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Identification of a New Promoter Upstream of the Murine Dihydrofolate Reductase Gene

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In vitro reactions identified a transcription initiation site located 740 nucleotides upstream of the dihydrofolate reductase translational start. Transcription from this site proceeded in the direction opposite to that of dihydrofolate reductase mRNA. Deletion mapping indicated that this new promoter can be separated from the dihydrofolate reductase promoter and that separation increased transcription at −740. Transcripts that initiate at −740 were also detected in cellular RNA, indicating that this is a bona fide transcription initiation site in vivo.

The DNA immediately upstream of the murine dihydrofolate reductase gene (*dhfr*) contains many transcription initiation signals (Fig. 1C). In addition to the major in vivo *dhfr* start site 55 nucleotides upstream of the +1 translation start codon (13), minor initiations occur at −115, −165, and −215 and between −500 and −550 (3, 12). The *dhfr* promoter also produces a series of RNAs that initiate between −247 and −308 and are transcribed in the direction opposite to that of *dhfr* mRNA (3, 5). Although the transcription pattern from this region is complicated, in vivo studies have suggested that sequences far upstream are not required for regulation of the *dhfr* gene (10). Therefore, previous in vitro transcription analyses used templates that did not extend upstream of −411 (8). In this study, we performed transcription reactions on larger templates and found a previously unidentified promoter upstream of the *dhfr* gene.

Identification of a novel RNA. A linear DNA template extending from −1010 to +376 of the murine *dhfr* gene was used in an in vitro transcription assay with HeLa nuclear extract. In addition to the expected 431-nucleotide runoff RNA initiating at the major in vivo *dhfr* start site, a 270-nucleotide RNA was produced (Fig. 1A, lane 1). Neither transcript was observed in reactions containing 2 μg of α-amanitin per ml (lane 2), indicating that both were products of RNA polymerase II.

To delimit the DNA sequences specifying transcription of the 270-nucleotide RNA, various templates were analyzed in parallel transcription reactions. RNA initiating at −55 was too short to be visualized with the −1010/+6 template, but the 270-nucleotide band was still observed (lane 3). The −867/−545 template yielded a 127-nucleotide product (lane 4), suggesting that the 270-nucleotide RNA initiates at approximately −740 and is transcribed in the direction opposite to that of *dhfr* mRNA.

Promoter deletion mapping of the −740 start site. To define more precisely the sequences required for −740 initiation, reactions were performed with templates having different extents of DNA surrounding the −740 region (Fig. 1B). A series of 5′ deletions (lanes 1 to 5) indicated that sequences upstream (with respect to the orientation of the −740 transcript) of −656 were not necessary for initiation of −740 (lane 4), but activity was lost with further deletion to −749 (lane 5). Deletion of sequences 3′ of the −740 initiation site indicated that sequences downstream of −749 could be deleted and activity was still retained (lane 7). The results obtained with the −749/−690 template indicate that the minimal promoter contains 50 base pairs 5′ and 9 base pairs 3′ of the start site.

Although the *dhfr* and −740 minimal promoters do not overlap, they do share common structural features. The 60-base-pair −740 promoter and the 80-base-pair *dhfr* promoter (P. J. Farnham and A. L. Means, submitted for publication) both include a binding site for transcription factor Spl (1) and span their transcription initiation sites. Moreover, as determined by densitometric scanning of the runoff transcripts produced from the −1010/+376 template (Fig. 1B, lane 1), the two promoters transcribe with equal efficiencies. They may compete for binding of transcription factors, perhaps Spl, since deletion of the *dhfr* promoter increases transcription from −740 (Fig. 1B, compare lanes 1 and 3) and deletion of the −740 promoter increases transcription from the *dhfr* initiation site (Farnham and Means, submitted).

Identification and characterization of cellular RNA initiating at −740. Because transcripts initiating at −740 were not detected in RNA from cells having a diploid complement of *dhfr* genes (data not shown), we developed a cell line (3T6 R1000) that has approximately 1,000 copies of the *dhfr* gene by exposure of 3T6 R50 cells (6) to increasing concentrations of methotrexate. Primer extension analysis of cytoplasmic RNA from 3T6 R1000 cells (Fig. 2, lane 6) results in bands corresponding to initiations at −740 and −746. The same bands at different relative intensities were seen when in vitro-transcribed RNA was used (lane 2). These results could indicate either microheterogeneity of initiation or partial failure of reverse transcriptase to extend to the exact 5′ end of the RNA. The primer extension results demonstrate that −740 corresponds to an in vivo initiation site and is not a splice site of a longer transcript.

