Journal of Cellular Biochemistry 88:165–180 (2003)

The 5′-Untranslated RNA of the Human dhfr Minor Transcript Alters Transcription Pre-Initiation Complex Assembly at the Major (Core) Promoter

Scott W. Blume,* Zheng Meng, Kedar Shrestha, Richard C. Snyder, and Peter D. Emanuel

Departments of Medicine, Biochemistry and Molecular Genetics, Anesthesiology, and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract The human dhfr minor transcript is distinguished from the predominant dhfr mRNA by an ~400 nucleotide extension of the 5′-untranslated region, which corresponds to the major (core) promoter DNA (its template). Based on its unusual sequence composition, we hypothesized that the minor transcript 5′-UTR might be capable of altering transcription pre-initiation complex assembly at the core promoter, through direct interactions of the RNA with specific regulatory polypeptides or the promoter DNA itself. We found that the minor transcript 5′-UTR selectively sequesters transcription factor Sp3, and to a lesser extent Sp1, preventing their binding to the dhfr core promoter. This allows a third putative transcriptional regulatory protein, which is relatively resistant to sequestration by the minor transcript RNA, the opportunity to bind the dhfr core promoter. The selective sequestration of Sp3 > Sp1 by the minor transcript 5′-UTR involves an altered conformation of the RNA, and a structural domain of the protein distinct from that required for binding to DNA. As a consequence, the minor transcript 5′-UTR inhibits transcription from the core promoter in vitro (in *trans*) in a concentration-dependent manner. These results suggest that the dhfr minor transcript may function in vivo (in *cis*) to regulate the transcriptional activity of the major (core) promoter. J. Cell. Biochem. 88: 165–180, 2003.

Key words: transcriptional regulation; Sp3; quadruplex; 5′-UTR; dihydrofolate reductase; G/C box; promoters arranged in series (tandem)

A small proportion of human dihydrofolate reductase (*dhfr*) transcripts initiate from an independently regulated minor promoter ~400 bp upstream of the major transcription start site [Masters and Attardi, 1985; Fujii and Shimada, 1989]. The minor transcript is enriched in the nucleus, and its specific function is unknown. The 5′-untranslated RNA sequence (5′-UTR) unique to the minor transcript corresponds to the sequence of the major (core) promoter DNA, which is not otherwise transcribed. This endows the minor transcript 5′-UTR with an unusual sequence composition, including in particular two series of tracts of contiguous guanines which correspond in part to the two G/C box consensus recognition elements of the core promoter DNA.

We hypothesized that the 5′-UTR of the *dhfr* minor transcript might be capable of directly influencing assembly of the transcription pre-initiation complex at the major (core) promoter. This could take place through any one of three plausible mechanisms: (1) formation of an intermolecular purine–purine–pyrimidine triplex structure [Morgan and Wells, 1968; Marck and Thiele, 1978; Letai et al., 1988] between the RNA and DNA; (2) specific recognition of single-stranded RNA by certain DNA-binding zinc finger proteins [Caricasole et al., 1996; Friesen and Darby, 1997; Lai and Blackshear, 2001]; or (3) adoption of a quadruplex-based structure by the RNA with affinity for certain regulatory proteins [Walsh and Gualberto, 1992; Schierer and Henderson, 1994; Frantz and Gilbert, 1995].

We have systematically characterized the potential for purine–purine–pyrimidine triplex

Grant sponsor: National Institutes of Health/National Cancer Institute; Grant number: CA75467.

*Correspondence to: Scott W. Blume, Tumor Inst. Room 508, 1824 6th Avenue South, Birmingham, Alabama 35294. E-mail: scott.blume@ccc.uab.edu

Received 1 August 2002; Accepted 1 August 2002

DOI 10.1002/jcb.10326

formation involving the human *dhfr* promoter sequence [Blume et al., 1992; Blume et al., 1997; Blume et al., 1999]. We demonstrated that synthetic G-tract-containing oligodeoxyribonucleotides could be used in an exquisitely sequence-specific manner to bind to either the distal or proximal G/C box regions of the *dhfr* core promoter. Spontaneous triplex formation of this type requires an antiparallel orientation of the third strand with respect to the purine-rich strand of the target, with which reverse-Hoogsteen hydrogen bonds are made [Kohwi and Kohwi-Shigematsu, 1988; Beal and Dervan, 1991; Lyamichev et al., 1991]. Generally, this requirement for antiparallel orientation would preclude two identical strands (such as the minor transcript 5′-UTR RNA and the coding strand of the core promoter DNA) from interacting in a triplex structure. However, because the distal G-tract region is perfectly symmetrical ($G_4CG_7CG_4$), it was conceivable that the minor transcript 5′-UTR could associate directly with the core promoter DNA in perfect antiparallel triple helical alignment. Such a triplex structure would be expected to block binding of regulatory polypeptides (e.g., Sp1 or Sp3) to the distal G/C box element, and consequently interfere with *dhfr* transcription [Hanvey et al., 1990; Gee et al., 1992; Noonberg et al., 1994].

We also found that the *dhfr* G-tract sequences exhibit a very strong tendency to self-associate to form inter- or intra-molecular quadruplex structures [Blume et al., 1997]. The quadruplex structure is characterized by a reciprocal pattern of Hoogsteen hydrogen bonds (1-NH: O-6 and 2-NH_2: N-7) amongst four guanine residues arranged in a square planar array (a total of eight hydrogen bonds per G-quartet [Zimmerman et al., 1975; Sen and Gilbert, 1988; Williamson et al., 1989; Cheong and Moore, 1992]. Quadruplex association is highly favored within physiological microenvironments, because the van der Waals radii of the monovalent alkali metal cations Na^+, and particularly K^+ (the predominant intracellular cation), fit well within the quadruplex helical core, where they coordinate to the internal carbonyl oxygens [Lee, 1990; Hardin et al., 1992; Wang and Patel, 1992]. The potential for quadruplex formation correlates roughly with G-content. RNA and DNA sequences much less G-rich than the *dhfr* minor transcript 5′-UTR are suspected of forming physiologically-relevant quadruplex structures in cells [Murchie and Lilley, 1992; Christiansen et al., 1994; Sun et al., 1999]. Quadruplex structures are also known to bind proteins. Some of these quadruplex-protein interactions are quite specific and of high affinity, and apparently of functional (natural or perhaps pathological) significance. Examples include proteins which bind telomeric DNA [Gualberto et al., 1995], the immunoglobulin switch region [Dempsey et al., 1999], or the fragile X repeat sequence [Uliel et al., 2000], proteins which promote quadruplex formation [Fang and Cech, 1993; Giraldo et al., 1994], nucleases which specifically cleave at quadruplex structures [Liu et al., 1995; Bashkirov et al., 1997], helicases that unwind quadruplex structures [Sun et al., 1998; Fry and Loeb, 1999], and transcription factor MyoD1 which binds a quadruplex structure formed by the creatine kinase enhancer sequence [Walsh and Gualberto, 1992]. We, therefore, suspected that the *dhfr* minor transcript might also adopt a quadruplex structure through its G-tract sequences, and that such a structure might facilitate interactions with specific transcriptional regulatory proteins.

It is also recognized that a number of DNA-binding transcription factors interact more tightly with one strand of DNA than the other [Gidoni et al., 1984; Buratowski et al., 1989]. In fact, a number of these proteins have now been demonstrated to be capable of binding single stranded RNA [Ladomery and Sommerville, 1994; Grondin et al., 1996; Hallier et al., 1996]. Within this list of DNA-binding factors with RNA-binding capability are several zinc finger proteins, including TFIIIA, WT1, ZNF74, and tristetraprolin. The zinc finger–RNA interaction has been examined from both molecular biological as well as structural perspectives. Since the 5′-UTR of the *dhfr* minor transcript corresponds precisely to the core promoter DNA, which includes two perfect consensus high affinity Sp factor recognition sequences [Blume et al., 1991; Fujii et al., 1992], and the zinc finger amino acid: base-specific contacts for the Sp family are thought to involve primarily the G-rich strand [Pavletich and Pabo, 1991; Choo and Klug, 1994; Desjarlais and Berg, 1994], it was conceivable that the minor transcript 5′-UTR might compete with the corresponding sequence of the core promoter DNA for binding of Sp1 and/or Sp3.

In this report, we have examined the potential of the 5′-untranslated RNA sequence of the *dhfr* minor transcript to alter transcription factor binding and regulate transcription initiation from the homologous major (core) promoter DNA sequence. We found that the minor transcript 5′-UTR interferes in *trans* with transcription pre-initiation complex assembly by selectively sequestering transcription factors intended to bind the core promoter DNA, and that these RNA–protein interactions occur through a distinct mechanism and involve a separate structural domain of the protein relative to that required for recognition and binding of DNA. These results suggest that the *dhfr* minor transcript may be capable of functioning physiologically in *cis* to regulate the transcriptional activity of the major (core) promoter.

MATERIALS AND METHODS

dhfr Core Promoter DNA

The human *dhfr* core promoter DNA sequence (−112 to +56) was obtained as described [Blume et al., 1991] from a subclone of pDHFR1.8 [Chen et al., 1984]. The core promoter fragment was excised from the plasmid, 3′-[^{32}P] end-labeled on either the coding or noncoding strand, isolated by native polyacrylamide gel electrophoresis, precipitated from a 1 M ammonium acetate solution, dried, and resuspended in distilled, deionized water.

dhfr Minor Transcript RNA

The portion of the *dhfr* minor transcript 5′-UTR sequence corresponding to the core promoter (−112 to +56) was synthesized in vitro from linearized plasmid template using Sp6 polymerase (Ribomax, Promega Corp., Madison, WI). Transcript quality was assessed by agarose gel electrophoresis. Gel-isolated transcript was quantitated by optical density. The radiolabeled preparation of the minor transcript 5′-UTR used to assess RNA conformation (Fig. 4) was obtained by internal incorporation of [alpha-^{32}P]-UTP.

HeLa Extracts

Transcriptionally active (for *dhfr*) HeLa whole cell or nuclear extracts were prepared as described [Manley et al., 1980; Dignam et al., 1983] or purchased from Promega.

