MOLECULAR AND CELLULAR BIOLOGY, Oct. 1986, p. 3433–3442
0270-7306/86/103433-10$02.00/0

Vol. 6, No. 10

Isolation and Characterization of Expressible cDNA Clones Encoding the M1 and M2 Subunits of Mouse Ribonucleotide Reductase

LARS THELANDER†* AND PAUL BERG

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Received 27 January 1986/Accepted 22 May 1986

Mammalian ribonucleotide reductase consists of two nonidentical subunits, proteins M1 and M2, which are differentially regulated during the cell cycle. We have isolated expressible cDNA clones of both subunits from an Okayama-Berg cDNA library made with mRNA from hydroxyurea-resistant, M2 protein-overproducing mouse TA3 cells. Expression of M2 protein could be demonstrated by electron paramagnetic resonance spectroscopy after transfection of COS-7 monkey cells with the plasmid. Electrophoresis and blot analyses of the parent and hydroxyurea-resistant TA3 mRNA revealed two M2 transcripts, a major one of 2.1 kilobases and a minor one of about 1.6 kilobases. Restriction endonuclease mapping of the corresponding cDNAs indicated that the two mRNAs differed only in the length of the 3′ untranslated ends. By contrast, there was only one mRNA corresponding to the M1 protein, and its mobility corresponded to about 3.1 kilobases. The hydroxyurea-resistant TA3 cells contained a 50- to 100-fold excess of the M2 mRNAs over that of the parent cells and a 10-fold excess of the M1 mRNA. However, a Southern blot analysis of the corresponding genomic DNA sequences showed that the M2 gene was amplified fivefold but the M1 gene was still single copy. The complete nucleotide sequence of the 2,111-base-pair-long M2 cDNA revealed an open reading frame coding for 390 amino acids, which corresponds to a molecular weight of 45,100. The mouse M2 protein sequence was quite homologous to the equivalent protein in the clam *Spisula solidissima*, while the homology to the smaller subunits of Epstein-Barr virus, herpes simplex virus type 2, and *Escherichia coli* ribonucleotide reductases were less pronounced.

The deoxyribonucleotides needed for DNA synthesis are made by a direct reduction of the corresponding ribonucleotides. As this is the first unique step leading to DNA synthesis, the reaction is regulated in a number of ways (17, 29). The enzyme responsible for ribonucleotide reduction, ribonucleotide reductase, consists of two nonidentical subunits; in mammalian cells these are referred to as M1 and M2. The M1 protein, which has been purified to homogeneity from calf thymus, is a dimer of molecular weight 170,000 and contains the binding sites for nucleoside triphosphates, which act as allosteric effectors (30). The M2 protein, which has been purified to homogeneity from hydroxyurea-resistant, M2-overproducing mouse TA3 cells, is a dimer of molecular weight 88,000 and contains stoichiometric amounts of a nonheme iron center and a tyrosyl free radical, essential for activity (31).

The activity of ribonucleotide reductase is cell cycle dependent, reaching its maximum during S phase (17, 29). It was recently demonstrated that the variation in holoenzyme activity is regulated by de novo synthesis and breakdown of the M2 subunit. By contrast, the M1 protein is expressed constitutively, and its level is constant and in excess throughout the cell cycle (10, 11). On the other hand, the M1 protein is only present in actively dividing cells and is absent in terminally differentiated cells that have stopped making DNA (9). This cell cycle-dependent regulation of M1 and M2 proteins is quite different from that in *Escherichia coli*, in which the two genes constituting the ribonucleotide reductase are arranged in one operon and their synthesis is coordinately controlled (4, 15).

To study the regulation of ribonucleotide reductase during the cell cycle at the gene level as well as the correlation between ribonucleotide reduction and DNA synthesis, we needed the genes for the M1 and M2 proteins. In this paper, we describe the isolation of what appear to be nearly full-length cDNA clones of both subunits by the Okayama-Berg method (23, 24). During this work, Caras et al. (3) described the cloning and sequencing of three overlapping cDNA clones encoding the mouse M1 protein. Our M1 protein cDNA clone contains 161 additional base pairs (bp) of sequence information extending in the 5′ direction.

