFR levels may reflect cell cycle-dependent DHFR expression since the enzyme is regulated during the cell cycle, with maximal DHFR mRNA and protein synthesis occurring in the S phase (18, 26, 34). The regulation of DHFR expression is apparently complex and may involve transcriptional or posttranscriptional control mechanisms, depending on the method of induction. To gain a more precise understanding of the control mechanisms involved in DHFR regulation, we constructed a human DHFR minigene and studied its regulation in a cellular expression system.

The structure of the human DHFR gene has been determined from studies on human DHFR genomic genes, cloned cDNAs, and mRNAs (2, 7, 35, 36, 38, 42, 48, 52). The human DHFR gene, like the mouse and hamster genes, is large (5, 13, 40). The gene covers approximately 30 kilobases (kb) of DNA on chromosome 5 and is formed from six short exons that are separated by large noncoding intervening sequences. The coding region of the gene is followed by a long 3′ nontranslated region which contains several polyadenylation sites where multiple mRNA species are terminated. The function of the multiple DHFR mRNA species, which are also found in mice and hamsters, is not known (31, 46). Although it is possible to transfect the genomic DHFR gene into cells (41, 50), it is difficult to manipulate large genomic genes with currently available recombinant DNA techniques. Therefore, several groups of workers have constructed a variety of DHFR minigenes by using mouse DHFR cDNA and genomic sequences (12, 20), as well as mouse DHFR cDNA and viral sequences (27, 30). These minigenes are capable of restoring DHFR gene function to a DHFR-deficient Chinese hamster ovary (CHO) cell line. In addition, a human DHFR minigene constructed from DHFR pseudogene and genomic sequences has been shown to produce DHFR mRNA in a transient expression system (7). Although these studies demonstrated that it is possible to obtain DHFR gene function by using a minigene construct, little has been done to characterize the regulation of DHFR genes in any of these systems.

In order to further our studies on DHFR regulation, human DHFR genomic DNA sequences were isolated by molecular cloning techniques. The 5′ and 3′ regulatory sequences from this cloned genomic DNA and the protein-encoding sequences from a cloned human DHFR cDNA (42) were used to construct a human DHFR minigene. This minigene, which eliminated all but one of the intervening sequences from the genomic gene, retained those DNA sequences which are necessary for DHFR gene function since it could restore DHFR activity to a DHFR gene-

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deleted CHO cell line (49). The DHFR minigene-transfected cells were analyzed to determine whether the transfected minigenes were modulated in a manner similar to the endogenous cellular DHFR gene.

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## MATERIALS AND METHODS

**Molecular cloning and minigene construction.** A human genomic bacteriophage library was constructed with DNA isolated from DHFR gene-amplified cell line MCF-7 (10, 21). Clones with DHFR gene sequences were identified by using the Benton-Davis filter hybridization technique (33) with human DHFR cDNA probes made from pHD84 (42). DNA sequences from the 5′ and 3′ ends of the genomic DHFR gene were used in the construction of the minigene. The majority of the protein-encoding sequence for the minigene came from cloned human DHFR cDNA pHD84 (42), which contained the entire protein-encoding region but lacked the 3′ nontranslated part of the gene. To construct the minigene, a 0.44-kb *Eco*RI-*Rsa*I fragment from pHD84 containing the 3′ end of exon 2 through the 5′ end of exon 6 was ligated to a 0.4-kb *Rsa*I-*Hin*dIII genomic fragment from exon 6 which encoded part of the 3′ nontranslated portion of the DHFR mRNA. Next, a 1.8-kb *Eco*RI fragment from the 5′ end of the genomic gene containing 1.25 kb of the 5′ flanking sequence, exon 1, intron 1, and the 5′ end of exon 2 was ligated to the 5′ end of the contract. Finally, a 5.3-kb *Hin*dIII-*Bgl*II fragment from the 3′ end of the genomic gene containing the remainder of exon 6 and the 3′ flanking sequence was ligated to the 3′ end of the minigene. Several constructs were made from the DHFR minigene. Hdhfr Δ5′-322 is a human DHFR minigene which begins at a *Pst*I site 322 base pairs (bp) 5′ to the start of transcription of the major DHFR mRNA (7, 36). Hdhfr Δ5′-113 starts at a *Sac*II site. Hdhfr Δ3′+728 is a minigene which terminates at a *Bgl*II site in the 3′ nontranslated region of the gene 728 bp from the start of transcription of the mRNA. The pMTVdhfr+intron construct was made by inserting a *Bam*HI-linked *Bal*I-*Eco*RI fragment containing the intron from the human DHFR minigene (intron 1) into the *Bgl*II site at the junction between the mouse DHFR cDNA and the simian virus 40 sequences in the 3′ nontranslated region of pMTVdhfr (30).

