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Characterization of the Promoter of the Human Ribonucleotide Reductase R2 Gene

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We describe here cloning and characterization of the promoter region of the human ribonucleotide reductase R2 gene. Primer extension and sequence data indicated that two different transcripts were produced via using two different promoter regions. Promoter activity of the 5′ flanking region of the first transcript was approximately 100-fold higher than controls, and that of the second transcript was approximately 30-fold higher than controls. Particularly, the proximal region of the first transcript, −125 to +1 bp, was responsible for approximately a 50-fold increase in promoter activity, compared to controls. This region had three CCAAT sequences, each of which contributed similarly to promoter activity. When all three CCAAT sequences were mutated, promoter activity declined 80%. In addition, the promoter region −125 to +1 bp was responsible for cell-cycle-specific expression. These data provided essential information concerning regulatory mechanisms of cell-cycle-specific expression of human ribonucleotide reductase R2.
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Ribonucleotide reductase (RR) (EC 1.17.4.1) catalyzes the direct reduction of all four ribonucleotides to the corresponding deoxyribonucleotides, a reaction essential for DNA synthesis (1). The activity of RR is mediated by at least two low molecular weight proteins: thioredoxin (2) and glutaredoxin (3–5). Mammalian RR is a heterodimer consisting of two nonidentical subunits, proteins R1 and R2. Protein R1 has a molecular weight of 2 × 84,000 Daltons and contains binding sites for nucleoside triphosphate allosteric effectors and ribonucleoside diphosphate substrates (6). Protein R2 has a molecular weight of 2 × 45,000 Daltons and has a non-heme iron center that generates and stabilizes a tyrosyl free radical essential for activity (7).

Protein R2 of mammalian RR was reported to be involved in the active proliferation of cells (8). Northern Blot indicated that expression of protein R2 is dominant at S phase of the cell cycle, and that the half-life of protein R2 is only 3 h. Since RR could be a key enzyme in regulation of cell proliferation (9), and protein R2 has been a candidate molecule for restraining growth of transformed cells (10), it seemed worthwhile to determine how the transcription of the protein is regulated in human cells. As one means to understand molecular regulation of protein R2 transcription, we isolated the human gene for the R2 protein of RR to study its promoter. The promoter region was characterized because protein R2 is expressed during specific states of the cell cycle. The data in this paper show that R2 transcription is under control of a strong promoter that is cell-cycle-specific.

Sequence data from this paper have been submitted to the NCBI/GenBank under Accession No. AF149206.

Abbreviations used: bp, base pairs; kb, kilobases; nt, nucleotide; RR, ribonucleotide reductase.

MATERIALS AND METHODS

Materials. T_4 DNA ligase and restriction enzymes were purchased from Promega (Madison, WI), and Taq polymerase and deoxynucleotides were from Perkin-Elmer (Norwalk, CT).

PCR cloning of human ribonucleotide reductase R2 subunit. The translated region of human ribonucleotide reductase R2 cDNA was amplified by PCR using two primers: 5′-ATGCTCTCCCTCCGTGTGCCG-3′ and 5′-TTAGAAGTCAGCATCCAAGGT-3′ (11). A neutrophil cDNA library was used as a template for PCR amplification (12).

Construction of human neutrophil genomic library. Human neutrophil genomic DNA was isolated using Qiagene DNA isolation kit (Valencia, CA). Sau3A-digested DNA with 6–15 kb was isolated using sucrose-gradient centrifugation method. The isolated DNA was ligated to lambda DASH II arms predigested with BamHI (Stratagene, La Jolla, CA), *in vitro*-packaged using Gigapack II Gold (Stratagene, La Jolla, CA), and amplified.

Isolation and sequencing of genomic clone. An amplified human neutrophil genomic DNA library was screened with random-primed ^{32}P-labeled full-length human ribonucleotide reductase R2 subunit

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cDNA (13). Several independent positive plaques were selected from 2×10^6 recombinants, and the plaques were purified by sequential platings. The nucleotide sequences of the amplified fragments were determined using the dideoxy chain termination method using modified T_7 DNA polymerase (Sequenase Version 2.0 DNA sequencing kit, U.S. Biochemical Corp., Cleveland, OH).