When equal amounts of nuclear and cytoplasmic RNAs were analyzed, the −740 RNA was detected predominantly in nuclear RNA (Fig. 2, lanes 4 and 5). However, in a cell, only 5 to 10% of the total RNA is in the nucleus. When the amounts of nuclear and cytoplasmic RNAs that correspond to an equal number of cells were compared (lanes 4 and 6), the −740 RNA was found to be equally distributed between the nucleus and the cytoplasm. We do not know the basis for the partitioning of this RNA; however, a similar distribution has been reported for several other transcripts (14).

As determined by comparison of signal intensities in

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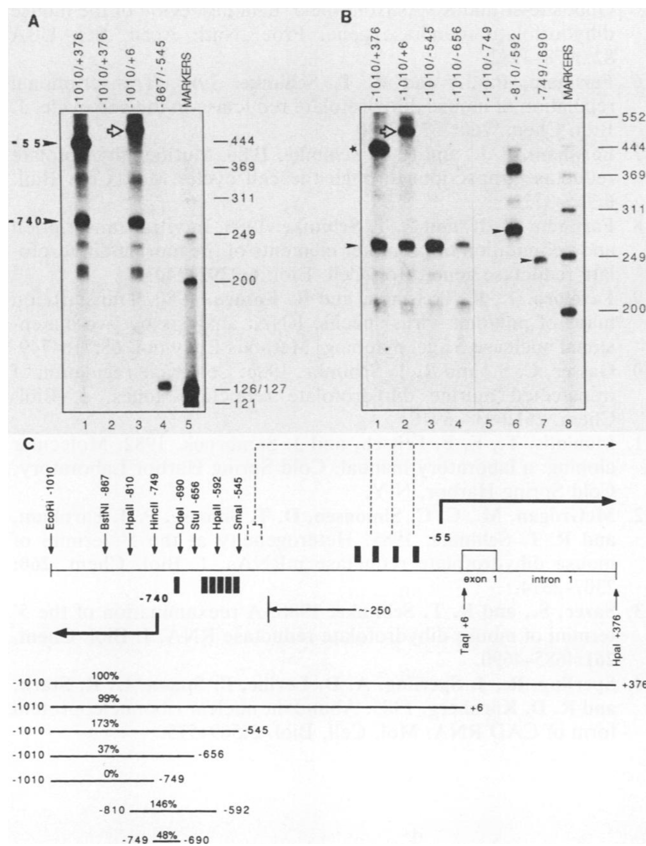

FIG. 1. (A) In vitro transcription from the *dhfr* 5′-flanking region. Runoff transcriptions were performed with 5 nM linear DNA template and 2.4 μg of HeLa cell nuclear extract (4) per μl at 24°C under previously optimized conditions (8). The 5′ and 3′ ends of the templates are indicated above each lane. The transcript initiating at the *dhfr* start site is labeled −55, and the novel 270-nucleotide transcript is labeled −740. The arrow beside lane 3 designates transcription from a secondary *dhfr* start site at approximately −500. The bands below the −740 transcript were not consistently present at high levels and were not further analyzed. Two micrograms of α-amanitin per ml was included in lane 2. Lane 5 contained DNA markers. (B) Promoter deletion mapping. Runoff transcriptions using HeLa nuclear extract were performed on the indicated templates. The expected positions of the transcript initiating at −740 are indicated by the arrowheads beside lanes 1, 6, and 7. The same-size runoff transcript was expected for lanes 1 to 5. The asterisk beside lane 1 indicates runoff transcription from the *dhfr* −55 start site, and the arrow beside lane 2 designates transcription from a *dhfr* −500 initiation site. The templates used in lanes 6 and 7 contained vector DNA at each end; therefore, the runoff products extended beyond the *dhfr* sequences. The numbers to the right of panels A and B indicate sizes (in base pairs) of the DNA markers. (C) Schematic of the *dhfr* promoter region. The restriction sites used to construct the different templates are indicated and are numbered relative to +1, the translation initiation codon of the *dhfr* gene. The major in vivo *dhfr* transcription initiation site is indicated by the large rightward arrow at −55. The newly identified start site described in this report is indicated by the large leftward arrow at −740. The dotted lines indicate the minor *dhfr* initiations discussed in the text. Opposite-strand transcription from the *dhfr* promoter is represented by the leftward arrow at −250. The 10 consensus Sp1 binding sites are represented by the filled boxes above or below the line. The orientation of the six Sp1 sites distal to *dhfr* is opposite to that of the four proximal sites. The in vitro transcriptional activity of the −740 promoter from the deleted templates relative to the activity from the −1010/+376 template is indicated. Transcriptional activity of each promoter construct was determined by densitometric scanning of autoradiograph bands and compensation for the number of guanines of each runoff product.

