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The HIP1 Binding Site Is Required for Growth Regulation of the Dihydrofolate Reductase Gene Promoter

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The transcription rate of the dihydrofolate reductase (DHFR) gene increases at the G_1/S boundary of the proliferative cell cycle. Through analysis of transiently and stably transfected NIH 3T3 cells, we have now demonstrated that DHFR promoter sequences extending from −270 to +20 are sufficient to confer similar regulation on a reporter gene. Mutation of a protein binding site that spans sequences from −16 to +11 in the DHFR promoter resulted in loss of the transcriptional increase at the G_1/S boundary. Purification of an activity from HeLa nuclear extract that binds to this region enriched for a 180-kDa polypeptide (HIP1). Using this HIP1 preparation, we have identified specific positions within the binding site that are critical for efficient protein-DNA interactions. An analysis of association and dissociation rates suggests that bound HIP1 protein can exchange rapidly with free protein. This rapid exchange may facilitate the burst of transcriptional activity from the DHFR promoter at the G_1/S boundary.

Cellular proliferation is controlled by a programmed series of events termed the cell cycle. Neoplasia can result when quiescent or differentiated cells escape cell cycle control and begin inappropriate proliferation. Therefore, understanding the checks and balances used by the cell to control entrance into S phase of the cell cycle is important in understanding the loss of proliferation control that leads to neoplasia. One widely used model system to study cell cycle progression is the reentry of quiescent cells into the proliferative cell cycle elicited by an increase in the level of serum growth factors. Murine 3T3 fibroblasts withdraw from the proliferative cell cycle into a quiescent G_0 state when the concentration of serum in the culture medium is low. The addition of high concentrations of serum to these quiescent cells initiates a series of events that results in the transduction of extracellular signals to the cell nucleus, with a concomitant change in the expression of growth-responsive genes. The first genes that are activated by the serum growth factors are termed immediate-early-response genes, many of which are transcription factors. Several hours later, at the transition from G_1 to S phase, late-response genes are activated, followed by the onset of DNA synthesis and the activation of histone gene expression. Among the late-response genes are the genes for DNA polymerase alpha, thymidine kinase, thymidylate synthase, carbamoyl phosphate synthase-aspartate carbamoyltransferase-dihydroorotase, and dihydrofolate reductase (DHFR). Many of these genes are involved in nucleotide biosynthesis; for example, the DHFR gene encodes an enzyme involved in the production of glycine, purines, and thymidylate.

Although much is known about the *cis* elements and *trans*-acting factors that are responsible for the activation of the early-response genes (see reference 32 for a review), few studies have focused on the activation of the late-response genes. Recently, several groups have analyzed the ability of the promoter regions of late-response genes to confer regulation on heterologous reporter genes. The promoter of the DNA polymerase alpha gene can confer growth regulation on a heterologous reporter gene (30); however, no single region could be identified that was responsible for this regulation. In contrast, the murine thymidylate synthase gene requires promoter elements as well as sequences in the coding region for proper regulation (22). Both the human (17) and murine (15) thymidine kinase promoters have been shown to confer growth regulation on heterologous genes. A specific element important for the growth regulation of the human thymidine kinase promoter is an inverted CCAAT box located between −64 and −83 (17, 24, 31). Cellular factors bind to this element, but the binding activity is similar in G_1-, S-, and G_2-phase extracts (17). CCAAT box binding factors are members of a multigene family (34), and it is not known whether the human thymidine kinase promoter is regulated by a novel or a previously characterized member of this family. Dou et al. (11) identified a site (CCCNCNNNCT) important for the growth regulation of the murine thymidine kinase promoter. Protein binding to this site increases as cells enter S phase; however, the protein that binds to this site has not been further characterized. Thus, progress has been made in identifying the *cis* elements that regulate several late-response genes, but little is known about the proteins that are involved.

The goal of our work is to understand the mechanisms by which the increase in DHFR expression at the G_1/S boundary is achieved. Results from two different synchronization techniques, mitotic selection (14) and serum stimulation of quiescent cells (33), demonstrated that DHFR expression is regulated at the transcriptional level. However, neither of these previous studies identified the elements responsible for the transcriptional increase at the G_1/S boundary, nor did they determine whether the promoter region is sufficient for the growth regulation. The regulatory regions of the murine DHFR promoter (26) have been extensively characterized by using an in vitro transcription system based on HeLa nuclear extract (13). Transcription analysis identified four important regulatory regions in the DHFR promoter, each of which binds protein. One region is 5′ of the initiation site and includes four 48-bp repeats extending from −40 to −217.

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Each repeat binds the transcription factor Sp1 and an unidentified protein. Protein also binds over the transcription initiation site (−16 to +11), in the 5′ untranslated region (+46 to +56), and near the exon 1/intron 1 boundary at two inverted CCAAT boxes (+101 to +116 and +141 to +154). The previous studies demonstrating that DHFR is regulated by a transcriptional increase at the G_1/S boundary and a thorough characterization of the promoter region provide the background for our current studies. We have now demonstrated that a DHFR promoter construct extending from −270 to +20 is sufficient for growth-regulated expression. Further, through mutational analysis, we have identified a site that is necessary for this growth regulation and have purified and characterized a protein, HIP1, that binds to this site.

MATERIALS AND METHODS

Plasmids. pMaeWTDHFR and pMaeNWDHFR contain sequences from −270 to +20 of the murine DHFR promoter cloned into the *Bam*HI site of pUC19. The plasmids differ in that the wild-type DHFR sequence from −11 to +11 (ATT TCGCGCCAAACTTGACGGC) in pMaeWTDHFR has been mutated to GCCCTATATCAAATCCAGTAAT in pMaeNWDHFR. *Sma*I-*Hin*dIII fragments from pMaeWTD HFR and pMaeNWDHFR were isolated, ligated to *Hin*dIII linkers, and inserted into pAAlucA at the *Hin*dIII site located at +30 in the luciferase cDNA (16) to create pWTluc and pNWluc. pEJluc and pOluc are identical to pWTluc except that specific bases have been mutated in the DHFR initiation region (see Fig. 5B for details). p636 was used to select for hygromycin B resistance (37). CH110 (Pharmacia) contains the simian virus 40 (SV40) early promoter driving the bacterial *lacZ* gene. pSVL contains the SV40 early promoter driving the luciferase cDNA (9). pST410 contains DHFR promoter sequences from −356 to +66 (28).

