The Bidirectionally Transcribed Dihydrofolate Reductase and rep-3a Promoters Are Growth Regulated by Distinct Mechanisms

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Abstract
The mouse dihydrofolate reductase (dhfr) gene possesses a bidirectional promoter that produces functional transcripts in the opposite direction. These opposite strand transcripts encode the Rep-3 gene product, a protein that has homology to DNA mismatch repair enzymes. The core of the bidirectional promoter consists of four consensus binding sites for the transcription factor Sp1. These binding sites have been shown to be important for basal transcription from both the rep-3a and dhfr promoters. Extensive characterization of the dhfr promoter has shown that growth-dependent regulation requires the E2F binding sites that flank the transcription initiation site. Here we show that endogenous rep-3a mRNA and the rep-3a promoter are growth regulated, in a manner very similar to the regulation of the dhfr mRNA and promoter region. However, we find that the E2F sites required for dhfr regulation are dispensable for regulation of the rep-3a promoter. Instead, we have shown that the rep-3a initiation region is critical for the G1/S phase-specific activation of this promoter. Gel mobility shift experiments indicate that a member of the Sp1 family of transcription factors binds to the rep-3a initiation region, suggesting that this family of transcription factors may play a role in cell growth control.

Introduction
The mouse dhfr gene was the first mammalian cellular gene shown to possess a bidirectional promoter that produces functional transcripts in the opposite direction (1, 2). Although the basal and regulated expression of the mouse dhfr gene has been extensively studied, relatively little is known about the bidirectionally transcribed rep-3 gene (reviewed in Ref. 3). dhfr is an essential gene, the protein product of which is required for the synthesis of glycine, purines, and thymidine. The Rep-3 gene product shares homology with proteins in bacteria, yeast, and humans that function in DNA mismatch repair, but no specific function has been elucidated for Rep-3. Transcription of the rep-3 gene is directed by two promoters; the majority of transcripts initiate 190–250 bp upstream of the dhfr transcription start site under the direction of the rep-3a promoter, and the remainder initiate about 700 bp upstream of dhfr from the rep-3b promoter. The unique architecture of the dhfr/rep-3a bidirectional promoter affords the opportunity to study the regulatory interplay between two closely spaced transcriptional units. The core of the bidirectional promoter is formed by 3[1/2] direct repeats of a 48-bp sequence, which contains a consensus binding site for the transcription factor Sp1. Analysis of the mouse dhfr promoter, both in transfection and in vitro transcription assays, revealed the critical role of Sp1 binding sites in basal transcription of the gene (4–7). Similarly, transfection experiments demonstrated that Sp1 binding sites are important for the basal transcription of rep-3a (7). In general, mutational analysis has indicated that the Sp1 sites closest to dhfr or rep-3a start sites are most influential in the basal transcription of that promoter.

Previous studies using mitotically selected cells established that levels of both dhfr and rep-3a mRNAs increase as cells enter S-phase (8). Numerous studies have shown that the dhfr promoter is transcriptionally regulated as cells enter S phase in preparation for DNA synthesis (reviewed in Ref. 3). Transcription increases 7- to 15-fold during the transition from mitosis to S-phase (9), which we will term cell cycle regulation, and about 12-fold during the transition from a quiescent state to S-phase (10), which we will term growth regulation. The mechanism by which the rep-3a mRNAs are regulated was not examined in these previous studies. We wished to compare the cis elements and transacting factors that regulate rep-3a transcription with those that regulate dhfr transcription. However, the elements required for the regulation of dhfr through the proliferative cell cycle have not been characterized. Instead, a transient transfection system using NIH 3T3 cells that are serum-starved and then stimulated has been used to characterize regulation of dhfr promoter fragments after serum stimulation of quiescent cells (10, 11). This system has numerous advantageous features: (a) a luciferase reporter gene is used which provides the sensitivity needed to measure relatively weak promoters, and the short half-life of the luciferase protein in mammalian cell lines (estimated to be 1–3 h) provides a low background in which to detect fluctuations in gene expression (12, 13); (b) because these constructs can be analyzed transiently, they are not subject to chromosome-position effects that have confused analysis of stably transfected dhfr constructs (see Ref. 3); and (c) homogeneous populations of cells in different phases of the growth cycle are easily obtained, allowing the fluctuation in levels of endogenous mRNA to be compared with changes in activity of the promoter-luciferase constructs. Thus, using this assay system, many different constructs can be analyzed rapidly and reproducibly.