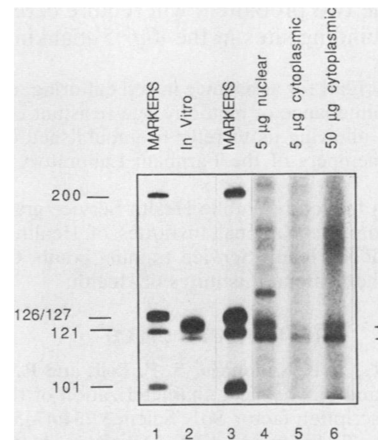

FIG. 2. Identification and localization of −740 transcripts in cellular RNA. Primer extension reactions were performed on RNA produced in vitro from the −1010/−545 template (lane 2) and nuclear (lane 4) and cytoplasmic (lanes 5 and 6) RNAs from mouse 3T6 R1000 cells. Lanes 1 and 3 contained DNA markers. Cytoplasmic RNA was prepared by the method of Maniatis et al. (11) as modified from Favaloro et al. (9), and nuclear RNA was prepared from nuclei by using the procedure previously described for whole-cell RNA (7). The primer is complementary to the region from −866 to −843. The arrowheads indicate RNAs that correspond to initiation at −740 and −746. The higher band in lane 4 was seen in all of the nuclear fractions tested but not in cytoplasmic RNA. As a control for the fractionation procedure, the ratio of *dhfr* transcripts in the cytoplasmic and nuclear fractions was determined. Less than 5% of the *dhfr* mRNA was in the nuclear fraction (data not shown). The numbers on the left are sizes (in base pairs) of the DNA markers.

RNase protection experiments, there are 50- to 100-fold more *dhfr* transcripts than −740 transcripts (L.J.S., unpublished data). Crouse et al. (3) performed transcription rate measurements in isolated nuclei from mouse cells using subclones of the *dhfr* gene and its 5′-flanking region and observed similar transcription rates from the *dhfr* promoter and from the region spanning −1550 and −750. Although the existence of the upstream promoter was unknown at that time, it is likely that they detected transcription from the −740 start site. If so, then the −740 promoter and the *dhfr* promoter are of approximately equal strength both in vivo and in vitro, suggesting that the difference in abundance of cellular *dhfr* mRNA and −740 RNA is due to posttranscriptional regulation.

In vitro transcription analysis of the human *dhfr* gene suggests that a transcript may initiate at −670 and transcribe in the direction opposite to that of *dhfr* (2). The region encompassing −670 in the human gene has a high degree of sequence similarity to the region surrounding −740 in the murine gene, suggesting that similar transcripts are present in both human and mouse cells.

Summary. We identified a new RNA polymerase II promoter located upstream of the *dhfr* gene that directs transcription to initiate at −740 and to proceed in the direction opposite to that of *dhfr* mRNA. The resulting transcript is distinct from the previously identified opposite-strand transcripts that are produced by the bidirectional *dhfr* promoter. The −740 promoter functions in cells, generating RNA that is distributed in approximately equal amounts in the nucleus and cytoplasm. Although the minimal sequence elements required for transcription from −740 are upstream of the minimal *dhfr* promoter, deletion analysis suggests that each promoter has a negative effect on the other. The close

proximity of the two promoters will require careful analysis of the protein-binding sites in the *dhfr* 5′-flanking region.

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