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Inappropriate transcription from the 5′ end of the murine *dihydrofolate reductase* gene masks transcriptional regulation

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ABSTRACT

Using the nuclear run-on assay we found that in proliferating cells the transcription rate in the 5′ end of the murine *dihydrofolate reductase* (*dhfr*) gene was approximately ten-fold higher than in the 3′ end of the gene, suggesting transcriptional attenuation within the *dhfr* gene. However, when the transcription rate was measured by pulse-labeling, the rate was uniform throughout the gene, and the 5′ *dhfr* signal was approximately ten-fold lower relative to a control gene signal than in the run-on assay. Previously, the activity of a *dhfr* promoter linked to a luciferase reporter gene was shown to increase about ten-fold at the G1/S-phase boundary following stimulation of serum-starved cells. To determine if the run-on procedure would detect growth regulation of the endogeneous *dhfr* gene, serum-starved and -stimulated NIH 3T3 cells were analyzed. Using a *dhfr* 5′ end probe no difference in transcription rate between these growth states was detected and the *dhfr* 3′ end probe did not detect signal above background. In a cell line that was amplified at the *dhfr* locus, the transcription rate in the 5′ end of the gene increased less than two-fold in stimulated cells, but the rate in the 3′ end of the gene increased five- to seven-fold. Therefore, the *dhfr* gene is growth regulated at the level of transcription, but the nuclear run-on assay was only able to detect a difference in transcription rate in the 3′ end of the gene in amplified cells. We suggest that isolation of nuclei may activate *dhfr* transcription complexes that normally are activated only at the G1/S-phase boundary.

INTRODUCTION

Dihydrofolate reductase is a key biosynthetic enzyme that is required for the *de novo* synthesis of glycine, purines, and thymidylate. The *dhfr* gene is expressed in all proliferating cells, thus the DHFR protein is a target in the treatment of hyperproliferative diseases including cancer. The response of the mouse, hamster, and human *dhfr* gene to growth stimuli has been the subject of many investigations (reviewed in 10). In general, *dhfr* expression increases when cells are induced to proliferate, such as after serum stimulation of quiescent cells, after viral infection, or after release from amino acid starvation or density arrest. Levels of *dhfr* mRNA decrease as cells withdraw from the cell cycle during differentiation or senescence.

Differing conclusions have been reached regarding the mechanisms that regulate changes in *dhfr* mRNA levels, thus generating an inconsistency in the literature. For example, Farnham and Schimke found a seven-fold increase in the *dhfr* transcription rate at the G1/S-phase boundary of the cell cycle (2). However, Feder *et al.* measured transcription rates that were essentially invariant throughout the cell cycle (11). Johnson and colleagues documented a four-fold transcription rate increase when quiescent cells were stimulated to re-enter the proliferative cell cycle (12, 13). These studies are difficult to reconcile because different cells, separation methods, and transcription rate assays were used. Because of these contradictory results, we undertook an in-depth transcription rate analysis of the *dhfr* gene.

Using the nuclear run-on assay, the transcription rate throughout the *dhfr* gene was examined in quiescent, serum-stimulated, and unsynchronized, proliferating cells in both non-amplified and amplified cell lines. Furthermore, the nuclear run-on assay was compared directly to two other assays that measure transcription rate: pulse-labeling of intact cells, and activity of a reporter gene linked to a promoter. We find that the nuclear run-on assay can be used to measure a transcription rate increase in the murine *dhfr* gene provided two conditions are met. First, the transcription rate must be measured in the 3′ portion of the gene, because the nuclear run-on signal generated from the 5′ end of the gene is high under all tested growth conditions. Second, a cell line that contains amplified copies of the *dhfr* locus must be used, because the *dhfr* 3′ end signal in non-amplified cells is below the limit of detection. Because of these limitations, some conclusions regarding the regulation of the *dhfr* gene that are based on nuclear run-on analysis should be re-evaluated. Furthermore, we caution that when nuclear run-on analyses of any gene suggest a lack of transcriptional regulation, an independent assay should be conducted to verify this result.

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MATERIALS AND METHODS

Plasmids

The coordinates of all *dhfr* clones are relative to the major transcription initiation site at +1. pSPCS− contains murine *dhfr* cDNA sequences from +10 to +719 and was created by insertion of a *Pst*I/*Bgl*II fragment from pdhfr11 (1) into the *Pst*I and *Bam*HI sites of pSP65 (Promega). The Exon 1, Exon 3+4, Exon 6, and Exon 4+5+6 clones were subcloned from pSPCS− and contain *dhfr* cDNA sequences from +10 to +125, +224 to +420, +564 to +719, and +311 to +719 respectively. Exon 1 contains a *Hin*dIII/*Bst*NI fragment, Exon 3+4 contains a *Bst*NI/*Bst*NI fragment, and Exon 6 contains a *Bst*NI/*Eco*RI fragment inserted into the *Hin*dIII and *Sma*I sites, the *Sma*I site, and the *Sma*I and *Eco*RI sites of pBSM13+ (Stratagene), respectively. Exon 4+5+6 was created by insertion of a *Sac*I/*Pvu*II fragment into the *Sac*I and *Sma*I sites of pSP64. The Intron1 clone contains genomic sequences from +127 to +275 and was created by insertion of a *Bst*NI/*Hin*dIII fragment from pSS625 (2) into the *Hin*cII and *Hin*dIII sites of pBSM13+. The Exon 2 clone contains genomic sequences from *Sau*3A (+275) to *Sau*3A (+592). The Exon 2 fragment was cloned into the *Bam*HI site of pUC9, then a *Eco*RI/*Hin*dIII fragment was transferred into pBSM13+. All the *dhfr* constructs used in nuclear run-on analysis were also cloned in pBSM13− so that single-stranded DNA containing either strand of the insert could be recovered. pBSd11 was created by inserting the *Pst*I fragment from pdhfr11 (1) into the *Pst*I site of pBSM13+.