DNase I Protection

DNase I protection assays were performed as described [Blume et al., 1991]. The labeled *dhfr* core promoter DNA fragment (~200,000 cpm, ~40 nM) was incubated with a transcriptionally active (for *dhfr*) HeLa extract (4.8 μg/μl final protein concentration) in the presence of a large excess of poly d(I-C) (300 ng/μl), under conditions otherwise conducive to transcription (24 mM Tris-Cl, pH 7.4, 60 mM KCl, 6 mM $MgCl_2$, 0.26 mM EDTA, 1.2 mM DTT, 2 mM creatine phosphate, 9% glycerol), except that ribonucleotides were withheld. Following incubation for 75 min at 30°C, samples were subjected to limited DNase I digestion (30 s on ice, 120 u/ml) which was terminated by addition of EDTA to 40 mM. Following extraction and precipitation, products were analyzed by electrophoresis on an 8% polyacrylamide, 8 M urea sequencing gel and the results visualized by autoradiography.

Gel Mobility Shift

The labeled *dhfr* core promoter DNA fragment (~50,000 cpm; ~10 nM) was incubated with transcriptionally active (for *dhfr*) HeLa extract (0.7–1.2 μg/μl) in the presence of the nonspecific competitor poly d(I-C) (240 ng/μl) for 30–55 min at room temperature, under conditions generally compatible with transcription pre-initiation complex assembly (10–40 mM Tris-Cl, pH 7.4, 50–60 mM KCl or 60–150 mM NaCl, 0.75–5.75 mM $MgCl_2$, 0.75–2.25% glycerol). Minor transcript RNA was included in some samples, as indicated in individual figure legends. Unless otherwise stated, the HeLa extract used as a source of transcription regulatory proteins was added last, and thus simultaneously exposed to the *dhfr* core promoter DNA, nonspecific competitor poly d(I-C), and minor transcript RNA. alpha- Amanitin (0.8 μM) was included in some experiments to ensure the absence of actively elongating transcription complexes and achieve a more homogeneous shift pattern. Samples were analyzed by electrophoresis on a native 5% polyacrylamide gel run at 40–70 V overnight, with recirculation of buffer as necessary. Gel and running buffers contained 15–40 mM Tris-acetate, supplemented with 40–112.5 mM sodium acetate and 0.75–3.0 mM $MgCl_2$.

Oligonucleotides

Oligonucleotides were prepared by automated phosphoramidite synthesis, eluted through reverse phase chromatography, and analyzed by polyacrylamide gel electrophoresis as described [Blume et al., 1992]. Stock solutions were lyophilized from methanol/H_2O and stored in distilled, deionized water.

The sequence of the C-tract-containing oligonucleotide complementary to the G-tract sequences of the minor transcript (used in Figs. 4and 5) was: 5′-$C_4GC_7GC_4$-3′. This C-tract oligonucleotide is expected to hybridize efficiently with both G-tract regions of the minor transcript (sequences nearly identical, 12 contiguous residues perfectly matched) under the conditions employed. The sequence of the G-tract oligonucleotide used to compete for intermolecular quadruplex interaction with the minor transcript RNA (Fig. 4) was: 5′-$G_4CG_7CG_4$-3′.

Antibodies

Anti-Sp1 (1C6), anti-Sp3 (D-20), anti-Egr-1 (588), anti-hTAF(II)130 (4A6), and anti-hTAF(II)250 (6B3) were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

In Vitro Runoff Transcription Assay

The core promoter/template DNA used was a restriction fragment of the human *dhfr* sequence extending from −112 to +524, which we [Blume et al., 1991] and others [Shimada et al., 1989] have previously established is sufficient to direct accurate transcription initiation, yielding the expected 524 nt run-off product representing the *dhfr* major transcript. The unlabeled core promoter/template (~45 nM) was incubated with transcriptionally active HeLa extract (final 1.3 μg/μl) for 75 min at 30°C in 18 mM Tris-Cl, pH 7.4, 45 mM KCl, 6 mM $MgCl_2$, 0.09 mM EDTA, 0.22 mM DTT, 0.22 mM PMSF, 9% glycerol, 500 μM each rNTP except UTP (25 μM plus [alpha-^{32}P]-UTP (3,000 Ci/mMol; Amersham Biosciences Corp., Piscataway, NJ) at 4 μCi per reaction), 1 mM creatine phosphate, and variable concentrations of the minor transcript RNA. Both the promoter/template DNA and minor transcript RNA were gel-purified to eliminate sources of non-specific transcriptional inhibition. Following extraction and precipitation, products of transcription were analyzed on an 8 M urea, 5% polyacrylamide sequencing gel.

RESULTS

Transcription Pre-Initiation Complex Assembly at the dhfr Major (Core) Promoter

High resolution DNase I protection assays were performed to depict the transcription pre-initiation complex on the human *dhfr* major (core) promoter (Fig. 1A). The end-labeled core promoter DNA fragment (−112 to +56) was incubated with a HeLa extract (transcriptionally active for *dhfr*) in the presence of a large excess of the non-specific double stranded DNA competitor poly d(I-C), under conditions otherwise conducive to transcription initiation, but without addition of ribonucleotides. On the coding strand, a large, nearly continuous region of protection (−55 to +20) was consistently observed which surrounded the *dhfr* major transcription start site and extended over the positions of each of the recognized consensus regulatory elements [Farnham and Schimke, 1986; Mitchell et al., 1986; Swick et al., 1989; Wade et al., 1995; Wells et al., 1996]. An incremental increase in KCl concentration (from 60 to 200 mM) was utilized to gradually perturb the protein–protein and protein–DNA interactions within this complex. Alterations in the location and intensity of DNase I hypersensitive sites delineated the boundaries of the individual transcription factor binding sites on the DNA. Stepwise addition of individual ribonucleotides necessary for synthesis of the nascent *dhfr* transcript also induced changes in the DNase enhancements consistent with activation of the complex (data not shown) [Cai and Luse, 1987; Yan and Gralla, 1997]. Thus, we suspect that this footprint represents at least a late intermediate in assembly of the transcription pre-initiation complex at the *dhfr* major (core) promoter.

The results of DNase I protection analysis, along with other key features of the core promoter sequence, are illustrated in Figure 1B. These results provide an image of the functional core promoter, and in particular, the protein-DNA contacts within the transcription pre-initiation complex. The distal (−49) G/C box matches the optimal consensus Sp1 decanucleotide perfectly, binds Sp1 with high affinity [Blume et al., 1991], and is essential for *dhfr* transcription [Fujii et al., 1992]. The proximal (−14) G/C box is positioned unusually near the

