Two YY-1-binding Proximal Elements Regulate the Promoter Strength of the TATA-less Mouse Ribonucleotide Reductase R1 Gene

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Ribonucleotide reductase is essential for DNA synthesis. In mammalian cells, the enzyme consists of two non-identical subunits, proteins R1 and R2. The expression of the mouse R1 and R2 genes is strictly correlated to S phase. Using promoter-reporter gene constructs, we have defined a region of the TATA-less mouse ribonucleotide reductase R1 gene promoter that correlates reporter gene expression to S phase. This is demonstrated in stably transformed cells both synchronized by serum starvation and separated by centrifugal elutriation, suggesting that the R1 gene expression during the cell cycle is mainly regulated at the transcriptional level. The region contains four protein-binding DNA elements, β (nucleotides −189 to −167), α (−98 to −76), Inr (−4 to +16), and γ (+34 to +61), together regulating promoter activity. The nearly identical upstream elements, α and β, each form three DNA-protein complexes in gel shift assays. We have identified YY1 as a component in at least one of the complexes using supershift antibodies and a yeast one-hybrid screening of a mouse cDNA library using the α element as a target. Transient transfection assays demonstrate that the α and β elements are mainly important for the R1 promoter strength and suggest that YY1 functions as an activator.

The enzyme ribonucleotide reductase is essential for de novo synthesis of deoxyribonucleotides. In this perspective, ribonucleotide reductase plays a central role in providing the precursors for DNA synthesis. Mammalian ribonucleotide reductase consists of two non-identical homodimeric subunits, proteins R1 and R2 (1). Protein R1 (large subunit) contains the active site and allosteric sites for regulating enzyme activity. Protein R2 (small subunit) carries a tyrosyl free radical essential for enzymatic activity. In quiescent or differentiated cells, none of the two subunits can be detected. In proliferating cells, the expression of protein R2 is S phase specific, whereas protein R1 shows a constant level throughout the cell cycle (2–4). However, both the R1 and R2 mRNA levels vary in parallel during the cell cycle with negligible levels during G0/G1 and maximal levels during S phase with a constant level throughout the cell cycle (2–4). However, both the R1 and R2 mRNA levels vary in parallel during S phase (5, 6). The R1 and R2 promoters are also activated in resting cells upon UV irradiation leading to nucleotide excision repair (7).

Genomic clones covering the mouse R1 and R2 genes have been isolated (8, 9). The R2 promoter contains a TATA-box and a proximal element, called α, that contains an NF-Y binding CCAAT motif (10).

The R1 gene consists of 19 exons that covers 26 kb. In contrast to the R2 promoter, the R1 promoter is TATA-less. DNase I footprinting assays proximal to the transcription start revealed two protected regions, α (nt −98 to −76) and β (nt −189 to −167) (8). These two regions are each 23 nt identical except for one nucleotide. Three DNA-protein complexes, A, B, and C, are formed when oligonucleotides corresponding to the α or β footprints are used in gel shift assays. Complexes A and B are present at constant levels during the cell cycle, whereas complex C increases in S phase cells. A comparison between the human and mouse R1 promoters revealed four conserved regions (11). These were α, β, an initiator element (Inr), and one downstream region, γ. The initiator element did not fully conform to the Inr consensus YYAN(T/A)YY (12), but a functional analysis showed that both the Inr and the γ regions were essential for cell cycle-regulated R1 promoter activity (11). Furthermore, it was shown that TFII-I binds to the R1 Inr, whereas the transcription factors YY1 or USF did not recognize the Inr.

A functional analysis of the R1 promoter using promoter-reporter gene constructs in stably transfomed cells indicated that α and β were not essential for cell cycle-regulated transcription (11). However, these assays only allowed a qualitative analysis of R1 promoter activity. Furthermore, in these experiments the major increase in R1 promoter activity came about 4 h after cells entered S phase, indicating that we were lacking an important regulatory DNA element. We have now made new promoter-reporter gene constructs defining an R1 promoter region that faithfully regulates S phase expression of the reporter gene. Using a transient transfection assay for a quantitative analysis of R1 promoter activity, we demonstrate that α and β are important for R1 promoter strength. Finally, our data suggest that YY1 functions as an activator in the R1 promoter by binding to the α and β footprints.