Cell culture

Cell lines used in this study include NIH 3T3, NIH 3T3WTLuc, NIH 3T6, 3T6 R50A, and 3T6 R1000. Cells were maintained in Dulbecco's modified Eagle's medium with high glucose supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. 5% defined-supplemented bovine calf serum was included in NIH 3T3, NIH 3T3WTLuc, and NIH 3T6 cultures, and 10% dialyzed fetal calf serum was added to 3T6 R50A and 3T6 R1000 cultures. Serum was obtained from Hyclone Laboratories. The NIH 3T3WTLuc cell line contains a stably-integrated *dhfr* promoter linked to a luciferase cDNA, and was created as described (3). The 3T6 R1000 cell line was developed by exposing 3T6 R50A cells (2) to increasing concentrations of methotrexate, beginning at 50 μM, and increasing in two-fold steps to 1 mM. The 3T6 R50 and 3T6 R1000 cultures were maintained in the presence of 50 μM and 1 mM methotrexate (Sigma), respectively.

Experiments with the NIH 3T3WTLuc cell line were performed as described previously (3). Cells were plated at 2×10^5 cells per 60 mm diameter dish or 1.6×10^6 cells per T225 flask in maintenance medium (5% serum). 1 h later the maintenance medium was replaced with medium containing 0.5% serum (starvation medium). After approximately 60 h the cells were stimulated by replacing the starvation medium with fresh medium containing 10% serum (stimulation medium). Luciferase assays were carried out as described previously (4). 3T6 R50A cells were starved and stimulated as follows: 1.6×10^6 cells per T225 flask were plated in maintenance medium, allowed to recover for 15−18 h, then were placed in starvation medium for 6 days. Using flow cytometric analysis we determined that a long starvation period was required to synchronize the 3T6 R50 cell line. The cells were stimulated by changing to medium containing 10% serum. Flow cytometric analysis was used to monitor the distribution of the cell population in the phases of the cell cycle as described previously (3).

Primer extension analysis

To prepare cytoplasmic RNA frozen cell pellets were resuspended in 1× lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris−HCl, pH 8.6, 0.5% NP-40, 1 mM DTT, 20 mM vanadyl-ribonucleoside complex), incubated on ice for 5 min, then the nuclei were removed with a 20 min spin in a microcentrifuge. The supernatent was combined with an equal volume of 2× PK buffer (0.2 M Tris−HCl, pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% SDS) and 200 μg/ml proteinase K was added. The samples were incubated at 37°C for 30 min, extracted with a 1:1 mixture of phenol and chloroform, ethanol precipitated, and rinsed with 70% ethanol.

Primer extension analysis was carried out as described previously (5) with the following modifications. 2 μg cytoplasmic RNA was hybridized at 60°C with 100 fmol of a primer complementary to *dhfr* sequences from +65 to +42. The samples were reverse transcribed at 45°C, and loaded on an 8 M urea−8% polyacrylamide gel.

Nuclear run-on assays

5 μg double-stranded plasmid DNA or single-stranded phagemid DNA was applied to each filter slot. Quantitation of DNAs was determined spectrophotometrically, then verified by visual analysis following electrophoresis on agarose gels. DNA in 100 μl was denatured by incubation at 65°C for 1 h with 10 μl 3 M NaOH. The samples were placed on ice, neutralized with 110 μl 2 M ammonium acetate, then applied to 0.45 μm nitrocellulose membrane (Schleicher and Schuell) that was prewashed with 10× SSC and assembled in a VacuSlot apparatus (American Bionetics). After rinsing each filter slot with 10× SSC, the membrane was dried at room temperature for 20 min, then baked at 80°C for 2 h under vacuum. To reduce non-specific hybridization, each filter strip contained a slot with the pRA plasmid (provided by Dr Craig Thompson) which contains a 1.6 kb fragment of a human 28S ribosomal gene. The pRA slots are not shown in the figures. In the NIH 3T3WTLuc experiments the stably-integrated *dhfr*-luc construct produced high background hybridization to the vector filter slot. Therefore, filter slots for those run-on experiments contained isolated fragments equivalent to 5 μg plasmid, and λ DNA was applied as the negative control DNA. Single-stranded DNA was rescued from XL1-Blue cells using R408 or MK1307 helper phage as described (6).

To harvest tissue culture cells the medium was removed, cells were rinsed with phosphate-buffered saline (PBS), then incubated with 0.5% trypsin−EDTA in PBS until the cells began to detach. The trypsin was inactivated with serum, and the loose cells were collected after banging the flasks. All subsequent steps were carried out at 4°C. The cells were pelleted at 1500×*g* for 5 min, resuspended in PBS, counted, then spun at 1500×*g* for 5 min. The pellet was resuspended in 1 ml RSB (10 mM Tris−HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) per 1×10^7 cells, and dounced 10 times in a Wheaton homogenizer fitted with a B-type pestle. The homogenate was centrifuged at 1500×*g* for 5 min, then resuspended in 0.5 ml RSB per 1×10^7 cells. 2 ml RSB with 0.5% NP-40 was added per 1×10^7 cells, and the mixture was dounced 5−10 times. The homogenate was centrifuged at 1500×*g* for 5 min, the liquid was carefully aspirated from the nuclear pellet, and the nuclei were resuspended in NFB (50 mM Tris−HCl, pH 8.3, 40% glycerol, 5 mM

$MgCl_2$, 0.1 mM EDTA) at a concentration of 1×10^7 cells per 100 µl. The nuclei were frozen at −70°C and used within 2 weeks.

