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Sequences Downstream of the Transcription Initiation Site Modulate the Activity of the Murine Dihydrofolate Reductase Promoter

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The murine dihydrofolate reductase gene is regulated by a bidirectional promoter that lacks a TATA box. To identify the DNA sequences required for dihydrofolate reductase transcription, the activities of various templates were determined by in vitro transcription analysis. Our data indicate that sequences both upstream and downstream of the transcription initiation site modulate the activity of the dihydrofolate reductase promoter. We have focused on two regions downstream of the transcription initiation site that are important in determining the overall efficiency of the promoter. Region 1, which included exon 1 and part of intron 1, could stimulate transcription when placed in either orientation in the normal downstream position and when inserted upstream of the transcription start site. This region could also stimulate transcription in *trans* when the enhancer was physically separate from the promoter. Deletion of region 2, spanning 46 nucleotides of the 5′ untranslated region, reduced transcriptional activity by fivefold. DNase I footprinting reactions identified protein-binding sites in both downstream stimulatory regions. Protein bound to two sites in region 1, both of which contain an inverted CCAAT box. The protein-binding site in the 5′ untranslated region has extensive homology to binding sites in promoters that both lack (simian virus 40 late) and contain (adenovirus type 2 major late promoter and c-*myc*) TATA boxes.

Dihydrofolate reductase (DHFR) is a housekeeping enzyme necessary for the de novo synthesis of glycine, purines, and thymidylate. The murine DHFR gene is cell cycle regulated at the transcriptional level, resulting in a transient increase in the transcription rate at the G_1/S boundary (16). The promoter region of the DHFR gene does not contain a TATA box, which is commonly used as a RNA polymerase II transcription initiation signal (23). Instead, the region directly upstream of the transcription initiation site consists of four copies of a 48-base-pair (bp) repeat, each of which contains a GC box that binds the transcription factor Sp1 (14). Six additional GC boxes, located 500 to 650 nucleotides upstream of the transcription initiation site, regulate transcriptional efficiency from an upstream promoter oriented in the direction opposite that of DHFR mRNA (32). The DHFR promoter is unusual in that it operates bidirectionally in vivo, producing DHFR mRNAs as well as a series of RNAs transcribed in the direction opposite that of the DHFR gene (10, 15).

We want to understand what DNA sequences and protein factors are responsible for regulation of the DHFR gene. Because the level of cellular DHFR mRNA is very low, it would be difficult to measure accurately the transcriptional efficiency of DHFR promoter deletions in vivo. With stable transfection systems, the chromosomal location of the transfected DNA is not controllable and can influence transcriptional activity. The replication origins in transient replicating vectors contain associated enhancer regions that can influence transcriptional activity. Thus, we have developed an in vitro transcription system for the DHFR promoter and have defined the minimal promoter region required for accurate transcription of the DHFR gene (18, 25). However, these studies did not quantitate the influence of flanking sequences on the efficiency of DHFR transcription. To define the sequences that contribute to the transcriptional efficiency of the DHFR promoter, we have now created a series of 5′ and 3′ promoter deletions and tested their transcriptional activities in nuclear extracts prepared from both human and mouse cells. We report here that regions both upstream and downstream of the transcription initiation site contribute to the efficiency of transcription from the DHFR promoter.

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MATERIALS AND METHODS

Plasmid constructions. The numbering of the DHFR promoter region has been changed from that used in previously published papers such that the transcriptional start site is +1. Construction of the murine DHFR subclones pR34 and pDSA7 (11), pSS625 (16), pRT10- (17), and pdpro19 and pdpro18 (23) has been described previously. Other plasmids containing regions of the murine DHFR gene were constructed as follows. pDRH1380 was created by insertion of an *Eco*RI (−955)-*Hpa*I (+429) fragment from pR34 into the *Eco*RI and *Sma*I sites of pBSM13+ (Stratagene). pST410 was created by insertion of a *Sma*I (−356)-*Taq*I (+61) fragment from pDSA7 into the *Sma*I and *Acc*I sites of pUC9. pBSpro19 and pBSpro18 contain sequences from −87 to +52 and −50 to +52, respectively, and were created by insertion of the *Eco*RI-to-*Xba*I fragments from pdpro19 and pdpro18 into the *Eco*RI and *Xba*I sites of pBSM13+. pBSpro19(ex+) and pBSpro19(ex−) were created by inserting a *Taq*I (+61 to +275) fragment from pSS625 into the *Acc*I site of pBSpro19 in either orientation. pBS(ex+)pro19 and pBS(ex−)pro19 were created by inserting the same *Taq*I fragment into the *Eco*RI site of pBSpro19 in either orientation. pDFX120, containing the DHFR sequences from −65 to +52, was created by insertion of a *Fok*I-to-*Xba*I fragment from pBSpro19 into pBSM13−. pDMM285 was created by insertion of a *Mae*I (−270 to +15) fragment from pSS625 into the *Sma*I site of pUC9. pSR320 was created by insertion of a *Pvu*II-*Rsa*I fragment (which includes DHFR sequences from −356 to −30 as well as vector DNA) from pSS625 into the *Sma*I site of pUC9. pHH381 was created by inserting a

DHFR *Hpa*II (−208 to +153) fragment from pSS625 into the *Sma*I site of pBSM13+.

Preparation of nuclear extracts. HeLa cells were grown in alpha-modified Eagle medium, 5% supplemented calf serum (Hyclone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml in Spinner flasks at 37°C and harvested during logarithmic growth (2×10^5 to 5×10^5 cells per ml). The cells were frozen (4) and stored at −70°C if nuclear extract was not prepared on the same day as the cell harvest. Nuclear extracts were prepared as previously described (13) except that buffers were made with Tris chloride (pH 7.9 at 4°C).

