Effect of Differential Polyadenylation and Cell Growth Phase on Dihydrofolate Reductase mRNA Stability*

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We have constructed tetracycline-responsive dhfr minigenes and transferred them to a Chinese hamster ovary cell DHFR-deficient deletion mutant to obtained cells in which dhfr transcription can be repressed by tetracycline (tet-off). DHFR mRNA half-life measured after the repression of transcription by tetracycline in these transfectants is about 1.5 h, which is significantly shorter than previously reported. In addition, we observed that DHFR mRNA is less stable in serum-starved cells than in exponentially growing cells. Given that the dhfr gene contains multiple polyadenylation sites, we analyzed the role of polyadenylation site usage on the stability of the resultant mRNA molecules. We found that DHFR mRNA is more stable when a strong polyadenylation site is used. Finally, we have observed that the relative lengths of the poly(A) tails for the different DHFR mRNA species correlated with their relative stability in growing versus resting cells.

Dihydrofolate reductase (dhfr) is a housekeeping gene encoding an enzyme required for the de novo biosynthesis of glycine, the purine ring, and thymidylate. DHFR expression is regulated in the cell cycle at the level of transcription (1–3), and there is evidence for post-transcriptional regulation during growth phase transitions (4–6). It is clear that regulated variations in mRNA stability can produce dramatic changes in mRNA levels without modification in the rate of gene transcription. For DHFR mRNA, the observed increase in dhfr transcription during the transition from G₀ to exponential growth is not sufficient to account for the increase of the mRNA levels under the same conditions (7). For this reason, we have examined the role of mRNA stability as a contributor to the relatively low levels of DHFR mRNA seen in resting cells. Toward this end, we have taken advantage of the tetracycline-responsive promoter developed by Gossen and Bujard (8). The tetracycline-responsive system allows for selective inhibition of transcription of the gene under the control of the responsive promoter without affecting the transcription machinery of the cell. Given that the hamster dhfr gene contains multiple polyadenylation sites (9) and that differential polyadenylation (pA) can affect the cell growth regulation of DHFR mRNA (5), we have analyzed the role of polyadenylation site usage in the stability of the resultant mRNA molecules. Using tetracycline-responsive dhfr minigenes, we have determined that DHFR mRNA half-life is significantly shorter than previously reported, that DHFR mRNA stability varies according to polyadenylation site usage, and that this mRNA is less stable in serum-starved cells.

MATERIALS AND METHODS

Cell Culture—Conditions for the monolayer culture of CHO cells have been previously described (10). CHO-K1, DG44 (10), and G7 cells (11) were grown in Ham's F-12 medium supplemented with 0.2% fetal calf serum (Life Technologies, Inc.) and incubated for 7 days.

Plasmid Construction—The starting construct, plasmid pDCH1P11, was constructed by cloning the Smal–HindIII fragment from hamster minigene pDCH2 (12), into the Smal–HindIII sites of the cloning vector pBSF2 (Promega). pDCH1P11 contains approximately 400 bp of the promoter region in the dhfr 5′–flank, the DHFR coding sequence including intron 1 as a sole intron, and the first polyadenylation site of the dhfr gene. To construct dhfr minigenes under the control of a modular promoter, we used the tetracycline-responsive system described by Gossen and Bujard (8). In this system, a hybrid phage-VP16 transcriptional activator (the tetracycline-responsive transactivator (tTA)), encoded by pUHD15-1 activates transcription of the gene of interest under the control of a chimeric promoter (provided by the vector pUHD10-3), consisting of basal elements of the cytomegalovirus early promoter preceded by operator sequences of the tetracycline resistance gene. In the presence of tetracycline, tTA cannot bind DNA, and the transcription of the subcloned gene is turned off (tet-off).