Transient transfections. NIH 3T3 fibroblasts were maintained in 5% CO_2 at 37°C in maintenance medium consisting of Dulbecco-Vogt's modified Eagle's medium (GIBCO) supplemented with 5% defined-supplemented bovine calf serum (HyClone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (GIBCO). Cells were passaged when subconfluent, by using 0.05% trypsin–EDTA (GIBCO). Calcium phosphate transfections were performed as described previously (25), with the following modifications. At 12 to 16 h before transfection, 2×10^5 cells were plated per 60-mm^2 dish; 20 μg of DNA (10 μg of promoter/luciferase construct, 1 to 2 μg of control CH110 DNA, and 8 to 9 μg of sonicated salmon sperm DNA) was precipitated in 450 μl of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM dextrose, 21 mM HEPES [pH 7.05])–50 μl of 1.25 M $CaCl_2$ for 30 min at room temperature. The calcium phosphate precipitate was added to the culture medium, and the cells were incubated for 8 h. The cells were rinsed with maintenance medium, treated for 4 min with 1 ml of 15% (vol/vol) glycerol in HEPES buffer, and allowed to recover for 1 h in maintenance medium. The medium was then changed to starvation medium (0.5% [vol/vol] serum) for 50 to 60 h and subsequently replaced with stimulation medium (10% [vol/vol] serum). Cells were harvested at the indicated time points. Two different DNA preparations of each construct were tested.

Stably transfected cell lines. NIH 3T3 cells were transfected with 18 μg of pWTluc or pNWluc plus 2 μg of p636, which confers hygromycin B resistance, as described above. One day after transfection, the cells were transferred to a 150-mm^2 dish containing 50 ml of maintenance medium. The following day, hygromycin B (Boehringer Mannheim) was added to the medium at 0.4 mg/ml. The medium was changed every third day until cell colonies could be seen. The colonies were harvested separately with 0.025% trypsin in phosphate-buffered saline (PBS), transferred to 12-well plates, and expanded as required. Hygromycin B was again added to the medium after the cells had been expanded from the 12-well plates. Seven pWTluc and twelve pNWluc clones were obtained. All clones expressed the luciferase cDNA. However, the luciferase activity between clones varied by more than 1,000-fold, probably because of the different sites of integration of the constructs into chromosomal DNA. Clones expressing various luciferase activities for both pWTluc and pNWluc were chosen for analysis. For the time course experiments, cells from three pWTluc and three pNWluc clones were plated at 2×10^5 cells per 60-mm^2 dish in maintenance medium. After the cells attached to the plates (about 1 h), the medium was changed to starvation medium for 50 to 60 h. The medium was then replaced with stimulation medium, and the cells were harvested at the given time intervals.

Reporter gene assays. Cells were rinsed twice with PBS, scraped from the plates in PBS containing 1 mM EDTA, and pelleted for 4 min at 4°C in a microfuge at 12,000 rpm. The cell pellets were resuspended in 100 μl of luciferase lysis buffer (Promega Corp.), and 10 to 20 μl was assayed by using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) with 100 μl of luciferin substrate (Promega Corp.). Transfection efficiencies were approximately 4%, as determined by in situ β-galactosidase assays (23). β-Galactosidase activity from an SV40 early promoter was measured in each extract from transiently transfected cells to control for large fluctuations in transfection efficiency (23).

Flow cytometric analysis. To monitor the progression of cells through the cell cycle, their DNA content was measured by flow cytometric analysis. Cells were rinsed with PBS, harvested with 0.05% trypsin–EDTA, and pelleted for 7 min at 1,500 rpm. The cells were resuspended in PBS, recentrifuged, fixed in 70% (vol/vol) ice-cold ethanol, and incubated on ice for at least 30 min. Immediately before analysis, the cells were pelleted by centrifugation at 1,500 rpm for 7 min and resuspended at 10^6 cells per ml in PBS containing propidium iodide (50 mg/ml) and RNase A (1 mg/ml). An Epics Profile II flow cytometer (Coulter Corp.) was used to generate histograms of cell number versus DNA content. G_0/G_1 cells contain 2N DNA content and therefore fluoresce half as much as do G_2/M cells, which have 4N DNA content. S phase is designated as the cells between the G_0/G_1 peak and the G_2/M peak.

Column preparation. Ten grams of heparin-Sepharose CL-6B (Pharmacia LKB) was swelled and washed as instructed by the manufacturer to yield a 30-ml column volume. After each purification, the heparin-Sepharose column was regenerated by washing with D* buffer (100 mM KCl, 40 mM Tris-HCl [pH 7.4], 6 mM $MgCl_2$, 20% [vol/vol] glycerol, 2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). An anti-Ku antibody column was prepared by using antibody N3H10, a mouse monoclonal immunoglobulin G2a (IgG2a) specific for the smaller subunit of Ku (18). IgG was partially purified from mouse ascites fluid by precipitation with 45% ammonium sulfate, dialyzed against 40 mM morpholinepropanesulfonic acid (MOPS; pH 8.0)–75 mM NaCl, and coupled to Affi-Gel-15 according to the manufacturer's instructions. About 2.3 mg of IgG was

coupled per ml of gel, as judged by Bradford protein assay. After each purification, the 2-ml Ku antibody column was eluted in D* buffer containing 2 M potassium isothiocyanate and regenerated with D* buffer. The third column was made by coupling sonicated salmon sperm DNA in 10 mM HEPES (pH 8.0) to an Affi-Gel-10 matrix (Bio-Rad) as instructed by the manufacturer for a DNA concentration of 1 mg/ml of column matrix. After each purification, the salmon sperm DNA column was eluted with D* buffer containing 1.0 M KCl and regenerated with D* buffer. The final column was a HIP1 DNA affinity column. The oligonucleotides 5'-AAT TCATTTCGCGCCAAACTTGACG-3' and 5'-AATTCGT CAAGTTTGGCGCGAAATG-3' were annealed and ligated to produce concatemers averaging 125 bp in length. These were then coupled to Affi-Gel-15 (Bio-Rad) at a concentration of 1 mg of DNA per ml of column matrix according to the manufacturer's instructions. After each purification, the column was regenerated by washing with D* buffer.

HIP1 purification. HeLa cells were grown in Joklik's modified minimal essential medium (GIBCO) with 5% (vol/vol) defined calf serum (HyClone) or in minimal essential medium alpha (GIBCO) plus 5% (vol/vol) fetal bovine serum (GIBCO) in suspension at 37°C. Cells were harvested at a density of 2×10^5 to 4×10^5 cells per ml and were frozen as described by Borelli et al. (3). Nuclear extract was made as described previously (10) except that buffer D contained Tris-HCl instead of HEPES. Nuclear protein (250 mg; from approximately 2×10^{10} cells) was loaded onto a 30-ml heparin-Sepharose column. The column was washed with 2 column volumes of D* buffer and then eluted sequentially with D* buffer containing 0.25, 0.4, and 1.0 M KCl. The fraction eluting at 0.4 M KCl was dialyzed to 0.1 M KCl and loaded onto the anti-Ku antibody column. This column was required to remove the large quantity of Ku protein present in the nuclear extract. If Ku protein was not removed, it competed for binding of HIP1 to the DNA affinity column. The flowthrough from the anti-Ku antibody column was passed through a 5-ml double-stranded salmon sperm DNA column. The flowthrough from the salmon sperm DNA column was loaded onto the HIP1 DNA affinity column. The column was washed with 2 column volumes of D* buffer and subsequently eluted first with D* buffer containing 0.4 M KCl and then with D* buffer containing 1.0 M KCl (these elution buffers also contained 0.1% Nonidet P-40). An equal amount of HIP1 activity was collected by reapplying the flowthrough from the DNA affinity column. The two eluates from the HIP1 DNA affinity column were diluted to 0.1 M KCl and passed over the column twice more to remove nonspecific DNA-binding proteins. The fractions were dialyzed to 0.1 M KCl and stored at −70°C.