MATERIALS AND METHODS

Cell cultures. Hydroxyurea-resistant, M2 protein-overproducing mouse mammary tumor TA3 cells and the corresponding parent cells (11) were grown as monolayer cultures in Dulbecco modified Eagle medium (DME) plus 10% fetal calf serum. The resistant cell line was routinely passaged in the presence of 2 mM hydroxyurea, but the cells were grown in the absence of drug for 72 h before the RNA preparation. The COS-7 cell line, which are African green monkey kidney cells transformed by replication-defective, T antigen-positive simian virus 40 (SV40) genomes (14), were obtained from the American Type Culture Collection (CRL 1651) and grown in the same medium.

Isolation of mRNA. Total cellular RNA was extracted from logarithmically growing cells by the guanidinium thiocyanate method (7), and mRNA was recovered after two cycles of adsorption and elution from an oligodeoxythymidylate [oligo(dT)]-cellulose column (2). About 400 μg of poly-

* Corresponding author.
† Present address: Department of Physiological Chemistry, University of Umeå, S-901 87 Umeå, Sweden.

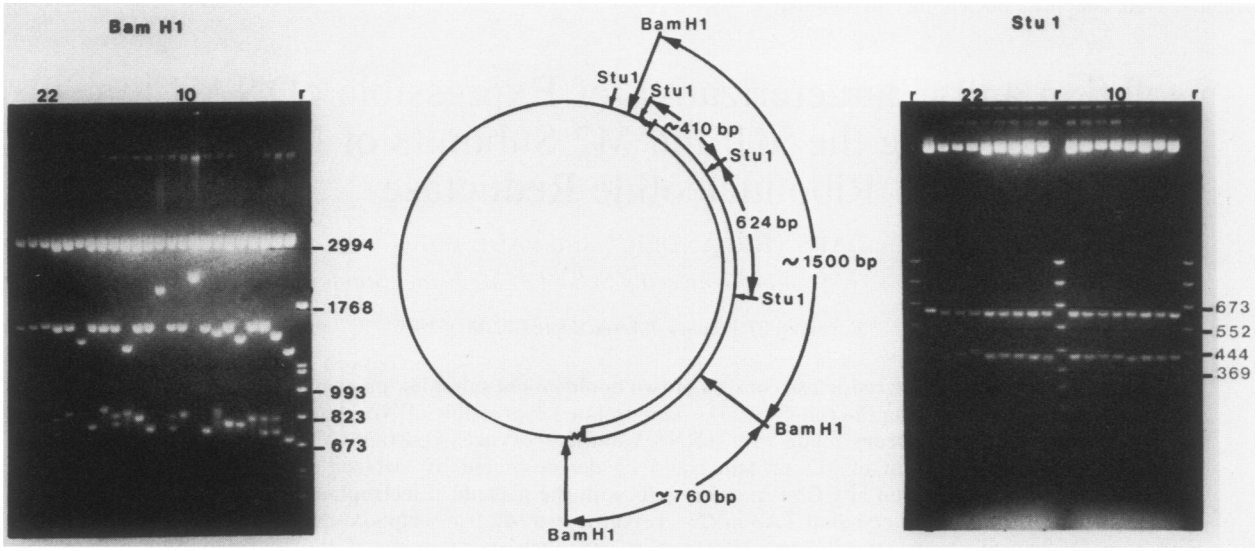

FIG. 1. Restriction endonuclease analyses of M2 protein cDNA clones. Plasmid DNA prepared from 24 recombinants with M2 cDNA inserts were digested with *Bam*HI (left) or *Stu*I (right) endonucleases and electrophoresed on 1.5 and 2% agarose gels, respectively. The DNA size markers were from *Hin*dIII or *Bst*NI endonuclease-digested SV40 DNA. The lanes are labeled with the numbers of specific clones or with r, indicating DNA size references. The diagram in the center summerizes the various enzyme cleavage sites that were deduced from the fully characterized cDNA sequences. The size of the *Bam*HI-digested vector fragment is 2,994 bp. Sizes are shown to the right of each panel (in base pairs).

adenylated [poly(A)$^+$] mRNA was obtained from 5×10^8 cells.