**Transfection into CHO cells.** In order to assay the functional activity of the DHFR minigene, DHFR$^-$ CHO cell line DG-21 was used (49). The cells were grown in improved minimal essential medium (IMEM) (44) supplemented with 2 μg of proline per ml, 10 μg of glycine per ml, 13.6 μg of hypoxanthine per ml, 3.88 μg of thymidine per ml, and 5% fetal calf serum (FCS) (GIBCO Laboratories) (nonselective medium) at 37°C in an atmosphere containing 5% $CO_2$. For transfection experiments cells were plated at a density of 250,000 cells per 100-mm dish. The next day, the medium was changed 4 h before 20-μg portions of various calcium phosphate-precipitated plasmid DNA constructs (22) were added to each dish. At 4 h after DNA was added, the cells were shocked with 20% glycerol in phosphate-buffered saline for 30 s (19). The glycerol was removed, and the cells were grown in nonselective medium for 2 days. The medium was then changed to selective medium (IMEM supplemented with proline and 5% extensively dialyzed FCS). The medium was changed every 2 to 4 days thereafter. Two weeks after transfection the DHFR$^+$ clones were isolated, or the plates were fixed, stained, and counted.

**Serum stimulation.** Cells were plated at a density of $1 \times 10^6$ to $2 \times 10^6$ cells per 150-cm$^2$ flask in IMEM containing 0.25% FCS. All IMEM preparations were supplemented with 2 μg of proline per ml. After 2 days the medium was changed to IMEM containing 0.1% FCS, and the medium was changed again after 2 days. On day 6, two-thirds of the cells were harvested, and one-third were stimulated by transferring them to IMEM containing 5% FCS. Stimulated cells were harvested after 16 h. DHFR levels were determined by using a [$^3$H]methotrexate (Moravek Biochemicals) binding assay (43), and total cellular protein levels were determined by a Bradford protein assay (Bio-Rad Laboratories) (4).

**Thymidine incorporation.** Cells were plated in six-well Linbro plates at a density of 20,000 cells per well and were grown as described above. At zero time the medium was changed to stimulating medium, and at different times cells were labeled for 1 h with 5 μCi of [*methyl*-$^3$H]thymidine (ICN) Pharmaceuticals Inc.) per well. At the end of incubation, the cells were harvested, and acid-precipitable [$^3$H]thymidine incorporation and total cellular protein concentration were determined.

## RESULTS

**Construction of the human DHFR minigene.** The human DHFR minigene shown in Fig. 1C was constructed from genomic and cDNA sequences. The cloned 5′ and 3′ genomic sequences shown in Fig. 1A and B provided the transcription initiation and polyadenylation sequences. Cloned DHFR cDNA sequence pHD84, which was isolated by Morandi et al. (42), provided the majority of the protein-encoding region. To construct the minigene, a genomic *Eco*RI fragment from the 5′ end of the gene containing 1.25 kb of DNA upstream from the start of transcription and the beginning of the protein-encoding region with the first intervening sequence was ligated to the *Eco*RI site in the cDNA. A genomic *Hin*dIII-*Bgl*II fragment from the 3′ end of the gene which included all of the known polyadenylation sites was then ligated to the *Hin*dIII site in the cDNA. This minigene should have directed the synthesis of DHFR mRNA which was indistinguishable from the DHFR mRNA produced in human MCF-7 cells.

**Transfection into DHFR-deficient CHO cells.** The ability of the DHFR minigene to function like the normal cellular DHFR gene was determined by stable transfection of a DHFR$^-$ CHO cell line which was isolated by Urlaub et al. (49). Since the DHFR genes were deleted from this cell line, these cells were able to grow only in media supplemented with glycine, hypoxanthine, and thymidine. Following transfection of the DG-21 cells with various DNAs by calcium phosphate-mediated gene transfer, cells with a functional DHFR gene were selected by growth in unsupplemented medium. CHO cells transfected with plasmid vector pUC-8 were used to determine the reversion rate for the DHFR$^-$ DG-21 cells to DHFR$^+$. As shown in Table 1, the reversion rate, which was determined by adding nine control experiments with pUC-8, was less than $1.7 \times 10^{-7}$.

Following transfection of DG-21 cells with the human DHFR minigene (Hdhfr), we found an average of 1,204 DHFR$^+$ colonies per $10^6$ cells, giving a transfection frequency of 0.12%. Since the human DHFR minigene can efficiently reconstitute DHFR gene activity in DHFR gene-deleted CHO cells, the DHFR minigene must contain most, if not all, of the DNA sequences required for DHFR gene expression. It should be noted that this transfection fre-

