S Phase-specific Transcription of the Mouse Ribonucleotide Reductase R2 Gene Requires Both a Proximal Repressive E2F-binding Site and an Upstream Promoter Activating Region*

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Ribonucleotide reductase (RNR) is essential for de novo synthesis of deoxyribonucleotides (dNTPs), which are required for DNA replication and repair. The active enzyme consists of two non-identical subunits called proteins R1 and R2. There are multiple levels of regulation of ribonucleotide reductase activity, which is highest during the S and G2 phases of an unperturbed cell cycle in mammalian cells. Previous reports in the literature have indicated that the S phase-specific transcription of the mammalian R2 gene is regulated by a transcriptional block, is dependent on the transcription factor E2F1, or is simply regulated by proteins that bind to promoter CCAAT boxes plus the TATA box. Here, we demonstrate that the S phase-specific transcription of the mouse R2 gene is dependent on an upstream promoter activating region (located at nucleotides nt −672 to −527 from the transcription start site) and one proximal promoter repressive element (located at nt −112 to −107) that binds E2F4. Binding to the E2F site is modulated by binding of nuclear factor-Y to an adjacent CCAAT element (nt −79 to −75). The upstream activating region is crucial for overall R2 promoter activity. Mutation of the E2F-binding site leads to premature promoter activation in G1 and increases overall promoter activity but only when the upstream activating region is present and intact. Therefore, E2F-dependent repression is essential for cell cycle-specific R2 transcription.
overproducing adenoviral vector (17). Therefore, R2 gene expression was reported to be controlled by E2F. However, expression of E2F1–3 in quiescent cells is sufficient to induce cells to re-enter the cell cycle (18). Accordingly, the activation of R2 gene expression could simply be a consequence of cells entering the S phase and not a direct effect of E2F binding to the R2 promoter.

The aim of this study was to identify protein-binding regions essential for promoter activity in the mouse R2 promoter. We also aimed to identify the mechanisms regulating S phase-specific expression of the R2 gene. DNase I footprinting was used to identify protein-binding DNA elements in the mouse R2 promoter. We then assayed their functional importance for S phase-specific transcription using R2 promoter-luciferase constructs and transient transfections in logaritically growing or serum-synchronized mouse cells as well as in serum-synchronized, stably transfected cells. Finally, chromatin immunoprecipitation assays were used to identify essential transcription factors that bind to the R2 promoter. Our studies show that S phase-specific transcription of the R2 gene is controlled by an upstream activating footprint region in combination with a proximal promoter repressive E2F4-binding element. Deletion of the upstream activating region nearly inactivated the promoter, whereas mutation of the S phase-binding site led to premature G1 phase promoter activation, but inactivated the promoter, whereas mutation of the E2F4-binding site. In the PCRs, the oligonucleotide pairs 5′-TCAAATCCGGCGGGGCTGGGCGG-3′ plus 5′-CCGGCCGGAGCG-3′ and 5′-CCTCGGACGCCGGCGGTTTCGGGC-3′ plus 5′-GCCACCACGCGCGGCGGCTCCAGAG-3′, respectively, were used with a DNA template consisting of pAC10 with mutated E2F-I and E2F-II sites.

The upstream footprint regions were deleted by using PCRs with the following oligonucleotides in the direct orientation (with the positions of the deletions shown with (_)): 3′, 5′-CGTTTCTCCCGCCGCTGGGC-3′ and 5′-GGG_GGGCAGGGTAGAAAGGA-3′. Oligonucleotide 5′-CATGGGCTGGAATCCGGTGCACCG-3′ plus 5′-CCGGCCGGAGCG-3′ was used in the reverse orientation. The PCR products were digested with XhoI and Smal and ligated into pAC10 digested with XhoI and AgeI. All constructs were verified by restriction analysis and sequencing of PCR-amplified regions.

Cloning of the Guinea Pig R2 Promoter—The guinea pig R2 promoter was cloned from a guinea pig kidney a genomic library (Stratagene 946110) using a 1100-bp DNA fragment of the R2 promoter (nt 10797–106) as a probe. The GenBank™/EBI Data Bank accession number for the guinea pig R2 promoter sequence is AY208181.

