Role of a Proximal NF-Y Binding Promoter Element in S Phase-specific Expression of Mouse Ribonucleotide Reductase R2 Gene*

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Cell cycle-regulated transcription of the R2 gene of mouse ribonucleotide reductase was earlier shown to be controlled at the level of elongation by an S phase-specific release from a transcriptional block. However, the R2 promoter is activated very early when quiescent cells start to proliferate, and this activation is dependent on three upstream sequences located nucleotide –672 to nucleotide –527 from the transcription start. In this study, we use R2-luciferase reporter gene constructs and gel shift assays to demonstrate that, in addition to the upstream sequences, a proximal CCAAT element specifically binding the transcription factor NF-Y is required for continuous activity of the R2 promoter through the S phase. When the CCAAT element is deleted or mutated, promoter activity induced by the upstream elements decays before cells enter S phase, and the transcriptional block is released. This is a clear example of how changing of a proximal sequence element can alter not only the quantitative but also the qualitative response to upstream transcription activation domains.

Ribonucleotide reductase (EC 1.17.4.1) is a key enzyme in DNA precursor synthesis reducing all four ribonucleotides to the corresponding deoxyribonucleotides (1, 2). Mouse ribonucleotide reductase is a heterodimer composed of the two homodimeric subunits, proteins R1 and R2, each inactive alone. Enzyme activity is cell cycle regulated with low or undetectable levels in G0/G1 and maximal activity in the S phase of the cell cycle (3).

The mouse R1 and R2 mRNA expression is S phase specific with very low or undetectable levels in G0/G1 cells, a pronounced increase as cells progress into S phase, and a decline when cells progress into G2 + M (4). Reporter gene constructs show that the R2 promoter is activated almost immediately after quiescent G0/G1, synchronized cells are released by serum readdition. Promoter activity then increases steadily, reaching its maximum at around 12 h after serum readdition (5). From the early promoter activation, the R2 gene could be classified as an immediate early response gene. However, in vitro studies demonstrated that this early activation only results in the synthesis of immature short R2 mRNA transcripts due to a G0/G1-specific transcriptional block located in the first intron of the R2 gene (5). This block is not released until cells reach S phase when full-length transcripts are synthesized. Reporter gene constructs containing the R2 promoter-1st exon/1st intron indicate that the transcriptional block is active also in vivo, and S phase-specific protein binding was identified to a DNA region just upstream from the block.3

The mouse R2 promoter contains a TTTAAA motif at position nt2 –24 and a CCAAT motif at position nt –75 upstream from the transcription start (6). DNase I footprinting analyses revealed four DNA-protein binding regions within the R2 promoter. The region most proximal to the transcription start (nt –93 to –56) includes the CCAAT box and is called α. The other three DNA-protein binding regions, called β, γ, and δ, are located very close to each other at positions nt –584 to –527, nt –623 to –597, and nt –672 to –637, respectively.1 Experiments using R2 promoter-luciferase reporter gene constructs suggested that the β, γ, and δ regions are required for the early proliferation specific activation of the promoter. Only basal transcription was observed in a construct lacking these regions but retaining the α region.

Gel shift experiments with nuclear extracts from synchronized BALB/3T3 mouse fibroblasts and an oligonucleotide representing the α region showed one specific, cell cycle-independent DNA-protein complex.1

The transcription factor NF-Y, also called CBF, is a ubiquitous heteromeric metaprotein composed of three subunits: A, B, and C (7–10). The C subunit, which was recently identified and cloned, is required together with the A and B subunits to form an NF-Y-DNA complex, containing all three subunits (10). NF-Y was shown to bind to the promoters of the major histocompatibility complex class I genes, the tissue-specific α-collagen gene, and the albumin gene (11–14).

In this study, we try to elucidate the functional importance of the α region within the R2 gene promoter and to identify the transcription factor(s) binding to it. The results show that the α region is required not only for basal transcription as suggested earlier but plays a pivotal role for the continuous activity of the R2 promoter through the S phase. Within the region, the CCAAT motif appears to be of major functional importance by its interaction with the transcription factor NF-Y.

MATERIALS AND METHODS

Cell Culture, Synchronization, Transfection, and Selection of Stable Transformants—Balb/3T3 cells (ATCC CCL 163) were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum. Synchronization of cells by serum starvation and readdition and flow cytometry to check cell cycle distribution was described earlier (5). Transfection of cells by electroporation was made using reporter gene DNA and pSV2neo, and stable transformants were selected in the presence of Geneticin base (Sigma) (5). Resistant clones were expanded, and the presence of transfected plasmid DNA was verified by PCR using specific primers for the

1 S. Björklund, D. Filatov, E. Skogman, M. Thelander, and L. Thelander, manuscript in preparation.

2 The abbreviations used are: nt, nucleotide; PCR, polymerase chain reaction.
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R2 promoter and luciferase cDNA (see below). Usually, two to three independent clones were tested for each particular construct.