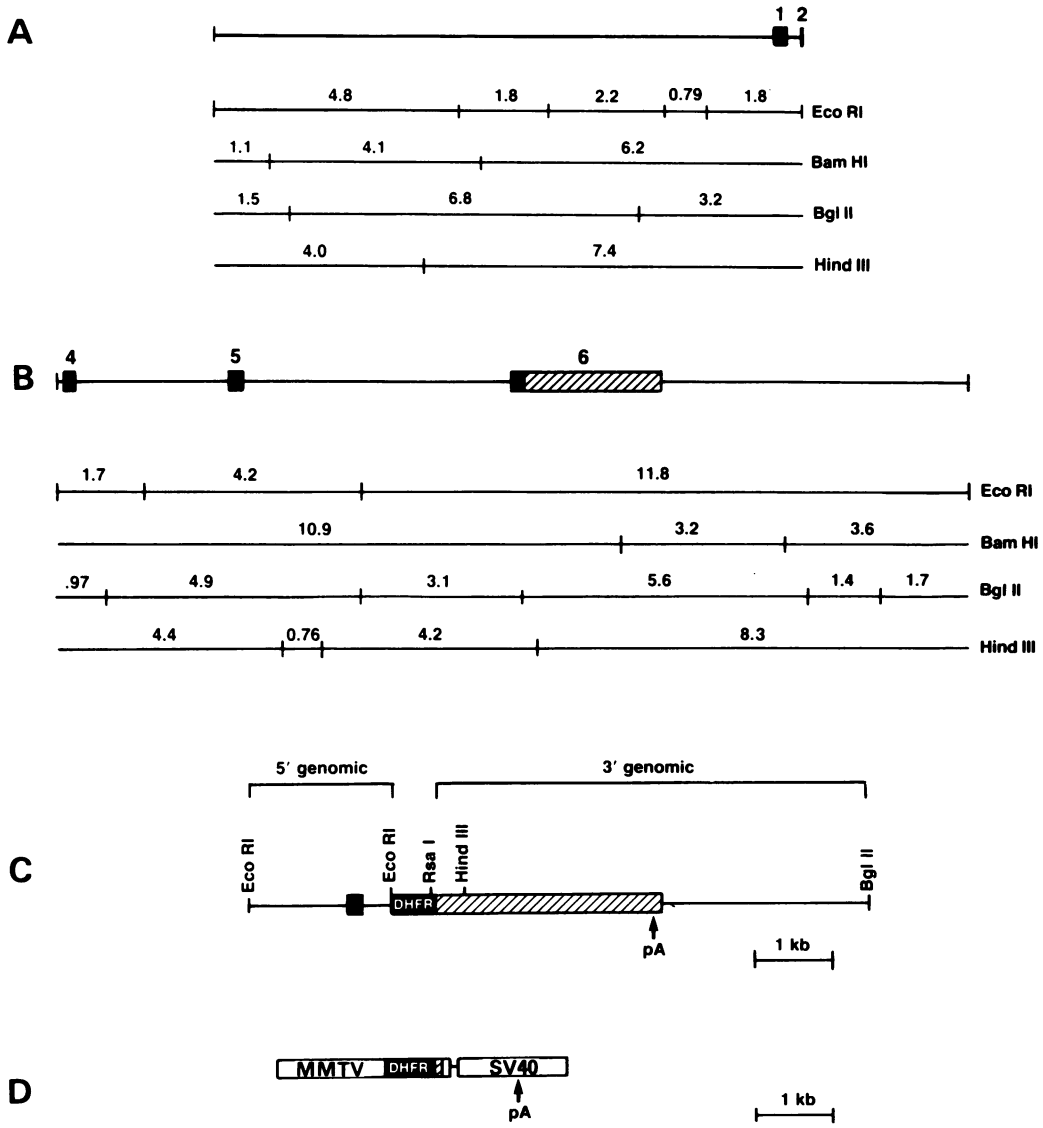


FIG. 1. Human DHFR minigene. (A) Genomic DNA from the 5′ end of the human DHFR gene used in the construction of the minigene. The top line shows the number and positions of the exons. Restriction maps of the enzyme sites used in the construction of DHFR minigenes are also shown; distances (in kilobase pairs) are indicated. (B) Genomic DNA from the 3′ end of the human DHFR gene used in the construction of the minigene. The cross-hatched area of exon 6 indicates the 3′ nontranslated region of the gene. (C) Structure of the human DHFR minigene. The thick black lines indicate translated regions, the cross-hatched line indicates the 3′ nontranslated region, and the thin lines indicates flanking and intervening sequences. The 5′ and 3′ genomic DNAs shown in panels A and B and the restriction sites used to construct the minigene are indicated. The position of the single polyadenylation site used in CHO cells is indicated by the arrow labeled pA. (D) Structure of pMTVdhfr. pMTVdhfr was constructed by Lee and co-workers from a mouse mammary tumor virus (MMTV) long terminal repeat viral promoter, mouse DHFR cDNA, and simian virus 40 (SV40) intervening and polyadenylation sequences (30). The homologous parts of pMTVdhfr and Hdhfr are the black coding regions labeled DHFR and the cross-hatched beginning of the 3′ nontranslated sequences.

TABLE 1. Frequency of conversion of DHFR$^-$ CHO cells to the DHFR$^+$ phenotype following calcium phosphate-mediated DNA transfer and selection in unsupplemented medium

| Transfecting DNA | No. of cells transfected ($\times 10^6$) | No. of experiments | No. of colonies per $10^6$ cells |
|---|---|---|---|
| Hdhfr | 4.4 | 4 | 1,204[a] |
| pMTVdhfr | 1.8 | 1 | 169 |
| Control (pUC-8) | 12.0 | 9 | <0.17[b] |

[a] The standard deviation in these experiments was ±314 colonies.
[b] Two colonies were found on a single plate. All other plates had no colonies.

quency was obtained without the addition of viral enhancer sequences to the human DHFR minigene.