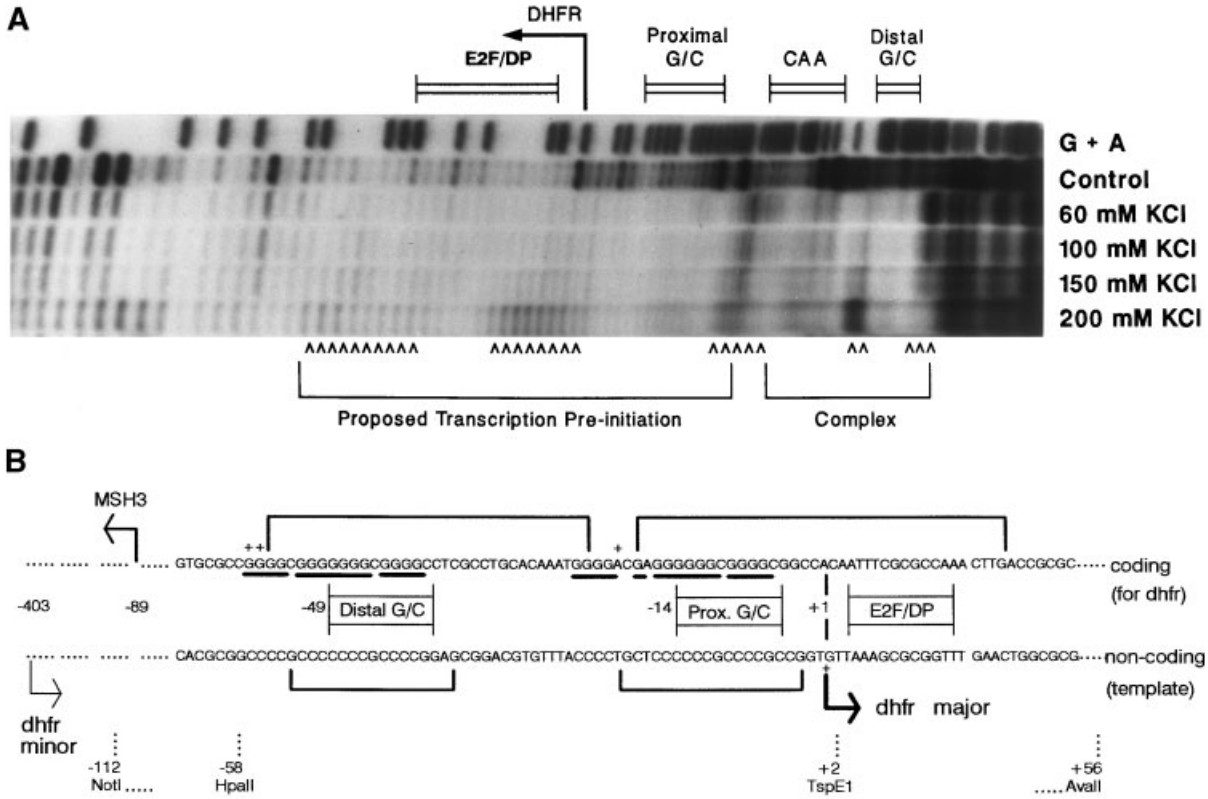

Fig. 1. Assembly of a transcription pre-initiation complex at the human *dhfr* major (core) promoter. **A**: DNase I protection pattern obtained following incubation of transcriptionally active HeLa extract (4.8 μg/μl) with the human *dhfr* core promoter DNA fragment (−112 to +56; 3′-end-labeled on the coding strand), in the presence of an excess of poly d(I-C) (300 ng/μl), and conditions otherwise conducive to transcription, except for the absence of ribonucleotides. A Maxam-Gilbert G+A reaction and control (no extract) DNase I digest are included for reference. The positions of the consensus recognition sequences (distal (−49) and proximal (−14) G/C box elements; E2F/DP initiator elements (+4)) and the major *dhfr* transcription start site (bent arrow) are indicated above. The consensus ''CAA element,'' also present in the homologous mouse and hamster *dhfr* promoters, is not known to bind a particular transcription factor directly, but is thought to serve a structural role in core promoter function [Pierce et al., 1992]. The location of the proposed transcription pre-initiation complex is bracketed below. Positions of hypersensitive sites (DNase enhancements) altered with increasing KCl concentration are marked with arrowheads. **B**: Summary of DNase I protection patterns on both strands of the core promoter DNA. Protected sequences are bracketed. The degree of protection is represented by relative height of the brackets, and is considerably greater for the coding strand than the non-coding (template) strand. DNase I enhancements (at 60 mM KCl) are represented by plus signs. The positions of the major and minor *dhfr* transcription start sites are indicated (bent arrows). The *dhfr* core promoter functions bidirectionally, also directing synthesis of the divergent MSH3 transcript which initiates 89 bp upstream of the major *dhfr* start site [Shinya and Shimada, 1994; Watanabe et al., 1996]. The positions of the consensus recognition sequences are shown (rectangles). The two series of contiguous guanine residues (G-tracts) on the coding strand, within which are embedded the two consensus G/C box recognition sequences for Sp factor binding, are underlined. The positions of restriction sites used in the course of these experiments are also indicated. Coordinates are given relative to the major *dhfr* transcription start site.

transcription start site, and is uncharacteristically spaced ~3.5 turns of the double helix away from the distal G/C box, placing the Sp factors bound to these two sites roughly on opposite topological surfaces of the DNA [Gidoni et al., 1984; Fujii and Shimada, 1989]. Each of these consensus G/C box elements is embedded within a larger series of contiguous guanine–cytosine base pairs. Note that these G-tract sequences are much longer (17–19 bp) than necessary to specify binding of the three-zinc-fingered Sp factors (3 bp per Zn finger = 9 bp). Also note that within the pre-initiation complex, the degree of DNase protection and the extent of protected sequence were considerably more limited on the non-coding (template) strand, even for the G/C box consensus sequences, indicating a much closer association of the promoter-bound transcription regulatory proteins with the coding strand.

Minor Transcript 5′-UTR Alters the Pattern of Protein Binding to the Major (Core) Promoter

Next, we sought to examine the effects of the minor transcript RNA on transcription pre-initiation complex assembly at the *dhfr* core promoter. In the course of these experiments, we found that the interactions between the minor transcript RNA, core promoter DNA, and nuclear regulatory proteins were best assessed through the use of mobility shift assays, whereby individual complexes within a larger population of molecules could be resolved.

In the first three lanes of Figure 2, the labeled *dhfr* core promoter DNA fragment was incubated with the minor transcript 5′-UTR (unlabeled) in the absence of nuclear extract. Formation of a stable three-stranded intermolecular complex was reproducibly observed (lane 1). This RNA–DNA complex was sensitive to RNase T1 (lane 2), as would be expected of a localized triple helical structure. However, the results of an extensive series of experiments have indicated that this RNA–DNA complex is a stabilized heteroduplex (formed by partial strand displacement) rather than a true triple helical structure (data not shown). Enhanced footprinting (evaluation of the DNase digestion pattern associated with an individual shifted band) failed to indicate protection of the G/C-tract regions as would be expected of a purine–purine–pyrimidine triplex structure; instead the complex was sensitive to RNase H. While it is yet possible that this unusual RNA–DNA interaction is of some physiological relevance, it is rapidly resolved upon addition of nuclear extract, and does not appear to be responsible for the alterations in protein binding to the core promoter observed in the presence of the minor transcript 5′-UTR (see below).

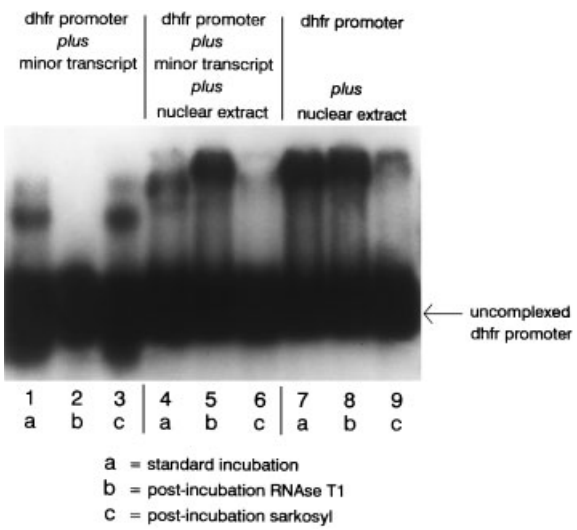

Fig. 2. Gel mobility shift analysis of the interactions between the *dhfr* major (core) promoter DNA, minor transcript RNA, and HeLa extract. The labeled *dhfr* core promoter fragment (~50,000 cpm; ~10 nM) was incubated with the homologous minor transcript 5′-UTR (~5 μM; **lanes 1–3**), transcriptionally active HeLa extract (1.2 μg/μl; **lanes 7–9**), or both (**lanes 4–6**). Samples also contained 240 ng/μl poly d(I-C), 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.75 mM $MgCl_2$. Following incubation for 55 min at room temperature, samples were electrophoresed on a native 5% polyacrylamide gel in which the gel and running buffer contained 15 mM Tris-Acetate, 112.5 mM sodium acetate, and 0.75 mM $MgCl_2$. Samples in lanes 2, 5, and 8 were digested with RNase T1 (25 u) for 15 min prior to electrophoresis. Samples in lanes 3, 6, and 9 were exposed to *N*-lauryl sarcosine (sarkosyl, 0.125% final concentration) for 15 min prior to electrophoresis. The minor transcript 5′-UTR significantly alters the pattern of protein binding to the core promoter (compare lanes 4 and 7), an effect, which is absolutely dependent upon the integrity of the RNA sequence.

In the last three lanes of Figure 2, the labeled *dhfr* core promoter DNA fragment was incubated with transcriptionally active HeLa extract in the absence of minor transcript RNA. A broad shifted band of high molecular weight was observed (lane 7), consistent with the formation of at least a partial pre-initiation complex. This protein–DNA complex was not altered by RNase T1 digestion (lane 8), but was essentially completely disrupted by the detergent sarkosyl (lane 9).

In the middle three lanes of Figure 2, the labeled core promoter DNA, minor transcript 5′-UTR RNA, and transcriptionally active HeLa extract were co-incubated. The pattern of regulatory protein binding to the *dhfr* core promoter was dramatically altered in the presence of the minor transcript 5′-UTR (compare lane 4 to lane 7). Note that the high molecular weight shift presumed to represent the transcription pre-initiation complex is replaced by bands of lower intensity and lower apparent molecular weight. However, upon digestion with RNase T1 (lane 5), the pattern of protein binding to the core promoter reverts to that observed in the absence of the minor transcript RNA. This indicates that the effect of the minor transcript 5′-UTR on protein binding to the core promoter DNA is absolutely dependent upon the integrity of the RNA sequence.

Disruption of the protein–DNA interactions by sarkosyl essentially eliminated all shifted bands, with no evidence of an underlying RNA–DNA complex (lane 6). Furthermore,

the alterations induced by the minor transcript RNA on protein binding to the core promoter were neither enhanced nor prevented by preformation of the RNA–DNA complex as in lane 1 prior to addition of the HeLa extract (data not shown). These results and those of a number of additional experiments have allowed us to conclude that the altered pattern of protein binding to the core promoter DNA in the presence of the minor transcript 5′-UTR is the result of selective sequestration of regulatory proteins by the minor transcript 5′-UTR RNA, rather than by a direct interaction of the RNA with the DNA.

Activity of the Minor Transcript RNA can be Mimicked by Single Stranded Oligodeoxyribonucleotides Corresponding to the G-Tract Sequences

A series of single stranded oligodeoxyribonucleotides, corresponding to the natural sequence of either the coding or non-coding strand of the *dhfr* core promoter, were tested for their ability to mimic the activity of the minor transcript RNA in altering transcription factor binding to the *dhfr* core promoter. The results shown in Figure 3 indicated that the effects of the minor transcript 5′-UTR could be reproduced, with somewhat lower selectivity, by oligonucleotides corresponding to either of the two G-tract regions of the *dhfr* promoter (or the homologous minor transcript 5′-UTR sequence), in either their natural or inverse orientation. By contrast, oligonucleotides corresponding to regions of mixed sequence composition, or C-tract-containing sequences of the non-coding strand, did not block protein binding to the *dhfr* core promoter DNA. Although these results were obtained using single stranded DNA oligomers as models, they strongly suggested that it might be the G-tract sequences within the minor transcript RNA which were primarily responsible for sequestering transcription factors that otherwise would bind the *dhfr* core promoter DNA.

dhfr Minor Transcript RNA Adopts a Series of G-Tract-Dependent Structures

We have previously established that the single stranded oligonucleotides representing the G-tract sequences of the *dhfr* core promoter exhibit a strong tendency to adopt highly stable quadruplex structures under physiological conditions (in the presence of K^+) [Blume et al., 1997]. We suspected, therefore, that the intact