The labeling, isolation, and hybridization of RNA was essentially as described previously (7). 1×10^7 nuclei were used per reaction. 210 µl nuclei in NFB and 60 µl 5× run-on buffer (25 mM Tris−HCl, pH 8.0, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM GTP, ATP, and CTP) and 30 µl [α-^{32}P]UTP (300 µCi, 3000 Ci/mmol) were mixed and incubated at 26−30°C for 10−30 min. 15 µl DNaseI (1−5 mg/ml) and $CaCl_2$ (final concentration 10 mM) were added and the reaction was incubated at 30°C for 10 min. 36 µl 10× SET buffer (10% SDS, 50 mM EDTA, 100 mM Tris−HCl, pH 7.4) and 200 µg/ml proteinase K were added, and the reaction was incubated at 42°C for 45 min. The reaction was extracted with an equal volume of phenol:chloroform (1:1), re-extracted with 100 µl 1× SET and precipitated with 2.1 M (final concentration) ammonium acetate and 667 µl isopropanol at −70°C for 15 min. After spinning 20 min in a microcentrifuge, the pellet was resuspended in 100 µl TE, and run over a Bio-spin 30 column (Biorad). The column was washed with 50 µl TE, the eluates were combined, brought to a total volume of 180 µl, and incubated on ice with 20 µl 2 N NaOH for 10 min. HEPES was added to a final concentration of 0.24 M, and the sample was precipitated with 880 µl ethanol for at least 1 h at −20°C. The sample was spun in a microcentrifuge for 15 min, then resuspended in 1 ml hybridization buffer (10 mM TES, 0.2% SDS, 10 mM EDTA, 250 µg/ml *Torula* RNA, 0.3 M NaCl, 1× Denhardt's). The incorporation of radiolabeled UTP into RNA was measured by scintillation counting. Typically, a reaction containing 1×10^7 cells incorporates 1×10^7 cpm. Filter strips were incubated with hybridization buffer without probe for at least 2 h at 65°C. The prehybridization solution was replaced with 2 ml hybridization solution containing probe. Typically 1×10^7 cpm were used per strip. However, in the regulation experiments, run-on material from an equivalent number of cells was used for starved and stimulated time points. The filters were hybridized at 65°C for 36−48 h, then were washed for 1 h at 65°C in 2× SSC, 30 min at 37°C in 2× SSC containing 10 µg/ml RNase A, and for 1 h at 37°C in 2× SSC. The filters were dried and exposed to X-ray film at −70°C. Hybridization of radioactive run-on RNA to individual filter slots was measured by scintillation counting in 5 ml cocktail. The signal in the vector slot was subtracted from the raw signals of the other slots, then the corrected signal was normalized as described in each table legend.

Elution of labeled RNA from filters was essentially as described by Rougvie and Lis (8). Filter slots containing run-on RNA hybridized to the *dhfr* Exon 1 plasmid were cut away from the rest of the filter, placed in 200 µl 1% SDS containing 2 µg tRNA. The filter and solution were boiled for 5 min. The solution was removed to another tube, 3 µg tRNA were added, and the eluted material was precipitated in ethanol. The NaOH treatment step was omitted from the run-on reactions that were to be eluted, and the filters were treated with 25 units/ml RNase T1 instead of RNase A.

dhfr-identical RNA was made *in vitro* by linearizing pBSd11 with *Bam*HI then transcribing with T3 bacteriophage polymerase (Promega) as described (6).

Pulse-labeling assays

3T6 R50 cells were plated in 100 mm diameter dishes at a concentration of 5×10^5 and grown for 2 days. To pulse-label the RNA, medium was removed from the plate, 0.8 ml conditioned media was mixed with 500 µl [5,6-^{3}H]uridine (500 µCi, 40−60 Ci/mmol) and added to the plate. Cells were incubated at 37°C for 30 min, the labeling medium was removed, and the plates were rinsed twice with 10 ml cold PBS. RNA was prepared by combining elements of protocols for isolation of total RNA from mammalian cells (9) and from the nuclear run-on protocol described above. 1 ml 1× lysis buffer (described above) was added, and the cells were scraped from the plate. The plate was rinsed with 0.5 ml lysis buffer, then 1.5 ml 2× PK buffer (described above) and 200 µg/ml proteinase K was added. The lysate was squeezed through a 21-gauge needle several times, incubated at 37°C for 30 min, extracted with phenol:chloroform (1:1), and precipitated in ethanol. The pellet was resuspended in 400 µl TE containing 10 mM each vanadyl-ribonucleoside complex and $MgCl_2$. 2 µg DNaseI was added, the mixture was incubated at 37°C for 30 min, EDTA and SDS were added to final concentrations of 10 mM and 0.2%, respectively, and the solution was extracted with phenol:chloroform. At this point, the pulse-labeled RNA was precipitated with isopropanol, run over a Bio-spin column, treated with NaOH, and precipitated as described above for the nuclear run-on assay. The preparation of filters, hybridization and washing protocols were as described above. To quantify the hybridization of [^{3}H]RNA to the filters, individual filter slots were cut apart, placed in scintillation vials, and the RNA was hydrolyzed by incubation in 800 µl 0.1 N NaOH at 65°C for 1 h. The solution was neutralized with 80 µl 1 N HCl, before scintillation cocktail was added.