DNase I Footprinting—The pSB2Ra plasmid used for DNase I footprinting of the proximal part of the R2 promoter was made by ligating an Rsal-PstI DNA fragment from the mouse R2 gene (nt 470 to +117) to the Smal and PstI sites of the pUC18 polylinker. Construction of the pSB2Rb plasmid used for DNase I footprinting of the distal part of the R2 promoter was done by ligation of a ClaI-RsaI DNA fragment from the R2 promoter (nt 1006 to –471) to the Accl and Smal sites of the pUC18 polyn linker. pSB2Ra and pSB2Rb were end-labeled at either the EcoRI or HindIII site in the pUC18 polyn linker with the Klenow fragment. DNA polymerase and [γ-32P]dATP. Isolation of the end-labeled fragments was done by separation on nondenaturing polyacrylamide gels and electroelution. Incubation of crude nuclear extracts and the DNA probes, DNase I digestion, and separation on 8 M urea and 6% polyacrylamide gels and electroelution. Incubation of crude nuclear extracts and the DNA probes, DNase I digestion, and separation on 8 M urea and 6% polyacrylamide gels were as described by Olson and Edlund (19).

The Ehrlich-Lettre ascites cells (American Type Culture Collection CCL77) were grown as spinner cultures in Dulbecco’s modified Eagle’s medium (Sigma) plus 10% heat-inactivated horse serum (Invitrogen). The cultures were maintained at a concentration of 0.1–1 × 10^6 cells/ml, representing a logarithmically growing culture. Crude nuclear protein extracts were prepared as described by Parker and Topol (20) with modifications (19). The protein concentration was usually between 5 and 9 mg/ml.

Transient Transfection Assays—To measure the promoter strength, murine Balb/3T3 cells (American Type Culture Collection CCL163) were transiently cotransfected with R2 promoter-luciferase constructs and a control plasmid, pRLSV40 (Promega), by the calcium phosphate precipi-
iteration method as described previously (14). The cells were cultured in Dulbecco's modified Eagle's medium plus 10% (v/v) heat-inactivated horse serum. The cells were harvested and lysed in passive lysis buffer (Promega) according to the instructions of the manufacturer. Firefly luciferase (expressed by the R2 promoter-luciferase constructs) and Renilla luciferase (expressed by the pRLSV40 plasmid) were assayed using the dual-luciferase reporter assay (Promega) and a TD-20/20 luminometer (Turner Designs). The firefly luciferase activities were then divided by the Renilla luciferase activities to normalize for differences in transfection efficiencies. The values obtained represent R2 promoter activity, where the activity of the 1500-bp promoter construct is called wild-type and was set to 100%. The activities of the different mutant promoter constructs were divided by the wild-type activity to obtain percent wild-type activity. The activity values are based on at least three experiments, and the S.E. is shown with error bars. To avoid saturation effects and to make certain that luciferase expression was proportional to the amount of transfected DNA, two to four different amounts of R2 promoter-luciferase reporter gene DNA were used for each construct in each experiment (usually 2–10 μg of DNA).

To study the expression of the various R2 promoter-luciferase constructs during the cell cycle, the cells were synchronized by serum starvation and release after the 14-h transfection by first incubating them in fresh medium containing 1% serum for 48 h and then in medium containing 20% serum. The cells were harvested in phosphate-buffered saline at different time points, and one-half of each sample was used for monitoring the cell cycle progression by flow cytometry as described previously (9). The other half of each sample was prepared for luciferase activity measurements as described above.

Stably Transformed Cells—Stably transformed mouse cells expressing the various R2 promoter-luciferase constructs were obtained by cotransfection with a pSV2neo plasmid using electroporation and selection with G418 as described (14).