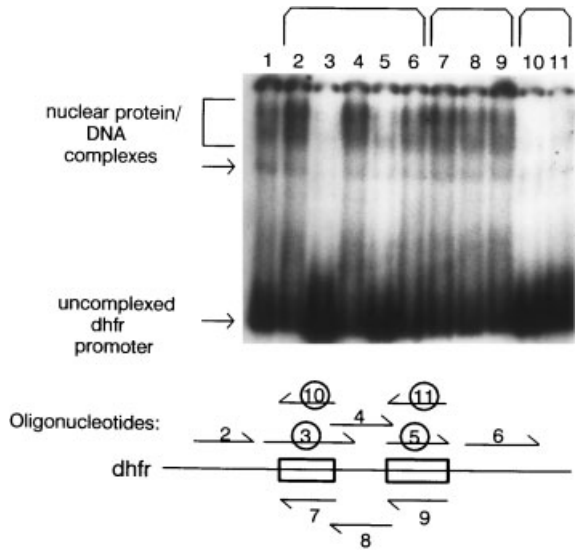

Fig. 3. Gel mobility shift analysis of alterations in protein binding to the *dhfr* major (core) promoter DNA induced by single-stranded oligodeoxyribonucleotides corresponding to the natural *dhfr* core promoter/minor transcript sequence. The labeled *dhfr* core promoter DNA fragment was incubated with transcriptionally active HeLa extract (0.7 μg/μl) in the presence of 240 ng/μl poly d(I-C) and 200 ng/μl yeast tRNA for 30 min at room temperature, following which samples were electrophoresed through a native 5% polyacrylamide gel. **Lane 1** is a positive control. Individual synthetic oligonucleotides (33 μM) representing the natural *dhfr* sequence were included in each of the remaining samples (**lanes 2–11**). The relationship of each oligonucleotide to the *dhfr* sequence is indicated in the diagram below. The long horizontal line represents the *dhfr* core promoter sequence, and the two rectangles represent the two G/C-tract regions. The small lines represent the oligonucleotides. Those arranged above the *dhfr* line correspond to the coding strand of the core promoter DNA (and the homologous minor transcript 5′-UTR sequence). Those below the line correspond to the non-coding strand of the core promoter. The half-arrowheads indicate the 5′–3′ orientation of each oligonucleotide sequence. Those oligonucleotides, which interfere with protein binding to the promoter DNA, are circled.

minor transcript RNA might also form G-tract-dependent quadruplex structures, and that these might contribute to the ability of this RNA sequence to alter transcription pre-initiation complex assembly at the core promoter. With two extensive series of tracts of contiguous guanine residues contained within the minor transcript 5′-UTR, intramolecular (folded) and/or intermolecular quadruplex interactions might be anticipated.

In Figure 4, the electrophoretic mobility of the labeled minor transcript RNA in a native gel was examined under variable microenvironmental conditions. Note the predominance of intramolecular folded structures (with increased mobility) in the absence of metal cations

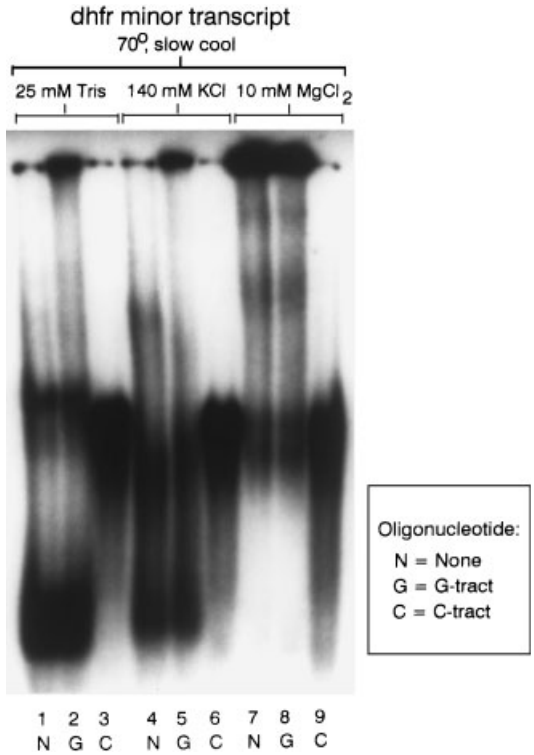

Fig. 4. The *dhfr* minor transcript 5′-UTR RNA adopts a series of altered conformations, all of which are dependent on the hydrogen bonding capacity of the G-tract sequences. Electrophoretic mobility of the internally labeled *dhfr* minor transcript 5′-UTR RNA sequence (− 112 to + 56; 0.7 μM) was evaluated following incubation under variable microenvironmental conditions: 25 mM Tris-Cl (pH 7.4) alone (**lanes 1–3**), Tris-Cl plus 140 mM KCl (**lanes 4–6**), or Tris-Cl plus 10 mM $MgCl_2$ (**lanes 7–9**). The samples were first heat denatured in the absence of metal cations, to disrupt pre-existing RNA secondary structure, then allowed to slow cool to room temperature. (Very similar results were obtained if the minor transcript RNA was allowed to equilibrate in buffer for 2 h or overnight without prior heat denaturation (data not shown)). The native 5% polyacrylamide gel and running buffer contained 1 × Tris-borate supplemented with 30 mM KCl and 10 mM $MgCl_2$. In lanes 2, 5, and 8, a G-tract oligonucleotide (33 μM) homologous to, and potentially capable of intermolecular quadruplex alignment with the G-tract sequences of the minor transcript RNA was included. In lanes 3, 6, and 9, a C-tract oligonucleotide (33 μM) complementary to, and capable of hybridizing with the G-tract sequences of the minor transcript RNA was included. Generally, intramolecular (folded) structures exhibit more rapid migration, intermolecular structures exhibit slower migration, and the relatively unstructured (single-stranded, random coil) form of the RNA migrates to approximately the middle of the gel.

(lanes 1 and 2), the virtual absence of single-stranded random coil forms (migration to approximately the middle of the gel) in the presence of K^+ (lanes 4 and 5), the appearance of a new higher shifted band, specifically in the presence of K^+ (consistent with an intermolecular quadruplex, lane 4), and loss of this band (by competition), with accumulation of a higher-ordered intermolecular quadruplex structure, upon addition of the G-tract oligonucleotide to the intact minor transcript RNA (in the well, lane 5).

Remarkably, the formation of each of these altered structures was completely blocked by addition of a C-tract oligonucleotide capable of hybridizing with the G-tract sequences of the minor transcript 5′-UTR (lanes 3, 6, and 9). These results indicate that the *dhfr* minor transcript 5′-UTR is capable of adopting a number of conformational alternatives, at least some of which are likely based on quadruplex interactions, and all of which depend on the hydrogen bonding potential of the G-tract sequences.

Selective Sequestration of DNA-Binding Factors by the Minor Transcript RNA

It appeared from the results of Figure 2 that the minor transcript 5′-UTR altered the pattern of protein binding to the *dhfr* core promoter through selective sequestration of regulatory proteins. By controlling the stringency of the assay through changes in the composition of the gel and running buffer, carefully titrating the relative concentrations of RNA and protein, and limiting the extent of the promoter DNA sequence involved, we were able to obtain results, which more precisely define the sequestration activity of the minor transcript 5′-UTR (Fig. 5).

A titration of protein binding to the full core promoter DNA by increasing the concentration of the minor transcript 5′-UTR (0.02–2.5 μM in lanes 2–5) revealed a gradual loss of higher molecular weight shifted bands, with appearance of a new shifted band of much lower effective molecular weight. We repeated the mobility shift analysis using two smaller restriction fragments of the core promoter from which either the *dhfr* or the MSH3 initiator element had been removed. The general pattern of shifted protein–DNA complexes and the changes in protein–DNA interactions induced by the minor transcript 5′-UTR were remarkably similar for each of these promoter DNA fragments. In the absence of the minor transcript 5′-UTR (lanes 6 and 9), two well-resolved protein–DNA complexes, designated “x” (upper, more intense) and “y” (lower), were reproducibly observed. Upon inclusion of the minor transcript 5′-UTR RNA, the y complex disappeared completely, while the intensity of x diminished to a much lesser extent (lanes 7 and 10). In each case, the

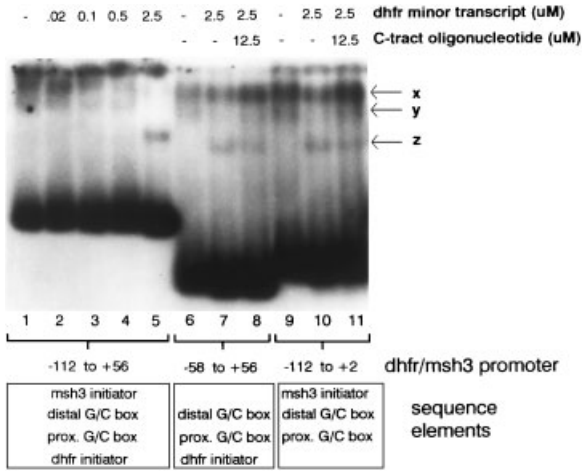

Fig. 5. The *dhfr* minor transcript 5′-UTR selectively sequesters transcription factors which otherwise would bind the core promoter DNA. High stringency gel mobility shift analysis was performed to assess the influence of the minor transcript RNA on the formation of specific transcription factor–promoter DNA complexes. Three different core promoter DNA fragments were utilized: NotIAvaII (**lanes 1–5**); HpaII-AvaII (**lanes 6–8**); and NotI-TspE1 (**lanes 9–11**). The sequence coordinates and consensus recognition sequences associated with each of these DNA fragments are indicated. For each of the promoter DNA fragments, incubation with transcriptionally active HeLa extract resulted in two shifts, labeled x and y. In lanes 2–5, the concentration of minor transcript RNA (added in *trans*) was titrated. Minor transcript RNA was also included at 2.5 μM in lanes 7–8 and 10–11. A C-tract-containing oligonucleotide complementary to the G-tract sequences of the minor transcript was included in lanes 8 and 11. The minor transcript potently sequesters the protein component of the y shift, but exhibits a lower affinity for the protein comprising the x shift. Formation of the z complex is actually favored in the presence of the minor transcript RNA.

decrease in intensity of the x and y complexes was accompanied by the appearance of a new lower shifted band "z". These results demonstrate the selectivity of the minor transcript RNA for sequestration of DNA-binding factors, with the greatest affinity for y, a lesser affinity for x, and relative resistance of the z factor to sequestration by the minor transcript 5′-UTR.