RESULTS

The nuclear run-on assay measures a higher transcription rate in the 5′ end of the *dhfr* gene than in the 3′ end

The transcription rate in the murine *dhfr* gene was assayed in proliferating NIH 3T3 cells using the nuclear run-on technique.

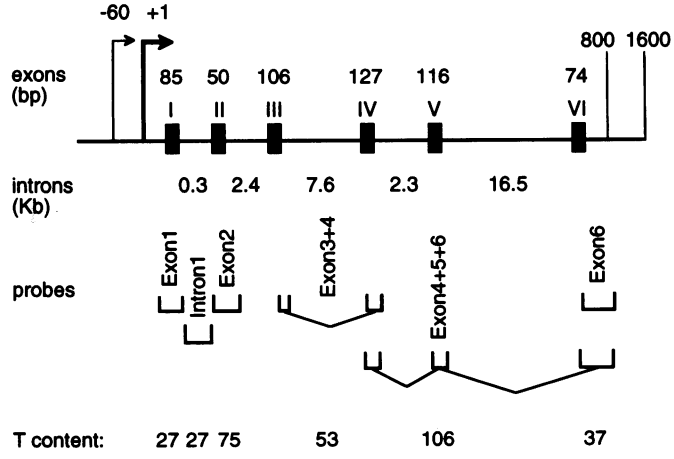

Figure 1. Schematic of the murine *dhfr* gene. The six Exons are represented as black boxes with the size in base pairs indicated above each. The sizes of the five introns are indicated in kilobase pairs below the line. We define Exons as translated sequences because this gene contains multiple 5′ and 3′ ends. The bent arrows represent the two predominant transcription initiation sites; approximately 85% of the transcripts initiate at +1, and the majority of the remainder initiate at −60. The two major polyadenylation sites are shown after Exon six with the size of the resulting transcript in nucleotides indicated above each site. The names and approximate positions of the inserts contained in the plasmids used in the nuclear run-on analyses are diagrammed with the number of thymidine bases contained in the coding strand of each insert listed.

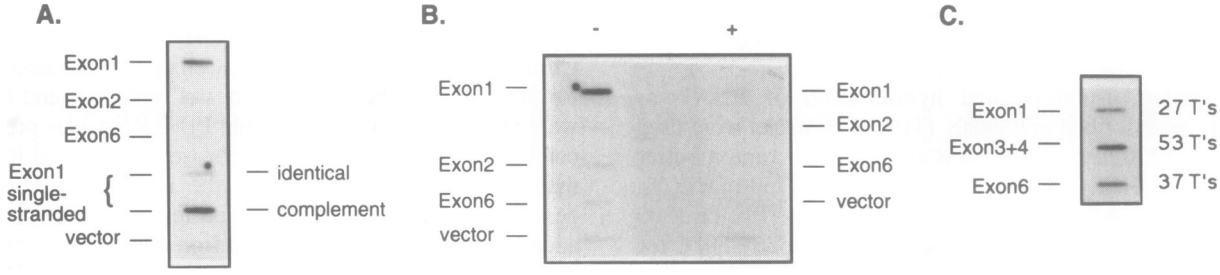

Figure 2. Nuclear run-on analysis of the *dhfr* gene in NIH 3T3 cells. Nuclei were isolated from unsynchronized, proliferating NIH 3T3 cells, engaged polymerases were allowed to extend in the presence of radiolabeled UTP, and RNA was prepared and hybridized to nitrocellulose filters as described in Materials and Methods. The filters contained denatured double-stranded plasmid DNA, except for the two slots that contained single-stranded phagemid DNA (denoted identical or complement). The inserts in the plasmids applied to the filter slots are diagrammed in Fig. 1. (**A**) The *dhfr* Exon 1 signal is strand-specific. (**B**) The *dhfr* Exon 1 signal is derived from RNA polymerase II transcription. Reactions were performed in the absence (−) or presence (+) of 2 μg/ml α-amanitin. (**C**) The *dhfr* plasmids hybridize to *dhfr* RNA with equal efficiency. A uniformly-radiolabeled *dhfr* RNA was synthesized *in vitro* with SP6 polymerase and hybridized to the filter.

We measured the transcription rate in different portions of the gene because this methodology has led to the identification of transcriptional block points in other cellular genes, including c-*myc*, c-*fos*, c-*myb*, and *adenosine deaminase*, as well as in the genomes of several viruses, including adenovirus and HIV (reviewed in 14, 15). Since transcriptional block points can be sites of regulation, we considered that some of the discrepancies in previous studies of *dhfr* regulation might be explained by the presence of a transcriptional attenuation site within the gene. Previous studies of the *dhfr* transcription rate employed either the entire cDNA or the promoter region as a probe; different probes were not compared directly, so that differences in the transcription rate across the gene would not have been detected.