DNase I protection assay. DNase I protection was performed as previously described (28) except that the amount of nonspecific competitor varied according to the purity of the protein fraction. Poly(dA-dT) · poly(dA-dT) was used at 3 μg for crude nuclear extract and 5 to 10 ng for purified protein. The reaction mixtures contained 100 μg of nuclear protein or 20 ng of purified HIP1, 1 ng of ^{32}P-labelled DNA probe, 24 mM Tris-HCl (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. Reaction mixtures were incubated for 10 min at room temperature. DNase I (0.25 to 2 μg) was then added, and the samples were incubated for 60 s. The reactions were terminated by addition of 4 μl of 0.25 M EDTA–1% (wt/vol) sodium dodecyl sulfate, diluted to 75 μl, phenol extracted, and ethanol precipitated. Electrophoresis was carried out on an 8 M urea–6% polyacrylamide gel. The DNA probe was the *Hin*dIII-*Bgl*I fragment of pST410 containing DHFR sequences from −206 to +65. The coding strand was labelled at the *Hin*dIII site with the large fragment of DNA polymerase I and [α-^{32}P]dATP as described previously (25).

Southwestern (DNA-protein) blotting. Fifty nanograms of purified HIP1 was electrophoresed through a 5 to 15% gradient polyacrylamide gel and blotted to nitrocellulose. The nitrocellulose was washed for 1 h in 60% (vol/vol) D* buffer and 5% (wt/vol) nonfat dry milk. The nitrocellulose was then probed with 50 ng of ^{32}P-labelled concatemerized oligonucleotides (identical to the oligonucleotides used for column chromatography) in 60% (vol/vol) D* buffer in a volume of 1 ml for 2 h. After 3 washes of 20 min each with 60% (vol/vol) D* buffer, the nitrocellulose was dried and exposed to film.

Mutagenesis of the HIP1 site. Mutations in the DHFR initiation site were obtained by using a Bio-Rad Mutagene in vitro mutagenesis kit (19). The *Eco*RI-*Hin*dIII fragment from pST410 was cloned into M13mp19. Phage was grown in *Escherichia coli* CJ236, a *dut ung* strain. Single-stranded DNA corresponding to the noncoding strand of the DHFR gene was isolated and annealed to the different oligonucleotides. For random mutagenesis, the oligonucleotide 5'-GATTTCGCGCCAAACTTGAC-3' was synthesized with 1.7% of each of the three incorrect nucleotides at each position, and the oligonucleotide 5'-GGTACACAGCTC AGGGCTGCGATTTCGCGCCAAACTTGACG-3' was synthesized with 2.5% of each of the three incorrect nucleotides at the underlined positions. Mutant O and mutant P (see Fig. 5B) were obtained by using the oligonucleotides 5'-CGATT TCGCGCCAAGTCCAGTAATAATCCTAGCG-3' and 5'-CAGGGCTGCGGCCCTATATCAAATCCAGTAATAATC CTA-3'. The oligonucleotides were used as primers for synthesis of a second strand of DNA. The double-stranded products were transformed into *E. coli* XL-1 Blue, and DNAs from the resulting plaques were sequenced to identify mutations. The *Eco*RI-*Hin*dIII fragments were recloned into pBSM13- (Stratagene).

Gel mobility shift assays. HIP1 was bound to DNA by incubating 5 ng of purified HIP1 with 5 ng of poly(dA-dT) · poly(dA-dT) and 1 ng of radiolabeled probe in 60 mM KCl–24 mM Tris-HCl (pH 7.4)–6 mM $MgCl_2$–5% Ficoll–0.12 mM EDTA–0.3 mM dithiothreitol for 5 min at room temperature. When competitions were performed, the competitor DNA was incubated with all reagents except the probe for 5 min, at which point the probe was added and the incubation was continued for 5 min. Modifications to this procedure are described in the figure legends. The probe was a 81-bp *Fsp*I-*Hin*dIII fragment of pMaeWTDHFR that spans the DHFR initiation site. The fragment was labelled at the *Hin*dIII site with [α-^{32}P]dCTP by using the large fragment of DNA polymerase I (25). The reaction mixtures were electrophoresed for 60 to 90 min at 180 V on a 4% polyacrylamide gel which had been preelectrophoresed for 60 min at 180 V. The gel buffer was 0.25× TBE (25).

RESULTS

The DHFR promoter can confer late growth response regulation on a heterologous gene. The first step in understanding the mechanisms by which the DHFR gene is transcriptionally regulated in response to changes in cell growth was to determine whether the DHFR promoter could confer regulation on a heterologous reporter gene. Sequences of the DHFR promoter from −270 to +20 (+1 is the

transcription initiation site) were cloned upstream of a luciferase cDNA to create plasmid pWTluc. This region of the DHFR promoter includes the four 48-bp repeats upstream of the initiation site as well as a protein binding site at the transcription initiation site. Transfection of proliferating 3T3 cells with pWTluc resulted in levels of luciferase activity that were 20-fold higher than the levels obtained after transfection of the promoterless luciferase vector DNA (data not shown). The ability of the −270 to +20 promoter fragment to confer growth regulation on the luciferase reporter gene was then examined by using serum starvation and stimulation. One hour after transfection of 3T3 cells with pWTluc, the cells were placed in starvation medium for 50 to 60 h. During this time, the cells entered quiescence, as monitored by flow cytometric analysis of their DNA content (Fig. 1B). The starvation medium was replaced with medium containing 10% serum, and samples were harvested every 2 h for analysis of both DNA content (Fig. 1B) and luciferase activity (Fig. 1A). Analysis of DNA content showed that the cells entered S phase approximately 10 h after release from serum starvation. Luciferase activity increased at 10 h and peaked between 12 and 14 h after serum stimulation. The peak of luciferase activity averaged 14-fold higher than the activity in starved cells. Transcriptional activity of the endogenous DHFR promoter in 3T6 cells increases at the G_1/S boundary 7-fold after mitotic selection (14) and 4- to 14-fold after serum stimulation (33). Thus, the increase in luciferase activity at the G_1/S boundary mirrored the increase in transcription of the endogenous DHFR gene and demonstrated that DHFR promoter sequences from −270 to +20 are sufficient to confer late growth response regulation on a heterologous reporter gene. Luciferase levels produced from an SV40 early promoter/luciferase cDNA construct were also examined after serum stimulation (Fig. 1A). Luciferase activity from this construct showed little variation through the cell cycle, demonstrating that the S-phase increase in luciferase activity was specific to the DHFR promoter and was not due to sequences in the reporter mRNA or protein.