Nuclear extracts were also prepared from murine F9 teratocarcinoma cells, grown in Dulbecco modified Eagle medium with high glucose, 10% dialyzed fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in 10% CO_2. The cells were grown initially on gelatin-coated plates (Difco gelatin, 0.1%), transferred to Spinner flasks, and grown in suspension. The cells were harvested for nuclear extract at approximately 2×10^6 cells per ml, which was still in the logarithmic growth phase.

In vitro transcriptions. The plasmids and restriction enzymes used to obtain the templates were as follows. pDRH1380 was digested with *Not*I and *Hin*dIII to obtain the −258/+429 template. pDMM285 was digested with *Pvu*II to obtain the −270/+15 template. pRT10- was digested with *Eco*RI and *Sph*I to obtain the −955/+61 template. pST410 was digested with *Pvu*II to obtain the −356/+61 template and with *Not*I and *Pvu*II to obtain the −258/+61 template. pSS625, pHH381, pBSpro19, pDFX120, and pBSpro18 were digested with *Pvu*II to obtain the −356/+275, −208/+153, −87/+52, −65/+52, and −50/+52 templates, respectively. pSR320 was digested with *Hae*II to obtain the −356/−30 template. pBSpro19(ex+), pBSpro19(ex−), pBS(ex+)pro19, and pBS(ex−)pro19 were digested with *Pvu*II to obtain template fragments that included DHFR sequences from −87 to +275, arranged as shown in Fig. 2. The templates were purified from polyacrylamide gels by electroelution (21) before use in the transcription reactions. The purified fragments were phenol extracted, ethanol precipitated, suspended in water, and quantitated by staining with ethidium bromide.

The standard in vitro transcription reaction contains 60 μg of nuclear protein and 5 nM of template DNA in a 25-μl reaction containing 6 mM $MgCl_2$, 25 μM GTP, 10 μCi of [α-^{32}P]GTP (410 Ci/mmol), 600 μM CTP, 600 μM UTP, 200 μM ATP, 24 mM Tris (pH 7.9 at 4°C), 12% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, 0.3 mM dithiothreitol, and 0.12 mM phenylmethylsulfonyl fluoride. All components except the nucleotide triphosphates were preincubated for 15 min at 24°C; then the nucleotide triphosphates were added, and the reaction was allowed to proceed for 15 min at 24°C. In experiments designed to test fragments for the ability to influence transcription from the DHFR promoter (see Fig. 3), the fragments were added to the reaction mixture 5 min before the promoter DNA. Other modifications of the standard conditions are noted in the text. Reactions were terminated by the addition of 75 μl of stop solution (0.1 M sodium acetate [pH 5.2], 0.4% [wt/vol] sodium dodecyl sulfate, 200 μg of protein-free *Escherichia coli* tRNA per ml) and then extracted with 100 μl of phenol. The phenol phase was reextracted with 100 μl of stop solution, the aqueous layers were combined, and the RNA was precipitated by the addition of 600 μl of ethanol. Samples were suspended in 10 μl of deionized formamide with tracking dyes and loaded onto 5% polyacrylamide–8 M urea gels. Products from the reactions were sized by comparison with molecular size markers.

DNA at 5 nM (which is in the linear range of the transcriptional response for all templates) was used to determine promoter activity. Because of the difference in size of the shortest (461 bp) and longest (1,472 bp) templates, the amount of DNA added ranged from 40 to 126 ng per reaction. To determine whether this difference in DNA concentration could influence the transcriptional activity, duplicate reactions were performed with and without the addition of a 400-bp vector fragment to bring the final DNA concentration to 126 ng per reaction. No differences in transcriptional activity were seen in the two types of reactions. This result confirmed our previous work, which had shown that the concentration of DHFR promoter-containing fragments, not the amount of DNA in the reaction, was important in determining transcriptional activity (18). The autoradiographs obtained after exposing the transcription gels to Kodak XAR-5 film were scanned with a LKB soft laser densitometer, and the peaks were integrated by using a Numonics Corp. 1224 electronic digitizer. The results were normalized to the number of guanines in each runoff transcript and presented as a percentage of the activity of the template that contained the most extensive DHFR sequences in each series of 5′ or 3′ deletions. Reactions were performed three to six times, and the activities determined from all experiments were averaged to give the values reported.

DNase I protection. The 635-bp *Hin*dIII-*Pst*I fragment of pSS625 was labeled on the coding strand with [α-^{32}P]dATP by the Klenow fragment of DNA polymerase (21). A 200-mg sample of nuclear extract was passed over a 15-ml heparin-Sepharose (Pharmacia) column. Protein eluting at 0.4 M KCl from the heparin-Sepharose column was added to a reaction (after dialysis to bring the KCl to 0.1 M) containing 1 ng of ^{32}P-labeled DNA, 1 μg of poly(dI-dC) · poly(dI-dC), 24 mM Tris (pH 7.4), 12% (vol/vol) glycerol, 60 mM KCl, 1.2 mM EDTA, 0.3 mM dithiothreitol, and 6 mM $MgCl_2$ in a total volume of 20 μl. Reactions were incubated for 10 min at 24°C. DNase I (0.1 μg) was then added, and the samples were incubated at 24°C for 60 s. The reactions were immediately terminated by addition of 4 μl of 0.25 M EDTA–1% sodium dodecyl sulfate (wt/vol), diluted to 75 μl with water, phenol extracted, and ethanol precipitated. Electrophoresis was carried out on an 8 M urea–6% polyacrylamide gel.

RESULTS

We have previously shown that the minimal DHFR sequences that are absolutely required for transcription in vitro extend from −65 to +15 relative to the transcription initiation site at +1 (25). However, these studies did not quantitate the relative transcriptional activity of the different promoter mutations. We have now used optimal conditions for transcription from the DHFR promoter (18) to compare the transcriptional activities of templates that extend different lengths 5′ and 3′ of the DHFR transcription initiation site. Our standard in vitro transcription assay monitors runoff transcription from a linear DNA template. A representative experiment comparing the transcriptional activities of 12 different templates is shown in Fig. 1A. The activities of many of the templates were also compared by primer extension from transcripts produced from circular templates. There was no difference in the relative activity of the templates in circular versus linear forms (data not shown).