The murine *dhfr* gene contains six Exons and covers approximately 30 kb (Fig. 1). Plasmids containing portions of *dhfr* cDNA or genomic DNA were applied to nitrocellulose filters and hybridized to RNA produced in the nuclear run-on reaction. In the run-on assay engaged polymerases elongate for a short distance (estimated to be 100−300 nt) in the presence of a limiting, labeled nucleotide triphosphate, and thus the density of polymerases in a portion of the gene at the time of nuclear isolation is measured. Transcription initiation does not occur in this assay; we have verified that conditions that block initiation *in vitro* do not decrease signal generated in the nuclear run-on assay (data not shown). Labeled RNA was detected hybridizing to the *dhfr* Exon 1 probe, but not to the Exon 2 or Exon 6 probes, suggesting that the transcription rate is higher at the extreme 5′ end of the *dhfr* gene than in more 3′ portions of the gene (Fig. 2). The difference in transcription rates between Exon 1 and Exon 2 (or Exon 6) could not be determined because the signal detected hybridizing to the downstream Exons was not reproducibly above background (defined as the signal detected hybridizing to a vector plasmid).

The results from several experiments verified the authenticity of the RNA hybridizing to the *dhfr* Exon 1 probe. First, single-stranded DNAs from the Exon 1 plasmid were used as probes to show that the nuclear run-on signal arises from transcription in the direction of the *dhfr* gene and not from anti-sense transcription (Fig. 2A). Second, when 2 μg/ml α-amanitin was included in the run-on reaction the Exon 1 signal was no longer detected, indicating that the RNA hybridizing to the Exon 1 plasmid is transcribed by RNA polymerase II (Fig. 2B). Third, when a uniformly-radiolabeled *dhfr* RNA was produced *in vitro* with SP6 polymerase and hybridized to a filter, the signal detected with each *dhfr* plasmid was proportional to the number of thymidines in the coding strand (Fig. 2C). Therefore, the hybridization efficiency is similar for the different *dhfr* probes.

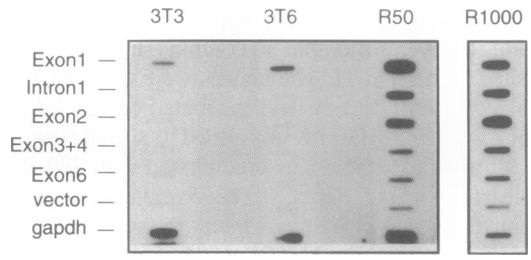

Figure 3. Nuclear run-on analysis in non-amplified and amplified cell lines. Nuclear run-on analysis was performed with unsynchronized, proliferating cells from the four cell lines listed above the panels. The R50 and R1000 cell lines were derived from the 3T6 cell line by growth in methotrexate and contain many copies of the *dhfr* locus. The inserts in the plasmids applied to the filter slots are diagrammed in Fig. 1.

Identification of transcriptional pause points in the *dhfr* gene

Nuclear run-on analysis also was conducted using other cell lines, including non-amplified lines such as NIH 3T6 and F9 teratocarcinoma. In both cell lines, the signal in Exon 1 was high and the signal in downstream Exons was near background levels (Fig. 3 and data not shown). Cell lines that contain amplified copies of the *dhfr* locus have been employed in many studies, and have been used to reproduce results obtained in the parental, non-amplified cell lines (10). The amplified lines also displayed a higher transcription rate in Exon 1 than elsewhere in the *dhfr* gene (Fig. 3), but the signals detected in downstream Exons were above background. In both the 3T6 R50 and 3T6 R1000 cell lines the transcription rate was 10- to 15-fold higher in Exon 1 than in Exon 6. This figure also illustrates the specificity of the Exon 1 signal; as the number of copies of *dhfr* increases from the 3T6 to the R50 to the R1000 cell line, the ratio of Exon 1 signal to *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) signal increases.

The nuclear run-on analysis allowed us to identify the general region of high transcription rate activity; the Exon 1 plasmid contains the first 125 bp downstream of the major transcription

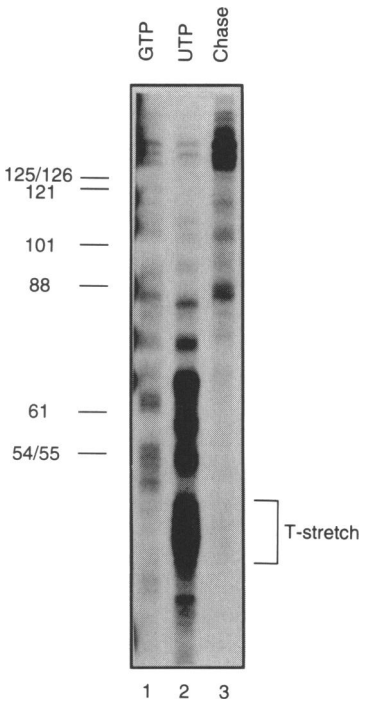

Figure 4. Localization of transcriptional pause points in the *dhfr* gene. RNA from nuclear run-on reactions that hybridized to filter slots containing the *dhfr* Exon 1 plasmid was eluted as described in Materials and Methods and run on denaturing polyacrylamide gels. The products in lanes 1 and 2 were produced in reactions that contained GTP or UTP, respectively, as the limiting nucleotide. The reaction that was eluted and run in lane 3 was identical to the reaction in lane 2, except that a 10 min chase with 240 μM UTP followed the labeling period. The positions of DNA size markers are shown to the left of the panel with the lengths in base pairs indicated.