Mutation of the HIP1 binding site eliminates DHFR growth regulation. Although the pWTluc construct was sufficient for growth regulation, it was not clear which DNA sequences were required for the regulation and which were required for basal transcription. The DHFR promoter sequences in pWTluc include four Sp1 binding sites (12) and a binding site (spanning the DHFR initiation site) for a protein called HIP1 (28). The Sp1 binding sites in the 48-bp repeats were unlikely candidates for growth regulation since Sp1 activates many promoters, such as the SV40 early promoter, that are not growth regulated (Fig. 1A). We therefore focused on the HIP1 protein binding site (28). A construct identical to pWTluc was made except that 19 of the 22 nucleotides of the HIP1 binding site were altered (mutant P in Fig. 5B). The CAA initiation site was left intact because these nucleotides are required for efficient transcription initiation in vitro (27). pNWluc therefore contains DHFR promoter sequence from −270 to +20 with a mutant HIP1 binding site at −11 to −3 and +2 to +11. This mutant site will no longer compete for binding to the wild-type sequence (see Fig. 5B).

We first determined the effects of this mutation on the strength of the promoter in exponentially growing 3T3 cells. In four separate transfection experiments, the luciferase activity from pNWluc averaged 113% of that from pWTluc. Next we determined the effects of the HIP1 site mutation on the growth regulation of the DHFR promoter in transient transfection assays (Fig. 1A). Mutation of the HIP1 site

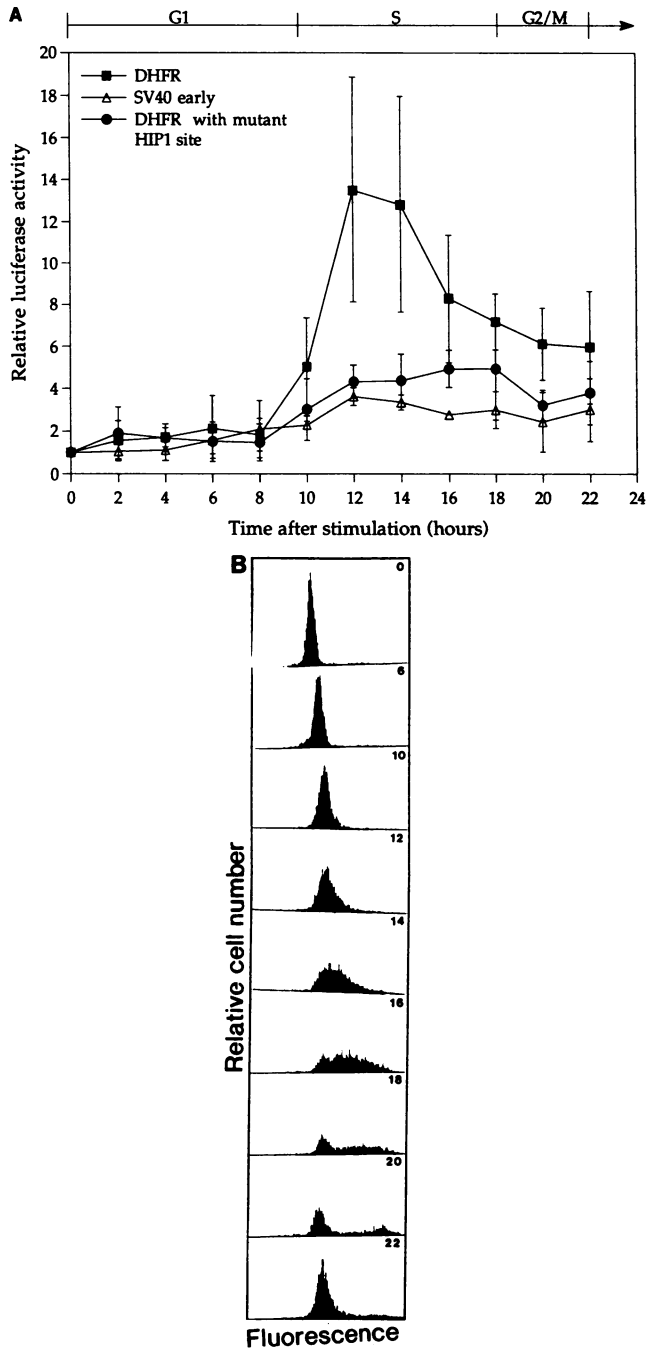

FIG. 1. Ability of the DHFR promoter to confer growth regulation on a heterologous gene in a transient transfection assay. 3T3 cells were transfected with constructs containing either the wild-type DHFR promoter, a mutant DHFR promoter, or the SV40 early promoter driving the luciferase cDNA. Cells were harvested for luciferase assays (A) and determination of DNA content (B) every 2 h after serum stimulation. Luciferase values are reported relative to the activity of the starved population; error bars indicate the standard error of the mean. For the 10- to 22-h time points, data for the wild-type DHFR construct are averages of five experiments, data for the mutant DHFR construct are averages of three experiments, and data for SV40 are averages of two experiments. For the 2- to 8-h time points, the data for all constructs are averages of two experiments.

dramatically reduced the increase in luciferase activity at the G_1/S boundary after serum stimulation of quiescent 3T3 cells. Two separate preparations of pNWluc DNA were used to eliminate the possibility of an inhibitor in the DNA preparation. The quality of the pNWluc DNA was also ensured by the fact that the pNWluc DNA produced the same level of luciferase activity as did the pWTluc DNA in exponentially growing cells. These results suggested that the HIP1 binding site is not necessary for basal promoter activity but is required for a maximal DHFR transcriptional increase at the G_1/S boundary of the cell cycle.

Because the transcriptional efficiency of transiently transfected DNA may vary over the time course of a serum stimulation experiment, we also created stable transfectants of pWTluc and pNWluc. Three different clones of each of the pWTluc and pNWluc cells were analyzed after serum starvation and stimulation. Cells were harvested for analysis of both DNA content (Fig. 2B) and luciferase activity (Fig. 2A) every 2 h for 22 h. In cells stably transfected with the wild-type DHFR promoter construct, the luciferase activity increased as the cells entered S phase (10 h after stimulation) and peaked an average of 11-fold higher than the luciferase activity in starved cells. As demonstrated by the flow cytometry data, a second S phase begins about 22 h after stimulation. Luciferase activity produced by the wild-type DHFR promoter again increased as cells entered the second S phase. In contrast, the luciferase activity from the cells stably transfected with the mutant DHFR promoter construct varied no more than twofold during the course of the experiment. The increase in luciferase activity from the wild-type DHFR promoter construct in a second cell cycle was not seen in the transient transfection assays, probably because the cells were too dense by 3 days posttransfection to enter a second cell cycle. From these experiments, we again concluded that the HIP1 binding site is required for the increase in luciferase activity at the G_1/S boundary, since the activity from the mutant construct does not vary significantly through the cell cycle.