Deletion analysis of the 5′-flanking region of the DHFR

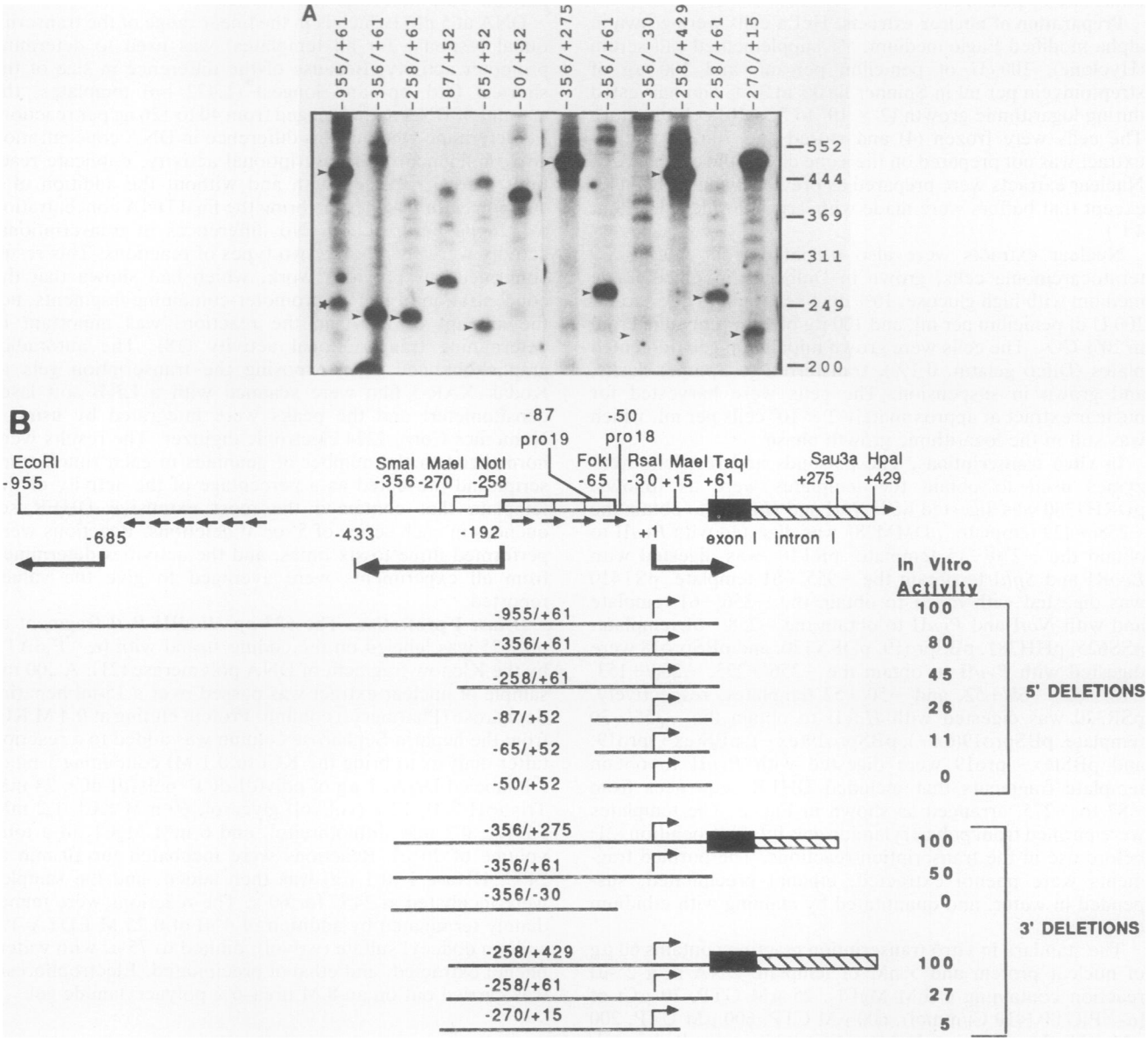


FIG. 1. Transcriptional activity of DHFR promoter deletions. (A) Runoff transcription reactions using HeLa nuclear extract, performed on 12 different templates. The 5′ and 3′ endpoints of the murine DHFR sequences are indicated above each lane. The runoff product initiating at the +1 start site is indicated by the arrowhead in each lane. Since the templates have different extents of vector DNA flanking the DHFR sequences, the sizes of the runoff products vary, and templates with the same DHFR 3′ sequences do not necessarily produce the same-size runoff RNA. The asterisk in the −955/+61 lane indicates runoff transcription from the newly identified −685 site site (32). The large transcripts in the −50/+52 and −270/+15 lanes are due to high levels of end-to-end transcription of the template DNA by RNA polymerase and are produced because most of the DHFR promoter elements have been deleted from these two templates. Positions of molecular size markers (in base pairs) are shown at the right. (B) The portions of various DHFR templates that correspond to murine DHFR genomic DNA. The templates contain additional vector DNA on one or both ends (not shown). The restriction sites used to construct the different templates are indicated on the schematic of the DHFR promoter region and are numbered relative to +1 as the transcription initiation site of the DHFR gene. The −87 and −50 5′ endpoints were created by BAL 31 deletions (23). The location of the major in vivo DHFR transcription initiation site (31) is indicated by the large rightward arrow at +1. The opposite-strand transcripts arising from the bidirectional DHFR promoter activity are indicated by the leftward arrow at −192. A newly identified transcription unit (32) is indicated by the leftward arrow at −685. The coding portion of exon 1 (■), part of intron 1 (▧), and tandem 48-bp repeats (□) are shown. The small arrows directly below the line represent the locations and orientations of 10 consensus GC boxes. Although previous sequence analysis had suggested that another GC box was present near the *Sma*I site at −356, more recent sequence analysis indicates that a consensus GC box is not present in this region. Three nucleotides were added to the sequence near the *Sma*I site, changing the positions of restriction sites referred to in previous publications. The in vitro transcriptional activity of each construct relative to the longest template in each series is indicated on the right. The numbers were obtained by averaging the results of three to six experiments. No reproducible differences were seen in the activities of the templates in human HeLa versus murine F9 nuclear extracts.