Primer extension. Primer extension was performed to identify the transcription initiation site of isolated gene. mRNAs were isolated from myeloid human cell lines U937 and HL-60, using FastTrack 2.0 mRNA kit (Invitrogen, Carlsbad, CA). Primer extensions (1st strand cDNA synthesis) were performed by reverse transcriptase using isolated mRNA. ^{32}P-labeled antisense primer corresponding to 6–38 nt of the translated region of the gene (1 nt is the first nucleotide of ATG) was hybridized to human mRNAs from cell lines U937 and HL-60 and extended with reverse transcriptase. The produced single strand DNA fragments were analyzed using 6% sequencing gel. To verify that primer extension products were specific to the sequence of the isolated genomic DNA, double strand DNAs were synthesized by DNA polymerase I (Klenow) fragment from primer extension products, ligated to TA vector, and sequenced.

Analysis of the promoter of R2 subunit of human ribonucleotide reductase. The sequence was analyzed by a computer program to identify potential transcription elements as described (14).

Determination of promoter activity of human R2 genomic gene. A series of reporter plasmids were prepared by inserting fragments of various lengths of the promoter region of the human R2 protein gene immediately upstream from a firefly luciferase reporter vector pGL2 (Promega, Madison, WI). Genomic DNA fragments were amplified by PCR using oligonucleotides, corresponding to the 5′-ends and 3′-ends of the isolated genes. The 5′-ends were 5′-CTTTTTTTCTTCTCTTTTTAA, 5′-TAGTTTGAAGGTTTACAAAG, 5′-GACCACCCCGCCAAAATGT, 5′-GCTGGAGGAGGTGCTTTCGGGAGGC, and 5′-GGGGCAAGCGCAGCCAAT. The 3′-end was 5′-GCTGGAGTGAGGGGTCGC. Amplified DNA fragments were ligated into pGL2 reporter vector. For transfections, HeLa and NT cells were cultured in Dulbeco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were grown to approximately 50% confluence in 100-mm petri dishes. The constructed reporter vectors were transfected using calcium phosphate into the cells. After transfection, cells were incubated for additional 48 h, harvested, and lysed. Luciferase assays of the lysates were performed according to manufacturer's protocol (Promega). The efficiency of transfection was carefully monitored by cotransfection with pSEAP-2 promoter vector, of which alkaline phosphatase was determined according to manufacturer's protocol (Clonetech, Palo Alto, CA).

Determination of promoter activity of a proximal DNA region containing three CCAAT sequences. To determine the role of three proximal CCAAT sequences in the expression of human ribonucleotide reductase R2 subunit, each individual or all CCAAT sequences were mutated to ACAAA using GeneEditor (Promega). These mutated reporter vectors were transfected into NT cells, as described above. After transfection, cells were incubated for additional 48 h, harvested, and lysed. Luciferase assays of the lysates were performed as described above. To determine whether the proximal promoter activity is cell-cycle-specific, the proximal DNA region containing three sequential CCAAT sequences was also transfected into NT cells, as described above. After the transfection, the cells grew up to 80% confluence, and were further incubated for 24 h in serum free medium to synchronize the cell cycle. Then, complete medium was added to the synchronized cells, and the activities of luciferase were determined over the times (0, 3, 6, 9, 12, 15, 18, 21, and 24 h). The stages of the cell cycle of synchronized cells were confirmed by flow cytometry (Beton-Dickinson, Benelux, NV), as described previously (15).

RESULTS

Isolation of a Genomic Gene of Human Ribonucleotide Reductase R2 Subunit and Sequencing of Its Promoter Region

Human neutrophil genomic library was screened using ^{32}P-labeled human ribonucleotide reductase R2 subunit cDNA, and several positive plaques were isolated. These positive plaques were verified by sequential screening with the same ^{32}P-labeled human R2 subunit cDNA. A positive plaque with approximate 9 kb insert was digested with EcoRI, and the resulting DNA fragments were subcloned into a sequencing vector (TA-vector). Each subclone was sequenced first using primers located at both ends of TA-vector, then further sequenced using internal primers. The sequence of one subclone (3 kb in size) showed complete homology to the 5′-untranslated region of human R2 subunit cDNA (Fig. 1). Several subclones also exhibited complete homology to the translated regions of hum R2 subunit cDNA (data not shown). The sequence data in Fig. 1 showed clearly that complete homology would exist between a proximal promoter region of the subclone and 5′-untranslated region of human RR2 subunit cDNA (11). Also, the sequence of this subclone contained the junction sequence for 1st exon and intron, at the same site demonstrated in the gene of mouse RR2 subunit (7).