**DHFR DNA, mRNA, and protein in minigene-transfected cells.** The results of a Southern transfer analysis of DNAs from three clonal lines of minigene-transfected CHO cells hybridized to a human DHFR cDNA probe are shown in Fig. 2A, lanes 4 through 6. The genomic DNA was digested with *Pst*I and *Hin*dIII, which gave an internal 1.8-kb minigene fragment that included 300 bp of 5′ flanking DNA and extended through the second polyadenylation site. A 0.7-kb CHO DNA fragment also hybridized to the human DHFR probe and was observed in DNA from nontransfected DG-21 cells (Fig. 2A, lane 2), as well as in DNA from minigene-

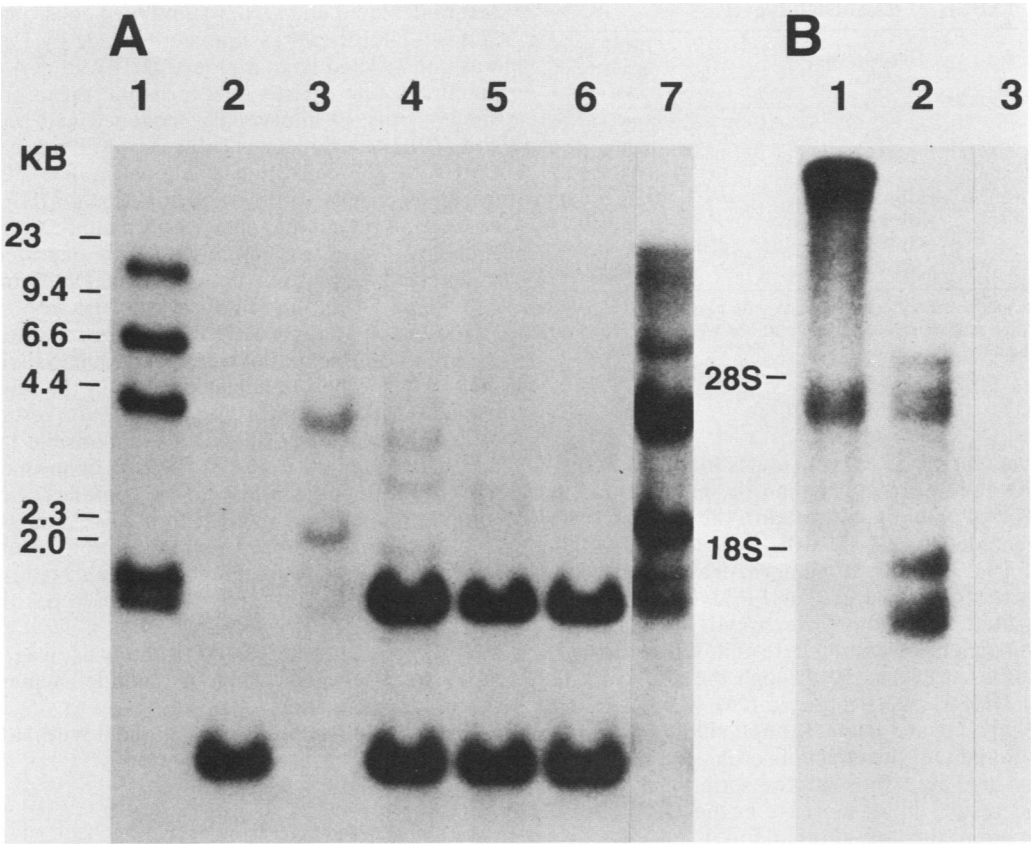


FIG. 2. Analysis of human DHFR DNAs and RNAs in minigene-transfected cells. (A) Southern blot hybridization of human DHFR DNAs in minigene-transfected cells. Various genomic DNAs were digested with restriction endonucleases, the fragments were separated on a 1% agarose gel, the DNA was blotted onto nitrocellulose paper, and the blot was hybridized to a $^{32}$P-labeled nick-translated human DHFR cDNA probe. Lane 1, Human MCF-7 DHFR gene-amplified DNA (2 μg) digested with *Eco*RI; lane 2, CHO DG-21 DNA (10 μg) digested with *Pst*I and *Hin*dIII; lane 3, human MCF-7 wild-type DNA (30 μg) digested with *Eco*RI; lanes 4 through 6, DNAs (10 μg) from clones Hdhfr-3, Hdhfr-7, and Hdhfr-6, respectively, of CHO DG-21 cells transfected with the human DHFR minigene digested with *Pst*I and *Hin*dIII; lane 7, a 5.5× exposure of lane 3. The functional DHFR gene had *Eco*RI-hybridizing fragments at 11.8, 6.6, 4.2, 1.8, and 1.7 kb which in wild-type MCF-7 DNA hybridized poorly to the labeled probe because of short coding regions. The more intense bands in wild-type MCF-7 DNA come from intronless, presumably nonfunctional DHFR pseudogenes which are found on *Eco*RI fragments that are 20, 4.0, 3.8, 2.3, and 2.0 kb long (8, 37, 47; M.E.G., unpublished data). These pseudogenes are not amplified concomitantly with the functional DHFR gene in gene-amplified cell lines. (B) Northern blot hybridization of human DHFR mRNA in minigene-transfected cells. The mRNA was isolated following disruption of the cells in guanidine isothiocyanate and centrifugation in CsCl. Polyadenylated mRNA (10 μg) was glyoxalated and separated on a 1.4% agarose gel (33). The RNA was then transferred to aminobenzyloxymethyl paper (1) and hybridized to $^{32}$P-labeled human DHFR cDNA probe pHD84. Lane 1, Human DHFR minigene-transfected CHO mRNA clone Hdhfr-3A (amplified); lane 2, human MCF-7 DHFR gene-amplified mRNA; lane 3, CHO cell line DG-21 mRNA.

