Transcription Factor YB-1 Mediates DNA Polymerase α Gene Expression*

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Y-box protein-1 involvement in cyclin A and B1 gene regulation has recently been demonstrated. A more generalized role of this protein for cell replication is hypothesized as numerous regulatory sequences of cell cycle-related genes contain putative binding sites. In the present study the DNA polymerase α (DPA) gene is identified as another YB-1-responsive gene with a Y-box and 3′ inverted repeat sequence, designated DPA RE-1, in the serum-responsive promoter region. Overexpressed YB-1 concentration-dependently trans-activated DPA gene expression in reporter assays and Western blotting as well as DNA binding analyses revealed binding of distinct endogenous proteins to the RE-1 with molecular sizes of 26, 32 and 52 kDa. Among these, YB-1 binding was confirmed using recombinant as well as endogenous proteins, with preferential single-stranded DNA binding. Early serum growth response in mesangial cells was accompanied by a nuclear YB-1 shift and nucleocomplex formation at the RE-1. Fine mapping of the DPA RE-1 sequence unraveled a dependence on co-factors for trans-regulation with gene activation in the context of a heterologous SV40 promoter but suppression in the context of the abbreviated homologous promoter sequence. A YB-1 knock down resulted in decreased DPA transcription rates and abrogated the serum-dependent induction of DPA transcription. These results link YB-1 with serum responsiveness of DPA gene expression and provide insight into the required sequence and protein binding context.

As eukaryotic cells progress through the cell cycle, multiple genes involved in DNA replication and nucleotide metabolism are coordinately up-regulated shortly before or at the onset of DNA synthesis. These genes among others encompass histones, dihydrofolate reductase, thymidine kinase and synthase, proliferating cell nuclear antigen, topoisomerase IIα, and DNA polymerase α (DPA) primase (1–3). A concordant up-regulation of the transcription factor Y-box protein 1 (YB-1) with topoisomerase IIα and proliferating cell nuclear antigen has been previously reported (4); however, a direct involvement of YB-1 in the transcriptional control of the aforementioned genes has not been investigated. DPA is a key component of the chromosomal replication apparatus and is regarded as the principal polymerase involved in eukaryotic DNA replication (5). A role for DPA primase has been found in the checkpoint that couples S phase to mitosis (6). Furthermore, Wahl et al. (7) demonstrated a significant up-regulation of DPA gene transcription during the activation of quiescent (G0 phase) to proliferating cells (G1/S phases). Steady state DPA mRNA levels, synthesis rates of nascent polymerase protein, and enzymatic activity all exhibit a substantial increase before the peak of in vivo DNA synthesis. The concerted increase of these three parameters is consistent with the regulation of this key DNA replication enzyme to a considerable extent at the transcriptional level. Studies performed by Wang and co-workers (7, 8) demonstrated that in serum-deprived cells, DPA mRNA, protein, and in vitro activity levels are low, whereas serum addition leads to a coordinate increase in parallel with the onset of DNA synthesis. Prior analyses of the GC-rich TATA-less DPA promoter sequence for cis-acting elements identified a serum response element that is activated in NIH 3T3 cells (8). This element was mapped to sequences −65/−17 relative to the transcriptional start site. The 28-bp sequence −45/−17 includes an inverted CCAAT box and enhances transcription 10-fold in cycling cells when compared with the minimal activity construct −17/−45 (8). Specific binding activities that trans-activate DPA gene transcription via this element include CTF1 (9) and CTF/NF-I (10). The importance of this sequence for DPA gene expression has also been demonstrated in the course of human cytomegalovirus infection. Human cytomegalovirus immediate-early protein 1 directly interacts with CTF1 and synergistically trans-activates DPA gene transcription via the inverted CCAAT box (9).

Inverted CCAAT boxes also constitute binding sites for Y-box-binding proteins (11). YB-1 binds to DNA as well as RNA in a sequence-specific fashion and is implicated in the transcriptional regulation of a variety of genes (12). Depending on the cellular context, YB-1 may act either as a transcriptional activator or repressor, even of the same gene (13).