initiation site. To define more precisely the transcriptional block point(s) we adopted a method described by Rougvie and Lis (8). Run-on RNA from R1000 nuclei that hybridized to the *dhfr* Exon 1 plasmid was eluted from the filter slot and electrophoresed on a denaturing polyacrylamide gel (Fig. 4). Our standard run-on reaction, with UTP as the limiting nucleotide, produced a series of discrete, labeled *dhfr* RNAs, ranging in size from 45 to 86 nt (lane 2). The highest concentration of 3′ ends mapped to a stretch of thymidine residues in the 5′ untranslated region (T-stretch). We next asked whether the same block points were recognized when another NTP was limiting in the run-on reaction. The Exon 1 signal was much higher than the Exon 6 signal on filter slots when either GTP or UTP was limiting (data not shown), but the eluted pattern from Exon 1 was clearly different (lanes 1 and 2). In particular, polymerase did not pause at the T-stretch when GTP was limiting. Thus we were unable to identify an attenuation site that was insensitive to the conditions of the assay, suggesting that there is no dominant structure or recognition site in the sequence that defines the pause points.

The elution method also enabled us to determine that the short RNAs generated in the run-on reaction were pause products. When a reaction identical to the reaction in lane 2 was chased with an excess of cold UTP (lane 3), the RNAs were elongated past the end of the Exon 1 probe. Pause sites have been identified and studied in other genes using *in vitro* transcription systems (16, 17). We also generated RNAs in a modified *in vitro* transcription system that resemble the RNAs we identified from the eluted run-on material (data not shown). However, we were unable to identify attenuated *dhfr* RNAs in cells using an RNase protection assay (data not shown). Thus, we have determined that the region of high transcription rate activity observed in the nuclear run-on reaction is contained within Exon 1, that polymerase pauses but does not terminate in this area, and that the precise pause points observed depend on the limiting nucleotide in the reaction.

Table 1. Growth regulation of the *dhfr* gene in NIH 3T3WTLuc cells

	I	II
	Fold induction (stimulated/starved)	
Luciferase assay *dhfr*-luc	3.93	7.80
Run-on assay *dhfr* Exon 1	0.42	0.78

NIH 3T3 cells that were stably transfected with a portion of the *dhfr* promoter linked to a luciferase reporter gene were used to measure the transcriptional activity of the murine *dhfr* promoter in serum-starved and -stimulated cells. The transcription rate was measured in side-by-side nuclear run-on and luciferase assays in two independent experiments. Measurements were taken at 0 h (starved) and 12 h (stimulated) following serum stimulation of cells that were starved for about 60 h. The *dhfr* Exon 1 run-on signals were normalized to the signal hybridizing to an actin probe to allow a comparison between the 0 h and 12 h signals.

The nuclear run-on and luciferase reporter assays yield different conclusions concerning the growth regulation of the *dhfr* gene

We next tested whether the nuclear run-on assay could detect transcriptional regulation of the *dhfr* gene in non-amplified cells. Previously, Means *et al.* showed that a *dhfr* promoter spanning from −270 to +20 confers serum responsiveness to a luciferase reporter gene in NIH 3T3 cells (3). A double point mutation in the promoter at −2 and −6 abolishes the G1/S-phase increase, and thus indicates a transcriptional mechanism for the serum response. We performed side-by-side nuclear run-on analysis and luciferase assays on NIH 3T3 cells that were stably transfected with the mouse *dhfr* promoter linked to a luciferase reporter gene. The luciferase assay measured increased activity of the *dhfr* promoter 12 h after serum stimulation of quiescent cells, at the G1/S-phase boundary (Table 1). However, the nuclear run-on assay failed to demonstrate a transcriptional increase of the endogenous *dhfr* gene. In fact, the transcription rate in *dhfr* Exon 1 actually decreased in stimulated cells relative to starved cells. The transcription rate in the 3′ end of the gene was still below the limit of detection, suggesting that no change in read-through of a transcriptional block had occurred. Run-on analyses in NIH 3T3 cells that were not stably transfected with the *dhfr*-luc construct also failed to demonstrate serum regulation of the endogenous *dhfr* gene (data not shown).

Comparison of the nuclear run-on and pulse-labeling assays in transcription rate analysis of proliferating 3T6 R50 cells

Due to the inability of the run-on assay to reproduce the results of the reporter gene assay we compared the nuclear run-on assay to another technique for measuring transcription rate: pulse-labeling of intact cells. The radioactive labeling of new transcripts occurs in intact cells during pulse-labeling and in isolated nuclei in the run-on procedure. Proliferating 3T6 R50 cells were used

Table 2. Transcription rate analysis of the *dhfr* and actin genes in the nuclear run-on and pulse-labelling assays

	run-on		pulse-labelling	
	I	II	I	II
Exon 1/Exon 6	14.2	12.5	1.30	1.01
Exon 1/actin	1.00	0.73	0.079	0.071
Exon 6/actin	0.080	0.070	0.081	0.096

Results from two nuclear run-on and two pulse-labelling analyses of proliferating 3T6 R50 cells are shown. To allow a comparison of transcription rates at the 5′ vs. 3′ ends, the *dhfr* signals were normalized according to the T-content of each probe.