Chromatin Immunoprecipitations—Logarithmically growing mouse Balb/3T3 cells were treated with formaldehyde, swelled, and lysed, and the nuclei were sedimented and lysed as described (21). The resulting chromatin solution was sonicated; and after microcentrifugation, 1 ml of solution (corresponding to ~6 × 10⁷ cells) was precleared by incubation for 15 min at 4 °C with 90 μl of a 50% suspension of protein A-agarose (Sigma). One μg of antibody was added to each aliquot of chromatin and incubated as described (21). Antibodies against E2F4 (sc-866X) and E2F1 (sc-251X) were purchased from Santa Cruz Biotechnology; antibody against NF-YB was kindly provided by Dr. Roberto Mantovani (Dipartimento di Biologia Animale, Università di Modena e Reggio, Modena, Italy); and antibody against transcription factor II-I was kindly provided by Dr. Ananda L. Roy (Department of Pathology, Tufts University School of Medicine, Boston, MA). Immunoprecipitations of the antibody-protein-DNA complexes were carried out with 30 μl of a 50% suspension of protein A-agarose/sample. Washing, elution, cross-link reversal, preparation of the samples for PCR analyses, and PCR analyses were as described (22). The following R2-specific oligonucleotide primers were used: 5′-CTGTGGCAGGCCCAATCAGCAGCCG-3′ (nt -281 to -260) and 5′-CACGCGGCAGAATGACGCTGC-3′ (nt 346 to 325); and 5′-ACAGATTAGTCAGCATTGCTGC-3′ (intron 5) and 5′-ACGTAGCCTCTTGTCCCAATCC-3′ (exon 6).

RESULTS

DNA-Protein Interactions in the Mouse R2 Promoter—An end-labeled RsaI-PstI R2 promoter DNA fragment (nt -470 to +117) and unfractonated nuclear extracts isolated from logarithmically growing Ehrlich-Lettre ascites carcinoma cells were used for DNase I footprint analyses of the region around the R2 transcription start site and the proximal region of the R2 promoter. Fig. 1A shows the partial DNase I digestion pattern of the RsaI-PstI fragment labeled at the 3′-end of the coding strand with (lane 2) and without (lanes 1 and 3) prior incubation with nuclear extract. One sequence of -38 nt (nt -93 to -56) containing a CCAAT box was specifically protected and is designated footprint α. This element was previously demonstrated to bind NF-Y (23). Dideoxy-A and dideoxy-C sequencing reactions were run alongside as size markers (data not shown). Assays in which the RsaI-PstI fragment was labeled at the 5′-end showed no additional protected regions (data not shown).

The distal part of the R2 promoter was analyzed for DNA-protein interactions by DNase I footprinting using a ClaI-RsaI DNA fragment (nt -1006 to -471) (Fig. 1B). Protection of three closely spaced regions was observed after labeling the 3′-end of the coding strand: nt -672 to -637, nt -623 to -597, and nt -584 to -527. Experiments with the same fragment labeled on...
**Fig. 2. Functional analyses of the upstream R2 promoter region.** A, relative promoter activities of different R2 promoter-luciferase constructs measured by transient transfection assays. The wild-type activity was set to 100%. The S.E. of measurement based on at least three transfections is shown for each construct. The different R2 promoter-luciferase constructs are indicated schematically. The TATA box is shown as a vertical bar, and the CCAAT box is shown as a vertical bar within the α region (open rectangle). The protected upstream region in the DNase I footprint analysis is shown as an open rectangle. The size and positioning of the DNA elements are drawn to scale. Arrows mark the transcription start site. **Vector** indicates the backbone pGL3-Basic vector alone. B, luciferase activities in serum-synchronized, transiently transfected cells. The ordinate shows the firefly luciferase activity in arbitrary light units, and the abscissa shows hours after serum re-addition. The insets show the flow cytometry profile corresponding to each time point for the wild-type cells. **C**, luciferase activities in serum-synchronized, stably transformed Balb/3T3 cells. Sma is identical to the 760-bp construct in A and B. Rsa is similar to the Δ760–470 construct in A and B, but lacks the upstream sequence from nt −1500 to −470. Stu is similar to the Δ760–130 construct in A and B, but lacks the upstream sequence from nt −1500 to −130. Independent clones of cells were first synchronized by serum starvation and then harvested at different time points after serum re-addition. At each time point, one fraction of the cells was assayed for luciferase activity and protein concentration, and the values were normalized to the luciferase activity at 0 h (Sma construct, 6.5 luciferase units/μg of protein; wild-type promoter, 11.9 luciferase units/μg of protein; Rsa construct, 0.06 luciferase/μg of protein; and Stu construct, 1.1 luciferase units/μg of protein). The remaining cells from each time point were analyzed by flow cytometry (insets).
the opposite strand showed no additional protected regions (data not shown). Gel shift assays using nuclear extracts and labeled oligonucleotides corresponding to DNA sequences within region \(-672\) to \(-527\) gave one specific major protein-DNA complex. No clear difference in pattern was observed when nuclear extracts from cells in different growth phases were used (data not shown).