Close inspection of the DPA gene sequences −45/−17 revealed the presence of an inverted CCAAT box on the opposite strand with an inverse repeat sequence extending from −37 to −10 bps relative to the transcription start site (see Fig. 3). These motifs exhibit striking similarities to a previously identified enhancer cell; AS, antisense; BrdUrd, bromodeoxyuridine; RE-1, response element-1; MBN, mung bean nuclease; IR, inverted repeat.

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‡ The abbreviations used are: DPA, DNA polymerase α; YB-1, Y-box-binding protein-1; rYB-1, recombinant YB-1 protein; MC, mesangial...
element (denoted RE-1 and R1, respectively) in the rat and human matrix metalloproteinase-2 promoters, which is a well-characterized binding site for YB-1 (14). The current study examined the potential YB-1 interaction with the inverted CCAAT-box and demonstrates that YB-1 functions as a positive trans-activator of DPA gene expression, underscoring the pivotal role of YB-1 in the regulation of cellular proliferation.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions

Rat mesangial cells (MCs) were established and characterized as described previously (15, 16) and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in humidified 5% CO2 in air. HK-2 cells were maintained as described previously (17).

Plasmids

pSG5-YB-1—The eukaryotic YB-1 expression vector (pSG5-YB-1) containing the complete human YB-1 open reading frame cloned into the expression vector pSG5 (Stratagene) was kindly provided by J. P.-Y. Ting (University of North Carolina) (18).

DNA Polymerase α Promoter Constructs—DNA polymerase α promoter/luciferase reporter plasmids were kindly donated by T. S.-F. Wang (8). Designations pDPALA5 (−1571) and pDPASL5 (−248) refer to plasmids that contain 1571 and 248 bps of the DPA upstream sequence, respectively, which is subcloned in pSV0A. pSV0A is a negative control plasmid also subcloned in pSV0A. DPA sequences −65/+45, −46/+45, and −9/+45 were subcloned into the multiple cloning restriction sites BglII and KpnI of reporter constructs pGL3-Basic and -Promoter (Promega).

Transient Transfection Studies

Transient transfection of MCs was performed with liposome preparation Tfx-50 (Promega) as described (19). Purified plasmid DNA was diluted in 1000 μl of RPMI 1640 medium, mixed with sterile Tfx-50 preparation (4.5 μl/μg of DNA), and incubated at room temperature for 15 min. MCs were grown to 60–70% confluence in 6-well culture plates and washed twice with phosphate-buffered saline. To each well 1 ml DNA/liposome mixture was added and incubated for 2.5 h at 37 °C with subsequent addition of complete 10% fetal calf serum/RPMI medium. In co-transfection experiments 1 μg of luciferase reporter plasmid was combined with 1 μg of pSG5-YB-1 plasmid DNA/well. The total DNA content was equalized by inclusion of pSG5 plasmid. As control for transfection efficiency, pSV40-βGal plasmid (1 μg/well; Promega) was included. Cell lysis, β-galactosidase, and luciferase assays were performed after 48 h. Luciferase assays were performed with 100 μl of the lysates as described previously (20). β-Galactosidase activity was measured using a commercial chemiluminescence assay (Promega). All transfections were performed in triplicate to quadruplicate and were repeated at least three times. Transfection results were averaged and are expressed as the mean ± 1 S.D.

Nuclear and Cytoplasmic Cell Extracts

Cells were grown to 80% confluence in tissue culture flasks, washed twice with ice-cold phosphate-buffered saline without calcium and magnesium, and scraped in 10 ml of cold phosphate-buffered saline. Nuclear and cytoplasmic cell extracts were prepared as described previously (13). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. Extracts were stored at −80 °C until performance of electrophoretic mobility shift analysis, Western, or Southwestern blotting.