Using the transient transfection assay, growth-regulated expression of dhfr has been shown to be dependent on the
E2F binding sites that flank the *dhfr* transcription site. At present, seven different but related proteins, collectively termed the E2F family, have been shown to bind to the consensus E2F sequence: TTTSSCGC (reviewed in Ref. 14). In the context of the *dhfr* promoter, the E2F sites are necessary but not sufficient to mediate a G1,S-phase increase in transcription (15, 16), suggesting that a factor binds to upstream sequences and cooperates with a member of the E2F family to activate growth-regulated transcription. We have now examined the growth regulation of the rep-3a mRNA using the serum-starvation and -stimulation assay. We find that, although the expression pattern of rep-3a mRNA is remarkably similar to the *dhfr* mRNA profile, the E2F binding sites required for the serum-dependent increase in *dhfr* transcription are dispensable for activation of rep-3a transcription. However, the rep-3a initiation region is required for the induction of rep-3a. Thus, although these two promoters share the same sequences and display similar expression patterns, they are regulated by distinct mechanisms.

**Results**

**The Expression of rep-3a mRNA Is Growth Regulated.** Although previous studies had established that rep-3a mRNAs are regulated during the proliferative cell cycle, changes in abundance of these mRNAs have not been examined during the transition from a quiescent state into the proliferative cell cycle. To determine whether endogenous rep-3a is growth regulated, NIH-3T3 cells were incubated in low levels of serum for 2 days to induce quiescence; medium containing a high concentration of serum was then added, and samples were harvested at various times after cells reentered the growth cycle. Analysis of DNA content by flow cytometry demonstrated that the cells entered quiescence during the starvation period and that S-phase began approximately 10 h after release from serum starvation in the absence of cycloheximide (data not shown). RNase protection assays were carried out with a uniformly radiolabeled probe complementary to a 5′ portion of rep-3a mRNA (Fig. 1). Although rep-3a initiates transcription at multiple sites, the protected fragment appears as a single band of 180 bp because the probe does not overlap the initiation region. rep-3a mRNA abundance was lower in quiescent cells than in proliferating cells (Fig. 1, Lanes 1 and 2). Following serum stimulation of quiescent cells, rep-3a mRNA levels increased as cells reentered the growth cycle and peaked as cells entered S-phase; this profile of rep-3a mRNA induction closely resembles the response of the *dhfr* gene (11). Levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were examined in the RNA samples used in Fig. 1, Lanes 1–13 to control for errors in quantitation and to ensure that mRNA degradation had not occurred (11). Although our analysis did not measure the level of each of the multiple rep-3a 5′-ends during the growth cycle, the relative abundance of the 5′-ends does not vary during a proliferative cell cycle (8). Therefore, we expect that the increase in the population of rep-3a mRNA reflects the increases in individual rep-3a mRNAs. As is characteristic of genes that are activated late in the cell cycle, the addition of cycloheximide to the medium during stimulation abolished the increase in rep-3a mRNA (Fig. 1, Lanes 8–13), suggesting that new protein synthesis is required for the induction. Therefore, a growth-responsive activator of rep-3a expression is not present in quiescent cells, or a repressor of rep-3a expression is not removed when cells are treated with the protein synthesis inhibitor. The serum-dependent increase in *dhfr* expression is also abolished in the presence of cycloheximide (11). Therefore, rep-3a can be characterized as a growth-responsive gene, and in this assay, responded in a manner indistinguishable from the *dhfr* gene.