transfected CHO cells. A comparison of the intensity of the DHFR minigene band in transfected cells with the intensity of the presumably single-copy 2.3-kb DHFR pseudogene (37) in wild-type MCF-7 human cells (Fig. 2A, lane 3) indicated that there were approximately 20 to 30 copies of the human DHFR minigene in the transfected CHO cells. Digestion of genomic DNA from minigene-transfected cells with other restriction enzymes indicated that the minigenes were probably integrated in tandem at one or possibly a few sites within the CHO DNA.

The polyadenylated mRNAs from the three clonal lines of minigene-transfected CHO cells, whose DNAs are shown in Fig. 2A, were studied by Northern transfer analysis. All clones contained barely detectable levels of human DHFR mRNA. The concentration of DHFR mRNA was increased by passing the cells in stepwise increasing concentrations of methotrexate, which resulted in amplification of the number of minigene copies per cell (12, 27). Gene-amplified sublines from the clonal lines of minigene-transfected cells were selected, and a Northern blot analysis of the polyadenylated mRNA from one of these cell lines is shown in Fig. 2B, lane 1. Most of the DHFR mRNA was a 3.8-kb species which corresponded to one DHFR mRNA found in human cell line MCF-7 (Fig. 2B, lane 2). In gene-amplified cell line MCF-7 there were an additional three DHFR mRNAs (1.1, 1.6, and 5.3 kb). The 3.8-kb DHFR mRNA is the predominant DHFR mRNA in most human cell lines (14, 42, 48).

Table 2 shows the basal DHFR levels, as determined by a [$^3$H]methotrexate binding assay, for several cell lines and clonal lines of minigene-transfected CHO cells. Although the levels of DHFR produced in minigene-transfected CHO cells varied from clone to clone (0.4 to 2.0 pmol/mg of protein), the enzyme levels were in the range found in wild-type human cell line MCF-7 (1.4 pmol/mg of protein). Cell line MCF-7 was the parental line for the DHFR gene-amplified cell line from which the genomic parts of the minigene were

TABLE 2. Basal DHFR levels

| Cell line | Transfected DNA | DHFR level (pmol/mg of protein)[a] |
|---|---|---|
| CHO pro-3 | | 7.32 ± 1.19 |
| MCF-7 | | 1.43 ± 0.19 |
| DG-21 | | 0.03 ± 0.02 |
| DG-21 | Hdhfr-3 | 0.38 ± 0.05 |
| DG-21 | Hdhfr-6 | 0.78 ± 0.10 |
| DG-21 | Hdhfr-7 | 2.00 ± 0.48 |
| DG-21 | pMTVdhfr-18 | 1.20 ± 0.46 |

[a] Each value is the mean ± the standard deviation for two or three samples. DHFR levels and total cellular protein concentrations were determined for each sample in duplicate.

derived. However, the basal enzyme levels in the minigene-transfected CHO cells were lower than the levels found in CHO pro-3 cells (7.0 pmol/mg of protein), the cell line from which DHFR gene-deleted DG-21 cells were derived (49).

**Regulation of DHFR levels in minigene-transfected cells.** Previous studies demonstrated that DHFR levels are altered by changes in growth conditions. The growth of cells maintained in serum-deficient medium is arrested. Upon replacement of serum, these cells move through the cell cycle in synchrony, and DHFR levels increase (26, 45, 51). Using intact cell pulse-labeling and nuclear runoff studies, Johnson and co-workers found that the effect of serum stimulation on DHFR levels is mediated through transcriptional control mechanisms and results in an increase in the DHFR transcription rate of approximately threefold (45).

The minigene-transfected CHO cells were analyzed to determine whether they respond to serum stimulation like previously studied mouse cells. First, the ability of the minigene-transfected cells to be synchronized by growth in serum-deficient medium was studied. In these experiments, cells grown in serum-deficient medium were stimulated to grow, and DNA synthesis was monitored by following [$^3$H]thymidine incorporation into DNA. The [$^3$H]thymidine incorporation curve shown in Fig. 3 indicates that serum-deprived minigene-transfected cells underwent a synchronous round of DNA replication following the addition of growth medium containing 5% serum, with maximum thymidine incorporation occurring 16 h after the medium change.