A 180-kDa protein binds specifically to the DHFR transcription initiation site. We next wanted to characterize the protein that mediates the growth regulation of the DHFR promoter. We have previously shown that a protein present in HeLa nuclear extract binds to the DHFR initiation site and protects nucleotides −16 to +11 from DNase I digestion (28). When DNA corresponding to these nucleotides is cloned in three tandem copies into a bacterial vector, protein still binds, indicating that these sequences are sufficient for binding (data not shown). We used this specific binding capacity to purify a protein (called HIP1) from HeLa cells. Because of the large number of cells required, it was not feasible to purify this protein from 3T3 cells.

The purification scheme for HIP1 is diagrammed in Fig. 3, and the extent of HIP1 purification is detailed in Table 1. The percentage of HIP1 binding activity in each fraction was determined by DNase I footprint analysis. In this particular purification, approximately 13% of the HIP1 binding activity was recovered from the HIP1 DNA affinity column, while 30% of the HIP1 activity flowed through the column, indicating a loss of 57% of HIP1 binding activity in this step. Other purifications resulted in recoveries of up to 30% of the HIP1 activity (data not shown). While these losses could be due to denaturation or degradation, some of the loss can be attributed to the separation of HIP1 from other protein(s) that stabilize HIP1 binding (27). The purified HIP1 fraction bound specifically to the DHFR transcription initiation site, as determined by DNase I footprinting (Fig. 4B). The

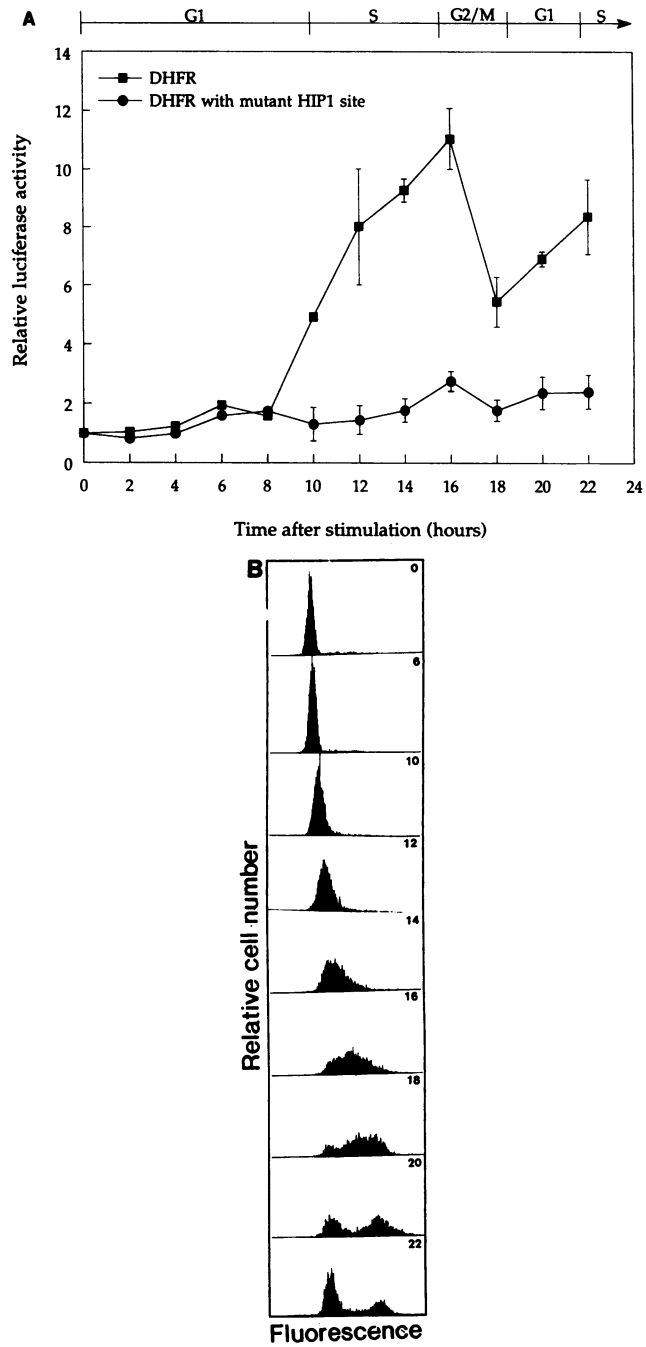

FIG. 2. Ability of the DHFR promoter to confer growth regulation on a heterologous gene in stably transfected cells. Three clones containing the wild-type DHFR promoter or the mutant DHFR promoter driving the luciferase cDNA were examined in serum starvation and restimulation experiments. Only two clones each were analyzed for the 2- to 8-h time points. Cells were harvested for luciferase assays (A) or flow cytometry analysis (B) every 2 h after serum stimulation. Luciferase values are reported relative to the activity of the starved population; error bars indicate the standard error of the mean.

boundaries of the footprint of the purified HIP1 are identical to those seen with use of crude nuclear extract on both the coding strand (Fig. 4B) and the noncoding strand (data not shown), suggesting that all components required for HIP1

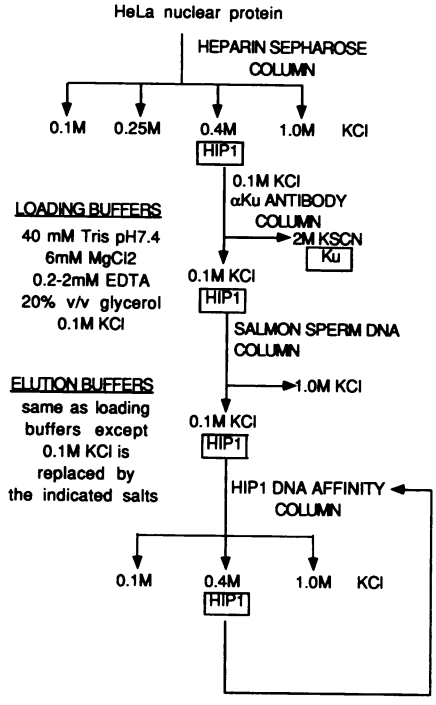

FIG. 3. Scheme for purification of HIP1 protein. The four columns used for HIP1 purification are indicated in the order of usage. The purification is described in detail in Materials and Methods. αKu, anti-Ku; KSCN, potassium isothiocyanate.