**The rep-3a Promoter Confers Growth Responsiveness on a Reporter Gene.** As a first step towards identifying the regulators of the rep-3a growth response, we asked whether some portion of the rep-3a gene was sufficient to confer serum inducibility to a heterologous gene. In proliferating cells, a *dhfr* promoter fragment spanning from −270 to +20 (relative to the *dhfr* transcription initiation site at +1) produced similar luciferase activities in either orientation (data not shown), indicating that the 290-bp fragment contained functional promoters for both *dhfr* and rep-3a. This fragment contains the *dhfr* start site and all of the initiation sites for the rep-3a gene. Previously, this fragment was shown to impart serum inducibility to a luciferase reporter gene when inserted in the *dhfr* direction (10). We next measured the activity of the rep-3a promoter in serum-starved and -stimulated cells. NIH 3T3 cells were transiently transfected with the rep-3a-luciferase construct, starved for 3 days, stimulated, and harvested at intervals after stimulation. The
activity of the rep-3a construct remained low until 10 h after stimulation, peaked at 14 h, and then declined (Fig. 2). The response of this rep-3a-luciferase construct was similar to the profile observed for the endogenous rep-3a mRNA, indicating that the cis targets for the serum-response regulators are contained within the 290-bp DNA fragment. The rep-3a serum-response curve was also nearly equivalent to the previously published profile for the dhfr construct (Fig. 2). Since these two promoters share both a common promoter region and comparable serum-response profiles, we hypothesized that dhfr and rep-3a may be growth regulated by the same mechanism.

**E2F Binding Sites Are Not Required for Growth Regulation of the rep-3a Promoter.** Because the rep-3a and dhfr expression patterns were comparable in the serum-starvation and -stimulation assays, it was possible that the same cis elements controlled G1,S-phase regulation of the bidirectional promoters. The E2F binding sites at the dhfr initiation sites have been shown to be critical for G1,S-phase activation of the dhfr promoter (10). Therefore, we wished to test directly whether E2F binding sites are necessary for the serum-dependent increase in rep-3a expression. This first requires identification and mutation of all sites in the rep-3a promoter that resemble E2F binding sites. In addition to the consensus E2F sites near the dhfr initiation site, a 7 of 8 match to an E2F site lies within the rep-3a initiation region. Although E2F binding has been demonstrated using the consensus sites near the dhfr initiation site (17), protein binding to the putative E2F site near the rep-3a initiation region has not been examined. Therefore, we assayed regions of the dhfr/repl construct for E2F binding activity in gel shift competition experiments using Friend cell nuclear extract and the dhfr initiation region as a probe (Fig. 3). Binding to the probe was competed by prior incubation of the extract with unlabeled probe (Fig. 3B, Lane 2) or with the entire promoter fragment (Fig. 3B, Lanes 4–5) but not with an oligonucleotide of the dhfr initiation region that contains mutations in the E2F sites (Fig. 3B, Lane 3) or with an oligonucleotide containing binding sites for the transcription factor Sp1 (Fig. 3B, Lanes 12–13). A BglI/FspI fragment of the dhfr/repl promoter containing the 3[1/2] copies of the 48-bp repeated sequence (see Fig. 3A for a schematic) did not compete for E2F binding (Fig. 3B, Lanes 9–11). However, the NotI/BglI fragment containing the rep-3a initiation region did compete binding (Fig. 3B, Lanes 6–8), albeit about 6-fold less effectively than the probe itself. Mutation of the best match to an E2F site within the rep-3a initiation region from TCTGCCGC to TCTCAAGC reduced the ability of the NotI/BglI fragment to compete E2F binding activity (Fig. 3B, Lanes 14–19). Thus, the most significant target for E2F binding activity in the dhfr/repl promoter region, besides the overlapping sites flanking the dhfr initiation region, is a region near the rep-3a initiation sites.

Using the serum-starvation and -stimulation assay, we next tested whether E2F sites are necessary for rep-3a growth regulation. Although the sequences near the rep-3a initiation region bind E2F with low efficiency, we decided to introduce a mutation into this region in the context of a previously described plasmid that already contains mutations in the dhfr proximal E2F sites (mutant E14 Ref. 10). The double mutant (2xmut) and wild-type -270/+20 constructs were tested in both orientations. When the luciferase reporter was under the direction of the dhfr promoter, the wild-type construct displayed a serum-dependent increase. Previously, mutations in the dhfr proximal E2F sites were shown to abolish growth-regulated transcription from the dhfr promoter. Therefore, as expected, the doubly mutated dhfr promoter was not induced in the presence of serum (Fig. 3C). Surprisingly, the mutations did not significantly alter the serum-response profile of the luciferase construct under direction of the rep-3a promoter (Fig. 3D). Thus, E2F binding sites are not required for growth regulation of the rep-3a promoter. Because no significant E2F binding activity remains in the mutated promoter fragment (as determined by binding assays in Friend cell nuclear extract), we conclude that E2F binding activity is not required for the rep-3a serum response.