Since previous studies have shown that the DHFR levels and DNA synthesis rate increase in parallel following release from serum deprivation (51), DHFR levels were determined for several clones of minigene-transfected cells in the serum-deprived state and at 16 h following the addition of growth medium to serum-deprived cells. Table 3 shows the DHFR levels for cells in the two growth states, as well as the relative increase in DHFR concentration between the two. Our data indicated that clonal lines of CHO cells transfected with the human DHFR minigene responded to the change in serum concentration with 2.4- to 3.7-fold increases in cellular DHFR enzyme levels. These increases were similar to the threefold increase in DHFR transcription rate in response to serum which was observed by Johnson and co-workers in DHFR gene-amplified mouse cells (45). Thus, the DHFR minigene exhibits regulation by serum in a manner similar to the regulation of the endogenous cellular gene.

In contrast, cells transfected with the construct pMTVdhfr showed no difference between the cellular DHFR levels of serum-depleted and serum-stimulated cells. pMTVdhfr (Fig. 1D) is a DHFR expression vector made by Lee et al. which was constructed from a mouse DHFR cDNA with a mouse mammary tumor virus long terminal repeat promoter and a simian virus 40 intervening sequence and polyadenylation site (30). As shown in Table 1, pMTVdhfr could rescue DHFR$^-$ CHO cells, and as shown in Table 2, pMTVdhfr-transfected cells synthesized functional DHFR protein. In addition, a thymidine incorporation curve similar to the one shown in Fig. 3 was found for serum-deprived pMTVdhfr-transfected CHO cells following the addition of medium containing 5% serum (data not shown).

The finding that the DHFR levels in cells transfected with the pMTVdhfr construct were not serum inducible suggested that DHFR gene sequences present in the human minigene but not in pMTVdhfr are responsible for serum modulation of DHFR levels. The sequences responsible for this regulation may be found in the 5′ flanking or promoter, intron 1, most of the 3′ nontranslated, or 3′ flanking regions. Although serum modulation is thought to involve transcriptional control mechanisms (45), other conditions which modulate DHFR levels, such as amino acid deprivation and contact inhibition (9, 32), are thought to involve posttranscriptional mechanisms. Thus, we believed that a survey of the entire DHFR gene through deletion analysis was necessary in order to determine which nucleotide sequences mediate serum responsiveness.

Figure 4 shows the results obtained with Hdhfr Δ3′+728,

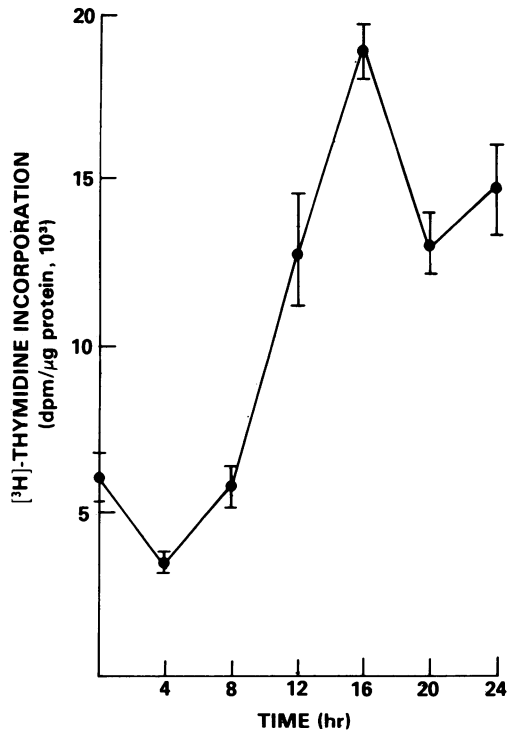


FIG. 3. [$^3$H]thymidine incorporation into serum-stimulated minigene-transfected cells. Minigene-transfected CHO cells (clone Hdhfr-6A) were grown in the presence of reduced serum and then stimulated as described in Materials and Methods. At different times, cells were incubated for 1 h with [$^3$H]thymidine, and the acid-precipitable [$^3$H]thymidine incorporated and total cellular protein concentration were determined for each sample. Each point represents the mean ± standard deviation for three samples.