gene. We first examined a series of 5′ deleted templates (Fig. 1). A gradual reduction in template activity was observed when increasing extents of the 5′ region were removed. Examination of the sequences removed in these 5′ deletions suggests that the six upstream GC boxes can be removed without significantly affecting template activity, since the −356/+61 template showed 80% activity. However, a template ending at −258 exhibited only 45% activity, about twofold less than that of the template ending at −356. The −356 to −258 region contains a sequence (TTTCTCGC) that is similar to a binding site for the transcription factor E2F located upstream of the c-*myc* P2 promoter (33). The −87/+52 template displayed only 26% activity, about twofold less than that of the −258/+61 template. Deletion of the −258 to −87 region removed a sequence (TTTCCCGC) that is identical to the E2F-binding site upstream of the c-*myc* promoter and also removed three of the four 48-bp repeats, each of which contains an Sp1-binding site. The significance of the E2F-binding sites is not clear because E2F activity is not normally detectable in HeLa nuclear extracts (29). The −65/+52 template showed only 11% activity, which was about twofold less than that of the −87/+52 template. This deletion removed an A-rich sequence adjacent to the remaining Sp1 site. Methidiumpropyl-EDTA footprinting on the DHFR promoter using partially purified Sp1 has shown a small region of protection over these A's in addition to protection of the GC box (19). Optimal Sp1 binding to the adjacent GC box may require this A-rich sequence just upstream. As we have shown previously (18), transcription is completely eliminated with the deletion of the proximal GC box, demonstrating that at least one Sp1-binding site is absolutely required. These results correspond well to the results of deletion studies of the hamster DHFR gene that found that the 5′ limit of the hamster promoter was 48 bp 5′ of the major transcription start site (8).

Deletion of sequences 3′ to the transcription initiation site. We next assayed two series of 3′ deleted promoter templates, with each member of a series having a similar 5′ end (Fig. 1). We found that two regions downstream of the transcription initiation site influenced the efficiency of transcription. First, a two- to fourfold decrease in transcriptional activity was observed when exon 1-intron 1 sequences were removed (compare −356/+275 with −356/+61 and −258/+429 with −258/+61). These results demonstrated that sequences within exon 1-intron 1 are important for optimal activity from the DHFR promoter. Second, deletion of 5′ untranslated sequences from +61 to +15 reduced the transcriptional activity to about 5% of the activity of the longest template having a similar 5′ end (compare −258/+61 with −270/+15). This result indicated that sequences between 15 and 61 bp downstream of the transcription initiation site are critical for optimal activity. As we have shown previously, a more extensive 3′ deletion that removes the transcription initiation site (−356 to −30) does not produce transcripts initiating the correct distance downstream of the proximal GC box, even though all four 48-bp repeats are retained (25).

Stimulation of transcription by the exon 1-intron 1 fragment. Deletion of the exon 1-intron 1 region resulted in a two- to fourfold reduction in the transcriptional activity of the DHFR promoter (Fig. 1). To determine whether this fragment possessed typical enhancer activity, we examined the transcriptional activity of a shortened promoter region before and after insertion of this fragment in either orientation both upstream and downstream of the transcription initiation site. First, the +61/+275 fragment was inserted 8 bp downstream of the 3′ end of the −87/+52 template,

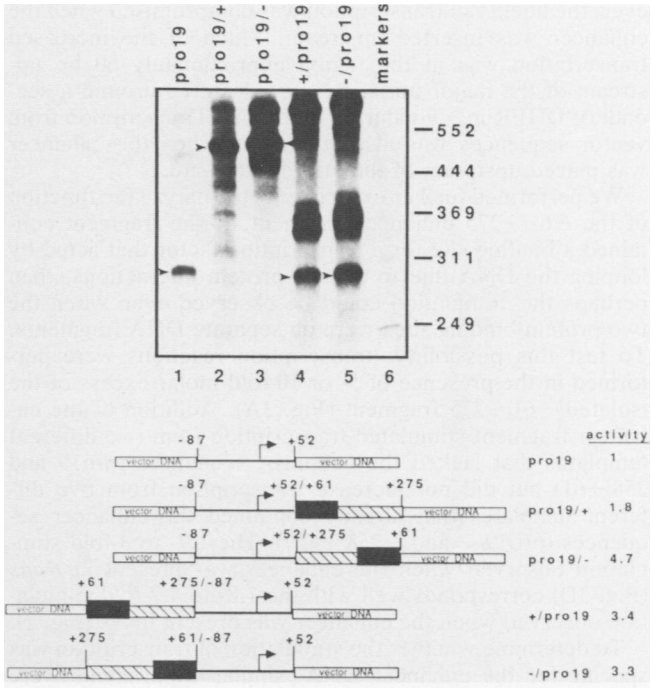


FIG. 2. Enhancer activity of the +61/+275 fragment. In vitro transcription reactions were performed on templates containing the −87/+52 sequences only (pro19) or with the +61/+275 fragment inserted in either orientation both upstream and downstream of the transcription initiation site. Symbols on the diagram are as in Fig. 1. The activity reported (relative to that of the pro19 template) is an average of five experiments, using three different sets of isolated fragments. The ranges of values obtained for promoter activities were as follows: pro19/+, 1.6 to 2.2; pro19/−, 2.6 to 5.9; +/pro19, 2.5 to 4.5; and −/pro19, 2.6 to 4. Arrowheads on the autoradiograph indicate transcripts initiating at the +1 start site. Positions of molecular size markers (in base pairs) are indicated at the right. The transcripts in lanes 4 and 5 that are near the 369-bp marker arose from initiations clustered around the secondary in vivo start site.