Construction of cDNA library. The cDNA library was constructed by using the pcDV1 vector-primer and the pL1 linker fragment by the procedure of Okayama and Berg (23, 24). The cyclized vector-cDNA preparation was used to transform competent *E. coli* MC1061 cells (6) with calcium chloride. About 8 μg of poly(A)$^+$ RNA from hydroxyurea-resistant, M2 protein-overproducing TA3 cells yielded 1.9×10^6 independent transformants. Sublibraries based on the size of the cDNA insert were prepared from the total cDNA library as described previously (24), with the following modifications. The pooled plasmid DNA was digested with *Sfi*I endonuclease and electrophoresed in a 0.8% low-melting-point agarose gel containing ethidium bromide, and the gel was sliced into five sections corresponding to cDNA insert sizes of 0.3 to 1.1, 1.1 to 2, 2 to 3, 3 to 5, and >5 kilobases (kb). After extraction from each gel slice, the plasmid DNA was recyclized with T4 DNA ligase and used to transform MC1061 cells.

Oligonucleotide probes. The amino acid sequence used to specify a 15-mer oligonucleotide for the M2 protein was obtained from a conserved region (Thr-Asn-Phe-Phe-Glu, position 355–359) found in a sequence alignment of the smaller subunits of Epstein-Barr virus, herpes simplex virus type 2, and clam and *E. coli* ribonucleotide reductase (28). Use of all possible codon combinations resulted in a mixture of 64 isomers. To obtain probes for the M1 protein, pure M1 from calf thymus was carboxymethylated and cleaved with CNBr, and the resulting peptides were separated by gel filtration on a Sephadex G-50 column in 20% acetic acid. Further separation was obtained by reverse phase high-pressure liquid chromatography (HPLC) with 0.1% trifluoroacetic acid and a gradient of acetonitrile. Finally, individual peptides were analyzed on a liquid-phase amino acid sequenator, and two favorable short sequences, Lys-Glu-Asp-Ile-Asp-Ala and Phe-Asn-Tyr-Ile-Asn-Pro, were chosen to specify two different 17-mer oligonucleotides containing 48 isomers. These sequences were later shown to correspond to residues 180–185 and 102–107 in the M1 protein (3).

Filter hybridization. Oligonucleotide probes were radiolabeled at their 5′ ends with [γ-^{32}P]ATP (specific activity, 7,000 Ci/mmol; ICN) and T4 polynucleotide kinase to a specific activity of 5×10^8 cpm/μg. Filters were prehybridized overnight in 5× SSC (20× SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 7.5× Denhardt solution (50× Denhardt contains 1 g each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin in 100 ml of water), 50 mM sodium phosphate, pH 7.0, 0.1% sodium dodecyl sulfate, and 0.25 mg of tRNA per ml and hybridized overnight in the same solution containing 130 ng of radioactive probe per ml. The filters were washed in 6× SSC–0.1% sodium dodecyl sulfate. All the procedures for the 15-mer and the 17-mer probe were done at 37 and 42°C, respectively.

Prehybridization and hybridization with the *Pst*I fragment of the clam ribonucleotide reductase clone 2 cDNA (28) was done at 50°C in the solution described above containing in addition poly(A) RNA (1 μg/ml). The filters were washed at 50°C in 2× SSC–0.1% sodium dodecyl sulfate.

When the *Pst*I fragment of the cloned M2 protein cDNA was used as the hybridization probe, the prehybridization and hybridization were done at 42°C with the same solution as for the clam cDNA with 50% formamide and the final wash was at 42°C in 0.1× SSC–0.1% sodium dodecyl sulfate. cDNA probes were labeled by nick translation or by the hexadeoxyribonucleotide method of Feinberg and Vogelstein (12) with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Amersham) to a specific activity of 1×10^8 to 2×10^8 cpm/μg, and they were heat denatured before use.

DNA sequencing. Restriction endonuclease fragments were subcloned in M13 vectors (22) and sequenced by the dideoxy terminator method (25).

DNA transfection of monkey cells. COS-7 cells were transfected with plasmid DNA by using DEAE-dextran and chloroquine as already described (33). About 120 μg of DNA

in 8 ml of DME medium containing 50 mM Tris hydrochloride, pH 7.4, and 400 μg of DEAE-dextran per ml were used per 15-cm dish containing approximately 4×10^6 cells. After incubation at 37°C for 48 h in DME medium containing 4% fetal calf serum, the cells were harvested by trypsinization, washed in Tris-saline, transferred to electron paramagnetic resonance (EPR) tubes, frozen in liquid nitrogen, and analyzed by EPR spectroscopy (11).