Primer Extension

Primer extension was performed to identify the transcription initiation site of the isolated gene. Three distinct bands were found (Fig. 2). Since the bands from primer extension result from various mRNAs (genuine, pseudo, non-specific mRNAs), each band was cloned and sequenced to verify their identities. The sequences of three bands demonstrated clearly that two bands (A and B) were confirmed as genuine transcripts of the R2 gene, and a band between A and B bands was a non-specific transcript. The sizes of two genuine transcripts were located at tcgCccg (first tsp) and tcgCgcg (second tsp) (Fig. 1). These data also agree with those analyzed by a computer program (14). The primer extension and sequence data indicated that two different transcripts of human RR protein R2 were produced, and that two different promoter regions might be involved in producing the different transcripts.

Analysis and Characterization of the Promoter Region of Human R2 Gene

The isolated genomic gene contained 3 kb of promoter region. To analyze the binding sites for putative transcription factors, a 1.2 kb DNA fragment was sequenced (Fig. 1). Several putative transcriptional bind-

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...........................AACACCATCAAACAGTAAGAGGGGTCCTTCTTG -1078

TAGTAGAGCGACAGCCACTTTTTCTTTTTCTTTTTTTCTTCTCTTTTTAATGGCAGAGGC -1018

GGGGTCTCACTATGTTGCCCAGGATGTCTCCAGCTCCTGGCCTCAAACAATCCTCCTGCC -958
SP-1
GTGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAACCGCGCCCAGCCACAGTTTGCAATT -898
                                    SP-1
CTTAAGGCAAGGGTGACAATAGGGGTCAGGGGTCTGACAGGAGACAGGATTTCTGTGGAA -838
                                                       C/EBP
AACTGCACCAAGGGCCTTCTCGCCATGTGCCGTAGTTTGAAGGTTTACAAAGGACTGCAC -778

ATTTTACATGAGTCATCTCAACGAACGCTCTCCTCACCGCATTAACAGTCCACGCGGTTA -718

CGAGTCCCATTTTACTCACGGGGACACCGAATCTGTAAGAAGCCTGGTCGCTTGTCCCAG -658

CAAAACGAGCCACGGGGCTCAGCGGCCTAACTTTTAGGCTGTAGGGTCCTCGCCGACCAC -598

CCCGCCAAAATGTCAGGCCTCGGGGCCTTGCACCCCCACCGCAGACACGGATCGAAAGGG -538
                   AP-2
TCGCAGCAACGCTCCCCCGCACCCAGGAGCGTTTTCCAGGCCTTTGCACCAACCTCGTTG -478

CTAAGCCCCTGCCCGGCGGCGGCCCGGCTGGAGGAGGTGCTTTCGGGAGGCGGGGCGCGG -418
              AP-2                                  SP-1
CGGGATCCTCTCGCGCCCGCGCTCCAATCGCTGCTCCTCACGCAATCCTAAACGGTTCCC -358
                        CCAAT
GGGCGAACGGCCCGCGCGCGCCAAGGCCGCCGAGACCCTCAGGGGCTGCCGTGGTCCCGC -298

GGATGTGGGGGCCTGGGCGGCGGCGCCCCGACCCAGCCAGCGGACGGGCCGGGGGGGGAA -238
                                                     AP-2
CGGGAGGTCCGGGGGGCGTCCACGGGGGTGTCCCCGGGGGTCTCCGGAAGCGCCGGCGGA -178