correctly positioning the +61/+275 fragment with respect to the transcription initiation site (Fig. 2). Comparison of the transcriptional activity of the −87/+52 template (pro19) with that of the new template (pro19/+) revealed that transcriptional activity was increased 1.8-fold after reinsertion of the exon 1-intron 1 sequences. This result corresponds well with the twofold reduction in transcription seen when the +61/+275 fragment was deleted from the template having a 5′ end of −356 (Fig. 1). Interestingly, insertion of the +61/+275 fragment downstream of the start site but in the opposite orientation (pro19/−) resulted in a 4.7-fold increase in transcriptional activity as compared with the −87/+52 template. This orientation-independent stimulation was suggestive of enhancer activity. Therefore, we tested whether the exon 1-intron 1 fragment could also confer transcriptional stimulatory activity in a position-independent manner. The same +61/+275 fragment was inserted upstream of the −87/+52 template in both orientations. Comparison of the transcriptional activity of these two templates with that of the −87/+52 template shows that the fragment stimulated transcription by 3.4-fold when placed upstream of the transcription initiation site in the 5′-to-3′ orientation (+/pro19) and by 3.3-fold when inserted upstream in the 3′-to-5′ orientation (−/pro19). Thus, the exon 1-intron 1 fragment functioned as an enhancer when present in either orientation both upstream and downstream of the transcription start site. How-

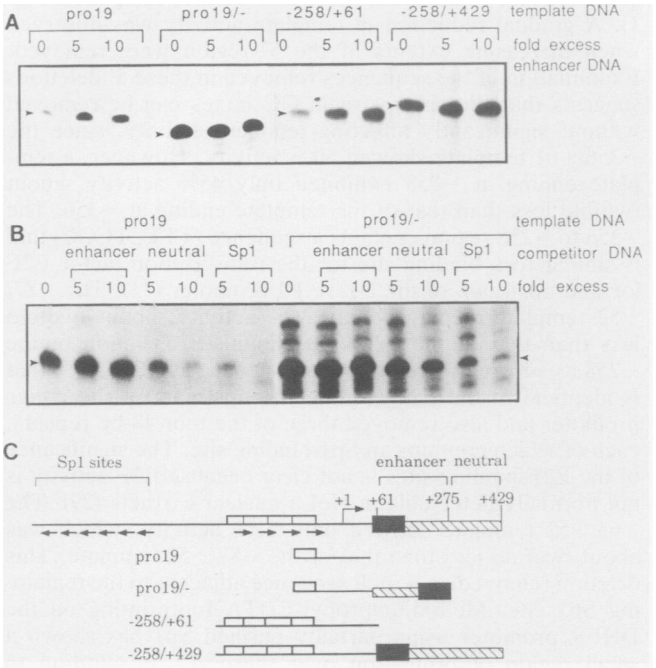
ever, the fidelity of transcription was compromised when the enhancer was inserted upstream. Much of the increased transcription was in the region approximately 60 bp upstream of the major initiation site, clustered around a secondary DHFR in vivo start site (23, 31). Transcription from vector sequences was also stimulated when the enhancer was placed upstream of the DHFR start site.

We performed further experiments to analyze the function of the +61/+275 enhancer fragment. If the fragment contained a binding site for a transcription factor that acted by looping the DNA due to protein-protein interactions, then perhaps the stimulation could be observed even when the two protein-binding sites were on separate DNA fragments. To test this possibility, transcription reactions were performed in the presence of 5- or 10-fold molar excess of the isolated +61/+275 fragment (Fig. 3A). Addition of the enhancer fragment stimulated transcription from two different templates that lacked the enhancer sequences (pro19 and 258/+61) but did not increase transcription from two different templates that already contained the enhancer sequences (pro19/− and −258/+429). The 1.7- to 3-fold stimulation observed when the enhancer was present in *trans* (Fig. 3D) corresponds well with the 1.8- to 4.7-fold stimulation observed when the enhancer was present in *cis* (Fig. 2).

To determine whether the stimulation of transcription was specific for the enhancer DNA, similar experiments were performed by using a fragment that should have no effect on DHFR transcriptional activity. The fragment containing intron sequences from +275 to +429 was chosen because deletion of this fragment did not change the transcriptional activity from the DHFR promoter (Fig. 1 and data not shown). A fragment containing six Sp1 sites was also tested, since it should compete for Sp1 binding and severely reduce transcription from the DHFR promoter. Addition of a fivefold excess of a neutral fragment slightly reduced transcriptional activity from both templates (Fig. 4B). This slight reduction in transcriptional activity was also seen when vector fragments were added to the reaction (data not shown) and probably represents nonspecific inhibition due to competition for general factors such as RNA polymerase. Tenfold excess of a neutral fragment reduced transcription to a greater extent. As expected, addition of the fragment containing Sp1 sites severely reduced transcription at 5-fold excess and almost eliminated it at 10-fold excess. Therefore, the only fragment that stimulated transcription in *trans* was the enhancer fragment. Addition of the enhancer resulted in 1.7- to 3-fold increase in transcription as compared with the activity of the template alone. However, when compared with a reaction in which the same amount of a neutral fragment was added, transcription in the presence of the enhancer was increased 4- to 13-fold. Very little stimulation was seen from templates that already contained the enhancer sequences in *cis*. These results were consistent with the idea that the stimulatory effects of the exon 1-intron 1 fragment are transduced via protein-protein interactions.