EXPERIMENTAL PROCEDURES

Promoter-Luciferase Reporter Gene Constructs—The luciferase reporter gene plasmid pGL3-basic (Promega) was opened with BglII and HindIII, and a new linker containing unique EcoRI and Esp31 sites was introduced upstream from the luciferase gene. Next, a 177-bp fragment from the mouse R1 5′-UTR (EcoRI-Esp31) was introduced into the linker. This construct, pGL3R1 177, was then opened with SacI and EcoRI, and a 5.5-kb SacI-EcoRI R1 promoter fragment was inserted to complete the pGL3R1 5.7 construct (Fig. 1). Shorter constructs are available online at http://www.jbc.org

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pGL3R1 3.7 and pGL3R1 420 (Fig. 1), were made by digesting the pGL3R1 5.7 with SacI-BglII and EcoICRI-EcoRV, respectively, followed by filling in sticky ends with the Klenow fragment and religation. The constructs pGL3R1 420 (−/−4/+6) and pGL3R1 420−γ(−33/+43) were made by digesting the pGL3R1 420 with XbaI and EcoRI, respectively. Primer pairs, 5′-GAAGAGGAAATTCGTGTAACACTC-3′ and 5′-GTCCTGCCGGTTCGCTTGGTACACTC-3′, were used in a PCR reaction to introduce a 10-nucleotide mutation in the IR element. The resulting PCR product was cut with EcoRI-XhoI and ligated into the opened pGL3R1 420 construct. The primer pairs, 5′-ATTCGATGGCATTAGGTGACGTTTCAACG-3′ and 5′-GTCCTGCCGGTTCGCTTGGTACACTC-3′, were used in a PCR reaction to introduce a 10-nucleotide mutation in the γ region. The resulting PCR product was cut with EcoRI-HindIII and ligated into the opened pGL3R1 420 construct. The pGL3R1 420-Int (−/−4/+6)−γ(−33/+43) was created by subcloning the EcoRI-HindIII fragment from the pGL3R1 420 (−/−4/+6) construct, opened with EcoRI-HindIII, into the opened pGL3R1 420 construct. The pGL3R1 420-Int (−/−4/+6) construct was digested with EcoRV and HindIII, and then the ends were filled in and religated, creating the pGL3R1 αβ construct. A series of constructs, pGL3R1 287 with or without mutations in the α footprint, were designed in the following way (Fig. 4B). pGL3R1 177 was opened in XhoI and EcoRI. A region from XhoI at −106 to nt +61 was amplified from pGL3R1 3.7 by a polymerase chain reaction using five different 5′ and 3′ primer pairs, 5′-CTAGACAAACTTCTAT-3′ and 5′-CTAGTA-TACCTTAGAGATTTAATCTGAGATA-3′ and 5′-CTAGTA- TACCTTAGAGATTTAATCTGAGATA-3′, containing the mutations shown in Fig. 4B and 5′-CTCCCTTCCCCTC- CCCGGACCGGTGAG-3′ at the 3′-end. The resulting fragment was digested with XhoI and EcoRI, purified on an agarose gel, and ligated into the opened pGL3R1 177 bp.

The pGL3-control plasmid contains a firefly luciferase gene under the control of SV40 promoter and enhancer. This plasmid was used to construct a hybrid gene that expresses luciferase mRNA containing R1 mRNA in the 3′-UTR. This construct (pGL3 +59) was designed as follows. Two 49-mers were annealed (13), 5′-CTAGACAAACTTCTAT- AATCTGAGATA-3′ and 5′-CTAGTA-TACCTTAGAGATTTAATCTGAGATA-3′ and 5′-CTAGTA-TACCTTAGAGATTTAATCTGAGATA-3′, containing the overlaps shown in the open XhoI site in the pGL3-control plasmid, and the 49-mer was subsequently ligated into the firefly luciferase 3′-UTR, upstream from the luciferase gene poly(A) signal.