Oligonucleotides—All oligonucleotides were synthesized on an Applied Biosystems 392 RNA/DNA synthesizer and purified by chromatography on Sephadex G-25 columns. Oligonucleotides used for construction of p19lucR2 1.0-α were 5′-CTTGCAGCAACTCAACCTCTCCGGCGCTGGGATCCGACCGC-3′ and 5′-GGGCGGCCGCCCCGGCCCGCCGGCTCCGGGGGCGG-5′; oligonucleotides were used for construction of p19lucR2 1.5J1 were 5′-TGAGCCCGAGACTTTGATTCGAAACACATCAAATCATGAGGCAAGAGGATAGACG-3′ and 5′-GGGCGGCCGCCCCGGCCCGCCGGCTCCGGGGGCGG-5′; oligonucleotides were used for construction of p19lucR2 1.5J2 were 5′-GAGGAGCCATTTGAATACTAAATGAGTATCTTGGACCCCGTAGGGC-3′ and 3′-GGGCGGCCGCCCCGGCCCGCCGGCTCCGGGGGCGG-5′; and oligonucleotides used in gel electrophoresis representing footprint sequence happened to contain a CCAAT motif like the native R2 promoter (Fig. 2). However, closer inspection showed that the J1 sequence (Fig. 2A, lane 17) ligated to the luciferase reporter gene. All plasmid constructs were verified by double-stranded DNA sequencing.

Luciferase Assay—Harvesting of cells and preparation of cell extracts for luciferase assays were performed as described (5). Luciferase activity was measured as the number of light units emitted during a 10-s period per μg of total protein, as determined by the Bradford assay (17).

Gel Shift Analysis and Antibodies—Crude nuclear extracts from BALB/3T3 cells were prepared at different time points after serum stimulation as described (18). Binding reactions were performed as described (15), and DNA-protein complexes were resolved through native low ionic strength 4% polyacrylamide gels (6.7 mM Tris-Cl, pH 7.9, 1 mM EDTA, 3.3 mM sodium acetate, 2.5% glycerol; acrylamide:bisacrylamide, 80:1). Antibodies against the NF-YA and NF-YB proteins were kindly supplied by Dr. Roberto Mantovani (Università di Milano, Italy). The NF-YA antibodies were the monoclonal YA7 made against the long recombinant NF-YA protein, and the NF-YB antibodies were a polyclonal YB antiserum made in rabbits against the complete recombinant NF-YB protein (12). The monoclonal antibodies were shown to have lower affinity for NF-Y than the polyclonal antiserum.

RESULTS

The α Region of the R2 Gene Promoter Is Required for Continuous Promoter Activity during the S Phase of the Cell Cycle—To investigate the functional role of the α region for R2 promoter activity, three R2 promoter-luciferase reporter gene constructs were made. In the first one, most of the α region including the CCAAT box was deleted, while the rest of the promoter containing the β, γ, and δ upstream regions was left intact. After electroporation of BALB/3T3 cells, stable transformants were selected, and the presence of the appropriate construct was verified by PCR. Cells synchronized by serum starvation were harvested at different time points after serum readdition and were subjected to luciferase activity measurements and flow cytometry (Fig. 1). There was a rapid increase in luciferase activity when cells passed from quiescence to proliferation much like the earlier observed increase with the intact R2 promoter-luciferase gene constructs p19lucR2 1.0-α and p19lucR2 1.5 (5). However, the p19lucR2 1.0-α reached its maximum already after 5–6 h and then declined to reach near basal levels at 16 h, unlike the intact promoter constructs, where the luciferase activity reached its maximum 12 h after serum readdition and remained almost constant at this level until 16 h. Therefore, the decline in promoter activity started at about the same time as the cell entered the S phase.

To study if the effects of deleting the α region were caused by the shortening of the promoter or a result of deleting specific DNA-protein binding areas, we decided to replace the α region with nonspecific DNA. A DNA fragment of the correct length was chosen from an upstream region of the R2 promoter (nt −1380 to −1343), which seemed to be of no importance for promoter activity (cf. the expression of p19lucR2 1.0 and 1.5 as mentioned above). To our surprise, this construct, p19lucR2 1.5J1, where the α region was replaced by the J1 DNA sequence (Fig. 2A), showed almost the same luciferase expression pattern as the intact R2 promoter constructs with enzyme activity, increasing steadily up to 16 h after serum readdition (Fig. 2B). However, closer inspection showed that the J1 sequence happened to contain a CCAAT motif like the native α region but was otherwise different.

We therefore made the p19lucR2 1.5J2 2 construct, which was identical to the J1 construct except that the sequence just
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The α Region Is Also Required for Basal Non-proliferation-dependent Transcription from the R2 Promoter—Deletion of the β, γ, and δ upstream regions from an R2 promoter-luciferase reporter gene construct abolished the early, proliferation-specific activation of the promoter. However, a proliferation-independent low basal activity was still detectable. To test if this region is required for promoter activity through the S phase but is dispensable for the early proliferation-specific activation.

The α Region Is Also Required for Basal Non-proliferation-dependent Transcription from the R2 Promoter—Deletion of the β, γ, and δ regions protected from DNase I cleavage in footprinting assays. The thick line indicates the deleted DNA fragment in footprint α. The numbering is from the R2 gene transcription start, which is +1 B. R2 promoter-controlled luciferase expression in Balb/3T3 cells stably transformed with the p19 lucR2 1.0-α and synchronized by serum starvation. C, cell cycle phase composition determined by DNA flow cytometry. ●, G1 phase cells; ○, S phase cells; X, G2 + M phase cells.