Electrophoretic Mobility Shift Analysis

Double-stranded probes (−45GGCGGAAAGTCCCGAGCCTTGGGGGAGCCCGCTGATTGGCTTTCAGGCTGGCGCCTGTCTCGGCCCCC) were generated by heating complementary synthetic oligonucleotides for 10 min at 95 °C in Tris-EDTA with subsequent cooling to room temperature over 6 h. All probes were radiolabeled by means of T4-polymerase kinase using [γ-32P]ATP and were purified on 14% polyacrylamide gels and eluted, and 6 × 10^4 cpm of labeled probe was included per binding reaction. Binding reactions were performed at 22 °C for 30 min in binding buffer (20 mM HEPES (pH 7.9), 20% glycerol, 0.1 mM NaCl, 0.2 mM EDTA) containing 0.2 mM poly(adenylic acid), 0.5 mM dithiothreitol, 300 μM acetylated bovine serum albumin, and 2 μg of poly(dI-dC) in a total volume of 25 μl upon the addition of nuclear or cytoplasmic extracts. Samples were electrophoresed on non-denaturing 4% polyacrylamide, 7.5% glycerol gels in a buffer containing 1× Tris borate/EDTA followed by autoradiography.

Recombinant YB-1 was prepared from a pRESET vector (Invitrogen) containing an insert coding for a hexahistidine T7 epitope-YB-1 fusion protein as described by Mertens et al. (14). For competition experiments, unlabelled oligonucleotides or non-specific DNA (500-fold molar excess) were added to the binding reaction 15 min before the addition of labeled oligonucleotides followed by a 30-min incubation period and subsequent separation on polyacrylamide gels. Relative binding affinities were determined by quantitation of shifted bands using a PhosphorImager system (Bio-Rad). For supershift assays, affinity-purified rabbit anti-YB-1 antibody raised against a C-terminal epitope (20) was added to the nuclear extracts 12 h before the addition of labeled oligonucleotides, and the binding reaction was incubated for 30 min at 20 °C.

Western Blot Analysis

Nuclear proteins (5 μg) were separated by SDB-10% PAGE before transfer to nitrocellulose membranes (Schleicher and Schuell) and probed with antisense oligonucleotides and small interfering RNA target YB-1 inhibit MC growth. A, cycling MCs were incubated with AS or scrambled control (CON) phosphorothiated oligonucleotides (21) at a concentration of 50 μM. Medium containing oligonucleotides was replaced every 24 h, and nuclear extracts from cells were prepared for Western blot analysis to detect YB-1. A significant 60% decrease of nuclear YB-1 concentration was only apparent with specific antisense oligonucleotide addition (lane 3). B, phosphorothiated oligonucleotides were added to the medium at concentrations ranging from 10 to 50 μM, and a direct cell count was performed. A concentration-dependent growth inhibitory effect up to 50% was detected. Representative results from three independent experiments performed with quadruplicate determinations are given (mean values ± S.D.; * p < 0.05; ** p < 0.01). C, knock down of endogenous YB-1 by small interfering RNA resulted in 80% reduced YB-1 mRNA levels (left panel) and 90% reduced protein levels (middle panel). At the same time, DNA synthesis, assessed by BrdUrd incorporation, was down-regulated by ~50% (right panel).
from 0.1 to 2.0

the YB-1 expression plasmid was titrated are expressed as the means normalized for transfection efficacy and ing via the proximal 248 bp. Data were activator of the DPA gene expression act-

similar 2-fold increase of reporter con-

structs, indicating that YB-1 is a was observed with both reporter con-

transfected in the absence and presence of the reporter construct pDPALΔ5 (~1571/ +45) was observed that was at most 2.3-fold.

Southwestern Blot Analysis

Southwestern blot analysis was performed as described (13) using MC nuclear proteins (50 μg) and radiolabeled DPA RE-1 oligonucleotide probes (106 cpm/ml).