The rep-3a Initiation Region Is Critical for the rep-3a Growth Response. To localize the regions of the rep-3a promoter that mediate the serum-dependent induction of rep-3a, the responsive 290-bp promoter was divided into three regions: the core containing the 48-bp repeats, the rep-3a initiation region, and the dhfr initiation region. FBrep contains only the core, FMrep contains the core plus the rep-3a initiation region, and MBrep contains the core plus the dhfr initiation region (Fig. 4A). The three new rep-3a promoter constructs were tested in the serum-starvation and -stimulation assay, along with the responsive, full-length rep-3a construct and the nonresponsive SV40 early construct, pGLpromoter (11). The fold induction, measured at 14 h for the core promoter construct FBrep, was similar to that measured for pGLpromoter (Fig. 4B); therefore, either the dhfr or rep-3a initiation region or both regions are essential for rep-3a serum responsiveness. Removal of the dhfr initiation region from the full-length construct did not diminish the rep-3a serum response (compare rep-3a and FMrep). However, deletion of the rep-3a initiation region rendered the FBrep construct nonresponsive. Therefore,
sequences in the rep-3a initiation region, but not the dhfr initiation region, are critical for mediating the serum induction of the rep-3a promoter.

The constructs tested in Fig. 4B map the rep-3a growth-responsive element to the region between –270 and –206. The responsive 2xmutrep-3a construct used in Fig. 3D had an endpoint at –257. Therefore, the essential element is contained with the 51-bp NotI/BglII fragment. rep-3a sequence was compared against the transcription factor SITES database compiled by the National Center for Biotechnology Information (18). However, no strong matches to any transcription factor binding sites lie within the 51-bp responsive element of the rep-3a promoter, other than the partial match to an E2F site, which was shown in Fig. 3D to be dispensable for rep-3a regulation. Gel mobility shift analysis was conducted using Friend cell nuclear extract and a probe isolated from the 2xmutrep plasmid encompassing sequences from NotI to BglII. Protein binding was detected (Fig. 5A, Lane 2) that was not competed by prior incubation with an oligonucleotide containing the dhfr E2F sites (Fig. 5D, Lanes 3 and 4) or a nonspecific oligonucleotide (HS-mut; Fig. 5D, Lanes 9 and 10) but was competed by sequences from the SV40 promoter containing Sp1 sites (SV Sp1; Fig. 5D, Lanes 5 and 6). There are no consensus Sp1 binding sites within the probe; however the sequence is very GC rich. Therefore, additional competitors were tested that contain Sp1 sites or other GC-rich factor binding sites. Binding was also competed by a fragment of the cad promoter containing two Sp1 sites (Fig. 5B, Lanes 6 and 7) but not by GC-rich oligonucleotides containing binding sites for AP-2 (Fig. 5A, Lanes 7 and 8) or EGR1 (Fig. 5B, Lanes 8 and 9). Thus, only GC-rich oligonucleotides that bind Sp1 can compete protein binding to the rep-3a probe. When an anti-Sp1 antibody was added to the binding reaction, a
regulation of the rep-3a promoter. Thus, these two promoters are controlled by distinct mechanisms.

In the 290-bp fragment that can confer growth responsiveness to a luciferase reporter gene in both the dhfr and rep-3a orientations, we found that the elements most critical for growth regulation were overlapping the transcriptional initiation sites of the dhfr and rep-3a transcripts. E2F binding sites lie next to the dhfr start sites and are critical for dhfr regulation. A binding site for a member of the Sp1 gene family lies within the rep-3a initiation region that is critical for growth regulation. There is no consensus Sp1 site in this region, but the sequences are extremely GC-rich; analysis of a series of points mutations is required before the exact binding site can be identified. The repeat region of the dhfr/rep-3a bidirectional promoter, which contains four consensus Sp1 binding sites, is not sufficient to mediate the rep-3a serum response, suggesting that not all Sp1 sites are the same. For example, the position of an Sp1 site with respect to the transcription initiation site may determine if an Sp1 family member serves a basal or growth-regulatory role in transcription initiation, or particular sequences at or adjacent to the binding site may influence binding of different Sp1 family members. We cannot definitively rule out that an as yet unidentified factor could regulate rep-3a.