The following dhfr minigenes were constructed. (a) pUD1S contained a 1826-bp AvrII–HindIII fragment from pDCH1P11 cloned into the XbaI site of vector pHUD10-3. By cutting with AvrII, which falls in the 5′–UTR of dhfr, the major transcriptional start of the dhfr gene is excluded. (b) pUD1 lacked the SV40 late polyadenylation sequence contributed by pHUD10-3. pUD1 was constructed by digesting pUDIS with SpI and NarI to remove the region containing the SV40 polyadenylation sequence from the vector, and the protruding ends were blunted with T4 DNA polymerase prior to the ligation. The first dhfr polyadenylation site (pA1) is retained in pUD1. (c) pUDD1 containing the major transcriptional start site from the hamster dhfr gene promoter. pUD1 was digested with EcoRI to remove a 447-bp fragment extending from the EcoRI site in the polyadenylating region of the vector to the beginning of dhfr exon 2. A 509-bp fragment from dhfr minigene pDCH1P11 was PCR-amplified using a 5′ primer of a modified sequence to produce an EcoRI site just upstream of the major transcriptional start of dhfr (5′-EcoRI, 5′-CGGCCGATATCTAATTTCGCG-3′) and a 3′ primer from a sequence in dhfr exon 2, located downstream of the EcoRI site (3′-EcoRI, 5′-CACCACAGTAAGAAACCTTCATG-3′). After digestion of the PCR fragment with EcoRI, the 472-bp product was cloned.

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§ The abbreviations used are: dhfr and DHFR, dihydrofolate reductase; CHO, Chinese hamster ovary; tet, tetracycline; pA, polyadenylation site; RT, reverse transcriptase; PCR, polymerase chain reaction; APRT, adenine phosphoribosyl transferase; UTR, untranslated region; bp, base pair(s); nt, nucleotide(s).
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between the EcoRI sites of pUD1. pUD1 contains the dhfr promoter region/transcriptional start site and the first dhfr polyadenylation site (pA1). (d) pUD123 contains all three hamter dhfr polyadenylation sites. pUD1 was digested with BamHI and HindIII to eliminate the sequence containing the first polyadenylation site from dhfr. A 1.4-kilobase fragment containing the dhfr polyadenylation site was PCR-amplified from plasmid pMG2 as template (13) using Pveo thermostable polymerase with proofreading activity. The 5′ primer was a sequence containing the BamHI site upstream of the first polyadenylation site (pA1) (5′-GAACCTGGATCCCTGCTGAC-3′), and the 3′ primer was a modified sequence containing a HindIII site downstream of the dhfr/polyadenylation site (pA3) (5′-GCGTCTAAGGTTAAGCTTACTACTGTTG-3′). After digestion of the PCR fragment with BamHI and HindIII, the digested sequence was cloned between the BamHI and HindIII sites of pUD1.

Transfection—Two micrograms of the tetracycline-controlled trans-activator (tTA) plasmid pUHD15-1 (8) were cotransfected with 0.4 μg of plasmid BPVNeo (14) into dhfr-CHO DG44 cells (10) by the calcium phosphate method (15) using calf thymus DNA (Amersham Pharmacia Biotech) as carrier. After 14 days of selection for G418 resistance (400 μg of the active compound per ml of medium; Life Technologies, Inc.), the surviving colonies were pooled and used as recipient cells (DG44-tTA) for transfection with the tetracycline-responsive dhfr minigenes. Permanent transfecants of dhfr minigenes were obtained by transfection with at least 20 μg of each construct and selection in calcium phosphate method. DHFR selection was performed in Ham’s F-12 medium without hypoxanthine supplemented with 7% dialyzed calf serum for 8 days. Several well isolated colonies were then picked using cloning rings. For each construct, nine individual clones (three for each amount of DNA used in the transfection) were analyzed by RT-PCR to determine DHFR mRNA levels in the absence of tetracycline or after 24 h of treatment with 1 μg/ml concentration of the antibiotic. The transient clone of each construct that exhibited a substantial tetracycline response and a high level of DHFR mRNA expression was chosen for further studies.

mRNA Analysis by RT-PCR—mRNA levels were determined by quantitative RT-PCR using total cell lysates as the starting material for the RT reaction. 5000 cells were plated in 35-mm diameter dishes in Ham’s F-12 medium; 15 h later, the cells were treated with tetracycline (1 μg/ml) for different periods of time. After the treatments, the cells were harvested simultaneously by trypsinization, washed once with ice-cold phosphate-buffered saline, and resuspended in 11.25 ml of diethyl pyrocarbonate-treated water. The samples were heated at 80 °C for 5 min and chilled on ice, and the remaining components of the RT reaction mixture containing 125 ng of random hexamers (Promega), 10 mM dithiothreitol, 20 units of RNasin (Promega), 0.5 mM dNTPs (Promega), 4 μl of 5′ RT buffer, and 200 units of Superscript RNase H− reverse transcriptase (Life Technologies, Inc.). The reaction mixture was incubated at 37 °C for 60 min. Five μl of the cDNA mixture were used directly for PCR amplification.