Chromatin Immunoprecipitation Assay

Confluent human kidney cells (HK-2) were grown to 80% confluence and serum-starved for 24 h. Subsequently cells were incubated with 10% fetal bovine serum for 3 and 6 h and subjected to chromatin immunoprecipitation, as described by the manufacturer of the chromatin immunoprecipitation assay kit (Upstate Biotechnology Inc.). Briefly, 106 cells were treated with 1% formaldehyde solution for 10 min at 37 °C to cross-link proteins and were resuspended in lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1). Extracts were blocked in TTBS (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) containing 2% BSA for 2 h at room temperature. Filters were incubated with primary polyclonal rabbit anti-YB-1 antibody (1:1000) followed by 3 washes with TTBS for 5 min each and incubation with secondary goat anti-rabbit IgG in TTBS. For DNA polymerase α detection, a polyclonal antibody raised in goat (N19, Santa Cruz) was used at 1:1000 dilution with bovine anti-goat IgG (Santa Cruz; 1:5000) serving as secondary antibody. The filters were washed 3 times with TTBS and developed with an ECL detection kit (Amersham Biosciences).

Mung Bean Nuclease Sensitivity Analysis

Mung bean nuclease treatment was performed as described by Norman et al. (17). The strictly double-stranded, asymmetrically end-labeled probe was prepared by digesting pT4-Luc-DPA RE-1 with BglII or KpnI; the resultant overhanging 5’ ends were dephosphorylated with calf intestinal alkaline phosphatase (Roche Applied Science) and end-labeled with 1γ-32P]ATP by means of T4 polynucleotide kinase (Roche Applied Science). The DNA fragment was released by BglII/KpnI digestion and gel-purified. About 106 cpm of probe was incubated in the presence of 1 μg of poly(dI-dC) with either rYB-1 (10 ng) or nuclear proteins (10 μg) in binding buffer A (without EDTA, supplemented with 4 mM MgCl2) in a total volume of 15 μl at 25 °C for 20 min. The reaction volume was diluted 5-fold, and 1 volume of mung bean nuclease buffer was added. Mung bean nuclease reactions were performed at saturating concentrations of enzyme, as previously determined by titration. 50 units of mung bean nuclease (100 units/μl Promo) were added to each reaction and incubated for 20 min at 37 °C, followed by termination with 240 μl of stop buffer (100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM EDTA, 0.1% SDS, 100 μg/ml proteinase K). After incubation in stop buffer at 37 °C for 15 min, reactions were phenol/chloroform-extracted once and precipitated. Samples were subjected to electrophoresis on 12.5% polyacrylamide urea gels with parallel lanes containing chemical sequencing reactions.

Antisense Oligonucleotide Experiment

Cycling MCs grown in complete medium with 10% fetal calf serum were incubated with phosphothiorated antisense (AS) or scrambled control oligonucleotides (Stanford University), as described previously by Duh et al. (21) at concentrations ranging from 10 to 50 μM. Medium containing oligonucleotides was replaced every 24 h. Direct cell count was performed with trypan blue staining for assessment of viable
cells. Three independent experiments were performed in quadruplicate. Results were averaged and are expressed as the mean ± 1 S.D.

**Knock Down of Endogenous YB-1 by Small Interfering RNA**

MCs were grown to 50% confluency on 10-cm plates in RPMI 1640 with 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin. Cells were transfected with the empty vector pSuperDuper (Oligo-Engine, Seattle, WA) or the pSuperDuper vector harboring the se-
cillin. Cells were transfected with the empty vector pSuperDuper (Oligo-
structs pDPAL
regulatory sequence of the human DPA gene transfected into
established using hybrid luciferase constructs harboring the 5
influences transcription of the DPA gene. A reporter assay was

**Protein Phosphorylation**

Protein phosphatase 2A was performed on lysates of MCs using a

**Statistical Analyses**

Statistical significance was determined for paired comparisons using

**RESULTS**

**Reduction of Endogenous YB-1 Inhibits Cell Proliferation**—We have hypothesized of a key role for YB-1 in cell proliferation in non-transformed cells. As a first step, an oligonucleotide antisense approach was chosen to reduce endoge-

**Proliferation Assay**

Proliferation of YB-1 knock down and control cells was measured by

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mobility as endogenous YB-1 protein that is detected by Western blotting using an anti-YB-1 antibody (lane 4), supporting the notion of YB-1 binding to this element. A similar banding pattern was observed with the antisense DNA strand (data not shown).