GGCTCCCGTCGGCTTGAAAATCGCGCGCGCCCGCGGCCAGCCTGGGTAGGGGCAAGCGCA -118
                      2nd ts
GCCAATGGGAAGGGTCGGAGGCATGCACAGCCAATGGGAAGGGCCGGGGCACCAAAGCCA -58
 CCAAT                        CCAAT                        CCAAT
ATGGGAAGGGCCGGGAGCCGGCGCGGGAGATTTAAAGGCTGCTGGAGTGAGGGGTCGCCC +3
                              TATA                1st ts  +1
GTGCACCCTGTCCCAGCCGTCCTGTCCTGGCTGCTCGCTCTGCTTCGCTGCGCCTCCACT +63

ATGCTCTCCCTCCGTGTCCCGCTCGCGCCCATCACGGACCCGCAGCAGCTGCAGCTCTCG
METLeuSerLeuArgValProLeuAlaProIleThrAspProGlnGlnLeuGlnLeuSer

CCGCTGAAGGGGCTCAGCTTGGTCGACAAGGAGAACACG/gtgagcccgcggggagggcc
ProLeuLysGlyLeuSerLeuValAspLysGluAsnThr

tgcgcaggggagggaggcagggaaagcgaaggctcctcactcacacgcgtctccccgcag

ccgccggctgagcggacccctcctggccagcaagaccgcagaggatcttcaggagcccag

gagtccgtgagtgccggctggcagagggtccaggtag
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FIG. 1. Nucleotide sequence of the promoter region of human ribonucleotide reductase (RR) R2 gene. Two transcription initiation sites (ts) are indicated by 1st and 2nd ts, respectively. The TATA-like box and three CCAAT sequences are underlined. Putative binding sites for transcription factors are also marked. See text for details.

ing sites were found, including SP1, C/EBP, AP-2 and CCAAT. A TATA box (TATAA sequence) could not be found in the proximal promoter region, but a TTTAAA sequence (TATA-like box) was found at nucleotide site −27. Based on the primer extension data (Fig. 2), two independent promoters are necessary to produce the two transcripts. Since the location of the TTTAAA sequence is downstream from the second tsp, this TTTAAA sequence could only be a transcriptional control element for the first tsp. The length between the first tsp and the TTTAAA sequence is consistent with its action as a TATA box. The second tsp is predicted to have other binding sites to initiate transcription. These sites might be those in the GC-rich region proximal to the second tsp.

Promoter Activity of the Human R2 Genomic Gene

Promoter activity of the 5′ flanking region of the first transcript was measured using reporter plasmids containing a luciferase gene. Genomic DNA fragments were amplified by PCR and ligated into the reporter vector as described in methods. Using calcium phosphate, the constructed vectors were transfected into