PCR was technically carried out as described (7) using 2 units of Taq polymerase (Perkin-Elmer) for each reaction and [α-32P]dATP to label the PCR product. The primers used were as follows: 5′-AAGAAGGGGAGACCTTTTCCCTC-3′ (5′ DEX1) in exon 1 or 5′-GCGAAGAATTGTTTGTAGGAGACG-3′ (5′ DEX2) in exon 2 and 5′-TCACAGGCTACACCATCTA-3′ (A3′Ex5A) in exon 5 for APRT mRNA, used as an internal control. PCR was performed for 22 cycles after 1-min denaturation at 94 °C; each cycle consisted of denaturation at 92 °C for 30 s, primer annealing at 59 °C for 75 s, and primer extension at 72 °C for 110 s. Five μl of each PCR sample were electrophoresed in a 5% polyacrylamide gel. The gels were dried, and the radioactive bands were quantified using a PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Northern Blot Analysis—Total RNA was extracted from the different clones using the UltraSpec™ RNA reagent (Biotex) in accordance with the manufacturer’s instructions. Twenty μg of total RNA were run in 0.8% formaldehyde agarose gels and transferred to a Zeta-Probe GT blotting membrane (Bio-Rad) using 50 mM NaOH as transfer solution. After hybridization with a DNA probe containing 858 bp from 189 bp upstream of the first polyadenylation site (pA1) to 162 bp downstream of the second polyadenylation site (pA2) of the hamter dhfr gene, generated by PCR. The PCR product was gel-purified, and 25 ng were 32P-labeled using the Prime-a-Gene system (Promega). The hybridization was performed in a solution containing 5× saline/sodium phosphate/EDTA, 5× Denhardt’s reagent, 50% formamide, 0.1% SDS, 5% dextran sulfate, 100 μg of sonicated herring sperm DNA/ml, and 2 × 106 cpm/ml probe at 42 °C overnight. The blots were washed once in 2× SSC plus 0.1% SDS and once in 1× SSC plus 0.1% SDS at 65 °C for 30 min each and dried and exposed to x-ray film.

Poly(A) Length Analysis—Poly(A) length analysis of the different minigenes was performed by RT-PCR following the method of Salle`s and Strickland (16) with modifications. Two micrograms of total RNA from each clone were used in the RT reaction with 1 μg of primer of an arbitrary sequence followed by oligo(dT)16. The RNA and the primer were heated at 80 °C for 5 min, cooled to room temperature (0.5 °C/ min), and then placed at 4 °C. The remaining components of the RT reaction mixture containing 10 μl diethiothreitol, 20 units of RNasin (Promega), 0.5 μl dNTPs, 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The RT reaction was performed for 5 min at 4 °C, 5 min at room temperature, 5 min at 37 °C, and 45 min at 42 °C. Five μl of the RT reaction were then used in the PCR, which was performed with a 5′ primer upstream of either the pA1, pA2, or pA3 site of the dhfr gene and the arbitrary sequence of the RT primer as 3′ primer. PCR was carried out as described above for 30 cycles, and the PCR products were electrophoresed and visualized by autoradiography. The length of the poly(A) tails was determined from the length of the smear of the PCR products.