**Recombinant YB-1 Binds to the DPA RE-1**—DNA binding studies were performed with recombinant YB-1 protein (rYB-1) and sense (SS1), antisense (SS2), and double (DS)-stranded DPA RE-1 probes. Two closely migrating complexes were detected by electrophoretic mobility shift analysis with all probes (Fig. 4A, lanes 2, 6, and 10). Quantitative densitometry of bands revealed a 5-fold higher affinity of rYB-1 for both single-stranded templates compared with the double-stranded probe. Specificity of binding reactions was confirmed by inclusion of homologous competitor DNA at 500-fold molar excess, leading to diminished bands (lanes 3, 7, and 11), whereas heterologous competitor DNA had only a minor effect on complex formation (lanes 4, 8, and 12).

**Endogenous YB-1 Binds to the DPA RE-1**—Similar DNA binding studies were performed with MC nuclear proteins. Nuclear proteins formed several distinct complexes with sense (SS1), antisense (SS2), and double stranded (DS) probes. After inclusion of anti-YB-1 antibody in the binding reaction, supershifts were apparent with the DS (lane 3) and SS2 probes (lane 9, indicated by *). At the same time complexes 10 and 11 were diminished. For the sense strand, a disruption of complex 5 was detected, and at the same time a novel high mobility complex appeared (lane 6, indicated by **).
indicated by “***”). Here, the antibody most likely prevented binding of YB-1 to the probe and no supershift appeared. These experiments indicate that endogenous YB-1 is a component of the nucleoprotein complexes formed with DPA RE-1 in all conformations tested and that it interacts with other DPA RE-1 binding factors.

Serum-dependent Changes of Complex Formation at the DPA RE-1—Because the sequence element −45/−17 of DPA is responsive to serum growth factor, DNA binding studies were performed with nuclear (NE) and cytoplasmic (CE) extracts from serum-starved (24 h) and serum-stimulated MCs. Formation of nucleo complexes was tested with radiolabeled double-strand (DS) and SS1 DPA RE-1 probes (A and B). Under these conditions three distinct bands were detected with DS DPA RE-1 that exhibited only minor changes in intensities after serum stimulation. With CE and DS probes a minor decrease of complex >6 was detected after 2 h serum stimulation. With SS1 DPA RE-1 probe, intensities of bands designated 1>, 2>, 4>, and 5> (Fig. 5B, compare lanes 1–4) were increased within 30 min of serum incubation. This increase was transient and lasted for less than 2 h. Reciprocal changes were observed with cytoplasmic extracts; that is, band intensities indicated by 8> (Fig. 5B, compare lanes 6–9) decreased within 30 min and increased again 24 h after serum stimulation.

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lanes 3 (end of the probe that also extended over the 3′ end). The absence of banding upon the addition of MBN to the double-stranded probe alone (Fig. 6A, lane 2) confirmed the double-stranded conformation of the probe. In the presence of rYB-1, MBN produced a distinct banding pattern at the 3′ end of the probe that also extended over the 3′ inverted repeat (IR) sequence (lanes 3 and 4), with no banding within the Y-box motif. Nuclear extracts from serum-starved and serum-stimulated MCs were obtained and included in the binding reaction. Whereas with nuclear extracts from serum-starved cells no clear banding was apparent, an increase of banding could be detected in the nuclear extracts from serum-stimulated cells. Here, prominent bands appeared within 30 min of serum stimulation that matched with the ends of the 3′ IR sequence (Fig. 6A, lane 9). After 1 h of serum stimulation these bands were still present and even enhanced in between the inverted repeat sequence (indicated by the symbol ‘>’), whereas they resolved after 12 h of serum stimulation (Fig. 6A, compare lanes 10–11). From these results it is concluded that with serum stimulation the DPA RE-1 motif undergoes conformational changes with extended regions of single strands. These changes may not be explained by the sole action of YB-1, and the most likely explanation is that YB-1, in conjunction with interacting partner protein(s), produces the distinct pattern observed after serum stimulation.