Hybridization of the minor transcript G-tract sequences to a complementary C-tract-containing oligonucleotide (which abrogated the altered structural conformations of the minor transcript RNA; refer to Fig. 4), tended to block the ability of the minor transcript RNA to sequester the DNA-binding proteins: shift x increased in intensity, shift y was partially restored, and the prevalence of the new shift z was diminished considerably (compare lanes 8 and 11 to lanes 7 and 10). This result indicates that the G-tract-dependent structural alterations are at least partially responsible for the sequestration activity of the minor transcript 5′-UTR.

Minor Transcript 5′-UTR Binds Sp3 Through a Structural Domain Distinct From the Zinc Fingers

From the results of Figure 5, we could conclude that the *dhfr* minor transcript 5′-UTR selectively sequesters two transcription factors (y > x) which otherwise would bind the *dhfr* core promoter, and that each of these transcription factors (as well as the z factor) must recognize target sequences present within the limited region common to the two smaller restriction fragments. The only consensus recognition elements common to these two core promoter DNA fragments are the distal and proximal G/C box sequences. Supershift experiments were carried out in an attempt to positively identify the protein components of the individual shifted bands (Fig. 6). Pre-incubation of the HeLa extract with a monoclonal antibody specific for Sp1 prior to addition of the labeled DNA caused the majority of the radioactivity associated with

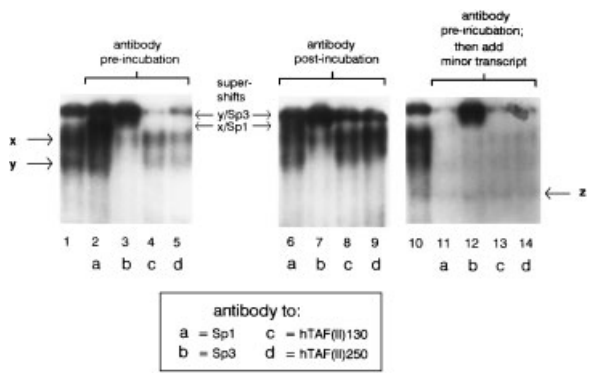

Fig. 6. Identification of the transcription factors sequestered by the *dhfr* minor transcript 5′-UTR. Supershift analyses of protein–DNA complexes were carried out under identical conditions to those described in the previous figure, using the HpaII-restricted *dhfr* core promoter fragment (− 58 to + 56). Antibodies were either pre-incubated with HeLa extract prior to addition of labeled promoter DNA (**lanes 2–5**, 11–14), or else added after formation of protein–DNA complexes (**lanes 6–9**). Lane 1 is a positive control (no antibody). The identities of the antibodies utilized and positions of the supershifted complexes are indicated. The y shift is a complex of Sp3 with the *dhfr* core promoter, whereas the x shift contains predominantly Sp1. Minor transcript RNA was included in samples in **lanes 10–14** to facilitate the formation of the z complex. Pre-incubation with the polyclonal antibody to Sp3 blocks sequestration of Sp3 by the minor transcript, but does not interfere with Sp3 binding to the *dhfr* core promoter, resulting in full maintenance of supershifted complex y (lane 12, compare to lanes 3 and 7).

band x to become supershifted (lane 2). Band y was not altered in intensity or migration by the antibody to Sp1. Preincubation of the HeLa extract with a polyclonal antibody specific for Sp3 caused all of band y and a part of the radioactivity from band x to become incorporated into a supershifted complex (lane 3). The same results were obtained if the anti-Sp1 and anti-Sp3 reagents were added after the proteins from the HeLa extract had been allowed to interact with the core promoter DNA (post-incubation protocol, lanes 6 and 7). Supershift assays using monoclonal antibodies to hTAF(II)130 or hTAF(II)250 (the TFIID components with which Sp factors would be expected to complex [Gill et al., 1994; Saluja et al., 1998]) revealed no specific, consistent changes in the x or y bands themselves. Thus, x represents predominantly a complex between the *dhfr* core promoter DNA and Sp1, whereas the y complex instead contains Sp3.

We also attempted to identify the protein component of the z shift, which is only reproducibly observed with inclusion of the minor transcript 5′-UTR (discussed further below). The z complex was unaffected by specific antibodies to Sp1, Sp3, or Egr-1 (lanes 11–14 and data not shown). Perhaps more importantly, however, we noted that the potency of the minor transcript 5′-UTR for sequestration of transcription factors was dramatically increased in the samples in which HeLa extracts were preincubated with antibodies (compare lanes 11–14 to lane 10). This was actually an inadvertent consequence of a modification in experimental protocol. For these samples, the HeLa extract (protein) was incubated with the minor transcript RNA for a brief period of time before the radiolabeled promoter DNA was added, whereas in the standard protocol, the HeLa extract would be simultaneously exposed to promoter DNA, the minor transcript RNA, and nonspecific competitor poly d(I-C).

It is remarkable that amongst the relative paucity of protein–DNA complexes in lanes 11–14, a very intense supershifted band is still observed with the antibody to Sp3. It appears that the anti-Sp3 reagent effectively blocks sequestration of Sp3 by the minor transcript 5′-UTR, but at the same time is permissive to binding of Sp3 to the core promoter DNA. This unanticipated but informative result indicates that binding of Sp3 to the *dhfr* core promoter DNA and its sequestration by the minor transcript RNA apparently involve separate structural domains of the protein. This experiment has been repeated and the result confirmed.

dhfr Minor Transcript 5′-UTR Inhibits Transcription From the dhfr Core Promoter in *Trans* in a Concentration-Dependent Manner

To assess the functional consequences of the alterations in transcription factor binding to the *dhfr* core promoter induced by the minor transcript 5′-UTR, in vitro transcription assays

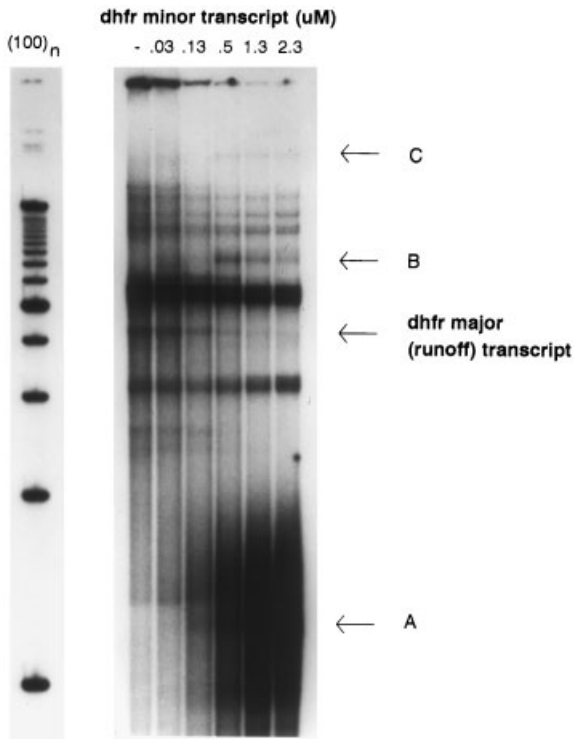

Fig. 7. The *dhfr* minor transcript RNA inhibits transcription initiation from the major (core) promoter in a concentration-dependent manner. An in vitro runoff transcription assay was performed as described in Materials and Methods, utilizing the *dhfr* promoter/template (−112 to +524), transcriptionally active HeLa extract, and variable concentrations of minor transcript RNA (0–2.3 μM; indicated above each lane). Both the promoter/template DNA and minor transcript RNA were gel-purified to eliminate sources of non-specific transcriptional inhibition. A 100 nt ladder is shown on the left (the lowest band visible is 200 nt). The position of the 524 nt product representing the *dhfr* major transcript is shown. We and others have previously characterized this template and confirmed that the 524 nt product is generated by authentic utilization of the major (core) promoter [Shimada et al., 1989; Blume et al., 1991]. The band marked 'A' represents the inadvertently end-labeled minor transcript RNA (232 nt) itself. The bands marked 'B' and 'C' apparently represent the transcripts of endogenous templates which are ordinarily repressed by the composition of transcription factors present in the extract, but which are derepressed in the midst of the titration by the minor transcript RNA.

were performed (Fig. 7). The conditions used were very similar to those of the gel mobility shift assays, but with inclusion of ribonucleotides (one radiolabeled), addition of a high energy phosphate source, and use of a longer, unlabeled *dhfr* core promoter/template DNA (− 112 to + 524) in place of the labeled *dhfr* core promoter fragment.

In the control sample, the appropriately-sized run-off product from the *dhfr* core promoter/template, representing the *dhfr* major transcript [Blume et al., 1991], was observed. With addition of the minor transcript RNA, the yield of the *dhfr* major transcript progressively declined. The concentrations of the minor transcript RNA required for inhibition of transcription from the *dhfr* core promoter correlated well with those necessary to alter the pattern of protein binding to the core promoter DNA sequence as assayed by mobility shift (refer to Figure 5). Thus, it appears that by selectively sequestering regulatory proteins, the *dhfr* minor transcript 5′-UTR is capable of modulating the activity of the *dhfr* core promoter in *trans*.

Another interesting observation can be gleaned from close examination of the results of the transcription assay. Two bands, labeled "B" and "C" in the figure, were not present in the positive control reaction, but appear suddenly midtitration, and then gradually decrease with higher concentrations of the minor transcript 5′-UTR. These bands apparently represent transcripts produced from unidentified templates endogenous to the extract, which are fully repressed by the milieu of transcription factors present in the extract, but which are derepressed by the sequestration activity of the *dhfr* minor transcript RNA. This result further illustrates the regulatory potential of the minor transcript 5′-UTR.