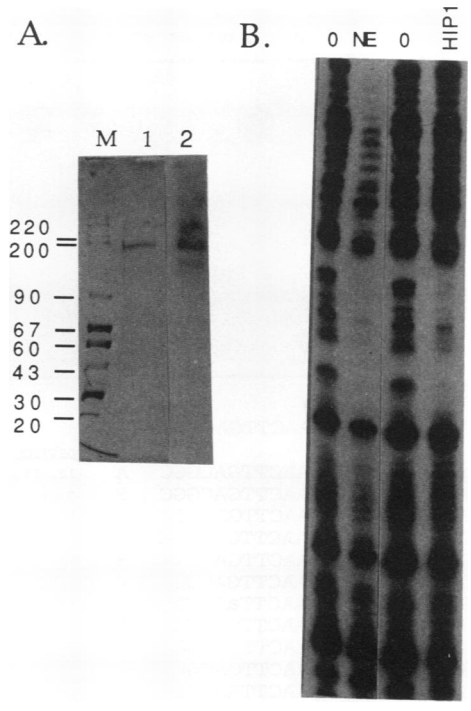

FIG. 4. Evidence that HIP1 is a 180-kDa protein that binds to the DHFR initiation site. (A) A portion (1.7%) of the HIP1 fraction from the DNA affinity column was electrophoresed on a 5 to 15% gradient polyacrylamide gel and stained with Coomassie brilliant blue dye (lane 1) or transferred to nitrocellulose and probed with concatemerized binding sites for HIP1 (lane 2). Lane M, size markers (indicated in kilodaltons at the left). The irregularly shaped spot above the 220-kDa marker was not reproducibly detected. (B) A DNA probe extending from −206 to +65 was treated with DNase I in the absence of protein (lanes 0) or in the presence of 100 μg of HeLa nuclear extract (lane NE) or 20 ng of purified HIP1 (lane HIP1).

binding activity have been purified. No other regions of the probe were protected by the HIP1 fraction.

Proteins that eluted from the DNA affinity column are shown in Fig. 4A, lane 1. The predominant protein, comprising greater than 90% of the proteins in this fraction (as judged by Coomassie brilliant blue staining and comparison with quantitative molecular weight protein markers), is approximately 180 kDa in size. To determine whether the 180-kDa protein bound to the DHFR initiation site, the protein fraction was electrophoresed and transferred to nitrocellulose. The nitrocellulose was then probed with radioactively labelled DNA corresponding to the DHFR initiation site. Autoradiography revealed that the 180-kDa protein bound to the DHFR initiation site (lane 2). This protein was not detected when a duplicate lane was probed with a binding site for the protein that binds from +46 to +56 in the DHFR promoter (21). A faint signal corresponding to a polypeptide with an apparent molecular size of 130 kDa can also be seen. Past purifications have shown that the 180-kDa HIP1 protein degrades to an apparent size of 130 kDa (data not shown). Therefore, we believe that the 130-kDa polypeptide is a proteolyzed product of the 180-kDa polypeptide.

TABLE 1. Purification of HIP1 protein[a]

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification factor (fold)	Yield (%)
Nuclear extract	250	6,000	22		
Heparin-Sepharose	40	6,000	150	6.8	100
Anti-Ku antibody	40	6,000	150	1	100
Salmon sperm DNA	40	6,000	150	1	100
DNA affinity	0.008	750	100,000	670	13

[a] Data are from a single preparation of HIP1; only slight differences occurred during other preparations. Total protein refers to the amount of protein in the fraction from each column that contained HIP1 activity. Total activity was determined by dividing the minimal volume needed to footprint 1 ng (25 fmol) of DHFR template into the volume of the column fraction. Specific activity was calculated by dividing the total activity by the total amount of protein. The purification factor is the increase in specific activity relative to the fraction from the previous column; total purification was 4,600-fold. Yield indicates the percentage of HIP1 binding activity recovered at each step. Protein concentrations were determined by spectrophotometric analysis using Bio-Rad protein reagents except for the final fraction, which was quantitated by comparison on polyacrylamide gels to quantitative molecular size markers.

HIP1 binds to the sequence TTCGCGCCA. We wanted to determine which sequences in the DNase I-protected region were important for HIP1 binding so that we could compare these sequences with the promoter regions of other late-response genes. Because binding sites identified by DNase I protection are often larger than the exact nucleotides which are important for the binding, a series of substitution mutants with mutations throughout the protected region was examined in a gel mobility shift assay (Fig. 5A). Incubation of purified HIP1 with a DHFR probe spanning the initiation site resulted in a band of slower mobility (lane b) than the free probe (lane a). In the remaining lanes, competitor DNA was incubated for 5 min with purified HIP1 prior to addition