To define more precisely the region of the exon 1-intron 1 fragment that was responsible for the stimulatory activity, another 3′ promoter deletion was tested. A template ending at +153 was compared with the templates having 3′ ends at +429 and +61. This new template contained all of exon 1 but only 12 bp of intron 1. The template ending at +153 had full activity (Fig. 4). Thus, the stimulatory sequences are between +61 and +153.

Identification of protein-binding sites. Our deletion experiments indicated that sequences in both the 5′ untranslated region and exon 1 were important for transcriptional activ-



D

template DNA	competitor DNA	5 fold excess a	5 fold excess b	10 fold excess a	10 fold excess b
pro19	none	1.00	-	1.00	-
	neutral	0.43	1.00	0.13	1.00
	enhancer	1.70#	3.95	1.70	13.0
	Sp1 sites	0.08	0.19	0	0
pro19/-	none	1.00	-	1.00	-
	neutral	0.59	1.00	0.29	1.00
	enhancer	0.93	1.57	0.73	2.50
	Sp1 sites	0.14	0.24	0.03	0.10
-258/+61	none	1.00	-	1.00	-
	enhancer	1.80*	-	2.98	-
-258/+429	none	1.00	-	1.00	-
	enhancer	1.13	-	1.09	-

FIG. 3. Stimulation of transcription by the +61/+275 fragment when present in *trans*. (A) 0-, 5-, and 10-fold molar excesses of the +61/+275 fragment over the concentrations of four different promoter templates were added to in vitro transcription reactions. The templates are indicated above the lanes and diagrammed in panel C. Run-off transcripts were sized by comparison with molecular size markers (not shown). (B) 0-, 5-, and 10-fold molar excesses (as compared with the promoter template DNAs) of the +61/+275 enhancer fragment, a +275/+429 neutral fragment, or a fragment containing six Sp1 sites were added to reactions containing a template that lacks the enhancer (pro19) or a template that contains the enhancer (pro19/−). The templates are diagrammed in panel C. Arrowheads indicate transcripts initiating at the +1 start site. The autoradiogram was overexposed to show the weak signals. A lighter exposure was used for quantitation (reported in panel D). (C) Templates used in panels A and B. Symbols are as in Fig. 1B. Locations of the enhancer, neutral, and Sp1-containing fragments are indicated. (D) Transcriptional activities from the different templates after addition of 5- or 10-fold excess of each fragment relative to the activity of the template with no excess DNA (column a) or to the activity of the template in the presence of the neutral fragment (column b). #, Average of two experiments (1.65 and 1.7) using different DNA preparations; *, average of two experiments (1.6 and 2) using different DNA preparations.

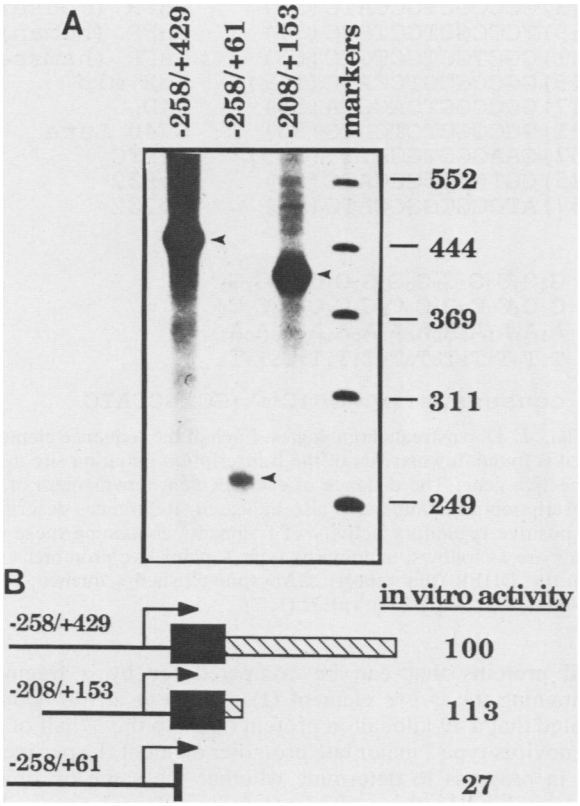


FIG. 4. Delimitation of the enhancer region. In vitro transcription reactions were performed on templates having similar 5' ends but differing in the extent of exon and intron sequences. Arrowheads indicate runoff transcription from the DHFR initiation site. Relative activities of the templates are averages of three experiments. The ranges of values obtained were as follows: −208/+153, 95 to 130; and −258/+61, 27 to 33.

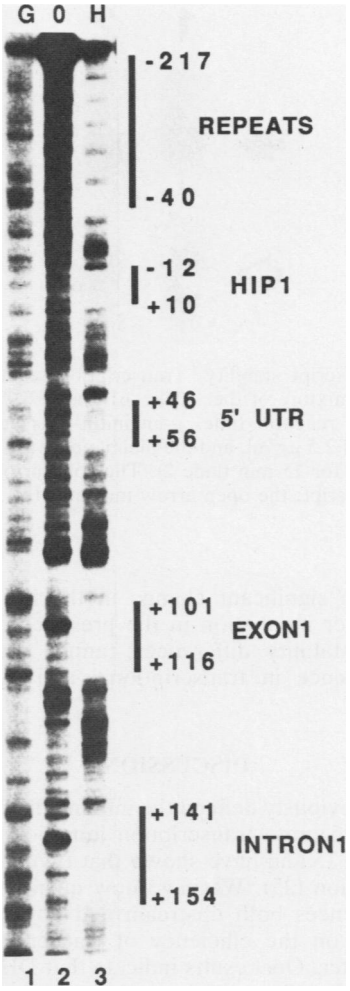


FIG. 5. Protein-binding sites downstream of the DHFR initiation site. The coding strand of pSS625 was assayed for DNase I cleavage in the absence (lane 2) and presence (lane 3) of 15 μg of protein eluting from a heparin-Sepharose column at 0.4 M KCl. Lane 1 shows the position of G nucleotides (22). Nucleotides protected from DNase I cleavage are indicated on the right. Proteins binding to the repeats and to the initiation site (HIP1) have been described previously (14, 25).