Analyses of R1 Promoter Activity in Stably Transformed Cell Lines—Balb/3T3 fibroblasts were cotransfected by electroporation as described previously (14) with R1 promoter-reporter gene constructs and a pSV2neo plasmid. Several independent clones of stably transfected cells were selected in the presence of Geneticin (G418) and G418-resistant luciferase activity, expressed as light units per microgram of protein (14, 15). Cells were synchronized by serum starvation as described earlier (5). At least two independent clones were analyzed for each construct. Logarithmically growing cells were separated on the basis of different cell size by centrifugal elutriation using a Beckman JE-6B elutriation system (2). A sample of each fraction was analyzed by flow cytometry to give cell cycle distribution and cell concentration. The remaining cells were lysed and assayed for luciferase activity, which in this experiment is given as light units per cell.

Transient Transfection Assays—To measure the promoter strength, Balb/3T3 cells were transiently cotransfected with R1 promoter-reporter gene constructs and a control plasmid, pRLSV40 (Promega), by the calcium-phosphate precipitation method (16). A typical transfection contained 2–5 µg of control plasmid, 2–5 µg of R1 promoter-reporter gene construct, and pUC18 to a total amount of 20 µg of DNA. This precipitate was incubated with 0.5×1010 cells for 14 h. Then, the cells were washed twice with phosphate-buffered saline, and fresh medium was added. This medium contained 10% heat-inactivated horse serum when exponentially growing cells were harvested 24 h later and 9.6% fetal bovine serum when the cells were synchronized by serum starvation. After 48 h of starvation, medium containing 20% fetal bovine serum was added back, and the cells were harvested at different time points. One-half of the cells were used for flow cytometry analyses and the other half was used for enzyme assays. Firefly luciferase expressed by the pGL3R1 plasmids and Renilla luciferase expressed by the pRLSV40 plasmid were assayed by the dual luciferase assay kit (Promega). Firefly luciferase activity was then divided by Renilla luciferase activity to normalize for differences in transfection efficiency between plates. This value reflects the R1 promoter activity.

Gel Shift Assays—Nuclear extracts were prepared from Balb/3T3 cells as described (17), and the protein concentration was measured by the Bradford method (15). The reaction mixtures were composed as described (11), and the antibodies against YY1, SRF, DP-2 (Santa Cruz Biotechnology), NF-YA, and NF-YB (kindly provided by Dr. Roberto Mantovani, Universita di Milano) were added last to the reaction mixture, followed by an incubation on ice for 1–2 h before the 15-min incubation at 30 °C. The following oligonucleotides annealed with a complementary oligonucleotide were used in the competition experiments: β footprint, 5′-TGGCCCACTCATATGCGCCG-3′; β′ footprint, 5′-TGGCCCACTCATATGCGCCG-3′; ε-Myc-260 YY1 oligonucleotide (18), 5′-GAAGAGGAAATTCGTGTAACACTC-3′. Oligonucleotides with the sequence shown in Fig. 4B annealed with a complementary oligonucleotide were used in gel shift experiments to determine how different mutations in the α footprint effect the protein-DNA interaction.

To study mRNA stabilization in vivo, we used both the yeast two-hybrid system and a mouse T-cell lymphoma Matchmaker cDNA library purchased from CLONTECH. The screening was essentially performed as recommended by the manufacturer. Five copies of the R1 α footprint were inserted into the polylinker of the three plasmids pHisI, pHisI-1, and pHisI-2, and plazci just upstream from the minimal promoter of the HIS3 and lacZ genes. A dual reporter yeast strain (YMEJ2) was established by integrating pHisI-1 and plazci into the genome. YMEJ2 grew poorly on SD−(His+)15 mx3-aminotriazole and not at all on SD−(His+)45 mx3-aminotriazole agar plates. The Matchmaker cDNA library, expressing mouse proteins fused to the GAL4 activation domain, was transfected into YMEJ2, and the yeast was plated on SD−(His+)45 mx3-aminotriazole agar plates. The expression plasmid was isolated from the cell cycle distribution from the yeast transformed into Escherichia coli JM109. A portion of the mouse cDNA was sequenced in each plasmid and used to search Genbank with Blastsearch.