Fig. 1. A, structure of the p19 lucR2 1.0-α construct with the R2 promoter α, β, γ, and δ regions protected from DNase I cleavage in footprinting assays. The thick line indicates the deleted DNA fragment in footprint α. The numbering is from the R2 gene transcription start, which is +1 B. R2 promoter-controlled luciferase expression in Balb/3T3 cells stably transformed with the p19 lucR2 1.0-α and synchronized by serum starvation. C, cell cycle phase composition determined by DNA flow cytometry. ●, G1 phase cells; ○, S phase cells; X, G2 + M phase cells.

The α region is sufficient for binding the NF-Y transcription factor and no other transcription factor is bind-
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The R2 promoter-reporter gene constructs clearly demonstrate the importance of the α region for S phase-specific expression of the R2 gene. In the absence of the α region, the early proliferation-induced activation of the promoter caused by protein binding to the upstream regions would not result in any production of mature R2 mRNA due to the G1 transcriptional block. Protein binding to the α region maintains a high promoter activity even after the S phase-specific release from the block, and this is a prerequisite of R2 mRNA expression.

It is obvious from the different reporter gene constructs that a CCAAT motif is sufficient to functionally replace the α region DNA. The fact that the anti-NF-YB antibodies completely inhibit the formation of any DNA-protein complex using the α oligonucleotide and a nuclear extract strongly indicates that NF-Y really binds to the α region and not some other CCAAT binding protein. In this context, it is interesting that NF-Y does not bind to the CCAAT motif present in the β fragment of the mouse R1 gene promoter (15) or to a CCAAT motif present in an inverted orientation in the β fragment of the R2 gene promoter, indicating that the CCAAT motif by itself is not always sufficient for NF-Y binding (data not shown). It has also been suggested that other interactions with DNA in addition to the CCAAT element are important for NF-Y binding (data not shown). It has also been suggested that other interactions with DNA in addition to the CCAAT element are important for NF-Y binding (10).

None of the three subunits of NF-Y shows any homology to known protein-protein binding motifs such as leucine zippers, coiled coils, or helix-loop-helix motifs (10). This ubiquitous, heteromeric DNA binding protein, which binds to the proximal part of many eukaryotic promoters, may interact both with upstream DNA binding transcription factors and with proteins involved in the formation of the pre-initiation transcription complex. However, the precise role of NF-Y in transcription activation is still not known.

By studying the function of NF-Y in the transcription of major histocompatibility complex class II genes, it was suggested that NF-Y is involved in the very first stages of pre-initiation and re-initiation of transcription at the promoter (12). NF-Y carries glutamine-rich activation domains thought to be involved in protein-protein interactions with other transcriptional activators. In the activation of the R2 promoter, a close interaction between NF-Y and the transcription factor(s) binding to the upstream β, γ, and δ regions may be required to maintain promoter activity into S phase and achieve productive R2 mRNA expression. Such a role of NF-Y would agree with the proposal that NF-Y bound to a promoter stabilizes the pre-initiation complex. Without NF-Y, the complex formed by the factors binding to the β, γ, and δ upstream regions may not be stable enough to survive into S phase when DNA replication occurs. On the other hand, the pre-initiation complex formed by NF-Y in the absence of the upstream factors does not respond to proliferation but only supports a low basal luciferase activity.

No measurable luciferase activity was observed in cells stably transformed with an R2-luciferase gene construct, lacking the α region and only containing the TTTAAA box and the TATAAA box and other components of the R2 TATA element does not match the consensus TATAAA but represents a rare variant (19).

Many promoters combine the function of a proximal element with that of a more distant enhancer. In the human thymidine kinase gene, most of the promoter activity is contributed by an upstream GC element and a proximal CCAAT element. Competition studies indicated that the protein binding to the
CCAAT was NF-F (20). In the thrombospondin 1 gene, serum-stimulated promoter activation is dependent on a serum response element located at nt –1280 and NF-Y bound to a CCAAT box at nt –65 (21). A somewhat similar arrangement is found in the serum-stimulated β-actin gene promoter, where the serum response element and the CCAAT box are closely located within a 50-base pair region centered at nt –75 with the NF-Y binding situated upstream from the serum response element (22, 23). However, there are no direct similarities between the reported serum response element consensus sequence 5′-CC(A+T)GG-3′ and the R2 promoter β, γ, and δ sequences. Furthermore, in the thrombospondin case, deletion of the upstream serum response element or the CCAAT both resulted in decreased serum response. In contrast, deletion of the CCAAT motif in the R2 promoter changes not only the level of activation but also the cycle-dependent pattern of promoter activation. This shows that alteration of a proximal promoter element can change the qualitative response to upstream transcription activation domains (cf. Ref. 24). A more complete understanding of the activation of the R2 promoter will require identification of the transcription factor(s) binding to the upstream region.

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