Serum-induced Binding of YB-1 to the Proximal DPA Promoter—Given these findings of YB-1 binding to the DPA RE-1 in vitro, further experiments were designed to directly test for YB-1 contacting this element in vivo; that is, by chromatin immunoprecipitation. Cells were serum-starved for 24 h and subsequent harvests without prior serum addition or after a 3- and 6-h serum incubation period. By adding an anti-YB-1 antibody and amplification of the proximal DPA promoter after serum induction, B, chromatin immunoprecipitation was performed with HK-2 cells that were serum-starved for 24 h and subsequently harvested without prior serum addition or after a 3- and 6-h serum incubation period. By adding an anti-YB-1 antibody and amplification of the proximal DPA promoter after serum induction, the junction to the CCAAT-box appeared. 12 h after serum stimulation a factor-induced shift of cytoplasmic YB-1 protein to the nuclear compartment and predominant binding of YB-1-containing complexes to the single-stranded DPA RE-1 element.

YB-1 Promotes Single-stranded DNA Conformation in the DPA Promoter—Previous studies suggest that YB-1 promotes strand separation in a sequence-specific fashion (18). Mung bean nuclease (MBN) sensitivity analysis was performed to detect single-stranded DNA regions within the DPA RE-1 motif. A strictly double-stranded probe containing the entire DPA RE-1 sequence was generated and asymmetrically labeled at the 5′ end. The absence of banding upon the addition of MBN to the double-stranded probe alone (Fig. 6A, lane 2) confirmed the double-stranded conformation of the probe. In the presence of rYB-1, MBN produced a distinct banding pattern at the 3′ end of the probe that also extended over the 3′ inverted repeat (IR) sequence (lanes 3 and 4), with no banding within the Y-box motif. Nuclear extracts from serum-starved and serum-stimulated MCs were obtained and included in the binding reaction. Whereas with nuclear extracts from serum-starved cells no clear banding was apparent, an increase of banding could be detected in the nuclear extracts from serum-stimulated cells. Here, prominent bands appeared within 30 min of serum stimulation that matched with the ends of the 3′ IR sequence (Fig. 6A, lane 9). After 1 h of serum stimulation these bands were still present and even enhanced in between the inverted repeat sequence (indicated by the symbol ‘>’), whereas they resolved after 12 h of serum stimulation (Fig. 6A, compare lanes 10–11). From these results it is concluded that with serum stimulation the DPA RE-1 motif undergoes conformational changes with extended regions of single strands. These changes may not be explained by the sole action of YB-1, and the most likely explanation is that YB-1, in conjunction with interacting partner protein(s), produces the distinct pattern observed after serum stimulation.

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Serum-induced Binding of YB-1 to the Proximal DPA Promoter—Given these findings of YB-1 binding to the DPA RE-1 in vitro, further experiments were designed to directly test for YB-1 contacting this element in vivo; that is, by chromatin immunoprecipitation. Cells were serum-starved for 24 h and subsequent harvests without prior serum addition or after a 3- and 6-h serum incubation period. By adding an anti-YB-1 antibody and amplification of the proximal DPA promoter after serum induction, the junction to the CCAAT-box appeared. 12 h after serum stimulation a factor-induced shift of cytoplasmic YB-1 protein to the nuclear compartment and predominant binding of YB-1-containing complexes to the single-stranded DPA RE-1 element.
subjected to the chromatin immunoprecipitation protocol as outlined under “Experimental Procedures” either without serum addition or after serum stimulation for 3 or 6 h. As can be seen in Fig. 6B in lane 4, after serum induction for 6 h, the DPA proximal promoter DNA was retrieved by immunoprecipitation of YB-1 and visualization of the PCR amplification product. In cells grown in the absence of serum (lane 2) or incubated with serum for 3 h (lane 3) no amplification product was visible. Negative and positive control reactions are shown in lanes 5 and 6. These findings indicate that YB-1 contacts the DPA proximal promoter within 6 h of serum induction. To assess DPA protein synthesis in serum-induced cells Western blotting for DPA was performed under similar conditions (Fig. 6C). Here, DPA protein was detected as a 180-kDa band 6 h after serum induction (lane 3). Furthermore, MCs with YB-1 knock down by small interfering RNA (compare Fig. 1C) were tested for DPA protein expression after serum induction. Here, DPA was not detected after serum induction (Fig. 6C, lanes 4–6). Taken together, these results indicate that YB-1 confers the serum-responsive up-regulation of DPA gene transcription and protein synthesis within 6 h.