**RESULTS**

Transcriptional Regulation of the R1 Promoter during the Cell Cycle—Using the improved luciferase vector, pGL3, we made a plasmid containing 5.7 kb of the R1 promoter in front of the luciferase gene. This construct, pGL3R1 5.7, and two shorter versions, pGL3R1 3.7 and pGL3R1 420 (Fig. 1), were all regulatied in the same way when independent clones of stably transformed cells were analyzed after serum starvation and readdition (Fig. 2 and data not shown). Now, the increase in luciferase activity showed a good correlation to the increase in S phase cells like the expression of R1 mRNA (5), indicating that the R1 gene is regulated mainly at the level of transcription.

The pGL3 control plasmid, containing the firefly luciferase gene under the control of the SV40 promoter, did not show an S phase correlated expression (data not shown). The observed increase in R1 promoter activity as cells entered S phase could be due to a serum proliferation response and not represent a true cell cycle regulation. To exclude this possibility, we elutriated logarithmically growing stably transformed cells containing the pGLR1 420 construct (Fig. 3). The fractions enriched in S phase cells clearly showed a higher promoter activity compared with fractions dominated by G1 cells.

Possible Control of R1 Gene Expression during the Cell Cycle by Posttranscriptional Mechanisms—It was earlier demonstrated that the addition of phorbol 12-myristate 13-acetate to mouse cells causes a rapid increase in the levels of R1 and R2 transcripts. This was reported to be the result of mRNA stabilization caused by protein binding to a specific regulatory 49-nucleotide element in the 3′-UTR of the mRNA. However, no studies on cell cycle-regulated R1 gene expression were reported (13). To study mRNA stabilization as a mechanism for regulating the R1 mRNA levels during the cell cycle, we introduced the putative regulatory 49-mer from the R1 gene into the luciferase reporter gene. The 49-mer was introduced upstream from the luciferase poly(A) signal in the 3′-UTR region of the luciferase gene; therefore, it is included in the luciferase mRNA. Our mosaic luciferase gene was under the control of the SV40 promoter and enhancer. This construct and a control plasmid without the 49-mer was introduced into Balb/3T3 cells, and stably transformed clones were synchronized by serum starvation. An excess of mRNA containing the 49-mer might lead to loss of regulation due to exhaustion of regulatory pro-
Therefore, clones with very different levels of mRNA (luciferase activity ranging from 151 LU/mg to 144499 LU/mg) were analyzed. Cells containing the control plasmid showed the same expression pattern as five different clones containing the 49-mer plasmid, and no clones showed any S phase-specific luciferase expression (data not shown).

The a and b Footprints Are Important for R1 Promoter Strength—Earlier R1 promoter-reporter gene constructs indicated that cell cycle-specific expression of the R1 gene required the Inr and γ elements, whereas there seemed to be no requirement for the a/b elements (11). This puzzled us since we could demonstrate S phase-specific binding to the a/b oligonucleotide (complex C) in gel shift assays (8). To analyze if the a and b footprints were important for the R1 promoter strength, we performed a series of transient transfection assays in logarithmically growing cells. The promoter activity of the different constructs in logarithmically growing cells was normalized against the 3.7-kb R1 promoter activity (pGL3R1 3.7). The result is the mean value (± standard deviation) from two to six independent experiments.

The diagram (Fig. 1) shows the functional assays of the R1 promoter region. To the left, the R1 promoter-luciferase constructs used to characterize the functions of different regions in the R1 promoter are shown. The filled-in boxes show the different known protein binding elements, β, α, Inr, and γ. An open box in such an element indicates where a mutation has been made. The large open box to the right represents the luciferase gene. The 5′-ends of the pGL3R1 5.7 and pGL3R1 3.7 are not drawn to scale. S, B, E, X, and 3I denote the restriction endonucleases SacI, BglII, EcoRV, XhoI, and Esp3I, respectively. To the right, results from transient transfection assays to measure promoter strength are given. The firefly luciferase activity controlled by the R1 promoter was divided by the Renilla luciferase control plasmid activity to quantify the R1 promoter activity. The promoter activity of the different constructs in logarithmically growing cells was normalized against the 3.7-kb R1 promoter activity (pGL3R1 3.7). The result is the mean value (± standard deviation) from two to six independent experiments.