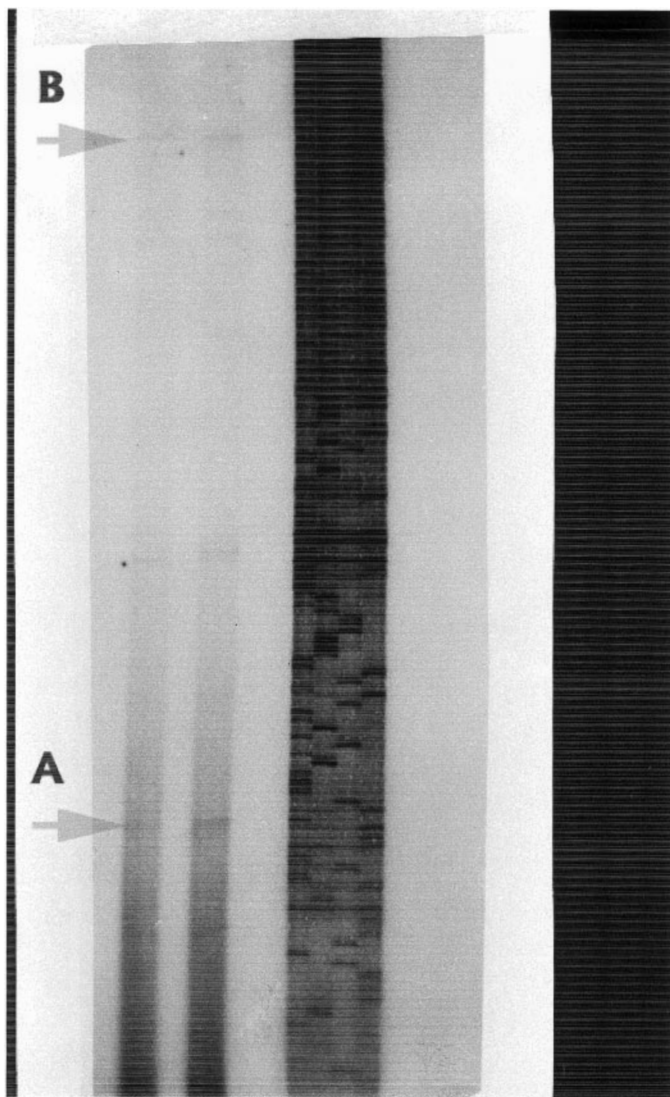

FIG. 2. Determination of transcription start points of human RR R2 gene by primer extension. Primer extension was performed using an oligonucleotide primer (5′-CGGGACACGGAGGGAGAGCAT) corresponding to the first seven amino acid residues of human ribonucleotide reductase R2 protein (11). Lanes 1 and 2 are extension products from U937 and HL-60 mRNAs, and GATC lanes represent the sequencing ladder of human ribonucleotide reductase R2 cDNA prepared using the same primer. Arrows at A and B indicate the two bands confirmed by sequencing as genuine transcripts of the R2 gene.

human fibroblasts, HeLa cells, and NT-2 cells. Promoter fragments of five different lengths were prepared and transfected (Fig. 3A). In all three cell types, the highest promoter activity was observed for the fragment −800 to +1 bp, and activity was as much as 100-fold more than controls (Fig. 3B). 80% of maximum activity occurred with the fragment −610 to +1 bp. The fragment −125 to +1 bp had more than half-maximum activity, approximately 50-fold more than controls, and contained the TATA-like box sequence for the first tsp and three CCAAT sequences.

Since the second transcript was detected in primer extension, the proximal promoter for the second transcript was studied using the same cell types and reporter vectors (Fig. 3B). The highest activities were found in the fragments −610 to −125 bp and −800 to −125 bp (Fig. 3C). Maximal activities for these fragments were 30 fold higher than controls, but were only one third that of maximal activity observed with the promoter for the first transcript. Also, the promoter for the second transcript did not contain TATA box sequence, but GC-rich DNA sequence.

Characterization of CCAAT Sequence Contribution to Promoter Activity

Since the proximal promoter region (−125 to +1 bp) of the R2 gene contributed at least 50% of maximal promoter activity, and contains three sequential CCAAT sequences, we investigated this region to determine their roles in promoter activity. Promoter fragments −125 to +1 bp were constructed with mutations in each of the three CCAAT sequences or in all three sequences (Fig. 4). As described above, the promoter fragments were inserted into reporter vectors and transfected into NT-2 cells. For each individual mutated CCAAT sequence, promoter activity was decreased approximately 20–40% compared to control (Fig. 4). The data indicate that each of the three CCAAT sequences contributed similarly to maximal promoter activity. When all three CCAAT sequences were mutated, <20% of promoter activity was detected compared to control (Fig. 4). Similar findings were observed when the constructs were transfected into human fibroblasts and HeLa cells (data not shown). The results indicate that the three CCAAT sequences may play a substantial regulatory role in producing the first transcript of human RR protein R2. Also, each CCAAT sequence confers a similar amount of promoter activity to the proximal promoter region.