RESULTS

Establishment of DHFR Stable Transfectants Responsive to Tetracycline—To study the stability of DHFR mRNA, we constructed several versions of dhfr minigenes under the control of a tetracycline-responsive promoter, which is a chimeric promoter consisting of several copies of tet operator sequences from Escherichia coli and the minimal immediate early promoter of human cytomegalovirus (8). The initial step was to establish stable transfecants that produced the tTA in CHO DG44 cells. After transfection of DG44 cells with plasmid pUHD15-1, encoding tTA, selection with G418 was applied, and the surviving colonies were pooled. This pool (DG44-tTA) was the recipient in transfections with tetracycline-modulatable dhfr minigenes. In the absence of tetracycline, the hybrid transactivator will specifically stimulate transcription of the promoters containing tet operator sequences. In the presence of the antibiotic, tTA will be unable to bind to the promoter sequences, disabling the expression of the gene under the control of this promoter. Transfectants that received the tetracycline-responsive dhfr minigenes were selected for DHFR activity in medium lacking hypoxanthine as well as tetracycline and subsequently individual clones (usually nine) were culled. To test for responsiveness to tetracycline inhibition, DHFR mRNA levels in the individual clones were analyzed after growth in the absence or in the presence of 1 μg/ml tetracycline for 24 h. This analysis was performed by quantitative RT-PCR using cell lysates as the starting material. In all cases, the clone showing the highest effectiveness of tetracycline regulation of dhfr gene expression was chosen for the determination of DHFR mRNA half-life in exponentially growing and serum-starved cells.

Actinomycin D Stabilizes DHFR mRNA—All of the dhfr constructs contain the complete protein coding sequence for DHFR initiated by the 300-nt dhfr intron 1 as the only intron; they differ at their 5′ and/or their 3′ ends. The first construct transfected in DG44-tTA cells was pUD1S, which contains the first of three dhfr polyadenylation sites followed by an SV40 late polyadenylation site. After selection for DHFR activity in medium lacking hypoxanthine, eight individual clones were analyzed for their responsiveness to tetracycline inhibition. All of
the clones analyzed were sensitive to tetracycline inhibition (Fig. 1A); clone A3-5 showed the greatest reduction (70-fold) in DHFR mRNA levels and was selected for further studies. The determination of mRNA levels was always performed by RT-PCR directly from cell lysates immediately after each treatment. That this determination is quantitative is shown in Fig. 1B. In this experiment, an increasing number of cells from clone A3-5 were subjected to RT-PCR, and the DHFR mRNA levels were determined by the ratio between the DHFR and APRT radioactive signals. The APRT mRNA being used as an internal endogenous gene control. The correlation coefficient between the number of cells plated and the level of DHFR mRNA detected was 0.98.

We proceeded to determine the half-life of DHFR mRNA for clone A3-5 in exponentially growing cells. We performed time course experiments by determining the remaining DHFR mRNA levels in cells treated with tetracycline for different periods of time. After the addition of tetracycline, DHFR mRNA decayed with exponential kinetics over the next 6 h with a half-life of 124 min (Fig. 2). APRT mRNA levels, used as an internal control in the RT-PCR, were not affected by the tetracycline treatment. The half-life of DHFR mRNA from pUD1S was also estimated from the time required to achieve a new steady-state level after induction. The DHFR mRNA half-life was calculated from the exponential curve fit calculated with Cricket Graph III.

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The DHFR mRNA half-life in clone A3-5 represented a decay
rate about 4 times greater than that measured for the endogenous gene in CHO UA21 cells in the presence of actinomycin D (17). We repeated these measurements of the decay rate of endogenous DHFR mRNA in CHO cells in the presence of actinomycin D using our RT-PCR methodology. The DHFR mRNA half-life was 8.5 h (Fig. 3) in agreement with the previous determination in CHO UA21 cells by Northern blot analysis. That difference between the tetracycline and the actinomycin D experiments could be due to the modifications in the 5'-UTR and 3'-UTR in the pUD1S minigene with respect to the endogenous gene or to the method used to inhibit transcription. Actinomycin D treatment has been reported to stabilize specific mRNAs against normal degradation (18, 19). We therefore measured the decay rate of the DHFR mRNA encoded by the tetracycline-responsive minigene in the presence of actinomycin D. As shown in Fig. 3, actinomycin D stabilized DHFR mRNA in clone A3-5, yielding a half-life of over 11 h, a value 5-fold greater than the half-life determined using tetracycline.