TABLE 3. Serum induction of DHFR levels

| Transfected cell line | DHFR level (pmol/mg of protein)[a] | | Relative increase (fold) |
|---|---|---|---|
| | Depleted | Induced | |
| Hdhfr-3A | 0.17 | 0.56 | 3.3 (3.3 ± 0.1)[b] |
| | 0.18 | 0.58 | 3.2 |
| | 0.18 | 0.59 | 3.3 |
| Hdhfr-5A | 0.40 | 1.00 | 2.5 (2.4 ± 0.1) |
| | 0.44 | 1.06 | 2.4 |
| | 0.50 | 1.16 | 2.3 |
| Hdhfr-6A | 3.62 | 11.93 | 3.3 (3.6 ± 0.2) |
| | 1.66 | 6.25 | 3.8 |
| | 1.82 | 6.46 | 3.6 |
| Hdhfr-7A | 4.96 | 8.97 | 1.8 (3.7 ± 1.7) |
| | 1.04 | 5.11 | 4.9 |
| | 1.35 | 6.09 | 4.5 |
| pMTV-12 | 0.27 | 0.26 | 1.0 (1.0 ± 0.1) |
| | 0.56 | 0.60 | 1.1 |
| pMTV-18 | 0.85 | 0.58 | 0.7 (0.8 ± 0.2) |
| | 2.44 | 1.99 | 0.8 |
| | 0.33 | 0.36 | 1.1 |
| | 0.85 | 0.65 | 0.8 |

[a] DHFR levels were determined in cells grown in serum-depleted medium and in serum-depleted cells 16 h after the addition of medium containing 5% FCS. DHFR levels and total cellular protein concentration were calculated in duplicate.

[b] The values in parentheses are means ± standard deviations.

a construct which ends 90 bp downstream from the termination of translation and which removes most of the 3′ nontranslated region and all of the 3′ flanking region of the minigene. Clonal lines transfected with Hdhfr Δ3′+728 responded to serum stimulation with increases in DHFR protein levels (2.3- to 3.8-fold) which were essentially identical to the increases found in cells transfected with the entire minigene (2.4- to 3.7-fold). Thus, the nucleotide sequences in the 3′ nontranslated and flanking regions of the DHFR gene are not necessary for serum modulation of DHFR levels.

In order to examine whether sequences present in intron 1 could play a role in serum modulation of DHFR gene expression, we constructed a minigene (pMTVdhfr+intron) in which the intervening sequence from Hdhfr was inserted into the 3′ nontranslated region of pMTVdhfr. Like the levels in the pMTVdhfr parent construct, the DHFR protein levels in cells transfected with this minigene were not inducible by serum stimulation (1.1- ± 0.1-fold increase). This finding suggests that the sequences within the first intervening sequence by themselves are not responsible for serum stimulation.

Figure 4 also shows the serum-stimulated increases in DHFR protein levels in cells transfected with two minigene deletions with different amounts of 5′ sequences removed. One minigene, Hdhfr Δ5′-322, contained 322 bp of DNA 5′ to the start of transcription, while the other, Hdhfr Δ5′-113, contained only 113 bp. Clonal cell lines transfected with Hdhfr Δ5′-322, which eliminated 929 bp of 5′ flanking sequences from the minigene, showed serum-stimulated increases in DHFR levels (2.2- to 4.0-fold) which were similar to the increases obtained with the entire minigene (2.4- to 3.7-fold). Thus, DHFR sequences upstream from position -322 are not required for serum modulation of DHFR expression. In contrast, clones of cells transfected with Hdhfr Δ5′-113 showed much more variability in response to serum stimulation (1.3- to 8.1-fold) than previously observed. One clonal line transfected with Hdhfr Δ5′-113 was essentially uninducible, while others were hyperinducible. Each line was tested at least twice, and the inducibility of an individual transfected clone (or lack thereof) was an intrinsic property of that clonal line and did not reflect differences in experimental conditions. These findings suggest that nucleotide sequences between position -322 and position -113 may be involved in serum-modulated DHFR expression.

## DISCUSSION

In this paper we describe an expression system which was developed to study growth modulation of the human DHFR gene. A stable transfection system was used because the regulatory processes have longer induction times than the lifetimes of transient expression systems. A human DHFR minigene was constructed because the large genomic DHFR gene cannot be manipulated for detailed expression studies. In order to construct the minigene, we used a combination of DHFR genomic DNA and cDNA sequences, which eliminated four of the five intervening sequences, covering approximately 25 kb of DNA. A single intervening sequence was retained because studies with other systems have suggested that one intron may be important for mRNA stability (23, 25) and because mouse DHFR minigenes without introns had a lower transfection frequency than minigenes with introns (20). Only DHFR sequences were included in the minigene because the addition of foreign DNA in regions other than the promoter appears to influence DHFR expression (28).

Our studies demonstrated that human DHFR minigene sequences can efficiently reconstitute DHFR gene activity in