Involvement of the Proximal Promoter Containing Three CCAAT Sequences in Cell-Cycle-Specific Expression

Since the proximal promoter (−125 to +1 bp) contributed substantially to maximum promoter activity, and the contained three CCAAT sequences were obviously involved in producing the first transcript of human RR protein R2, we investigated whether the promoter containing this sequence participated in cell-cycle-specific expression. A chimeric reporter vector was constructed to include the proximal promoter region of the RR2 gene (−125 to +1 bp). NT cells were transfected with this construct and subsequently syn-

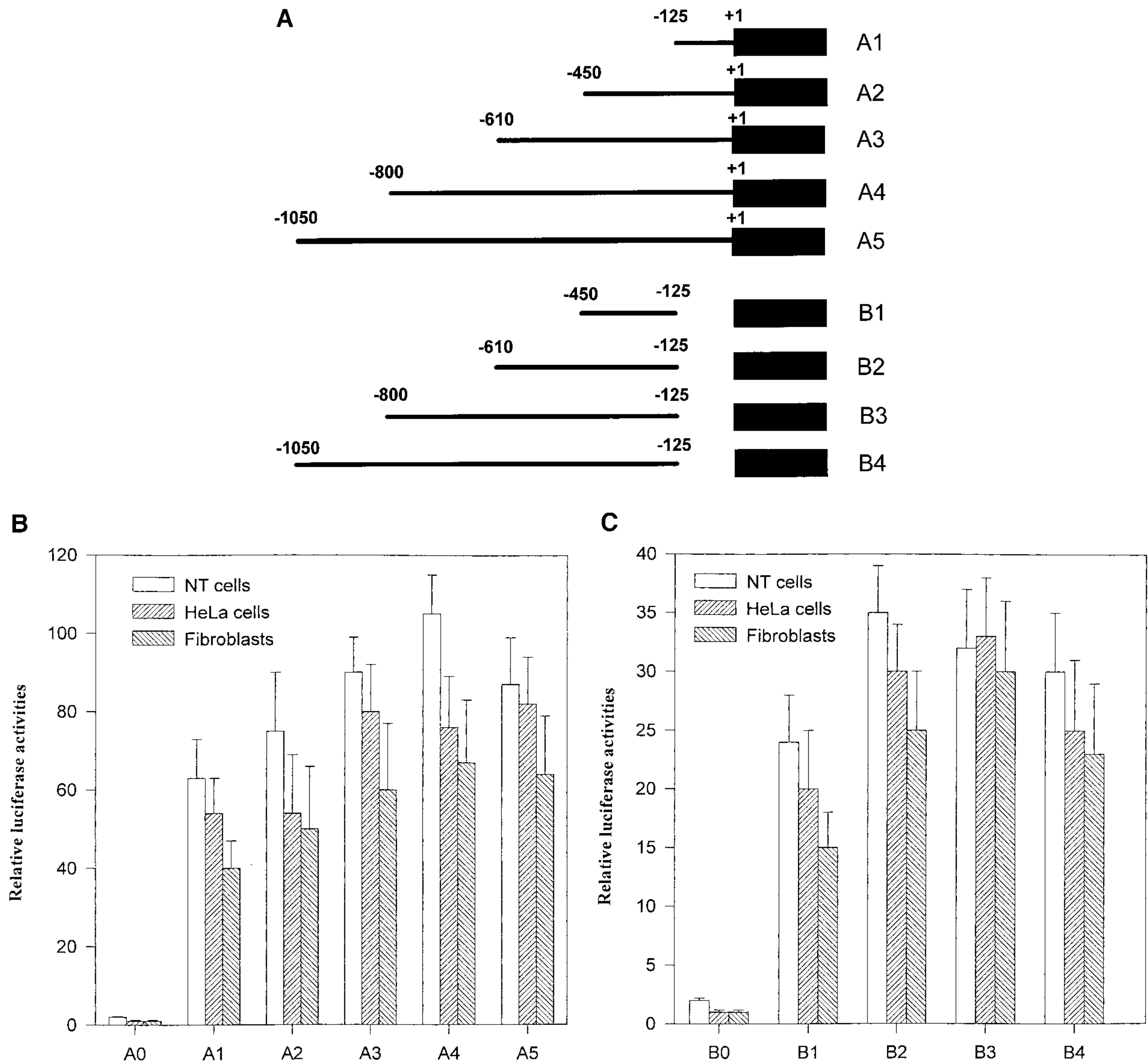

FIG. 3. Human RR R2gene promoter activity. (A) Nine different fragments of the RR R2promoter of increasing length (lines A1–A5 and lines B1–B4) were inserted into the reporter plasmid pGL2 for luciferase as described under Materials and Methods. The numbers represent the positions of the fragments of the RR R2gene relative to the first transcription initiation site. Control is the reporter plasmid without an insert. (B) Relative luciferase activities of the promoter constructs A1-5 transfected into NT cells, HeLa cells, and normal human fibroblasts. Control (Ao) is the reporter plasmid without an insert. (C) Relative luciferase activities of the promoter constructs B1-4 transfected into NT cells, HeLa cells, and normal human fibroblasts. Control (Ao) is the reporter plasmid without an insert.