The diagram (Fig. 2) shows cell cycle-regulated luciferase activity in synchronized Balb/3T3 cells stably transformed with the pGL3R1 3.7 or pGL3R1 420 constructs. Cells were first synchronized by serum starvation and then harvested at different time points after serum readdition (5). At each time point, one fraction of the cells was assayed for luciferase activity and protein concentration (top panel), and the values were normalized to the luciferase activity at 0 h (pGL3R1 3.7), 1628 LU/µg protein and pGL3R1 420, 906 LU/µg protein). The remaining cells from each time point were analyzed by flow cytometry (lower panel). The two experiments showed the same degree of synchrony, and for simplicity, only the values from the experiment with the pGL3R1 420 are plotted. ●, G0/G1 cells; ○, S phase cells; ×, G2 + M cells.

The α and β Footprints Are Important for R1 Promoter Strength—Earlier R1 promoter-reporter gene constructs indicated that cell cycle-specific expression of the R1 gene required the Inr and γ elements, whereas there seemed to be no requirement for the α/β elements (11). This puzzled us since we could demonstrate S phase-specific binding to the α/β oligonucleotide (complex C) in gel shift assays (8). To analyze if the α and β footprints were important for the R1 promoter strength, we performed a series of transient transfection assays in logarithmically growing cells. First, we made a deletion analysis of the...
R1 promoter. These constructs were cotransfected with an SV40-driven Renilla luciferase gene to normalize for transfection efficiency. No significant difference in promoter strength was seen as the promoter was shortened from 5.7 kb to 420 bp in our R1 promoter-reporter gene constructs (Fig. 1). The deletion of the β footprint (pGL3R1 287) did not cause any dramatic reduction in promoter strength; in contrast, a mutation in the remaining α footprint caused a 50–60% decrease in promoter strength (pGL3R1 287 (−85/−81) compared with pGL3R1 287). The importance of the Inr and γ regions was again clearly demonstrated as the promoter activity decreased to about 20%, when either region was mutated in the presence of the α and β footprints. Interestingly, a construct containing only the α and β footprints, pGL3R1 α/β, completely lacking the Inr element and γ region, showed a higher promoter activity than the empty pGL3 vector. In contrast, a promoter lacking both functional α/β footprints and Inr element, pGL3R1 177, was as inactive as the empty vector.

After this crude functional characterization of the promoter regions, we wanted to make a more detailed study on the α footprint. Therefore, additional constructs were made with point mutations within the α footprint (Fig. 4B). As in the previous experiments, the constructs were cotransfected with a plasmid containing an SV40-driven Renilla luciferase gene to normalize for transfection efficiency. The results from a series of transient transfection experiments, summarized in Fig. 4, show that all our point mutations in the α footprint decreased the promoter activity in logarithmically growing cells to about 40–55% of wild-type activity with mutations in the center of the footprint being somewhat less active than mutations in the 5'- or 3'-ends.

The different regions of the α footprint may bind different proteins important under different cell cycle phases. Therefore, we synchronized the cells by serum starvation after the transient transfection (Fig. 5 at 0 h). Again, mutations in the center of the α footprint (−91/−86 and −85/−81) lowered the promoter activity more than mutations in the 5'- or 3'-ends. The synchronized cells were then released by serum readdition and harvested at different time points. The promoter activity of the wild-type construct was low in G0/G1 cells and started to increase in S phase cells (Fig. 5). A maximal promoter activity was reached after 20 h when there was a maximal number of S phase cells. This value was 3.4 times higher than the 0-h value. The mutated α footprint constructs all showed the same pattern, but maximal promoter activity was only comparable with the wild-type G0/G1 value (Fig. 5). This indicates that the α footprint is important for total R1 promoter activity.