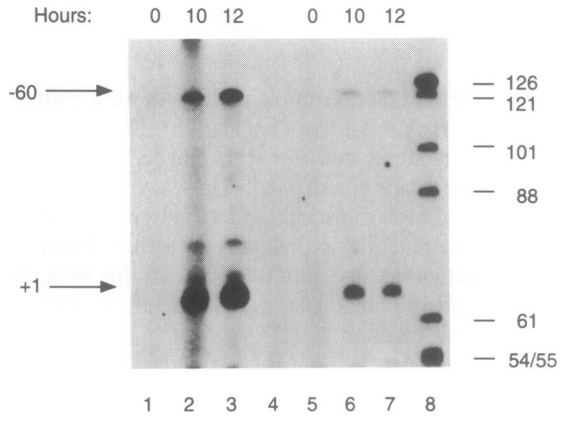

Figure 5. Primer extension analysis of *dhfr* mRNA in serum-starved and -stimulated 3T6 R50 cells. 2 μg cytoplasmic 3T6 R50 RNA from starved (0 h) or stimulated (10 and 12 h) cells were analyzed as described in Materials and Methods. The RNA samples were prepared from the same cells that were analyzed by nuclear run-on in Table 3. The reaction in lane 4 contained 2 μg yeast tRNA. DNA size markers were run in lane 8. The bands that correspond to the major (+1) and minor (−60) *dhfr* transcription initiation sites are indicated with arrows.

because pulse-labeling analysis cannot detect *dhfr* transcription in non-amplified cells. Again, we observed the large difference in transcription rates between the 5′ and 3′ ends of the *dhfr* gene in the run-on assay (Table 2). However, a 30 min pulse-labeling reaction revealed a very similar transcription rate at the two ends of the *dhfr* gene. We also compared the *dhfr* signals to the actin signal generated in the two assays. While the Exon 6 to actin ratio was similar in both assays, the Exon 1 to actin ratio was about ten-fold higher in the run-on than in the pulse-labeling assay. These results suggest that the high *dhfr* Exon 1 signal detected in the run-on assay is artifactual, and that the Exon 6 signal is a more accurate measure of the *dhfr* transcription rate. We propose that the process of isolating nuclei from cells allows polymerases at the 5′ end of the *dhfr* gene to be inappropriately released in the run-on assay. Radioactive RNA is generated from these released polymerases which then pause when the concentration of limiting nucleotide falls below a critical level. These complexes appear to be released upon isolation of nuclei whether cells are quiescent, stimulated, or cycling.

Nuclear run-on analysis of the *dhfr* gene in serum-starved and -stimulated 3T6 R50 cells

Because the run-on signal in the 5′ end of the *dhfr* gene was always high regardless of growth state, we asked whether the nuclear run-on assay could measure a change in the transcription rate in the 3′ end of the *dhfr* gene. To measure run-on signal in the 3′ end of the *dhfr* gene amplified cells must be used; we used the 3T6 R50 cell line because it was used in previous pulse-labeling experiments to document a seven-fold transcription rate increase of the *dhfr* gene at the G1/S-phase boundary (2). We developed a serum-starvation and -stimulation regimen for 3T6 R50 cells that is similar to the protocol we employed with the NIH 3T3 cells, except that the cells were starved for a longer period of time. Flow cytometric analysis indicated that the 3T6 R50 cells synchronously exited G0 phase following serum stimulation with kinetics similar to the NIH 3T3 cells (data not shown) and primer extension analysis revealed that endogenous *dhfr* mRNA levels increased following serum stimulation (Fig. 5). The results of nuclear run-on analysis from starved and stimulated cells is shown in Table 3. The fold-induction measured in *dhfr* Exon 1 was less than two-fold, but the transcription rate increase observed in the 3′ half of the gene was five- to seven-fold. Therefore, the *dhfr* gene is transcriptionally regulated in 3T6 R50 cells following serum stimulation of quiescent cells. We conclude that the nuclear run-on assay can measure a transcription rate increase in the murine *dhfr* gene, but the rate cannot be measured at the 5′ end of the gene, and thus an amplified cell line must be studied.

Table 3. Growth regulation of the *dhfr* gene in 3T6 R50 cells

	I	II
	Fold induction (stimulated/starved)	
dhfr Exon 1	1.95	1.75
dhfr Exon 4 + 5 + 6	6.46	4.82

The transcription rate was measured in the 5′ and 3′ ends of the *dhfr* gene in serum-starved and -stimulated 3T6 R50 cells in two independent experiments. Measurements were taken at 0 h (starved) and 12 h (stimulated) following serum-stimulation of cells that were starved for 6 days. To allow a comparison of the 0 h and 12 h signals, the *dhfr* signals were normalized to the signal hybridizing to the *gapdh* probe.

DISCUSSION

In all cell lines we have examined, the nuclear run-on assay measured a much higher transcription rate in the 5′ end of the murine *dhfr* gene than in the 3′ end. This finding could be interpreted as evidence for transcriptional attenuation near the Exon 1/intron 1 boundary within the *dhfr* gene. However, we do not favor this attenuation hypothesis for the following reasons: (1) the transcription rate throughout the *dhfr* gene was uniform when measured by pulse-labeling analysis of intact cells; (2) although we could isolate short, paused *dhfr* RNAs from the nuclear run-on reaction, we were unable to identify attenuated products in cellular RNA. Thus, the results obtained in cells are different than those obtained using the nuclear run-on procedure.

Comparison of the signals generated in the run-on and pulse-labeling assays with 3T6 R50 cells shows that the *dhfr* Exon 1 signal is disproportionally high relative to the signal that is detected with an actin probe (Table 2), but that the Exon 6 to actin ratio is relatively constant in both assays. We suggest that the signal detected by the Exon 6 probe is a more accurate measure of the transcription rate of the *dhfr* gene. This finding is consistent with the idea that during either nuclear isolation,

or the run-on reaction, the transcription complexes formed at the *dhfr* promoter are altered such that during the run-on reaction the polymerases are inappropriately released from the *dhfr* promoter. The polymerases then pause at discrete sites based on the sequence of the gene and the nucleotide composition of the reaction. Due to a very limiting concentration of the labeled nucleotide, the high transcription rate region is confined to the extreme 5′ end of the *dhfr* gene.