**Functional Analyses of the Mouse R2 Promoter**—Fig. 2A shows a schematic representation of the R2 promoter with the upstream footprint region (nt \(-672\) to \(-527\)) and the \(\alpha\) region with the CCAAT box (nt \(-93\) to \(-56\)) and the TATA box (nt \(-29\) to \(-24\)). We have previously shown that a 1500-bp mouse R2 promoter is sufficient for cell cycle-specific transcription of an R2 promoter-luciferase construct transfected into mouse cells (9). To narrow down the regions needed for R2 promoter activity, we created a number of different R2 promoter deletion-dependent luciferase constructs, taking advantage of existing restriction endonuclease sites. The promoter strength of the different constructs was tested in transient transfection assays using logarithmically growing mouse Balb/3T3 cells and cotransfection with a Renilla luciferase reporter gene construct to monitor transfection efficiencies. A linear response was observed between luciferase activity and the amount of transfected DNA using 1–10 \(\mu\)g of DNA for each construct. Fig. 2A shows the promoter strength of these deletion constructs compared with that of the wild-type promoter. A 760-bp promoter
still containing all identified footprint regions had full promoter activity compared with the 1500-bp wild-type promoter. In contrast, a promoter with a 290-bp fragment deleted between −760 and −470 bp upstream of the transcription start site (Δ760−470) showed only 30% of the wild-type promoter activity. This result shows that the region −760 to −470 bp upstream of the transcription start site, closely corresponding to the upstream footprint region, is needed for full R2 promoter activity. A promoter with a deletion all the way from nt −760 to −130, i.e., still containing the CCAAT and TATA boxes, showed even lower promoter activity (15% of the wild-type activity). The backbone vector of all constructs, the pGL3-Basic vector (Promega), showed almost no activity by itself in the luciferase assay.

The cell cycle expression pattern of the different constructs was tested in experiments in which transiently transfected cells were synchronized by serum starvation, followed by re-addition of serum. The cell cycle expression patterns and expression levels were the same for the wild-type promoter and the 760-bp promoter fragment (Fig. 2B). Again, the promoter activity was dramatically decreased in the Δ760−470 construct compared with the wild-type promoter, and the low activity made it difficult to clearly observe an S phase-specific expression pattern. The Δ760−130 construct showed no significant cell cycle-related expression pattern, and the promoter activity was very low. Very similar cell cycle expression patterns were observed for serum-synchronized Balb/C3T3 clones stably transformed with the R2 promoter-luciferase constructs, confirming the relevance of the transient expression experiments (Fig. 2C).

To analyze the function of region −760 to −470 in more detail, we constructed a series of stepwise deletions in our R2 promoter-luciferase construct starting −760 bp from the transcription start site (Fig. 3A). Deletion constructs Δ1 and Δ2 (covering nt −760 to −690) had no effect on promoter activity compared with the wild-type promoter. However, deletion constructs Δ3−7 (covering the region between nt −760 and −505) all showed decreased promoter activity (∼30% compared with the wild-type activity). This result indicates that the region important for promoter activity is located downstream of nt −690. Mutating the region from nt −623 to −597 by replacing it with a fragment of unrelated DNA resulted in a similar decrease in promoter activity (20% of the wild-type activity), underscoring the importance of region −690 to −470. The influence on the cell cycle expression pattern of two of the deletions affecting promoter activity is shown in Fig. 3B. Deletion constructs Δ4 and Δ6 were chosen as representatives of the affected deletion constructs and showed the same pattern as the Δ760−470 construct. Similar results were obtained with deletion constructs Δ3, Δ5, and Δ7 (data not shown). In contrast, but in accordance with the promoter strength results, deletion constructs Δ1 and Δ2 had the same pattern as the wild-type promoter (data not shown). Mutating region −623 to −597 almost abolished promoter activity. In conclusion, the functional analyses of the R2 promoter region strongly support the importance of the upstream footprint region (nt −672 to −527).