In the course of measuring mRNA decay rates in the presence of actinomycin D, we noted that DHFR mRNA produced by a dhfr minigene was less stable than that produced by the endogenous gene (Fig. 3). G-7 cells are CHO transfectants carrying the dhfr minigene pDCH1P (11), which is driven by the dhfr promoter but contains only the first of the three dhfr polyadenylation sites (9). The half-life of DHFR mRNA in G-7 cells using actinomycin D was 2.6 h (Fig. 3) versus 8.5 for the endogenous gene in CHO cells. This result suggested that the different stability in actinomycin D of the endogenous DHFR mRNA and the mRNA in G-7 cells could reside in the different 3' ends of the mRNA molecules. The greater DHFR mRNA half-life determined for clone A3-5 using actinomycin D could be due to the presence of the SV40 polyadenylation signal included as part of the original vector.

An SV40 Polyadenylation Site Stabilizes DHFR mRNA—To test the possibility that differences in polyadenylation site usage could be involved in DHFR mRNA stability, we eliminated the sequence that includes the SV40 polyadenylation site in pUD1S. We transfected this new construct, pUD1, that maintained only the first polyadenylation site from the dhfr gene, into the DG44-tTA pool. Following the same methodology as with clone A3-5, clone B1-4 was chosen, since it gave the highest response to tetracycline inhibition of dhfr gene expression (100-fold). Time course experiments after the addition of tetracycline were performed to determine DHFR mRNA half-life in this clone. As shown in Fig. 4, clone B1-4 exhibited a DHFR mRNA half-life of 50 min, compared with 124 min when the SV40 polyadenylation site was present. Thus, deletion of the SV40 polyadenylation site caused a decrease by half in stability of DHFR mRNA.

The transcriptional start of the dhfr gene was absent in these tetracycline-responsive minigenes (clones A3-5 and B1-4). With the idea that sequences in the 5'-UTR could affect mRNA stability, we constructed a third minigene that contained the major transcriptional start from the hamster dhfr gene in addition to the first polyadenylation site. This minigene,
pUDD1, was transfected into the DG44-tTA pool, and clone C3-1, with the highest response to tetracycline inhibition (54-fold), was chosen to determine DHFR mRNA stability. DHFR mRNA half-life was 39 min in clone C3-1, close to the value of 50 min produced by pUD1 in clone B1-4 (data not shown). Thus, DHFR mRNA was not stabilized by inclusion of 5′-UTR sequences.

**dhfr Polyadenylation Sites 2 and 3 Stabilize the mRNA**—The endogenous CHO dhfr gene contains three polyadenylation sites (9) that could be affecting the stability of the resulting mRNA molecules. For this reason, we constructed a fourth minigene, pUDD123, that contains the sequence corresponding to the three polyadenylation sites (pA1, pA2, and pA3) from the hamster dhfr gene (as well as the dhfr 5′-UTR). This new construct was transfected into the DG44-tTA pool, and clone D3-3, with a 37-fold tetracycline deinduction, was chosen to determine DHFR mRNA stability. As shown in Fig. 5, the DHFR mRNA half-life for this clone was 94 min. The restoration of the three polyadenylation sites from the endogenous dhfr gene increased DHFR mRNA half-life 2-fold with respect to the transfectants containing only the first dhfr polyadenylation sites (39- and 50-min half-lives).