Candidates for another growth-regulating factor include the c-myc proto-oncogene which regulates the carbamoylphosphate synthase (glutamine-hydrolyzing/aspartate carbamoyltransferase/dihydroorotase (cad) gene (22), and H1TF-2, Oct-1, and Hinf-M, which regulate transcription of histone subtypes H1, H2b (23), and H4 (24), respectively. However, consensus binding sites for these factors are not present in the rep-3a initiation region, nor could we detect binding of non-Sp1 family members to the rep-3a responsive element.

Other investigations have recently linked the Sp1 family to growth regulation. For example, the nuclear oncogene, v-Rel, enhances Sp1-driven transcription (25, 26), and the retinoblastoma protein-mediated control of transcription from c-fos, c-myc, and transforming growth factor β1 occurs through Sp1 (27, 28). Also, we have shown that v-RAF-induced transcription from the rep-3b promoter (the minor promoter of the rep-3 gene) and the mdr1 promoter is mediated via Sp1 binding sites (29). Common mechanisms by which eukaryotic genes are transcriptionally regulated in vivo include adjusting the abundance of critical transcription factors, modulating the activity of preexisting factors via posttranslational modification, or altering the nature of protein-protein interactions. We have shown that the amount of binding to a variety of Sp1 sites does not change after serum stimulation of quiescent 3T3 cells (17, 22, 29). These data suggest that serum-induced signals do not modulate the abundance of DNA binding-competent protein, but rather that mitogenic signals may enhance the transcription potential of a prebound Sp1 family member. Since the increase in rep-3a mRNA at the G,S-phase boundary is abolished by the addition of cycloheximide to the culture media, an attractive model is that a protein that either interacts with or modifies an Sp1 family member is lacking in quiescent cells. Sp1 is believed to directly contact the general transcription machinery, as well as other activator proteins. For example, TAF250, a TFIIID-associated protein critical for cell cycle progression, mediates Sp1-activated transcription (30). Also, Sp1 can be regulated via posttranslational modifications to its transactivation domain, such as O-linked glycosylation and phosphorylation on serine and

Discussion

To better understand proliferation control, and ultimately to understand how normal cells lose these controls in the process of tumorigenesis, we have studied the regulation of genes whose expression is linked to cell proliferation. We have previously shown that the dhfr promoter is regulated by a member of the E2F family of transcription factors (11, 17). Although other evidence suggests that E2F does not control all G,S-phase activated genes (11, 17, 22), because rep-3a is bidirectionally transcribed from the same promoter region as dhfr, and is induced at the G,S-phase boundary in a similar manner to dhfr, we expected that the E2F family might control rep-3a expression. However, we found that the E2F sites that are essential for the serum induction of the dhfr promoter are not required for growth
threonine residues, both of which correlate with an increase in transactivation potential (31, 32). The precise means by which serum mitogens lead to alterations in the activity of Sp1 family members awaits further investigation.

In summary, although dhfr and rep-3a do share a core promoter for basal transcription, the key elements that regulate dhfr and rep-3a during the growth cycle are different. Continued analysis of the E2F and Sp1 families of transcription factors will provide insight into the complex signal transduction pathways that lead to G1/S-phase-specific gene expression.

Materials and Methods
DNA Constructs. The previously described wild-type dhfr luciferase construct contains sequences from −270 to +20
(relative to the \textit{dihfr} transcription initiation site at +1) inserted into the \textit{Hind}III site of the pAAlac4 vector, which contains the luciferase cDNA (10). The wild-type \textit{rep-3a} luciferase construct contains the same DNA fragment upstream of the luciferase cDNA, but in the opposite orientation. The 2xmutdihfr and 2xmutrep-3a were created by PCR using primers homologous to +4 to +20 and -257 to -236, except for a double point mutation in the latter primer that changed the sequence at -243 and -242 from GC to AA, and a DNA template that contains mutations in the \textit{dihfr} proximal E2 sites (mutant EJ); Ref. 10. The PCR primers also contained 12-bp \textit{Hind}III linkers to facilitate cloning of the PCR-generated fragment into the pAAlac4 vector at the \textit{Hind}III site in both orientations. The FBrep, FMrep, and MBrep constructs contain sequences from FspI (-39) to BglII (-206), FspI (-39) to Mael (-270), and Mael (+20) to BglII (-206), respectively, cloned into pAAlac4 at the \textit{Hind}III site with \textit{Hind}III linkers.