ity. To determine whether these regions contained binding sites for transcription factors, DNase I protection assays were performed on DNA fragments extending from −356 to +275 (Fig. 5). The protein preparation used in these DNase I footprint experiments was obtained by passing HeLa nuclear extract over a heparin-Sepharose column. The DHFR transcriptional activity eluted at 0.4 M KCl; 60 μg of this fraction produced the same levels of DHFR transcription as did 60 μg of the crude nuclear extract (A. L. Means, unpublished results). When the 0.4 M KCl fraction was used in DNase I footprint reactions, protected regions could be seen at −217 to −40, −12 to +10, +46 to +56, +101 to +116, and +141 to +154. Previous studies have shown that purified Sp1 binds to the repeats (14) and that a protein (HIP1) binds to the initiation site (25). We have now detected three protein-binding sites in the downstream sequences that confer stimulatory activity. The protein binding at +46 to +56 may be responsible for the fivefold reduction in transcription when the region from +15 to +61 is deleted. The protected regions from +101 to +116 and +141 to +154 both contain similar sequences, and it is likely that the same protein binds to both sites (see Discussion). The protein binding to these sites may be responsible for the enhancer activity in the +61 to +153 region.

Transcript stability. It was possible that differences in transcriptional activities of templates with downstream deletions could arise from differential stability of transcripts due to removal of a RNA stabilization signal. Therefore, we also examined transcript stability in the transcription mixture. DHFR downstream sequences have been deleted in templates missing the +15 to +275 region. However, the +61/+275 element increased transcription when present in an orientation- and position-independent manner and when present in *trans*; thus, the +61/+275 element is not a transcript stabilization sequence. To examine the effects of deletion of the +15 to +61 region, the stabilities of two transcripts that are identical except for the +15 to +61 region were compared. We used 10 nM −270/+15 template and 2 nM −258/+61 template so that approximately equal signals would be detected from each template. The two templates were added to a single reaction mixture to control for different recovery of the transcripts. After the standard transcription reaction, α-amanitin was added and incubation continued for an additional 15 min (Fig. 6). Since α-amanitin blocks RNA polymerase II-directed transcription, the stability of the previously synthesized RNA could be examined.

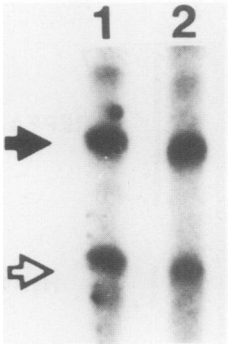


FIG. 6. Transcript stability. Transcription reactions were performed with a mixture of the −258/+61 and −270/+15 templates. After a 15-min reaction time, α-amanitin was added to a final concentration of 2.5 μg/ml, and the incubation was terminated (lane 1) or continued for 15 min (lane 2). The solid arrow indicates the −258/+61 transcript; the open arrow indicates the −270/+15 transcript.

There was no significant change in the ratio of the two transcripts after incubation in the presence of α-amanitin. Thus, RNA stability differences cannot account for the fivefold difference in transcriptional activity of the two templates.

DISCUSSION

We have previously defined the minimal DHFR sequences required for accurate transcription initiation as the region from −65 to +15 and have shown that two proteins bind in this 80-bp region (25). We have now quantitated the influence of sequences both upstream and downstream of the initiation site on the efficiency of transcription from the DHFR promoter. Our results indicate that DHFR transcriptional activity is influenced not only by sequences 5′ to the proximal GC box but also by sequences within the 5′ untranslated region and by sequences downstream of the translation start codon. We have shown that deletions that remove transcribed sequences do not influence transcript stability. Thus, although these downstream regulatory sequences identified by our deletion analysis are located in regions that are not normally thought to be required for transcriptional regulation, they do appear to modulate promoter efficiency.

The downstream sequences involved in transcriptional regulation of DHFR include the 5′ untranslated region and the +61 to +153 region. Deletion of 5′ untranslated sequences from +15 to +61 resulted in a fivefold reduction of transcriptional activity. DNaseI footprint analysis of this region indicates that a protein binds near the 3′ end of this sequence, spanning nucleotides +46 to +56. This region of the 5′ untranslated sequences is conserved among the mouse (23), hamster (26), and human (5) DHFR genes. However, no experiments examining the importance of this downstream region in the hamster or human DHFR genes have been reported. Inspection of the sequence of this region has revealed homologies to elements downstream of the following promoters (Fig. 7): adenovirus type 2 major late promoter, simian virus 40 (SV40) late, c-*myc*, carbamoyl phosphate synthetase-aspartate transcarbamoylase-dihydroorotase (CAD), and rpL32. In all cases (except for the CAD promoter), these elements have been shown to contain positive modulators of transcription. The rpL32 elements

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 (43)CCCCGCTGCCATC(55)     DHFR (mouse)
 (59)TCCCGCTGCTGTC(71)     DHFR (human)
 (51)CGCTGCTGCTGTC(63)     DHFR (hamster)
(149)GGCCGCGTCCATC(161)    Ad2 mlp
 (47)CCCCGCTCAGCGA(59)     CAD
 (21)GCCGGCTGTCACG(33)     SV40 Late
(367)GAAGGCTGGATTT(379)    C-MYC
 (25)GGTGGCTGCCATC(37)     rpL32
 (57)ATCGGCGGCCATC(69)     rpL32