chronized by serum starvation for 24 h. After serum was reintroduced, cells were harvested every 3 h for analysis of luciferase activity. As shown in Fig. 5, activity steadily increased from baseline at 0 time to a maximum at 12 h, and then decreased gradually by 24 h. Maximal expression of luciferase activity coincides with S-phase in the cell cycle, based on the time measured by flow cytometry (data not shown). A similar pattern of luciferase activity was observed when HeLa cells were transfected with the proximal promoter construct (data not shown). These data indicate that the proximal promoter region (−125 to +1 bp) of the RR2 gene may be involved in cell-cycle-specific transcriptional regulation.

DISCUSSION

Ribonucleotide reductase (RR) is a key protein for synthesis of deoxyribonucleotides from ribonucleotides. Since deoxyribonucleotides are required for DNA replication, the protein has been extensively investigated to understand its role in cell cycle regulation and

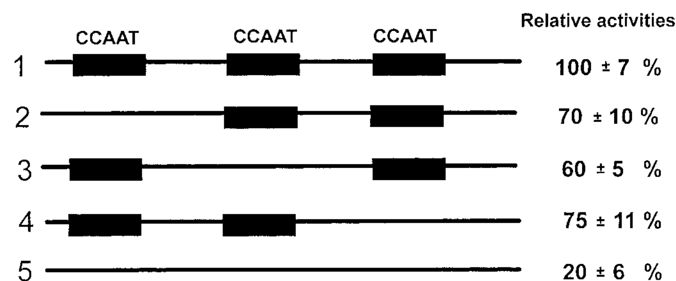

FIG. 4. Effect of CCAAT box mutation on proximal promoter activity. The proximal promoter sequence is shown at the top, with the three CCAAT sequences underlined. Promoter constructs are depicted with CCAAT sequences indicated by black-filled rectangles. Three constructs have CCAAT sequence mutations (CCAAT to ACAAA). NT cells were transfected with the different constructs as described under Materials and Methods, and percentage relative luciferase activity for each construct is indicated at the right.

to design inhibitors. The enzyme is composed of protein subunits R1 and R2, but protein R2 may be more relevant clinically (16). Expression of protein R2 is tightly controlled in the cell cycle, and its overexpression results in cells with transformed characteristics (17). To understand R2 expression mechanisms, the human R2 gene promoter region was isolated and characterized in this paper. The isolated gene contained two different promoter activities for two transcripts. The sequences of these transcripts were completely homologous to 5′-untranslated region of human ribonucleotide reductase R2 cDNA. Since pseudo-genes contain various mutations, primer extension products from pseudo-genes would also retain these mutations. Two transcripts (A and B bands) did not contain any mutation, thereby being generated from the genuine gene. Also, the location of bands between A and B bands exhibited small disparity in U937 and HL-60 (Fig. 2).