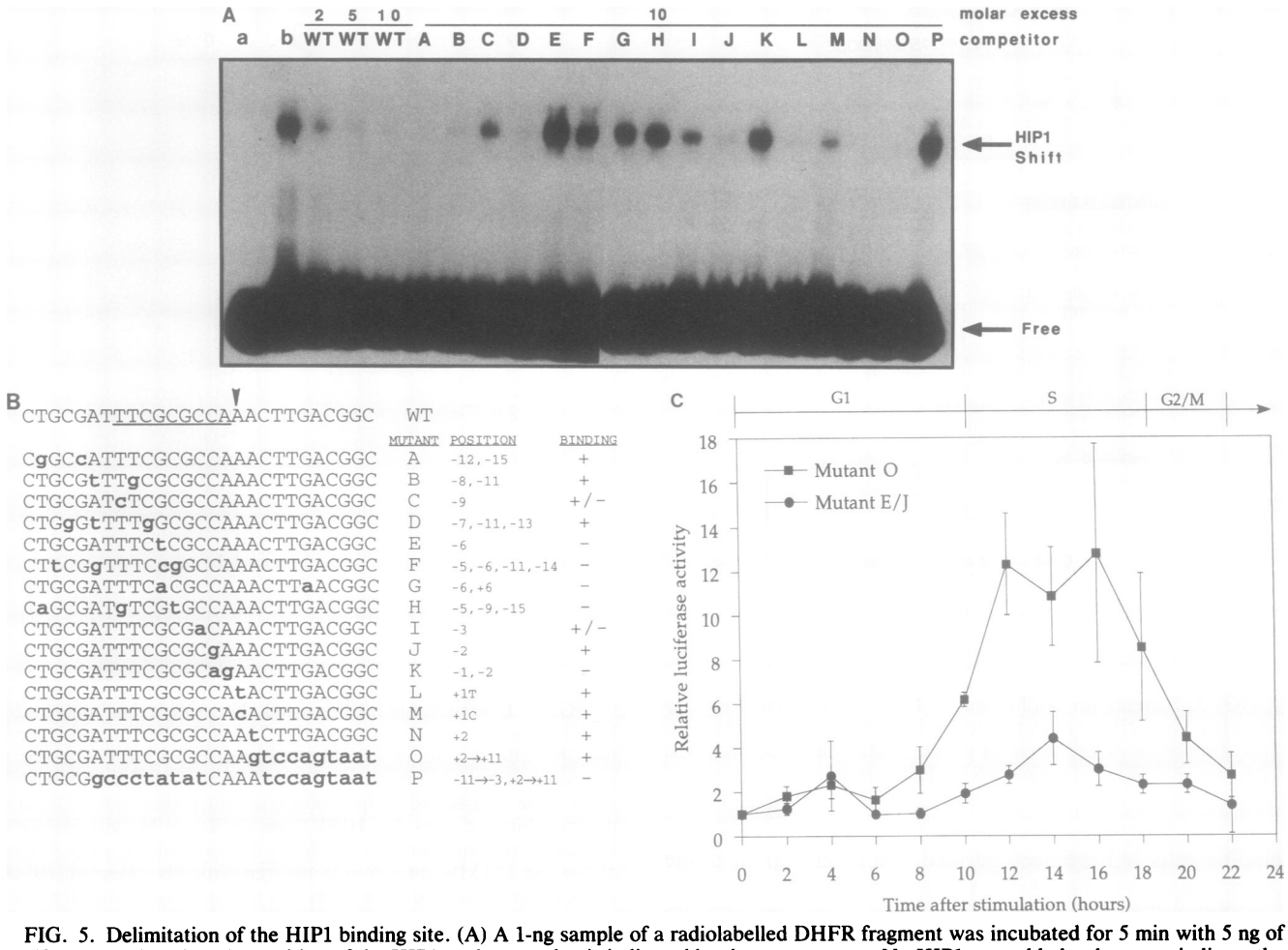

FIG. 5. Delimitation of the HIP1 binding site. (A) A 1-ng sample of a radiolabelled DHFR fragment was incubated for 5 min with 5 ng of purified HIP1 (lane b). The position of the HIP1-probe complex is indicated by the upper arrow. No HIP1 was added to lane a to indicate the position of the free probe. In the next three lanes, a 2-, 5-, or 10-fold molar excess of the unlabelled wild-type (WT) DHFR fragment was used, as indicated. The remaining lanes represent similar reactions except that specific nucleotides were mutated in the DNA fragments used as competitors (see panel B). The presence of the HIP1 shifted band indicates an inability of the mutant probe to compete with the wild-type probe and suggests that the mutated nucleotides are important for HIP1 binding. (B) Positions of the mutations (boldface lowercase letters) used in the gel mobility shift analysis. The arrowhead denotes the transcription initiation site. Mutant P corresponds to the bases changed in the mutant DHFR/luciferase construct analyzed in Fig. 1 and 2. +, the mutant sequence can compete for binding of HIP1 to the wild-type (WT) site; −, the mutant sequence can no longer compete for HIP1 binding to the wild-type site; +/−, there was partial competition for HIP1 binding to the wild-type site. The positions that were determined to be important for binding are underlined in the wild-type sequence. (C) Comparison of HIP1 binding and DHFR promoter regulation. 3T3 cells were transfected with constructs containing DHFR promoter mutations driving the luciferase cDNA. The mutant E/J promoter contains a double-point mutation at −2 and −6 and does not bind HIP1 in a gel mobility shift competition assay, whereas the mutant O promoter has positions +2 to +11 mutated and does bind HIP1 in a gel mobility shift competition assay (B). Cells were harvested for luciferase assays every 2 h after serum stimulation. Luciferase assays are reported relative to the activity of the starved population; error bars indicate the standard error of the mean. The data are averages of three experiments using different DNA preparations.

of the radiolabeled wild-type probe. Five minutes after addition of the probe, the reaction mixtures were loaded onto gels. A 2-fold molar excess of the wild-type sequence partially inhibited binding to the labelled probe, while 5- and 10-fold molar excesses severely reduced HIP1 binding. A 10-fold molar excess of each of the mutants was then tested for the ability to compete for binding of HIP1 to the wild-type sequence in a gel mobility shift assay. The competitors having mutations between −15 and −11 were able to compete for binding as efficiently as did wild-type DNA, as did a competitor containing a mutation of all nucleotides between +2 and +11 (mutant O), suggesting that the sequences at these positions were not involved in HIP1 binding. However, competitors having mutations in the central portion of the protected region were unable to compete for binding to the wild-type sequence. Substitutions that reduce but do not abolish binding occur at positions −9 (mutant C) and −3 (mutant I). Substitutions that abolish competition for HIP1 binding occur at positions −6 (mutant E), −5, −6, −11, and −14 combined (mutant F), −6 and +6 combined (mutant G), −5, −9, and −15 combined (mutant H), and −1 and −2 combined (mutant K). These mutants all had substitutions between −9 and −1. It is likely that the nucleotides flanking the −9 to −1 region are protected from DNase I cleavage because of the large size of the HIP1 protein. Thus, we conclude that the determinants of HIP1 binding to the

DHFR promoter include the sequence TTCGCGCCA (Fig. 5B). Sequences similar to the HIP1 binding site can be found in the promoters of other late-response genes such as DNA polymerase alpha (−2 CCCGCGCCA +7), murine thymidine kinase (−80 TTCGCGGGCA −71), human thymidine kinase (−102 TTGGCCGCA −110), and mouse thymidylate synthase (−59 AGAGCGCCA −51) genes.

Having determined more precisely the HIP1 binding site, mutants of the DHFR luciferase constructs were examined in the transient transfection assay (Fig. 5C). Specific mutants were chosen to determine whether the DHFR regulation pattern correlated with the binding of HIP1 to the DHFR promoter or with changing the 5′ end of the mRNA in pNWluc. Activity from pOluc, which is identical to pWTluc except in positions +2 to +11, increased at the G_1/S boundary and peaked with an average of 13-fold above values for starved cells (Fig. 5C). Although these changes do not affect HIP1 binding (Fig. 5B, mutant O), the mRNA produced from pOluc should be identical to the mRNA from the mutant pNWluc (mutant P) which did not have the wild-type expression pattern (Fig. 1A). pE/Jluc is also identical to pWTluc except that the −2 C and −6 G have been changed to −2 A and −6 T. The mRNA produced from this promoter should be identical to the mRNA from pWTluc, since all of these changes are upstream of +1. This double-point mutant did not compete in the gel shift assay, and expression increased only an average of fourfold above values for starved cells by late S phase. These data indicate that changing the 5′ end of the mRNA does not affect DHFR promoter regulation, whereas competition in the gel shift assay does correlate with regulation at the G_1/S boundary.

HIP1 rapidly dissociates from its binding site. We next further analyzed HIP1 binding to DNA, since the characteristics of specific protein-DNA interactions may suggest further insight into the mechanisms by which a factor regulates transcription. For example, at 25°C, TFIID dissociates from its binding site with a half-life of 1 h, whereas Sp1 dissociates very rapidly, with a half-life of 1 min (35). Gel shift analysis was used to examine a time course of HIP1 binding to and dissociation from DNA. Purified HIP1 was added to reaction mixtures containing the radiolabelled DHFR probe, and the binding reaction mixtures were loaded onto a gel after various lengths of time (Fig. 6, lanes 1 to 6). Maximal binding of HIP1 to DNA was seen at 30 s. Continuing the binding reaction for as long as 30 min did not increase the amount of binding.