G4G3G0G4G9G0G2G7G1G1G2G1G1
C3C4C7C4C0C9C0C1C6C5C1C1C6
A1A1A1A0A0A0A0A0A1A1A5A0A1
T1T0T1T1T0T0T7T1T1T2T1T7T1

consensus: (g/c) (g/c) C (g/c) GCTGCCATC
```

FIG. 7. Downstream homologies. Each of the sequence elements listed is found downstream of the transcription initiation site in the respective gene. The distance of each element downstream of the transcription initiation site is also indicated. References describing the positive regulatory activity of fragments containing these elements are as follows: adenovirus type 2 major late promoter (Ad2 mlp) (9); DHFR (this report); CAD (unpublished sequence, PJF); SV40 late (2); c-*myc* (34); rpL32 (1, 7).

bind proteins that can be competed for by a fragment containing the c-*myc* element (1). Cohen et al. (9) demonstrated that a 40-kilodalton protein binds to the 3′ half of the adenovirus type 2 major late promoter element. Experiments are in progress to determine whether the same or similar proteins bind to these elements from different genes. The SV40 late promoter is very similar to the DHFR promoter. In addition to the downstream homologies, the DHFR and SV40 late transcription initiation sites have extensive homology, and the same (or similar) protein binds to both sites (25). Preliminary data suggest that protein binding at the initiation site of DHFR is influenced by protein binding at the +46 to +56 site (A. L. Means, unpublished data). In the DHFR promoter, the protected regions are separated by only 36 bp, suggesting that direct protein-protein interactions are possible.

We have also shown that the +61 to +153 region contains a positive modulator element and two protein-binding sites. The mechanism by which protein binding to DNA distant from the initiation site influences transcription is a central question in the study of gene expression. The DNA looping hypothesis invokes protein-protein interactions that bring activating regions of distally bound proteins nearer to the initiation site and therefore nearer to RNA polymerase (28). Proteins bound to DNA both upstream and downstream of the transcription initiation site could interact in this model. To address the mechanism of transcriptional enhancement by the +61 to +153 region, we constructed templates that differed only in the position and orientation of an exon 1-intron 1 fragment. The exon 1-intron 1 fragment stimulated transcription when present in either orientation when placed upstream or downstream of the transcription initiation site. Therefore, this fragment has orientation- and position-independent enhancer activity. Transcription start sites upstream of the major in vivo start site (near a minor in vivo start site and in vector sequences) were utilized when the enhancer was inserted upstream of the promoter. The enhancer may be located in a downstream position in the wild-type DHFR gene to ensure a greater fidelity of transcription initiation as well as to increase transcriptional efficiency. It may thus behave aberrantly when placed in an

upstream location. The +61/+275 fragment also stimulated transcription when present in *trans*, preferentially from templates that lacked the enhancer. This observation suggests that the +61/+275 fragment may contain a binding site for a positive-acting factor that interacts cooperatively with a protein binding at another site in the DHFR promoter. This model would require that protein-protein interactions occur even when the binding sites lie on two different DNA fragments, similar to the results reported for the enhancer activity of the DNA-binding zeste protein that regulates the *Drosophila white* gene (3). While this manuscript was in preparation, similar evidence for protein-protein interactions was obtained by Müller et al. (27), demonstrating that an enhancer for SV40 or cytomegalovirus can stimulate in vitro transcription in *trans*. However, the enhancer was noncovalently linked to the promoter via the protein streptavidin or avidin. Müller et al. (27) believe that their results support a model for enhancer action that involves looping of the DNA, although they cannot rule out the possibility that a factor scanning along the DNA could jump over an obstacle such as avidin. Our experiments more directly support the looping model, since the enhancer and promoter were on physically separate DNA fragments. Although we cannot rule out all other possibilities (such as the stimulation in transcription being due to the sequestration of an inhibitory DNA-binding factor), we believe that our data demonstrating the enhancer activity of exon 1, the increase in minor starts when the enhancer is upstream of the promoter, and the stimulation of transcription in *trans* suggest that the stimulatory effect is due to protein-protein interactions. The differences in stimulatory activity in the different orientations and positions may be due to structural differences in the sequences in the DNA loop. For instance, the region upstream of the initiation site is extremely G+C rich. DNA sequences with a high G+C content are reported to be less flexible than other DNA (20). Because the *trans* stimulation does not require DNA bending, one might assume that the maximum stimulation would be observed in these reactions. However, the fact that the binding sites are on two separate pieces of DNA likely reduced the efficiency of the protein-protein interactions.

Similar influences on transcriptional activity by downstream elements located close to transcription initiation sites have been reported for other promoters. Several of these elements have been tested for enhancer activity. Deletion of an element in the first intron of the rpL32 gene reduces transcription to 10 to 20% of maximum efficiency. Unlike the exon 1 fragment of DHFR, the intron element of rpL32 did not stimulate transcription when inserted in the opposite orientation at the correct position, nor could it be moved upstream of the initiation site (7). A segment of the first exon of the c-*myc* gene has also been shown to function as a positive modulator, but only in its sense orientation and 3′ of a nearby promoter (34). An element located downstream of the bovine leukemia virus long terminal repeat functions as a positive modulator in both orientations, but only downstream of the initiation site (12).

We have shown that protein binds in exon 1-intron 1 in the regions from +101 to +116 and from +141 to +154. These two binding sites contain similar sequences that are +101(GGATTGGC)+108 and +141(GGTATTGGC)+149, suggesting that the same protein binds to the two sites. Interestingly, these sites contain inverted CCAAT elements. Although this element has been shown to function in an inverted orientation (24), it is usually found 50 to 100 bp upstream of a transcription initiation site. Many different proteins exist that bind to CCAAT elements (6, 30). Further work is required to determine whether any of the previously identified CCAAT-box-binding proteins regulate the DHFR gene or whether yet another member of the gene family is responsible for the enhancer activity.

We have shown that the DHFR promoter is composed of several elements, each of which contributes to transcriptional activity in nuclear extracts prepared from logarithmically growing cells. Having now identified the regions that modulate transcriptional activity, we can examine protein binding to these sites in extracts prepared from cell cycle stage-specific cells. We hope that results from these experiments will further our understanding of the mechanisms by which DHFR is transcriptionally regulated through the cell cycle.

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