**Polyadenylation Site Usage in the Different Clones**—To determine whether DHFR mRNA stability was affected by the cell growth phase, we analyzed DHFR mRNA half-life for the different constructs in serum-starved cells. Cells from the different clones were maintained in Ham’s F-12 medium supplemented with 0.2% fetal calf serum for 7 days; resting cells produced in this way maintained their viability and resume growth synchronously upon serum addition (7). The resting cells were then treated with tetracycline for different periods of time to determine the mRNA half-life (Fig. 7). For clone A3-5 (pUD1S, containing pA1 and the SV40 site) and clone C3-1 (containing pA1 only), the inhibition of DHFR transcription by tetracycline in starved cells was delayed for a period of 2 h (Fig. 7A). DHFR mRNA half-life was determined in the region of the plot corresponding to the exponential decay, obtaining a value of 60 min. This represents a 2-fold reduction in DHFR mRNA half-life as compared with exponentially growing cells (124-min half-life). For clones B1-4 and C3-1 (containing pA1 only), the DHFR mRNA half-lives in starved cells were 50 min and 37 min, respectively (Fig. 8, B and C), not different from the DHFR mRNA half-life in exponentially growing cells (half-lives of 50 min and 39 min, respectively). In starved cells of clone D3-3 (containing pA1, pA2, and pA3), the half-life was 65 min (Fig. 7D) versus 94 min in exponentially growing cells. These results suggested that the presence of a strong polyadenylation site, such as the SV40 polyadenylation site or the second and/or third polyadenylation sites from the dhfr gene conferred cell growth regulation on DHFR mRNA.
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Poly(A) Length of DHFR mRNA in Exponentially Growing and Starved Cells—Deadenylation of mRNA often precedes degradation (21, 22); thus, we might expect to see a correlation between the average length of the poly(A) tail and the mRNA half-life. To determine the poly(A) length in DHFR mRNA, a method based on RT-PCR was used (16). The RT reaction was performed using a primer with two parts: a 3′ oligo(dT) sequence that can initiate polymerization along the length of the poly(A) tail and a 5′ arbitrary sequence to be used for subsequent PCR. The PCR was carried out with a 5′ primer specific for DHFR and a 3′ primer corresponding to the arbitrary sequence of the RT primer. Under these conditions, using different 5′ primers upstream of the different polyadenylation sites, the length of the smear of the amplified products indicates the length of the poly(A) tail in each case. We analyzed the poly(A) length for polyadenylation sites in RNA molecules from exponentially growing cells and starved cells. For the construct pUD1S (clone A3-5), the poly(A) tail at the SV40 polyadenylation site was 120 nt in exponentially growing cells and was reduced to 40 nt in starved cells (Fig. 8B). Using construct pUDD123 (clones D3-3), the poly(A) tail was 60 nt in exponentially growing cells and 15 nt in starved cells (Fig. 8B); and the poly(A)3 tail was 145 nt in exponentially growing cells and 35 nt in starved cells (Fig. 8C). In the case of constructs pUD1 and pUDD1 (clones B1-4 and C3-1), containing only pA1, the poly(A) tails were 65 and 60 nt, respectively, in exponentially growing cells, and they remained practically unchanged in starved cells (Fig. 8A). Thus, molecules polyadenylated at dhfr sites 2 and 3 and the SV40 site are longer in exponentially growing cells compared with starved cells; the same molecules are also more stable in exponentially growing cells (Figs. 2 and 6 versus Fig. 7). In contrast, molecules polyadenylated at dhfr site 1 were of the same length in exponentially growing cells and starved cells, and their degradation rates were the same in these two conditions (Figs. 4, 5, and 7).

DISCUSSION

Our experimental approach to the measurement of RNA stability made use of some methodologies that have not yet been widely applied in the measurement of mRNA stability and that confer some advantages. First, we used tetracycline-responsive minigene, such that transcription of the target gene was specifically inhibited upon the addition of tetracycline to the transfectant cells. This method has an advantage over the use of treatments that stop all cellular transcription, since it has been known for some time that general transcriptional inhibitors such as actinomycin D can affect mRNA stability (Refs. 18 and 19 and references therein). Indeed, we show here...
that actinomycin D treatment extends the half-life of DHFR mRNA more than 5-fold (Fig. 3). Second, our quantitative RT-PCR assay was performed directly on cell lysates upon completion of each culture incubation. This elimination of an RNA extraction step made it simpler to make multiple measurements and probably reduced the variability associated with differential extraction efficiency. The reproducibility of this assay is indicated not only in the reconstruction experiment shown in Fig. 1 but also in the near constancy of the control APRT mRNA levels seen in Figs. 2–6. Finally, we measured the stability of these transgene transcripts in permanent transfecteds rather than by transient transfection. In the latter case, there is the possibility that tens of thousands of gene copies per cell will produce enough transcripts to overload the RNA degradation machinery of a cell. This danger is avoided in the cloned permanent transfecteds we used, which represent homogeneous populations carrying modest numbers of gene copies.

One conclusion reached here was that DHFR mRNA is more short lived than previously thought, with a half-life of about 1.5 h in exponentially growing cells. Previous experiments performed using actinomycin D had yielded values from 8 to 16 h depending on the cell line used (17, 24). As mentioned above, these results can be ascribed to mRNA stabilization by the treatment. Other experiments found half-lives of 6–16 h using in vivo pulse-chase radioactive labeling in dhfr-amplified cells (6, 23, 24). The long labeling times used in most of these experiments may have precluded the detection of molecules with half-lives of 1 h. Alternatively, differences in the experimental systems may underlie the apparent discrepancy.