Comparison of the Mouse, Human, and Guinea Pig R2 Promoter Sequences Corresponding to nt −760 to −470 in the Mouse Promoter—Comparison of the human and mouse R1 promoters revealed highly conserved nucleotide sequences important for transcriptional regulation (24). No such obvious homologies were observed between the human and mouse upstream R2 promoter regions. To increase the predictive value of such a sequence comparison, we cloned and sequenced the guinea pig R2 promoter region because the guinea pig is only distantly related to human and mouse. In Fig. 4, the homologies in the nucleotide sequences of the human, mouse, and guinea pig upstream R2 promoter regions are indicated, as well as the positions of the footprints. Clusters of conserved nucleotides are present; but surprisingly, a long DNA element, completely conserved in all three species, is present within Δ2, a deletion that had no effect on promoter activity (Fig. 3A).

Mutation of Putative Proximal E2F-binding Sites in the R2 Promoter Results in Increased Overall Promoter Activity and Premature G1 Phase Activation—To investigate if the transcription factor E2F binds directly to the R2 promoter as was suggested previously (17), we looked for the core consensus sequence CCGGCG or CGCGGCG (25) in the promoter. Four putative E2F-binding sites (E2F-I, E2F-II, E2F-III, and E2F-IV) were identified. These sites were mutated in R2 promoter-
luciferase constructs, and the activities of the corresponding promoters were measured. Fig. 5A shows the positions of the putative E2F-binding sites in the mouse R2 promoter and the relative promoter activity (compared with the wild-type activity) of promoters with all four sites mutated (mE2F-I–IV) or the sites mutated pairwise (mE2F-I+mE2F-II and mE2F-III+mE2F-IV). When all four putative E2F sites were mutated, the promoter activity increased ~2-fold compared with the wild-type activity. Mutation of E2F-I plus E2F-II resulted in the same 200% active promoter, whereas mutation of E2F-III
plus E2F-IV resulted in a promoter with wild-type activity. These results indicate that an inhibitory transcription factor binds to the putative E2F-I and/or E2F-II site.

Fig. 5B shows a comparison of the cell cycle expression patterns of the wild-type promoter, a promoter with mutated E2F-I plus E2F-II, and a promoter with mutated E2F-III plus E2F-IV. The wild-type promoter and the mE2F-III/mE2F-IV promoter showed the same S phase-specific expression pattern. In contrast, the mE2F-I/mE2F-II promoter showed increased promoter activity both in G1 and S phase cells, with the activity increase being most pronounced in G1 phase cells. This relative increase in the activities of the wild-type and mutant promoters is presented in Fig. 5C, where all luciferase values for a certain construct were divided by the luciferase value at 0 h for the same construct. The S phase-specific increase in promoter activity is clearly seen for the wild-type and mE2F-III/mE2F-IV constructs, whereas it is less pronounced for the mE2F-I/mE2F-II construct. Accordingly, the most important contribution of the putative E2F-I and E2F-II sites to the S phase-specific expression of the R2 promoter seems to be inhibition of promoter activity during G1 phase.

Deletion of the Upstream Activating Footprint Region Eliminates the Activating Effect of the E2F Site Mutations—Considering the importance of the upstream activating footprint region for R2 promoter activity, we wanted to know whether mutating the putative E2F-I and E2F-II sites would result in an overactive R2 promoter even if the upstream activating region was deleted. Fig. 6A compares the activity of a promoter with region 760 to 470 deleted with that of a promoter with region 760 to 470 deleted plus the mutated E2F-I and E2F-II sites. The promoter activity of both constructs was ~30% of the wild-type activity. Furthermore, combining a mutation in the upstream footprint region (nt −623 to −597) with mutations of the four putative E2F sites also resulted in ~30% of the wild-type promoter strength. The S phase specificity of the latter construct was completely abolished (Fig. 6C). These results demonstrate that the upstream activating region is necessary to activate the R2 promoter and that, when this region is deleted or mutated, mutating the repressive putative E2F-binding sites cannot increase promoter activity.
Comparison of the Proximal R2 Promoter Regions in Mouse, Human, and Guinea Pig Shows a Conserved Putative E2F-binding Site—Fig. 6B shows a sequence comparison of the proximal parts in the mouse, human, and guinea pig R2 promoters. They all contain the variant TTTAAA form of a TATA box and, in addition, one (mouse and guinea pig) or three (human) CCAAT boxes. A conserved putative E2F-binding site corresponding to E2F-II is indicated with an arrow, and the conserved sequences are in boldface. The putative E2F-I site is also indicated, but this site is conserved only in mouse and human, but not in guinea pig. The putative E2F-III and E2F-IV sites found in the mouse R2 promoter are not conserved in human and guinea pig (data not shown).