The promoter activities of the first and second transcripts were 100 and 30 fold more than control, respectively. Especially, the promoter for the first transcript had a proximal region of approximately 125 bases that increased promoter activity approximately 50 fold more than control. This proximal promoter region included a TATA-like box, three CCAAT sequences, and putative binding sites for SP-1 and AP-2. The TTTAAA sequence (TATA-like box) in the human RR2 gene is identical to the TATA-like sequences of the mouse RR2 gene (18) and the human glutaredoxin gene (13). The promoter activity of each of the three CCAAT sequences conferred approximately one third of the proximal promoter activity. The three CCAAT box sequences showed cumulative activity with approximately equal contribution from each. In contrast, the mouse gene promoter contains one rather than three CCAAT sequences (18). Since the promoter activity of the first transcript was higher than that of the second transcript, it is possible that the first transcript is the dominant form in human cells. However, the two transcripts in the experiment of our primer extension exhibited no significant difference in their intensity. Therefore, the production of the second transcript seems to be not only influenced by its promoter, but also affected by undefined regions of the gene. Nevertheless, the activity of promoter (measured by luciferase assay) of the second transcript was weaker than that of the first transcript, and the second transcript utilized a different region of the promoter. A putative TATA box for this promoter was not present. However, the region of this promoter exhibiting the highest activity contained binding sites for SP-1, CCAAT, and AP-2 (Fig. 3B). The second transcript has another point to be discussed in view of its translation, since this transcript contained three additional ATG codons (initiation codon) in three CCAATTGG sequences (Fig. 1). These additional ATG codons may lead to yielding different proteins via initiating translation at those ATG sites rather than original ATG codon (marked in bold in Fig. 1). However, the different proteins are not likely

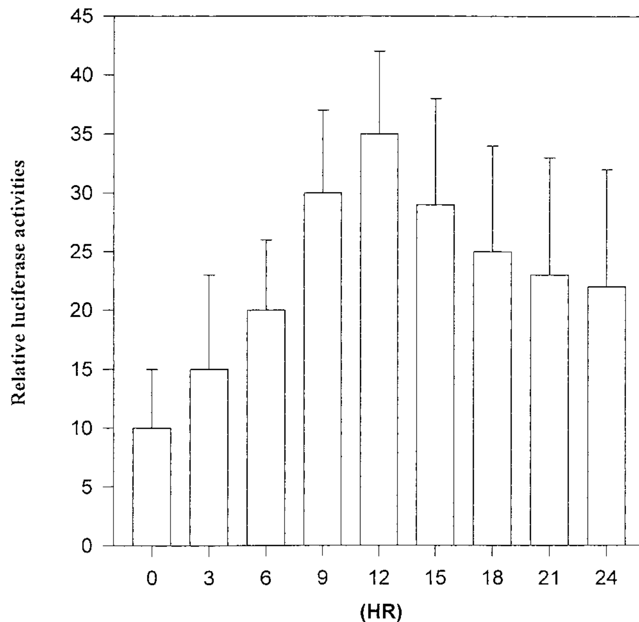

FIG. 5. Promoter activities of the proximal promoter region of human ribonucleotide reductase in synchronized cells. The proximal promoter fragment −125 to +1 bp was inserted into the reporter plasmid pGL2 for luciferase, and NT cells were transfected with this construct. Cells were synchronized by growth in serum-free media for 24 h. Serum was re-introduced at time 0, and luciferase activity was measured at the times indicated.

to exist, since it was reported in eukaryotes that the second or third ATG from the 5′ end is occasionally recognized (19), and such alternative products have not been reported so far. Future experiments are necessary to explore the role of these factors in producing the 2nd transcript, and to investigate translational mechanism of the second transcript.

We demonstrated here that activity of the proximal promoter for the first transcript of human ribonucleotide reductase R2 subunit was cell-cycle-specific. One explanation is that factors such as NF-Y/CAAT and CTF-1/NF-1 could bind to regulatory sites in the proximal promoter (20). It is not yet known how such factors are themselves produced or activated at a specific stage of the cell cycle. Another explanation is that advocated for mouse R2. During transcription a cell cycle phase-specific blocker could exist, producing truncated or incomplete transcripts except during S-phase (18). The blocker's effects could be eliminated by its release from a proposed binding site, located at a cctaccG sequence in the first intron of the mouse R2 protein gene (G indicates the position of the proposed transcriptional blocker). If this mechanism is the major means of controlling cell-cycle-specific expression of R2, the proposed binding site for the transcriptional blocker should be conserved. The first intron of the human R2 gene did not have this sequence (not shown), implying that other mechanisms are responsible for cell-cycle-specific regulation. Data from this paper, including the first reported sequence of the promoter of human ribonucleotide reductase R2 subunit and characterization of its transcripts and their promoter activities, should facilitate studies to characterize new mechanisms of cell-cycle-specific regulation of human ribonucleotide reductase R2 subunit.

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