A second conclusion from this work is that DHFR mRNA is less stable in resting cells than in growing cells and that this growth-related difference is dependent on the polyadenylation site used. Kaufman and Sharp (5) have previously reported that the addition of an SV40 late polyadenylation site to a dhfr minigene conferred cell growth regulation on dhfr gene expression in transfected mouse cells, as measured by DHFR protein expression. The relatively stronger SV40 site and the SV40 polyadenylation site downstream of an apparently weak one results in the much less frequent use of the weak upstream site (Fig. 6). Apparently, once a downstream site is used an upstream site is bypassed. The additional 3′-UTR destabilizes DHFR mRNA. The additional 3′-UTR sequence could be acting by binding sequence-specific stabilizing factors (27) or by providing stable secondary structures.

It is interesting to note that the introduction of a strong polyadenylation site downstream of an apparently weak one results in the much less frequent use of the weak upstream site (Fig. 6). Apparently, once a downstream site is used an upstream site can no longer be used. This situation is consistent with a coupling between transcription and polyadenylation, an idea supported by the recent finding that RNA polymerase II is required for polyadenylation (28–30). This interpretation implies that when a single weak site is the only one present, the many transcripts that bypass it never become polyadenylated and are presumably rapidly degraded.

Northern blot analysis of transfected cells carrying a dhfr minigene with all three of the natural polyadenylation sites (pUDH123) showed that polyadenylation site 2 was used predominantly. In contrast, in transcripts of the endogenous dhfr gene, site 3 is most often used, and site 2 is least often used.

Transfection per se does not explain this discrepancy, since transfecteds carrying a complete 25-kilobase pair genomic version of the dhfr gene faithfully reproduced the polyadenylation pattern of the endogenous gene (12). Thus, the difference is likely to reside in the structure of the minigene compared with the genomic gene, namely the lack of introns 2–5 in the minigene. Nesic et al. (31) found that sequences within the last intron of the human triosephosphate isomerase gene were necessary for efficient 3′ end formation at the unique polyadenylation site in that transcript. In the case of the dhfr minigene, many intron sequences are absent; the sole remaining intron 1 may not contain such activating sequences, or they may be too far upstream (e.g. the only 3′ splice site has four exons interposed). The dhfr case is more complicated, since there are multiple polyadenylation sites that apparently respond differently to the absence of intron sequences in the minigene. From the intensity of the signals in the Northern blots and the RT-PCR analyses, it appears that dhfr polyadenylation site 2 and the SV40 polyadenylation site are used quite efficiently despite the lack of introns 2–5.

Poly(A) length can be a determinant of mRNA stability; a shortening of the poly(A) tail is often the first step in mRNA degradation for eukaryotic mRNAs (Ref. 25 and references therein). Contrary cases have also been reported: mRNA degradation without concomitant poly(A) shortening (32) and mRNA stability despite extensive deadenylation (33). We found that the relative lengths of the poly(A) tails for the different DHFR mRNA species correlated with their relative stability in growing versus resting cells.

Poly(A) tail shortening of the DHFR mRNA occurred in resting cells in dhfr transcripts polyadenylated at the SV40 site or at dhfr polyadenylation sites 2 and 3. Transcripts in these cells also have lower stability in this serum-starved condition. On the other hand, for constructs that contain the first dhfr site as the only polyadenylation site, the short poly(A) length remains unchanged despite the cell growth phase, and the low DHFR mRNA stability is similarly unaffected by the growth conditions. These results suggest that polyadenylation site usage plays a role in the stability and regulation of the resultant DHFR mRNA molecules.

Serum-starved cells exhibit a low rate of dhfr transcription compared with growing cells, but this rate is not low enough to account for the even lower relative steady-state levels of DHFR mRNA (7). The rather rapid turnover of DHFR mRNA in resting cells revealed here, i.e. a half-life of approximately 60 min, helps explain this difference. Upon growth stimulation, there is an increase in both the transcription rate of the gene and the stability of the mRNA, resulting in an increase in the mRNA levels for DHFR as cells prepare for ensuing DNA synthesis.

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