**Trans-regulation of the DPA RE-1 by YB-1 Is Dependent on the Immediate 5′ Sequence and Proximal Promoter Element**—The transfection studies outlined above indicate a concentration-dependent trans-activation of the DPA gene by YB-1. The sequence requirements for YB-1 trans-regulation were tested using luciferase reporter constructs. Given the previous description of protein binding to the −65/−45 sequence motif, three constructs were designed using reporter plasmids pGL3basic and pGL2prom. Construct −65/+45 harbors the entire Y-box element including the 3′ IR and the immediate 5′ sequence (schematically depicted in Fig. 7, A and B). In construct −45/+45 only the 5′ adjacent sequence is omitted, whereas in construct −9/+45 the complete Y-box and 3′ IR are deleted. Plasmid pGL3basic does not harbor an additional proximal promoter element (A), whereas in plasmid pGL2prom the regulatory sequence tested acts in conjunction with a heterologous SV40 promoter element (B). Transfections were performed in the absence or presence of pSG5-YB-1 at 1 μg/well, and normalized luciferase activities were determined in four independent experiments, each performed in triplicate. A construct pGL3basic −65/+45 exhibited a high basal transcriptional activity that was decreased by 75% after omission of the immediate 5′ sequence −65/−45. The absence of significant promoter activity was detected with promoter sequence −9/+45. YB-1 overexpression led to a trans-repression of reporter gene activity by 60% with constructs pGL3basic −65/+45 and pGL3basic −45/+45. B, contrary to these findings a 3-fold increase of luciferase reporter gene activity by overexpressed YB-1 was detected with construct pGL2prom −65/+45. This trans-activation by YB-1 was dependent on the presence of the sequence located immediately 5′ to the Y-box element, as no YB-1 trans-regulation was found with construct pGL2prom −45/+45.
the absence and presence of pSG5-YB-1 at 1 µg/well, and normalized luciferase activities were determined in four independent experiments, each performed in triplicate.

Construct pGL3basic −65/+45 exhibited a high basal transcriptional activity that was decreased by 75% after omission of the immediate 5′ sequence −65/−45. The absence of significant promoter activity was detected with promoter sequence −9/+45. Unexpectedly, co-transfection with YB-1 led to a trans-repression of reporter gene activity by 60% with constructs pGL2prom, pGL2prom −45/+45, and pGL2prom −9/+45. B, to test for the relevance of YB-1 in the serum response of the proximal DPA promoter, cells were either manipulated with a construct that harbors the empty pSuperDuper plasmid (CON) or a pSuperDuper plasmid that harbors a target sequence for YB-1 (YB-1 siRNA). The luciferase reporter gene activity driven by sequences −65/+45 of the DPA gene was increased 2-fold when fetal calf serum (FCS) was added for 12 h. This induction was not observed in cells with YB-1 knock down.

**DISCUSSION**

YB-1 is a prominent member of the highly conserved Y-box binding factor family with an ever-increasing number of target genes (12). Our findings add the DNA polymerase α gene to this list, which is in accord with previous reports indicating that YB-1 is a crucial regulator of DNA replication (23) and cell proliferation (24). Wahl et al. reported (7) that in actively cycling cells, DNA polymerase α mRNA, protein, and in vitro activity levels are constitutively expressed through the cell cycle. However in serum-deprived cells, these parameters are low, and the addition of serum results in their coordinate increase in parallel with the onset of the DNA synthesis. In...
electrophoretic mobility shift assays with nuclear extracts prepared from MCs that were incubated for different periods with serum, the binding pattern to the DPA RE-1 element changed dramatically within 30 min of serum incubation, indicating that it coordinates the early serum response. The binding of YB-1 to the DPA RE-1 was also demonstrated by chromatin immunoprecipitation; however, complex formation was observed 6 h post-serum addition and not within 3 h of serum incubation. In accord with this delayed transcriptional regulation, DPA protein expression increased after the same period of serum incubation.