Because the putative E2F-II site is clearly conserved in all three species, we wanted to know whether this site alone is responsible for the observed repressive activity. Therefore, we mutated the E2F-I and E2F-II sites separately. Mutating the E2F-I site alone had only a marginal effect on the promoter activity (−120% compared with the wild-type activity). In contrast, mutating the E2F-II site alone resulted in 200% active promoter compared with the wild-type promoter (data not shown). Based on these results, we conclude that the conserved E2F-II site is responsible for the G1 repression of the R2 promoter. Interestingly, a protected region including E2F-II (nt −112 to −107) can be observed in the footprint experiment shown in Fig. 1A.

Interplay between the Transcription Factors That Bind to the Proximal Promoter CCAAT Box and Putative E2F Elements—The putative E2F-II site is situated close to the NF-Y-binding CCAAT box in the proximal part of the mouse R2 promoter. Mutating the CCAAT box to CTAGT was previously shown to decrease R2 promoter activity to −20% compared with the wild-type activity without changing the S phase-specific expression profile (16). We now combined the mutated CCAAT box with the mutated E2F-I and E2F-II elements in one reporter gene construct and recorded the effects on the promoter strength and cell cycle expression pattern. As seen earlier, mutating E2F-I and E2F-II alone increased promoter activity to −200%, whereas mutating the CCAAT box alone decreased promoter activity to −20% compared with the wild-type activity (Fig. 7A). To our surprise, combining the mutated E2F-I and E2F-II sites with a mutated CCAAT box resulted in 150% active promoter compared with the wild-type promoter. Shown in Fig. 7B are the cell cycle expression patterns of the wild-type promoter, a promoter with mutated E2F-I and E2F-II elements in one reporter gene construct and recorded the effects on the promoter strength and cell cycle expression pattern.
II + mCCAT promoter showed increased promoter activity both in G1 and S phase cells, with the activity increase being most pronounced in G1 phase cells. In sharp contrast to these results was the S phase-specific but very low activity of the R2 promoter containing a mutated CCAAT box and intact E2F-binding sites (Fig. 7B). Therefore, mutating the putative E2F sites results in approximately the same promoter activation with or without an intact CCAAT box, whereas in the presence of intact E2F sites, the CCAAT box is very important for promoter activity.

**E2F4 Binds to the Mouse R2 Promoter in Vivo—**Individual E2F/DP complexes recognize the same nucleotide sequence because they all have a very similar DNA-binding domain (18). Therefore, it appeared less likely that gel shift assays using an oligonucleotide probe corresponding to the E2F-II sequence would identify which of the transcription factors in the E2F family binds to the R2 promoter. Instead, we turned to a chromatin immunoprecipitation assay to determine which E2F bound to the R2 promoter in vivo. In this assay, we used logarithmically growing mouse Balb/3T3 cells and antibodies against the E2F1 and E2F4 transcription factors. As shown in Fig. 8 (A and B), a significant enrichment of the R2 promoter could be detected using antibody against E2F4 in the immunoprecipitation step. As a positive control, we observed R2 promoter enrichment also using antibody against the CCAAT box-binding NF-Y protein (Fig. 8A). Negligible quantities of R2 promoter were observed using an unrelated antibody (anti-transcription factor II-I) or in a sample to which no antibody was added. Likewise, a very weak band was observed with anti-E2F1 antibody relative to the band obtained with anti-E2F4 antibody (Fig. 8B). No chromatin enrichment could be detected in a control region situated 3000 bp downstream of the transcription start site in the R2 gene (Fig. 8C). Based on these results, we propose that the specific G1 repression of R2 promoter activity is a result of binding the repressive E2F4 transcription factor.