To examine the time required for HIP1 to dissociate from its binding site, HIP1 was incubated with a radiolabelled fragment containing the DHFR initiation site for 5 min. This binding was then challenged with a 50-fold molar excess of unlabelled DHFR fragment for 15 s, 30 s, 1 min, 5 min, and 15 min (Fig. 6, lanes 8 to 12, respectively) before the samples were electrophoresed. Lane 7 contained a reaction mixture in which no competitor DNA was added. As can be seen in lane 8, HIP1 protein completely dissociated from the labelled probe after only 15 s of challenge with the unlabelled fragment. Thus, HIP1 protein rapidly associates and dissociates from its binding site.

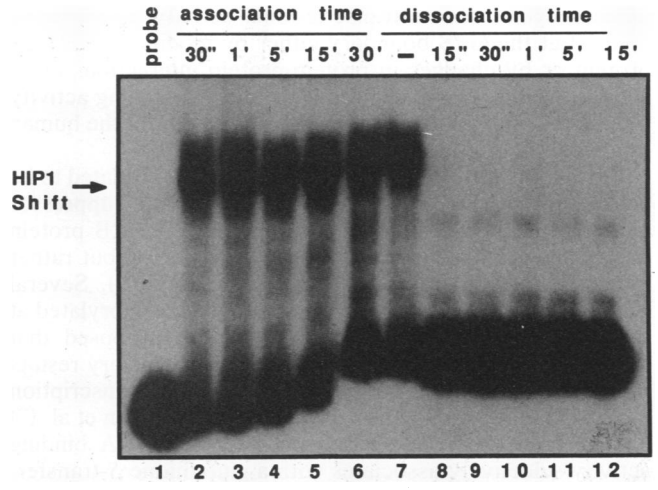

FIG. 6. Rapid association and dissociation rates of the HIP1 protein. Purified HIP1 protein and the radiolabelled DHFR probe were used in gel mobility shift assays. HIP1 was omitted from lane 1 to indicate the position of the free probe. For lanes 2 to 6, HIP1 was incubated with the DNA probe for the indicated times (", seconds; ', minutes) prior to loading of the gel. For lanes 7 to 12, either no competitor (lane 7) or a 50-fold excess of cold DHFR fragment (lanes 8 to 12) was added to the reaction mixture at the indicated times prior to loading the gel.

DISCUSSION

The transcription rate of the DHFR gene increases at the G_1/S boundary in murine fibroblasts that have been synchronized by mitotic selection (14) or serum starvation (33). We have now shown that DHFR promoter sequences extending from −270 to +20 are sufficient to confer similar growth regulation on a heterologous gene and that a double-point mutation at positions −2 and −6 severely diminishes this growth expression pattern. These results suggest that the protein binding site that spans the DHFR initiation site is necessary for the specific increase in transcriptional activity at the G_1/S boundary. However, we do not yet know whether this protein binding site is sufficient to confer regulation on a different promoter. Experiments to test the sufficiency of the element for growth regulation will be complicated by the fact that the HIP1 binding site spans the initiation site. Insertion of the DHFR initiation site into another promoter may result in aberrant initiations that complicate the interpretation of its role in growth regulation. Because it is likely that the exact position of this element in a promoter is important, a series of heterologous promoter constructs will need to be analyzed to determine whether the −16 to +11 region is sufficient to confer growth regulation on a heterologous promoter.

The transcription initiation regions of both the hamster (2) and mouse (28) DHFR promoters have been shown to bind protein in HeLa nuclear extract. We have now purified a protein that binds to the murine transcription initiation site. The predominant 180-kDa protein in the purified fraction bound to an oligonucleotide containing DHFR sequences from −11 to +9. Although the region protected from DNase I cleavage spans 27 bp, competition experiments with mutant sites indicated that the 9 bp just upstream of the initiation site are the most critical for protein binding.

How might HIP1 function to increase the transcription of the DHFR promoter at the G_1/S boundary? The simplest possibility is that the DNA binding activity of HIP1 increases at the G_1/S boundary, either by an increase in the amount of HIP1 or by a modification that increases the affinity of HIP1 for its binding site. Protein binding to the growth-regulatory CCCNCNNNCT element in the murine thymidine kinase promoter displays this form of regulation (11). Alternatively, HIP1 binding could be constant through

the cell cycle, but its transcriptional activity could be increased at the G_1/S boundary either by modification of the protein or by changes in protein-protein interactions. This activity would be similar to the constitutive binding activity seen at the growth-regulatory CCAAT element of the human thymidine kinase promoter (17).

Regulation at the G_1/S boundary has been postulated to be controlled by the retinoblastoma (RB) tumor suppressor gene that encodes a 105-kDa protein (20). The RB protein itself does not display sequence-specific binding but rather interacts with other DNA-binding proteins (5, 7). Several groups have shown that the RB protein is phosphorylated at the G_1/S boundary (4, 6, 8). It has been proposed that phosphorylation of RB protein at the G_1/S boundary results in its release from a complex with cellular transcription factors, leading to induction of S phase. Chittenden et al. (7) have demonstrated that a sequence-specific DNA binding activity selectively associates with a glutathione *S*-transferase–RB fusion protein. Analysis of the specific DNA sequences that are bound by this protein complex suggest that the transcription factor E2F or proteins with similar DNA binding properties interact with the RB protein. Several of the sequences that bind to this protein complex are very similar to the 9-bp region of the HIP1 site shown to be important for binding in our competition assays. Perhaps HIP1 activity is regulated by release from an RB-HIP1 protein complex at the G_1/S boundary, as suggested for E2F (1, 5). Although the HIP1 site is similar to the E2F site (2), the proteins do not appear to be the same. First, HIP1 is much larger than the 54-kDa E2F (38). Second, E2F DNA binding activity increases fivefold within 4 h after serum stimulation (29). This increased E2F DNA binding activity mirrors the activation kinetics of early-response genes such as c-*myc* which are regulated by E2F (36) but is in marked contrast with the activation kinetics of the DHFR promoter (Fig. 1 and 2). Two E2F-protein complexes have been identified (29). The DNA binding activity of one complex peaks 4 to 6 h after serum stimulation and thus does not correlate with DHFR activation. The second complex is most prevalent between 16 and 20 h after serum stimulation, at which point the DHFR promoter activity is decreasing. This complex contains cyclin A and is thought to be an inactive form of E2F. Thus, it is unlikely that free E2F or either of the E2F-protein complexes could account for the increase in DHFR transcriptional activity at the G_1/S boundary.

In conclusion, we have identified a site in the DHFR promoter that is required for the increase in transcriptional activity at the G_1/S boundary. We have purified a protein, HIP1, that binds to this site and have characterized the sequences that are important for binding of purified HIP1. Analysis of binding of this protein to the DHFR promoter indicated extremely rapid association and dissociation kinetics. This particular characteristic of HIP1 binding may provide a rapid response to changes in the activity of the HIP1 protein at the G_1/S boundary. We are currently investigating the mechanisms by which HIP1 activity is regulated and how this regulation is tied to the control of cellular proliferation.

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