DISCUSSION

We hypothesized that the 5′-untranslated RNA sequence unique to the *dhfr* minor transcript might be capable of influencing transcription pre-initiation complex assembly at the homologous core promoter DNA. We have demonstrated in fact that the pattern of protein binding to the *dhfr* core promoter is dramatically altered in the presence of the minor transcript, and that this effect is absolutely dependent upon the integrity of the RNA sequence. Although evidence for a direct interaction between the minor transcript RNA and the core promoter DNA was obtained, this appears not to represent the reverse-Hoogsteen hydrogen-bonded purine–purine–pyrimidine triplex we had postulated, nor is it this interaction which is responsible for the alterations in protein binding to the core promoter DNA. Rather, it appears the 5′-UTR sequence of the *dhfr* minor transcript selectively sequesters certain transcription factors which otherwise would bind the *dhfr* core promoter DNA. A marked selectivity in this sequestration activity is evident, with the minor transcript displaying the highest affinity for Sp3, and a considerably lower affinity for Sp1. In addition, the binding of an unidentified transcription regulatory protein designated "z" to the core promoter is actually enhanced in the presence of the minor transcript. As a consequence of the alterations in transcription factor binding, the *dhfr* minor transcript 5′-UTR effectively inhibits transcription initiation from the *dhfr* core promoter in vitro in a concentration-dependent manner.

Initially, we used oligonucleotides as models to investigate the mechanism by which the *dhfr* minor transcript 5′-UTR RNA selectively sequesters transcription factors. We found that the effect of the *dhfr* minor transcript 5′-UTR on protein binding to *dhfr* core promoter DNA could be mimicked (though with less selectivity) by oligonucleotides representing the G-tract sequences contained within the minor transcript 5′-UTR, but not by C-tract containing oligonucleotides, nor mixed sequence oligonucleotides corresponding to the remainder of the natural *dhfr* promoter sequence. We had already established that these G-tract-containing oligonucleotides adopt very stable quadruplex structures, based on Hoogsteen hydrogen bonding patterns of guanine–guanine self-recognition, which are highly favored under physiological conditions. Indeed, the minor transcript RNA itself exhibits a series of altered structural conformations which are absolutely dependent upon the hydrogen bonding capacities of the G-tract sequences, and sequestration of transcription factors by the minor transcript 5′-UTR is partially abrogated by hybridization to an oligonucleotide complementary to the G-tracts of the minor transcript RNA. Thus, these quadruplex structures apparently contribute to the ability of the minor transcript 5′-UTR to sequester transcription factors. We suspect that the sequences naturally flanking the G-tracts

within the minor transcript 5′-UTR help to define the structural characteristics of this particular quadruplex [Keniry et al., 1997; Smirnov and Shafer, 2000], and are likely responsible for the much greater selectivity of the minor transcript RNA for sequestration of transcription factors (Sp3 > Sp1 > z) compared with the G-tract oligonucleotides.

A polyclonal antibody which binds an epitope at the carboxy-terminus of the Sp3 molecule completely blocks sequestration of the transcription factor by the minor transcript RNA, but is permissive to binding (through zinc finger interactions) the G/C box elements of the *dhfr* core promoter DNA. Although a number of zinc finger-containing transcription factors have been attributed the capability of binding single-stranded RNA [Caricasole et al., 1996; Hallier et al., 1996; Lai and Blackshear, 2001], it appears that the minor transcript RNA sequesters Sp3 (and presumably Sp1, though with lower affinity) through a distinct mode of interaction, involving a separate structural domain of the protein. The anti-Sp1 reagent does not have the same effect, however, this antibody recognizes an internal rather than C-terminal epitope.

The z complex is occasionally observed as a very light shift in the absence of added minor transcript RNA, and clearly represents a protein–DNA interaction which does not directly involve the minor transcript RNA. However, binding of the z factor to the *dhfr* core promoter DNA is greatly enhanced in the presence of the minor transcript 5′-UTR. Apparently by sequestering Sp3 and Sp1, the minor transcript RNA increases the accessibility of the core promoter DNA to this factor, which is otherwise at a competitive disadvantage for binding the core promoter. There certainly are precedents for competition between Sp family members and heterologous transcription factors when consensus recognition sequences overlap [Ackerman et al., 1991; Huang et al., 1997]. The identity of the z factor remains unknown. The results of enhanced footprinting experiments have indicated that the z factor exhibits a much more limited area of contact with the promoter DNA sequence, not centered on the consensus G/C boxes, and not at all typical of the Sp factors (data not shown).

The differential affinity of the minor transcript RNA for Sp3 versus Sp1 might be of significant functional consequence. Although the Sp3 molecule contains glutamine-rich transcription activation domains homologous to those of Sp1, it appears that additional structural determinants unique to Sp3 tend to interfere with the positive activity of its glutamine-rich domains [Dennig et al., 1996; Kumar and Butler, 1997; Fandos et al., 1999]. However, the activity of Sp3 as a transcriptional regulator is very much dependent upon the promoter context into which it is placed. From studies published to date, it appears that promoters containing multiple G/C box sequences are repressed by Sp3, whereas those with a single G/C box element are not repressed, and may even be activated by Sp3 [Majello et al., 1997; Noti, 1997]. While it has been reported that Sp3 represses the *dhfr* promoter in a cotransfection/reporter assay [Birnbaum et al., 1995], it is important to realize that those experiments were performed using the hamster *dhfr* promoter sequence, which contains two repeats of the core promoter architecture, and two additional G/C box elements positioned immediately upstream of these sequences (for a total of four G/C boxes [Mitchell et al., 1986; Swick et al., 1989; Wells et al., 1996]). The human *dhfr* core promoter sequence is not repeated, but does contain an awkwardly positioned second G/C box at − 14, which is without homology in either the mouse or hamster *dhfr* promoters [Farnham and Schimke, 1986; Mitchell et al., 1986]. The unusual spacing between the two G/C box elements in the human promoter may not facilitate the usual cooperation observed between multiple Sp factors organized in series and spaced multiples of ~10 bp apart [Gidoni et al., 1985; Takahashi et al., 1986]. Thus the function of Sp3 in the context of the human *dhfr* core promoter is not yet certain.

Possible Natural Regulatory Function of the dhfr Minor Transcript

Our in vitro data have demonstrated the capability of the 5′-UTR sequence unique to the *dhfr* minor transcript to modulate transcription preinitiation complex assembly at the *dhfr* core promoter in *trans*, through selective sequestration of regulatory polypeptides. We interpret these results as indicative of the potential of the minor transcript to contribute in *cis* to the physiological regulation of *dhfr* major (core) promoter activity in vivo (see proposed model in Figure 8). There are three major factors, which should be considered in extrapolating these

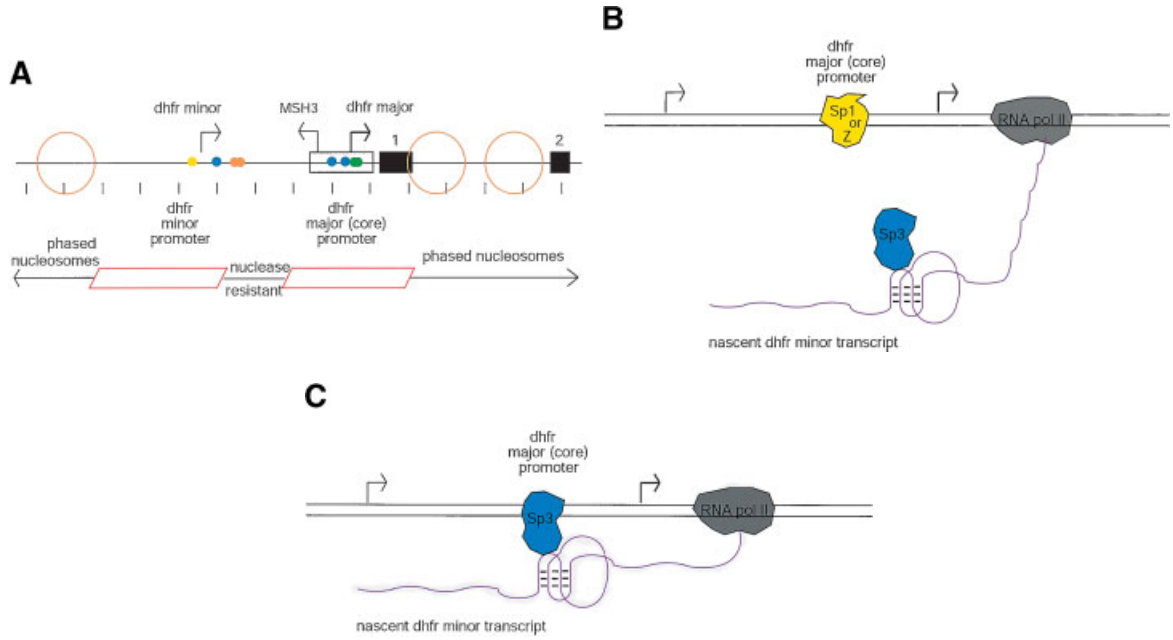

Fig. 8. Proposed model for natural regulatory function of the human *dhfr* minor transcript. **A**: Architecture of the promoter region of the human *dhfr*/MSH3 locus (drawn to scale). The *dhfr* major and minor and MSH3 transcription start sites are represented by bent arrows. The bidirectional core promoter is represented by an open rectangle. *dhfr* exons 1 and 2 are illustrated as filled black rectangles. Chromatin structure: the positions of nuclease hypersensitive regions of chromatin are indicated by red parallelograms; the positions of nucleosomes are indicated by large orange circles. Tic marks represent 100 base pairs. Consensus transcription factor binding sites for the *dhfr* major (core) and minor promoters are indicated by colored dots: blue = G/C box; yellow = G/C box (inverted); green = E2F/DP; orange = c-Myc. **B** and **C**: The *dhfr* minor transcript may function in vivo to selectively remove (sequester) or to facilitate (recruit) binding of Sp3 > Sp1 > z to the core promoter DNA, and thereby modulate the transcriptional activity of the major (core) promoter. The heavy and light bent arrows represent the *dhfr* major and minor transcription start sites, respectively, with the core promoter sequence (the site of assembly of the transcription initiation complex for synthesis of the predominant *dhfr* mRNA) in between. The model depicts the nascent minor transcript (purple line) during its synthesis by RNA polymerase II. The 5′-untranslated region of the minor transcript is inherently attached to the *dhfr* locus through the elongating RNA polymerase II transcription complex downstream, and likely resides in the immediate vicinity of the core promoter DNA (its template). Formation of G-tract-dependent quadruplex structure by the minor transcript 5′-UTR apparently contributes to its affinity for Sp family transcription factors. An intramolecular (folded) quadruplex structure (with reciprocal guanine–guanine hydrogen bonds) is depicted within the minor transcript 5′-UTR. Neither the precise organization of this structure nor its geometrical alignment with either of the G/C box sequences of the core promoter can be ascertained at this time. **(B) Sequestration**. As demonstrated in vitro, the minor transcript RNA may selectively remove or prevent binding of Sp factors to the core promoter DNA. The greater relative affinity of the minor transcript for Sp3 might result in preferential occupation of the G/C box elements by Sp1, or, by effectively sequestering both Sp3 and Sp1, the minor transcript may indirectly foster binding of the z factor to the *dhfr* core promoter DNA. **(C) Recruitment**. Since the interaction between the minor transcript RNA and Sp3 appears to involve a structural domain of the protein distinct from that required for recognition of consensus DNA sequences, it is conceivable that the minor transcript RNA may directly facilitate binding of Sp3 > Sp1 to the *dhfr* core promoter DNA.

in vitro results to an in vivo mechanism for control of *dhfr* expression.