The Transcription Factor YY1 Binds to the α and β Footprints—A data bank search was performed for sequences in the α or β footprints that could be recognized by already known transcription factors. The result suggested that YY1 should bind to both the α and β footprints, since they both contain the YY1 consensus binding sequence AANATGG. This was tested using a labeled oligonucleotide corresponding to the β footprint and a crude nuclear extract from mouse fibroblasts in gel shift.

**Fig. 4.** Transient transfection assays analyzing the function of the α footprint in exponentially growing cells. A, the firefly luciferase activity controlled by the R1 promoter was divided by the Renilla luciferase control plasmid activity to quantify the R1 promoter activity. The promoter activity of the mutant constructs was normalized against the wild-type promoter activity. The bars show the mean value (± standard deviation) of two independent experiments. B, the pGL3 287 construct is defined as the wild-type promoter, carrying the correct DNA sequence in the α footprint. The different mutations in the α footprint are listed below the wild-type sequence.

**Fig. 5.** Transient transfection assays analyzing the function of the α footprint in synchronized cells. The top panel shows the firefly luciferase activity divided by the Renilla luciferase in cells harvested at different time points after serum readdition. The mean value (± standard deviation) of two independent plates are illustrated. ○, pGL3R1 287 wild type; □, −79/−77; ×, −85/−81; —, −91/−86; ▽, −97/−93. The bottom panel shows the distribution of cells in different growth phases at the different time points for one of the parallel cultures (pGLR1 287 wild type): ○, G0/G1 cells; □, S phase cells; ×, G2/M cells. The remaining cultures were also analyzed by flow cytometry and showed a similar synchrony.
assays (Fig. 6). All three previously reported complexes, A, B, and C, were competed with unlabeled β footprint oligonucleotide. An unlabeled oligonucleotide corresponding to a YY1 binding DNA element in the c-Myc promoter clearly competed complex A but not complexes B and C. Next, we used a supershift antibody that recognizes YY1. This antibody specifically inhibited the formation of complexes B and C, indicating that YY1 is involved in the formation of these complexes (Fig. 7). Antibodies recognizing SRF or DP-2 did not inhibit the formation of or supershifted any complex (Fig. 7). The antibodies recognizing the CCAAT binding transcription factor NF-Y, subunit a or b, did not affect any of the three complexes (data not shown).

The mutations inserted in the α footprint in the previously described functional analyses were also analyzed in gel shift assays. Oligonucleotides corresponding to the different mutated α footprints (Fig. 4B) were labeled and mixed with crude nuclear extracts from logarithmically growing cells. The −79/−77, −85/−81, and −91/−86 mutations all overlap the known consensus binding sequence of YY1. As expected, complex B could not be formed or was severely affected by all these mutations (Fig. 8). Complex A was only affected in the mutation −91/−86. Complex C (the S phase-specific complex) was affected by all mutations, but only to a small extent by the −97/−93 mutation. The different DNA-protein complexes most likely consist of several different proteins, since a new complex b’ appeared with all the four mutated α oligonucleotides (Fig. 8).

**Yeast One-hybrid Screening to Identify Transcription Factors Binding to the α Footprint**—In an attempt to identify other transcription factors that participate in complexes A, B, and C, we performed a yeast one-hybrid screening using five copies of the α footprint as the target element. After establishing a yeast strain containing both the pHis-1 and pLacZ reporter genes, 238,000 independent clones were screened, and 13 clones survived on the selective medium and expressed β-galactosidase. The plasmids expressing the DNA binding activity in these yeast clones were isolated, used to transfect E. coli and subsequently sequenced. Four of the clones expressed the DNA binding domain of YY1 fused to GAL4AD.