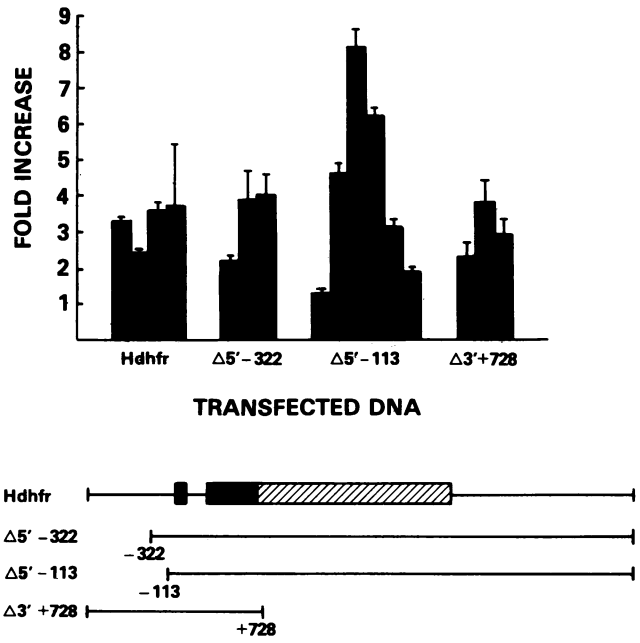


FIG. 4. Increase in DHFR levels following release from serum deprivation. The fold increases in DHFR protein levels (see Table 3) are plotted as a bar graph. Each bar represents the results obtained from a clonal line of transfected cells and indicates the mean ± standard deviation for, general, three samples, each done in duplicate. Schematic representations of Hdhfr (see Fig. 1C) and minigene deletion constructs, which are described in Materials and Methods, are shown at the bottom.

DHFR-deficient cells without the addition of viral enhancer sequences. Although a high transfection frequency is one measure of DHFR gene activity, it gives little information on whether the gene is functioning like the normal cellular gene. Therefore, several parameters of DHFR minigene-transfected cells were examined. First, the human DHFR mRNA transcribed in the transfected cells was analyzed. In contrast to the multiple DHFR mRNA species found in human cell lines (14, 42, 48), only a single mRNA is found in minigene-transfected CHO cells. This 3.8-kb human DHFR mRNA found in transfected CHO cells corresponds in size to one of the human DHFR mRNAs. This size similarity suggests, but does not prove, that most of the DHFR mRNA in transfected cells has been correctly initiated and terminated. Although the amount of DHFR protein produced in the transfected cells varies among independently isolated clones, the level of enzyme produced by the transfected cells is within the same range as the level produced by wild-type MCF-7 cells. However, since each transfected cell contains multiple (20 to 30) integrated minigene copies, the minigenes apparently do not function as efficiently as the endogenous DHFR gene. A previous study also found no direct correlation between the number of mouse DHFR minigene copies and the level of DHFR protein produced (12).

The experiments described in this report demonstrated that human DHFR minigene-transfected cells increase their DHFR levels in response to serum stimulation in a manner similar to the previously studied mouse cells (45). Thus, the human DHFR minigene apparently contains nucleotide sequences which are responsive to serum modulation. Several minigene deletion constructs were used to determine which DHFR gene sequences are responsible for the serum-modulated expression of this gene. An analysis of cells transfected with two of these constructs showed that neither deletion of the 3′ end of the minigene nor transfer of the intron into pMTVdhfr has an effect on serum-stimulated DHFR expression. Thus, neither the 3′ nontranslated and flanking sequences of the DHFR gene nor the first intervening sequence acting alone appears to be involved in this regulation. An analysis of cells transfected with a 5′ minigene deletion mutant showed that nucleotide sequences upstream from 322 bp 5′ to the start of transcription apparently have no effect on serum modulation. However, removal of the nucleotide sequences between positions -322 and -113 causes cells transfected with this minigene to show highly variable responses to serum stimulation. The most plausible explanation for the hypervariability in serum-modulated DHFR expression is that the removal of the 5′ sequences allows the DHFR minigene to come under the influence of its chromosomal location. All DNA sequences governing serum modulation were not removed since some clonal cell lines transfected with this minigene are still serum inducible. Additional minigene deletion constructs will be required to map more precisely which DNA sequences are involved in serum-modulated DHFR expression.

The DHFR promoter, like other promoters, has a complex structure. The gene does not have the classic transcription initiation sequences in proximity to the 5′ ends of the major mRNAs (7, 13, 39). In mice there are 3.5 to 4 copies of a 48-bp repeated DNA sequence immediately 5′ to the start of transcription (13, 39). The significance of these repeated sequences is not clear, but transcription occurs bidirectionally from these DNA sequences (11, 17). In addition, each of these repeating units contains a nucleotide sequence which corresponds to the Sp1 transcription factor binding site (16). In contrast, the human DHFR gene contains only 1 to 1.5 copies of the homologous repeated DNA sequence (7, 52). These repeated sequences which are probably critical for DHFR regulation remain intact in Hdhfr Δ5′-113. Other workers have found electrophoretically variant nucleosomes associated with the first exon of the transcriptionally active mouse DHFR gene and have shown that these variants decrease 10-fold within two nucleosomes upstream and downstream of this region (3). Although the role of the variant nucleosomes in DHFR expression is unknown, it is interesting that Hdhfr Δ5′-113 removes DNA in this region of the DHFR gene.

In this paper we describe a DHFR gene expression system which duplicates many functions of the endogenous gene. Most importantly, the DHFR levels in minigene-transfected cells are responsive to serum modulation. An analysis of cells transfected with minigene deletion mutants supported previous cellular studies which indicated that serum-modulated DHFR expression is transcriptionally regulated. The DHFR expression system described in this paper should be useful in further dissecting the regulation of the DHFR gene.

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