**DISCUSSION**

Our results demonstrate that the S phase-specific expression of the mouse R2 promoter is mainly regulated by repression of promoter activity in G1 phase cells by E2F4. This repression is released in the beginning of S phase, allowing full penetration of the upstream activating promoter region. The S phase regulation of the mouse RNR R2 gene through repression of promoter activity during G1 phase is reminiscent of the transcriptional regulation of the yeast RNR genes. In *Saccharomyces cerevisiae*, S phase transcriptional regulation occurs via Mbp1/Swi6 binding to MCB promoter elements (13, 26). Binding of Mbp1/Swi6 activates transcription in G1/S, whereas it represses transcription in other cell cycle phases. Cells lacking MBP1 are viable, but constitutively express genes involved in DNA synthesis. Mbp1 has been suggested to be a functional analog of the E2F/DP1 family in higher eukaryotes (27). In contrast to the yeast RNR genes, which are transcriptionally up-regulated after DNA damage through the Mec1/Rad53/Dun1 pathway, the transcription of the mouse R2 gene is not affected by DNA damage (9, 13). Instead, a novel R2 gene, p53R2, is induced by the DNA damage-signaling factor p53 (10). One reason for this difference between yeast and mammalian cells may be that the yeast R2 protein, like the p53R2 protein, lacks the KEN box, which mediates degradation of the mouse

**Fig. 8. Experimental verification of the mouse R2 promoter as an E2F4 target.** Cross-linked chromatin from mouse Balb/3T3 cells was immunoprecipitated with antibodies against E2F4, E2F1, NF-YB (positive control), and transcription factor (TF) II-I (negative control) or processed with no antibody. Purified DNA was analyzed by PCR using primers specific for the R2 promoter region (nt 281 to 130) (A and B) or part of exon 6 (C) as indicated schematically. A sample representing 0.3% of the chromatin used as starting material for the immunoprecipitation is also shown (Input). M, molecular size markers. Arrowheads indicate the expected positions of the R2 promoter fragment.
R2 protein by the Cdh1-anaphase-promoting complex active in late mitosis and in G_{0}/G_{1} phase cells (8).

Our R2 promoter model is clearly different from the proposed E2F1-regulated promoter model, in which the transcription domain of E2F1 stimulates the promoter activity of genes involved in DNA synthesis once it is released from the retinoblastoma protein in G_{S} (17, 18). We observed no promoter activity with a 130-bp R2 promoter-luciferase gene construct that still contained the E2F-binding element and the α region with the CCAAT box and the TTATAA box, a result that contradicts the E2F1 model. Furthermore, we observed an up-regulation of promoter activity (most pronounced in G_{1} phase) when the E2F element was mutated, provided that the reporter gene construct also contained a functional upstream footprint region. It is interesting that the combined loss of E2F1–3 in mutant mouse embryonic fibroblasts leads to failure to induce known E2F target genes such as dihydrofolate reductase, thymidine kinase, and DNA polymerase-α in response to serum. However, transcription of the R2 gene is not affected, again demonstrating that it is not a target for the activating E2F transcription factors (28). In contrast, R2 gene expression is de-repressed in G_{1} phase in primary cells lacking the two pocket proteins p107 and p130, the primary partners of E2F4 in G_{1} phase (29).

The E2F-II site in the mouse R2 promoter accounted for most of the observed up-regulation when mutated; and therefore, we believe that this site is a genuine E2F-binding site. Furthermore, it is conserved in mouse, human, and guinea pig. Not only is the core consensus sequence conserved, but also additional flanking sequences are conserved in the three species. How does E2F4 repress R2 promoter activity in G_{0}/G_{1} phase cells? E2F4 is a poor transcriptional activator, and its subcellular localization is regulated during the cell cycle, where it is found in the nucleus only in G_{0} and G_{1}. It lacks the nuclear localization signal that is present in the activating E2F1–3 transcription factors and instead has a nuclear export signal. However, association with the pocket proteins induces its nuclear localization during G_{0}/G_{1}, where active repression is performed by different corepressor molecules associated with the pocket proteins, such as histone deacetylases, ATP-dependent chromatin-modifying enzymes, and histone methyltransferases (18, 30). Recent data suggest that the rat R2 promoter is a histone deacetylase-independent target of retinoblastoma protein-mediated repression. The repression may instead be a result of SWI/SNF-dependent chromatin remodeling (30).