First, in vivo, the nascent minor transcript RNA will be at least transiently attached to the *dhfr* core promoter DNA through the elongating RNA polymerase II complex further downstream. Thus synthesis of the minor transcript inherently places the 5′-UTR sequence in the immediate vicinity of the core promoter DNA (which is in fact its template).

Second, because binding of Sp3 to the minor transcript RNA and to the core promoter DNA appear to involve distinct domains of the protein, it may be possible that a single molecule of Sp3 (or perhaps Sp1) could interact simultaneously with both the RNA and DNA. Thus, what appears as sequestration in *trans* in an in vitro experiment, could actually equate to recruitment in *cis* under natural circumstances inside the cell. Whether through recruitment or sequestration, we would propose that the minor transcript 5′-UTR might facilitate physiologically-relevant changes in identity of the polypeptide transcription factors (e.g., Sp1 vs. Sp3

vs. z factor) bound to the *dhfr* core promoter DNA [also see DeLuca et al., 1996].

Finally, although we have focused on the G-tracts, it is very possible that additional specific interactions between the non-G-tract sequences of the *dhfr* minor transcript 5′-UTR and the transcriptional regulatory apparatus may also take place and contribute to the regulation of transcription initiation from *dhfr* major (core) promoter in vivo.

Synthesis of the minor transcript is under independent control of its own distinct promoter, which is positioned within a separate region of nucleosome-free, DNase hypersensitive (open) chromatin relative to the major (core) promoter [Shimada and Nienhuis, 1985; Shimada et al., 1986]. Even the organization of regulatory sequence elements of the minor promoter differs from that of the major (core) promoter. Of particular note are the absence in the minor promoter of the E2F/DP elements, and the addition of two non-consensus E-box sequences which have been shown to bind c-Myc in vitro and in vivo [Mai and Jalava, 1994; Boyd et al., 1998].

The minor transcript represents only a small proportion (~1%) of *dhfr* mRNA molecules, but is enriched (~11-fold) in the nucleus [Masters and Attardi, 1985; Fujii and Shimada, 1989]. Furthermore, the extended 5′-untranslated region is expected to decrease the efficiency of translation of the minor transcript [Kozak, 1987; Gray and Wickens, 1998]. Collectively, these circumstances (independent regulation, low prevalence, nuclear enrichment, and low translational efficiency) suggest that the potential function of the *dhfr* minor transcript as a regulatory molecule to modulate the transcriptional activity of the core promoter might be more important than its capacity to serve as an alternative template for DHFR polypeptide synthesis.

This model is consistent with the emerging realization that nuclear events (e.g., transcription) are orchestrated in a highly spatially/structurally organized manner [Stein et al., 2000].

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health/National Cancer Institute grant CA75467 (SB).

REFERENCES

Ackerman SL, Minder AG, Williams GT, Bobonis C, Yeung CY. 1991. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc Natl Acad Sci USA 88:7523–7527.

Bashkirov VI, Scherthan H, Solinger JA, Buerstedde JM, Heyer WD. 1997. A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. J Cell Biol 136:761–773.

Beal PA, Dervan PB. 1991. Second structural motif for recognition of DNA by oligonucleotide-directed triple-helix formation. Science 251:1360–1363.

Birnbaum MJ, Vanwijnen AJ, Odgren PR, Last TJ, Suske G, Stein GS, Stein JL. 1995. Sp1 transactivation of cell cycle regulated promoters is selectively repressed by Sp3. Biochemistry 34:16503–16508.

Blume SW, Snyder RC, Ray R, Thomas S, Koller CA, Miller DM. 1991. Mithramycin inhibits Sp1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. J Clin Invest 88:1613–1621.

Blume SW, Gee JE, Shrestha K, Miller DM. 1992. Triple helix formation by purine-rich oligonucleotides targeted to the human dihydrofolate reductase promoter. Nucleic Acids Res 20:1777–1784.

Blume SW, Guarcello V, Zacharias W, Miller DM. 1997. Divalent transition metal cations counteract potassium-induced quadruplex assembly of oligo dG sequences. Nucleic Acids Res 25:617–625.

Blume SW, Lebowitz J, Zacharias W, Guarcello V, Mayfield CA, Ebbinghaus SW, Bates P, Jones DE, Jr., Trent J, Vigneswaran N, Miller DM. 1999. The integral divalent cation within the intermolecular purine–purine–pyrimidine structure: A variable determinant of the potential for, and characteristics of the triple helical association. Nucleic Acids Res 27:695–702.

Boyd KE, Wells J, Gutman J, Bartley SM, Farnham PJ. 1998. c-Myc target gene specificity is determined by a post-DNA-binding mechanism. Proc Natl Acad Sci USA 95:13887–13892.

Buratowski S, Hahn S, Guarente L, Sharp PA. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56:549–561.

Cai H, Luse DS. 1987. Variations in template protection by the RNA polymerase II transcription complex during the initiation process. Mol Cell Biol 7:3371–3379.

Caricasole A, Duarte A, Larsson SH, Hastie ND, Little M, Holmes G, Todorov I, Ward A. 1996. RNA binding by the Wilms tumor suppressor zinc finger proteins. Proc Natl Acad Sci USA 93:7562–7566.

Chen M, Shimada T, Moulton AD, Cline A, Humphries RK, Maizel J, Nienhuis AW. 1984. The functional human dihydrofolate reductase gene. J Biol Chem 259:3933–3943.

Cheong C, Moore PB. 1992. Solution structure of an unusually stable RNA tetraplex containing G and U quartet structures. Biochemistry 31:8406–8414.

Choo Y, Klug A. 1994. Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions. Proc Natl Acad Sci USA 91:11168–11172.

Christiansen J, Kofod M, Nielsen FC. 1994. A guanosine quadruplex and two stable hairpins flank a major