**DISCUSSION**

Our data from promoter-reporter gene constructs in stably transformed cells indicate that all elements required for a correct cell cycle regulation of the mouse R1 gene expression can be found within the proximal 420 bp of the R1 promoter. No indication for post-transcriptional regulation of cell cycle-dependent R1 gene expression was observed. Earlier data suggested that the α and β footprints were not required for the cell cycle regulation of the R1 promoter (11).
fection assays now clearly show that the α and β footprints are important for the overall R1 promoter activity. In the transient transfection assays in synchronized cells, the mutations in the α footprint did not significantly alter the expression pattern during G1-S transition, but the maximal level of mutant promoter activity in S phase was only comparable with the activity of the wild-type construct in quiescent cells. This level of promoter activity does not give rise to measurable levels of R1 mRNA in resting cells (5).

Gel shift assays with supershift antibodies, competition experiments, and yeast one-hybrid screening showed that the transcription factor YY1 binds to the α and β footprints and forms complex B. This complex was earlier shown not to vary during the cell cycle (8). YY1 is a ubiquitous transcription factor with many functions. It can activate, repress, and initiate transcription (19). In the R1 promoter, YY1 normally does not initiate transcription at the α and β footprints, since S1 nuclease analysis has shown that the transcription start is at the R1 Inr and not located to the two footprints (8). Furthermore, YY1 does not bind to the R1 Inr (11). However, there is a possibility that in our transient transfection assays, YY1 may initiate transcription at the α/β footprints in the R1 promoter when the Inr and γ elements are deleted. It has been shown in in vitro transcription experiments that YY1 can initiate transcription on supercoiled plasmids in the presence of only TFII B and RNA pol II (20, 21). It is not unlikely that this can occur inside cells as they are transfected with supercoiled plasmids.

YY1 activates or represses transcription by binding to DNA elements that often show a very complex structure. Repression may occur as a result of interaction with histone deacetylases (22). There are often several factors that can bind to the element, sometimes with overlapping binding sites. YY1 can inhibit the binding of SRF (23, 24) but also facilitate the binding of SRF (25). It has been shown that NF1 and YY1 both can activate the human p53 promoter by alternative binding to a composite element (26). To make these elements even more complicated, c-Myc has been shown to regulate YY1 activity via protein-protein interactions to DNA-bound YY1 (27, 28).

A comparison with different YY1-binding elements showed that the R1 α and β footprints are nearly identical to a serum response element in the skeletal α-actin promoter (SRE1 nt −93 to nt −77) (23). This element contains a consensus binding site, 5’-CCAAATATGG-3’, for SRF. However, this site is not present in the R1 α and β footprints, and SRF does not bind to the α/β footprints (Fig. 7). We took advantage of linker-scanning mutations in the SRE1 of the skeletal α-actin promoter in an attempt to characterize the functional role of YY1 binding to the R1 promoter. There are three different mutations in the SRE1 that binds only SRF, only YY1, or neither YY1 nor SRF (23). The mutation that inhibits only YY1 from binding to the SRE1 was also found to inhibit the binding of YY1 to the R1 α footprint (−79/−77) (Fig. 8). In contrast, the mutation in the SRE1 that only blocks SRF from binding to the element not only inhibited complex A but also YY1 from binding to the α footprint (−91/−86). From these results, we cannot draw any conclusions regarding the function of complex A. However, the results suggest that YY1 functions as an activator in the R1 promoter. Complex C appeared to be severely affected by any mutation across the α footprint except for (−97/−93). Both the competition gel shift experiments and the gel shift with the YY-1 antibody suggest that the formation of complex C is dependent on complex B (YY1). The appearance of a new DNA–protein complex b’ in all the gel shifts using a mutated α oligonucleotide indicates the participation of more than one protein in the complexes or multiple DNA-protein interactions.

We can only speculate on how YY1 activates the R1 promoter at this stage. One mechanism can be to facilitate the formation of the S phase-specific complex C. However, it is interesting that YY1 is required for cell cycle-regulated transcription from E2F-1-dependent promoters (29). At the same time, NF-Y was reported to have a similar function as YY1. We did not find that NF-Y binds to the α or β footprints, ruling out the possibility of a common factor between the mouse R1 and R2 promoters. A further characterization of the proteins that bind to the α and β footprints may clarify how YY1 activates the R1 promoter.

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