Although YB-1 was originally cloned as a CCAAT binding factor (25), there does not seem to be an absolute requirement for this motif, and many YB-1 binding sites contain either an imperfect CCAAT-box or lack one altogether, suggesting an important role for flanking sequences in DNA recognition by this protein. Sequence analysis of the DPA regulatory sequence revealed a striking similarity to the MMP-2 response element-1, an element that is highly conserved in evolution from rat to human (20). Seven of nine nucleotides of both elements match within the incomplete Y-box motif, and there are extended similarities within the 3′ adjacent sequence; that is, both elements harbor an inverted repeat sequence with 6 of 10 nucleotides conserved between both elements. These sequence similarities further stress the importance of the sequence context for DNA binding by this protein and the notion that binding sites may not be anticipated by a short sequence algorithm. However, the precise mechanism of induction is unique to each gene. Previously, binding activities to the inverted CCAAT box at −45/−17 in the DPA promoter have been described (9). Southwestern blotting suggested that there are at least two additional proteins with molecular sizes of 32 and 26 kDa that directly interact with the DPA RE-1, the identity of which remain unsolved and are the focus of ongoing studies. Data base searches of this region for transcription factor binding sites showed potential binding sites for activating protein 1, E2F, Sp1, and STAT (signal transducers and activators of transcription) factors.

Sequence specific binding of YB-1 and other Y-box proteins to single-stranded DNA has been demonstrated previously (12, 22, 26–28). MacDonald et al. (18) proposed a model of YB-1 action on the HLA class II DRA promoter that includes conformational DNA changes with regions of single strands. We have also demonstrated a similar ability to induce single strands in the MMP-2 RE-1 (14). In the DPA promoter MBN sensitivity analysis showed that strand separation within the IR of the DPA RE-1 differed significantly with nuclear proteins from serum-starved cells as compared with nuclear extracts from serum-stimulated MC. These results are in accord with the DNA binding studies performed with MC nuclear and cytoplasmic extracts, where a time-dependent change of binding could be detected that was predominantly found with the single-stranded probes. Furthermore, the different patterns obtained with recombinant YB-1 versus nuclear extracts from serum-stimulated cells may have different reasons. (i) With serum induction proteins may be activated that partner with YB-1 and may guide YB-1-dependent unwinding of DNA, as has been shown previously for transcription factor AP-2 (14). In favor of this explanation is the absence of any pattern similarity in the MBN analysis between recombinant YB-1 and nuclear extracts after serum stimulation. Alternatively, (ii) posttranslational modifications of YB-1, e.g. phosphorylation, may account for differences in sequence recognition by YB-1; however, such differences have not yet been described. Last, (iii) there could be additional, yet unidentified proteins with similar DNA melting capabilities as YB-1 and different sequence recognition motifs. Future work will be aimed at elucidating the underlying mechanism(s), as it seems to be a common finding with YB-1-regulated target genes. Overall, the pattern is in accordance with a DNA stem-loop conformation that is stabilized by protein contact(s) at the DNA. The absolute requirement for YB-1 in the serum-response of DPA gene transcription was demonstrated by means of YB-1 knock down in MCs. Here, the stimulatory effect of serum on the promoter was abrogated, and DPA protein was no longer detectable.

In conclusion, our data identify YB-1 as a novel trans-activator of the DPA gene acting via a defined sequence element, the DPA RE-1, in the immediate proximal promoter sequence. Both the immediate 5′ adjacent sequences as well as the overall proximal promoter sequence are required for trans-activation of the gene, indicating complex protein partnering that is effective in early serum response.

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