\textbf{Cell Culture.} NIH 3T3 cells were maintained in DMEM (GIBCO) supplemented with 5% defined-supplemented bovine calf serum (HyClone), 100 units/ml penicillin, and 100 \mu g/ml streptomycin. Cells to be harvested for RNA analysis were placed in starvation medium (0.5% serum) for 45 h and then were stimulated by replacing the starvation medium with stimulation medium (10% serum). For the samples used in Fig. 1, 30 min prior to stimulation, 10 \mu g/ml cycloheximide was added to one-half of the cultures. Calcium phosphate transfections were performed as described previously, except that a total of 1 x 10\textsuperscript{6} cells were plated per 60-mm dish (11).

\textbf{RNase Protection Analysis.} Cytoplasmic RNA was prepared as described previously (11). A uniformly labeled RNA probe complementary to \textit{rep-3a} mRNA was created by linearizing the pRT10+ plasmid (8) by digestion with NotI and transcribing the template with SP6 bacteriophage polymerase and (\alpha-\textsuperscript{32}P)GTP as described (33). Cytoplasmic RNA (20 \mu g) and 2.5 x 10\textsuperscript{4} cpm probe were ethanol precipitated, resuspended in 8 \mu l formamide; and 2 \mu l 5X hybridization buffer (200 \mu M PIPES (pH 6.4), 2 mM NaCl, and 5 \mu M EDTA) was added. The sample was incubated at 85°C for 5 min and then at 56°C for 3 h. Unhybridized RNA was digested by incubation with 300 \mu l digestion buffer (10 \mu M Tris-HCl (pH 7.5), 5 \mu M EDTA and 300 \mu M NaCl) containing 40 \mu g RNase A and 0.3 units RNase T1 for 30 min at 30°C. Digestion reactions were terminated by addition of 50 \mu g proteinase K and 10 \mu g of 20% SDS and incubated for 15 min at 37°C. The samples were extracted with an equal volume of a 1:1 mixture of phenol and chloroform and ethanol precipitated before resolving the hybrids by denaturing gel electrophoresis.

\textbf{Gel Mobility Shift Analysis.} E2F gel shift assays were performed as described (34); the probe was a 30-bp double-stranded oligonucleotide spanning from -20 to +9 of the mouse \textit{dihfr} promoter (see sequence of fragment D described below). Protein binding to \textit{rep-3a} promoter fragments was assayed by incubating 3-6 \mu g of Friend cell nuclear extract with 2 \mu g poly (dl-dC) poly (dl-dC) in a total volume of 19 \mu l containing 10 \mu M HEPES (pH 7.9), 10% glycerol, 0.05% NP40, 0.5 \mu M EDTA, 0.5 \mu M DTT, 50 mM KCl, and 6 mM MgCl\textsubscript{2} for 5 min on ice. Probe (1 ng) in 1 \mu l of water was then added, and the incubation continued for 10 min on ice. All reactions were electrophoresed as described previously (34). When competition assays were performed, a 20-, 50-, or 100-fold molar excess of the competitor DNA was incubated with the extract prior to the addition of the probe. Competitor DNAs included: (a) D: the -20 to +9 region of the \textit{dihfr} promoter that contains E2F sites. The sequence of this region is AATTCTGCATT-TCCGCGCAACTTGTGACG; (b) SVSp1: a fragment containing three consensus Sp1 binding sites from +54 to +84 of the genome of simian virus 40. The sequence is GATCCT-GGCGGAACTGCGGAGTTAGGGCGGG; (c) AP-2: an oligonucleotide purchased from Promega (Madsen, WJ). The sequence is GATCGAATCTAGCCCGCTG-GCCCTG; (d) HSmut: an oligonucleotide derived from a binding site for a heat shock transcription factor but mutated such that no protein binds to this element. The sequence is TCGATTTCACAACCTGATCATGGCC; (e) CAD Sp1: an oligonucleotide containing two consensus Sp1 binding sites derived from -43 to -75 of the cad promoter (35). The sequence is GAGGGCGGCGCCGACGTAGGGCGGGAC; and (f) Egr1: an oligonucleotide containing a consensus binding site for the Egr family of transcription factors. The sequence is CGGCTGACGT-CAGGCGGGCGAC. In some reactions, 1.5 \mu l of an anti-Sp1 antibody (Santa Cruz) was added 3 min after the probe.

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\textbf{References.}  