Because initiation does not occur in the run-on assay, the complexes at the *dhfr* promoter probably are transcriptionally engaged. We have shown that inclusion of sarkosyl or high salt in the run-on reaction does not decrease the signals detected by the *dhfr* plasmids. These conditions block initiation *in vitro* (18, 19). In eukaryotic cells, the phenomenon of transcriptionally engaged and paused polymerase was first shown by Lis and colleagues at the *Drosphila melanogaster hsp70* promoter (8). They have since found that several other *Drosphila* promoters are also transcriptionally engaged and paused at a promoter-proximal location, suggesting that this may be a common phenomenon (20). Groudine and co-workers have also characterized a pause site at +30 in the human c-*myc* gene (21).

The difference in transcription rate between the 5′ and the 3′ ends of the *dhfr* gene as measured by the run-on assay is similar in magnitude to the transcription rate increase of the *dhfr*-luc construct at the G1/S-phase boundary, suggesting that isolation of nuclei may activate *dhfr* transcription complexes that normally are activated specifically at the G1/S-phase boundary. In support of this hypothesis, in the non-amplified 3T3 WTLuc cell line, the signal detected with a *dhfr* 5′ end probe did not vary with the growth state of the cell. Yet in the same cells, the activity of the *dhfr*-luc construct increased at the G1/S-phase boundary. Despite the constitutively high 5′ *dhfr* signal observed in the run-on assay, we have used this assay to show that the *dhfr* gene is regulated at the level of transcription. An amplified cell line was used in the regulation studies because the run-on assay lacks the sensitivity to measure the transcription rate of the *dhfr* 3′ end in non-amplified cells. We found that the transcription rate in the 3′ end of the *dhfr* gene increased 5- to 7-fold following serum stimulation of starved 3T6 R50 cells. The fold induction was similar to the increase observed at the G1/S-phase boundary in proliferating 3T6 R50 cells when the transcription rate was measured by pulse-labeling analysis (2).

The high *dhfr* 5′ end signal that is observed regardless of growth state of the cells suggests that nearly complete transcription complexes are present on the *dhfr* promoter at all times. Miller and co-workers have shown that Sp1, which is required for *dhfr* transcription, is bound to the *dhfr* promoter regardless of growth state (personal communication). During formation of the transcription complex, Sp1 likely tethers the rest of the transcription complex to the DNA of the *dhfr* promoter via interactions with TFIID (22–25). The E2F site that overlaps the major *dhfr* transcription initiation site is necessary for the transcription rate increase of the *dhfr* promoter following stimulation of serum-starved cells (3). The E2F DNA binding activity is a heterodimer that also binds the retinoblastoma protein, p107, p130, and several different cyclins (reviewed in 26). We do not yet know which components of the cell cycle regulatory complexes are present in the *dhfr* transcription complex, and how the composition of the complex may change with the growth state. Perhaps a repressor protein that keeps *dhfr* expression low during most of the cell cycle is perturbed by the run-on assay. Conversely, the assay may obviate the need for a specific activator of *dhfr* transcription. These possibilities are not mutually exclusive.

In the light of these results, we would like to review several previous studies of the *dhfr* gene that employed the nuclear run-on assay (see 10 for a more complete discussion). Schmidt and Merrill used the run-on assay (27) to measure a 7- to 30-fold decrease in transcription rate of most of the *dhfr* gene following differentiation of a mouse muscle cell line. However, the transcription rate at the extreme 5′ end of the gene was unchanged by differentiation. Schmidt and Merrill's documentation of the high, unchanging transcription rate at the 5′ end of the *dhfr* gene closely parallels our findings. Yoder and Berget (28) concluded that the human *dhfr* gene is post-transcriptionally regulated following infection of cells with adenovirus because nuclear run-on assays did not detect a change in transcription rate after infection. Yet, pulse-labeling studies did show an increase in synthesis of *dhfr* mRNAs. Given our findings on the shortcomings of the nuclear run-on assay we would give more credence to the results of the pulse-labeling analysis. Feder *et al.* (11) separated neonatal thymocytes into cell cycle stage-specific populations using centrifugal elutriation, then measured transcription rate of the *dhfr* gene with the nuclear run-on assay. Using a probe that contained the 5′ end of the *dhfr* gene, they found that the transcription rate did not vary with cell cycle position, in agreement with the results we have presented in this paper. However, because of our results using the *dhfr* 3′ end probe, we feel that the conclusion of Feder and co-workers that the *dhfr* gene is not transcriptionally regulated during the cell cycle should be re-evaluated.

In summary, the transcription rate analysis of the *dhfr* gene is subject to several constraints. Either pulse-labeling or run-on assays must be carried out in amplified cell lines, and the rate must be measured in the 3′ end of the gene in the run-on assay. We have found that a useful alternative to these procedures is to transfect cells with a luciferase reporter gene linked to the promoter and to follow activity of the reporter gene. Although we do not know whether similar problems apply to the analysis of other genes, the assay we have used is a standard protocol that has been used by many investigators. We encourage others to verify the results of nuclear run-on analysis with an independent assay, particularly when run-on analysis suggests a lack of transcriptional regulation.

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