cleavage site in insulin-like growth factor II mRNA. Nucleic Acids Res 22:5709–5716.
DeLuca P, Majello B, Lania L. 1996. Sp3 represses transcription when tethered to promoter DNA or targeted to promoter proximal RNA. J Biol Chem 271:8533–8536.
Dempsey LA, Sun H, Hanakahi LA, Maizels N. 1999. G4 DNA binding by LR1 and its subunits, nucleolin, and hnRNP D, a role for G–G pairing in immunoglobulin switch recombination. J Biol Chem 274:1066–1071.
Dennig J, Beato M, Suske G. 1996. An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. EMBO J 15:5659–5667.
Desjarlais JR, Berg JM. 1994. Length-encoded multiplex binding site determination: Application to zinc finger proteins. Proc Natl Acad Sci USA 91:11099–11103.
Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.
Fandos C, Sanchez-Feutrie M, Santalucia T, Vinals F, Cadefau J, Guma A, Cusso R, Kaliman P, Canicio J, Palacin M, Zorzano A. 1999. Glut1 glucose transporter gene transcription is repressed by Sp3. Evidence for a regulatory role of Sp3 during myogenesis. J Mol Biol 294: 103–119.
Fang G, Cech TR. 1993. Characterization of a G-quartet formation reaction promoted by the beta-subunit of the Oxytricha telomere-binding protein. Biochemistry 32: 11646–11657.
Farnham PJ, Schimke RT. 1986. In vitro transcription and delimitation of promoter elements of the murine dihydrofolate reductase gene. Mol Cell Biol 6:2392–2401.
Frantz JD, Gilbert W. 1995. A novel yeast gene product, G4p1, with a specific affinity for quadruplex nucleic acids. J Biol Chem 270:20692–20697.
Friesen WJ, Darby MK. 1997. Phage display of RNA binding zinc fingers from transcription factor IIIA. J Biol Chem 272:10994–10997.
Fry M, Loeb LA. 1999. Human werner syndrome DNA helicase unwinds tetrahelical structures of the fragile X syndrome repeat sequence d(CGG)n. J Biol Chem 274: 12797–12802.
Fujii H, Shimada T. 1989. Isolation and characterization of cDNA clones derived from the divergently transcribed gene in the region upstream from the human dihydrofolate reductase gene. J Biol Chem 264:10057–10064.
Fujii H, Shinya E, Shimada T. 1992. A GC box in the bidirectional promoter is essential for expression of the human dihydrofolate reductase and mismatch repair protein 1 genes. FEBS Lett 314:33–36.
Gee JE, Blume S, Snyder RC, Ray R, Miller DM. 1992. Triplex formation prevents Sp1 binding to the dihydrofolate reductase promoter. J Biol Chem 267:11163–11167.
Gidoni D, Dynan WS, Tjian R. 1984. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature 312:409–413.
Gidoni D, Kadonaga JT, Barrera-Saldana H, Takahashi K, Chambon P, Tjian R. 1985. Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. Science 230:511–517.
Gill G, Pascal E, Tseng ZH, Tjian R. 1994. A glutamine rich hydrophobic patch in transcription factor Sp1 contacts the dTAF(II)110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. Proc Natl Acad Sci USA 91:192–196.
Giraldo R, Suzuki M, Chapman L, Rhodes D. 1994. Promotion of parallel DNA quadruplexes by a yeast telomere binding protein: A circular dichroism study. Proc Natl Acad Sci USA 91:7658–7662.
Gray NK, Wickens M. 1998. Control of translation initiation in animals. Ann Rev Cell Dev Biol 14:399–459.
Grondin B, Bazinet M, Aubry M. 1996. The KRAB zinc finger gene ZNF74 encodes an RNA-binding protein tightly associated with the nuclear matrix. J Biol Chem 271:15458–15467.
Gualberto A, Lowry J, Santoro IM, Walsh K. 1995. Parameters that influence the extent of site occupancy by a candidate telomere end-binding protein. J Biol Chem 270:4509–4517.
Hallier M, Tavitian A, Moreau-Gachelin F. 1996. The transcription factor Spi-1/PU.1 binds RNA and interferes with the RNA binding protein p54nrb. J Biol Chem 271: 11177–11181.
Hanvey JC, Shimizu M, Wells RD. 1990. Site-specific inhibition of EcoRI restriction/modification enzymes by a DNA triple helix. Nucleic Acids Res 18:157–161.
Hardin CC, Watson T, Corregan M, Bailey C. 1992. Cation-dependent transition between the quadruplex and Watson–Crick hairpin forms of d(CGCG3GCG). Biochemistry 31:833–841.
Huang RP, Fan Y, Zhengyu N, Mercola D, Adamson ED. 1997. Reciprocal modulation between Sp1 and Egr-1. J Cell Biochem 66:489–499.
Keniry MA, Owen EA, Shafer RH. 1997. The contribution of thymine–thymine interactions to the stability of folded dimeric quadruplexes. Nucleic Acids Res 25:489–492.
Kohwi Y, Kohwi-Shigematsu T. 1988. Magnesium ion-dependent triple-helix structure formed by homopurine–homopyrimidine sequences in supercoiled plasmid DNA. Proc Natl Acad Sci USA 85:3781–3785.
Kozak M. 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15: 8125–8148.
Kumar AP, Butler AP. 1997. Transcription factor Sp3 antagonizes activation of the ornithine decarboxylase promoter by Sp1. Nucleic Acids Res 25:2012–2019.
Ladomery M, Sommerville J. 1994. Binding of Y-box proteins to RNA: Involvement of different protein domains. Nucleic Acids Res 22:5582–5589.
Lai WS, Blackshear PJ. 2001. Interactions of CCCH zinc-finger proteins with mRNA: Tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. J Biol Chem 276: 23144–23154.
Lee JS. 1990. The stability of polypurine tetraplexes in the presence of mono- and divalent cations. Nucleic Acids Res 18:6057–6060.
Letai AG, Palladino MA, Fromm E, Rizzo V, Fresco JR. 1988. Specificity in formation of triple-stranded nucleic acid helical complexes: Studies with agarose-linked polyribonucleotide affinity columns. Biochemistry 27:9108–9112.
Liu Z, Lee A, Gilbert W. 1995. Gene disruption of a G4-DNA-dependent nuclease in yeast leads to cellular senescence and telomere shortening. Proc Natl Acad Sci USA 92:6002–6006.
Lyamichev VI, Voloshin ON, Frank-Kamenetskii MD, Soyfer VN. 1991. Photofootprinting of DNA triplexes. Nucleic Acids Res 19:1633–1638.

Mai S, Jalava A. 1994. C-myc binds to 5′ flanking sequence motifs of the dihydrofolate reductase gene in cellular extracts: Role in proliferation. Nucleic Acids Res 22: 2264–2273.

Majello B, DeLuca P, Lania L. 1997. Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. J Biol Chem 272:4021– 4026.

Manley JL, Fire A, Cano A, Sharp PA, Gefter ML. 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc Natl Acad Sci USA 77: 3855–3859.

Marck C, Thiele D. 1978. Poly(dG)–poly(dC) at neutral and alkaline pH: The formation of triple stranded poly(dG)–poly(dG)–poly(dC). Nucleic Acids Res 5:1017–1028.

Masters JN, Attardi G. 1985. Discrete human dihydrofolate reductase gene transcripts present in polysomal RNA map with their 5′ ends several hundred nucleotides upstream of the main mRNA start site. Mol Cell Biol 5:493–500.

Mitchell PJ, Carothers AM, Han JH, Harding JD, Kas E, Venolia L, Chasin LA. 1986. Multiple transcription start sites, DNase I hypersensistive sites, and an opposite strand exon in the 5′ region of the CHO *dhfr* gene. Mol Cell Biol 6:425–440.

Morgan AR, Wells RD. 1968. Specificity of the three stranded complex formation between double stranded DNA and single stranded RNA containing repeating nucleotide sequences. J Mol Biol 37:63–80.

Murchie AIH, Lilley DMJ. 1992. Retinoblastoma susceptibility genes contain 5′ sequences with a high propensity to form guanine-tetrad structures. Nucleic Acids Res 20: 49–53.

Noonberg SB, Scott GK, Hunt CA, Hogan ME, Benz CC. 1994. Inhibition of transcription factor binding to the HER2 promoter by triplex-forming oligodeoxyribonucleotides. Gene 149:123–126.

Noti JD. 1997. Sp3 mediates transcriptional activation of the leukocyte integrin genes CD11C and CD11B and cooperates with c-Jun to activate CD11C. J Biol Chem 272:24038–24045.

Pavletich NP, Pabo CO. 1991. Zinc finger–DNA recognition: Crystal structure of a Zif268–DNA complex at 2.1 A. Science 252:809–817.

Pierce AJ, Jambou RC, Jensen DE, Azizkhan JC. 1992. A conserved DNA structural control element modulates transcription of a mammalian gene. Nucleic Acids Res 20:6583–6587.

Saluja D, Vassallo MF, Tanese N. 1998. Distinct subdomains of human TAFII130 are required for interactions with glutamine-rich transcriptional activators. Mol Cell Biol 18:5734–5743.

Schierer T, Henderson E. 1994. A protein from Tetrahymena thermophila that specifically binds parallel-stranded G4-DNA. Biochemistry 33:2240–2246.

Sen D, Gilbert W. 1988. Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. Nature 334:364–366.

Shimada T, Nienhuis AW. 1985. Only the promoter region of the constitutively expressed normal and amplified human dihydrofolate reductase gene is DNAse I hypersensitive and undermethylated. J Biol Chem 260:2469–2474.

Shimada T, Inokuchi K, Nienhuis AW. 1986. Chromatin structure of the human dihydrofolate reductase gene promoter. Multiple protein-binding sites. J Biol Chem 261:1445–1452.

Shimada T, Fujii H, Lin H. 1989. A 165 base pair sequence between the dihydrofolate reductase gene and the divergently transcribed upstream gene is sufficient for bidirectional transcriptional activity. J Biol Chem 264: 20171–20174.

Shinya E, Shimada T. 1994. Identification of two initiator elements in the bidirectional promoter of the human dihydrofolate reductase and mismatch repair protein 1 genes. Nucleic Acids Res 22:2143–2149.

Smirnov I, Shafer RH. 2000. Effect of loop sequence and size on DNA aptamer stability. Biochemistry 39:1462.

Stein GS, van Wijnen AJ, Stein JL, Lian JB, Montecino M, Choi J, Zaidi K, Javed A. 2000. Intranuclear trafficking of transcription factors: Implications for biological control. J Cell Sci 113:2527–2533.

Sun H, Karow JK, Hickson ID, Maizels N. 1998. The Bloom's syndrome helicase unwinds G4 DNA. J Biol Chem 273:27587–27592.

Sun D, Lopez-Guajardo CC, Quada J, Hurley LH, Von Hoff DD. 1999. Regulation of catalytic activity and processivity of human telomerase. Biochemistry 38:4037–4044.

Swick AG, Blake MC, Kahn JW, Azizkhan JC. 1989. Functional analysis of GC element binding and transcription in the hamster dihydrofolate reductase gene promoter. Nucleic Acids Res 17:9291–9304.

Takahashi K, Vigneron M, Matthes H, Wildeman A, Zenke M, Chambon P. 1986. Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter. Nature 319:121–126.

Uliel L, Weisman-Shomer P, Oren-Jazan H, Newcomb T, Loeb LA, Fry M. 2000. Human Ku antigen tightly binds and stabilizes a tetrahelical form of the Fragile X syndrome d(CGG)n expanded sequence. J Biol Chem 275: 33134–33141.

Wade M, Blake MC, Jambou RC, Helin K, Harlow E, Azizkhan JC. 1995. An inverted repeat motif stabilizes binding of E2F and enhances transcription of the dihydrofolate reductase gene. J Biol Chem 270:9783–9791.

Walsh K, Gualberto A. 1992. MyoD binds to the guanine tetrad nucleic acid structure. J Biol Chem 267:13714–13718.

Wang Y, Patel DJ. 1992. Guanine residues in d(T2AG3) and d(T2G4) form parallel-stranded potassium cation stabilized G-quadruplexes with anti glycosidic torsion angles in solution. Biochemistry 31:8112–8119.

Watanabe A, Ikejima M, Suzuki N, Shimada T. 1996. Genomic organization and expression of the human MSH3 gene. Genomics 31:311–318.

Wells J, Held P, Illenye S, Heintz NH. 1996. Protein–DNA interactions at the major and minor promoters of the divergently transcribed *dhfr* and rep3 genes during the Chinese hamster ovary cell cycle. Mol Cell Biol 16:634–647.

Williamson JR, Raghuraman MK, Cech TR. 1989. Monovalent cation-induced structure of telomeric DNA: The G-quartet model. Cell 59:871–880.

Yan M, Gralla JD. 1997. Multiple ATP-dependent steps in RNA polymerase II promoter melting and initiation. EMBO J 16:7457–7467.

Zimmerman SB, Cohen GH, Davies DR. 1975. X-ray fiber diffraction and model-building study of polyguanylic acid and polyinosinic acid. J Mol Biol 92:181–192.