Transient transfection as a method has the drawback of studying promoter activity in a partially chromatin-free environment. Therefore, it is difficult to investigate chromatin remodeling as a mechanism of transcriptional regulation using this method. However, there is at least one report using transient transfections as a method for studying nucleosome positioning, indicating that some chromatin formation does occur even in transient transfections (31). Our results from transient transfection experiments were also supported by experiments with stably transformed cells, where both types of experiments demonstrated very similar cell cycle expression patterns.

Mutating the CCAAT box alone in the R2 promoter resulted in only 20% active promoter compared with the wild-type promoter. In contrast, mutating both the CCAAT box and the adjacent E2F-II site resulted in up-regulated promoter activity comparable to the activity seen in constructs with only the E2F site mutated. The E2F-II site in the R2 promoter is situated very close to the CCAAT box, which is known to be important for R2 promoter strength. Binding of the trimeric NF-Y complex has been reported to bend the DNA helix 60–80°, and this complex spans 24–26 nucleotides on each DNA strand (32). Therefore, our data are compatible with the hypothesis that binding of NF-Y to the R2 promoter impairs binding of E2F4 to the E2F-II site and facilitates its release during S phase.

E2F transcription factors have been implicated in the regulation of the mouse R2 gene (17) prior to this study. However, up to now, no direct binding of E2F to the R2 promoter was demonstrated. Interestingly, cell cycle regulation of the tobacco RNR small subunit gene is also mediated by E2F-like elements (33). The two E2F elements identified in the tobacco R2 gene are involved in up-regulation of the promoter at the G_{1}/S transition, and mutation of both elements prevents any significant induction of the RNR promoter. In addition, one of the E2F elements, sharing homology with the animal E2F/cell cycle-dependent element motif, behaves like a repressor outside of S phase (33).

A few studies have also been performed with the human R2 promoter (34–36). Zhou and Yen (36) described the cloning of the human R2 gene and analysis of the promoter. Two different transcription start sites were identified, resulting in two different species of mRNA. The mRNAs would result in two R2 proteins that differ in length by 60 amino acid residues, and such proteins have not yet been observed in extracts from human cells. Comparison of the human and mouse R2 promoters shows conserved features, but also differences. Therefore, it was interesting to us that the human R2 gene (cloned in our laboratory with the same sequence as the one in the human genome data base) could be expressed in a functional R2 protein in a mouse cell and vice versa. This result shows that the mouse R2 promoter can activate transcription of the mouse R2 gene in a human cell. Likewise, the human R2 promoter can activate transcription of the human R2 gene in a mouse cell (data not shown). These results suggest that the human and mouse R2 genes are regulated in a similar fashion.

Using a computer search, Zhou and Yen (36) suggested some putative transcription factor-binding sites in the human R2 promoter. They did not, however, identify the E2F-binding site shown in this study to be conserved in the mouse, human, and guinea pig R2 promoters and to regulate the S phase specificity of the mouse R2 promoter. The longest promoter construct used by Zhou and Yen contained 659 bp from the transcription start site, which means that the putative human upstream promoter activating region was not present in their construct. Zhou and Yen performed deletion studies with the human R2 promoter and confirmed the importance of at least two of the three identified CCAAT boxes, in agreement with our study of the mouse R2 promoter (16, 23). It should also be mentioned that another study on the human R2 promoter confirmed the importance of the CCAAT boxes (34), but the sequence of the R2 promoter used was different from the sequence upstream of the R2 gene in the human genome sequence (NCBI Protein Database) and was also different from the sequence of our cloned human R2 promoter.

We still do not understand the relation between the positive effect on promoter activity of NF-Y binding to the CCAAT box and the transcription factor(s) that bind to the upstream footprint region. However, the limited size of the upstream footprint region (145 bp) now makes it possible to proceed with the identification of the proteins that bind there.

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