RESULTS

Isolation of mouse M2 protein cDNA clones. Mouse M2 protein has a subunit molecular weight of 44,000 (31), corresponding to 1.2 kb of protein coding sequence. Therefore, the initial screening was carried out on the sublibrary with cDNA inserts 1.1 to 2 kb long. Attempts to obtain amino acid sequence information for oligonucleotide synthesis by analysis of a sample of homogeneous M2 protein suggested that the amino terminus was blocked and the mouse protein sequence was not readily available for construction of a suitable oligonucleotide probe. Instead, we synthesized a mixture of synthetic oligonucleotides based on the amino acid sequence homologies at corresponding regions of the M2 protein equivalents of clam, herpes simplex virus type 2, and Epstein-Barr virus ribonucleotide reductases (Materials and Methods). We also used a 610-bp cDNA encoding part of the M2 protein equivalent of clam ribonucleotide reductase (clone 2) (28).

About 1.7×10^5 colonies were screened, and 29 clones hybridized with the clam clone 2 cDNA probe. Of these, 23 clones also hybridized to the oligonucleotide probe. DNA was isolated from each clone by the alkaline lysis method (20), and after digestion with *Sal*I or *Bam*HI endonucleases, the size of the cloned inserts was estimated by gel electrophoresis. Four clones, all showing the same restriction enzyme digestion pattern, contained an insert about 2 kb long, which was the upper limit of the size-selected sublibrary. Therefore, 5×10^4 colonies from the sublibrary containing cDNA inserts 2 to 3 kb long were screened with a 1.4-kb long *Pst*I fragment from the insert of one of the four earlier clones. In this screening, 3% of all colonies were positive, and the size of their inserts was analyzed by gel electrophoresis of minilysates from 24 clones after digestion with *Bam*HI (Fig. 1). All inserts contained an internal *Bam*HI site; the 5′ end was represented by a fragment of about 1.5 kb in 16 of the 24 clones and the 3′ end by fragments of about 0.8 kb showing the same type of fuzzy and heterogeneous size bands due to heterogeneity in the poly(dA:dT) segments observed earlier by Okayama and Berg with their globin cDNA clones (23). Digestion of the inserts with *Stu*I endonuclease, an enzyme which later was shown to release a fragment of about 410 bp from the 5′ end as well as an internal fragment of 624 bp, indicated that these 16 clones had very similar 5′ ends (Fig. 1). To further characterize the cDNA of clone 10, the plasmid DNA was digested with *Xho*I endonuclease at the sites which flank the cDNA insert (24). The cDNA segment was recovered after electrophoresis in low-melting-point agarose gels and used as a probe to detect M2 protein mRNA and genomic DNA sequences and for restriction endonuclease mapping and DNA sequencing.

Isolation of mouse M1 protein cDNA clones. The M1 protein of mammalian ribonucleotide reductase has a polypeptide molecular weight of 84,000 (30), corresponding to a protein-coding sequence of 2.4 bp. Considering the size of the M2 cDNA clones, the sublibrary containing the 3- to 5-kb-long cDNA inserts was screened by hybridization with two different synthetic oligonucleotide probes based on the known partial amino acid sequence of pure calf thymus M1 protein (Materials and Methods). The screening of 5.6×10^4 colonies with one of the oligonucleotide mixtures indicated that about 0.3% of the colonies were positive. Similar hybridization screening with the other oligonucleotide mixture focused our attention on 18 clones. The insert of cDNA was characterized further by gel electrophoresis after digestion of their plasmid DNAs with *Xho*I. Fifteen of the eighteen clones contained cDNA inserts of approximately 3.05 kb. Further analysis of these clones with *Bam*HI endonuclease yielded the 3′-terminal region as a 530-bp fragment, an internal fragment of 1,373 bp, and the 5′ terminus in a fragment of about 1,350 bp. The six clones yielding the longest *Bam*HI fragments corresponding to the

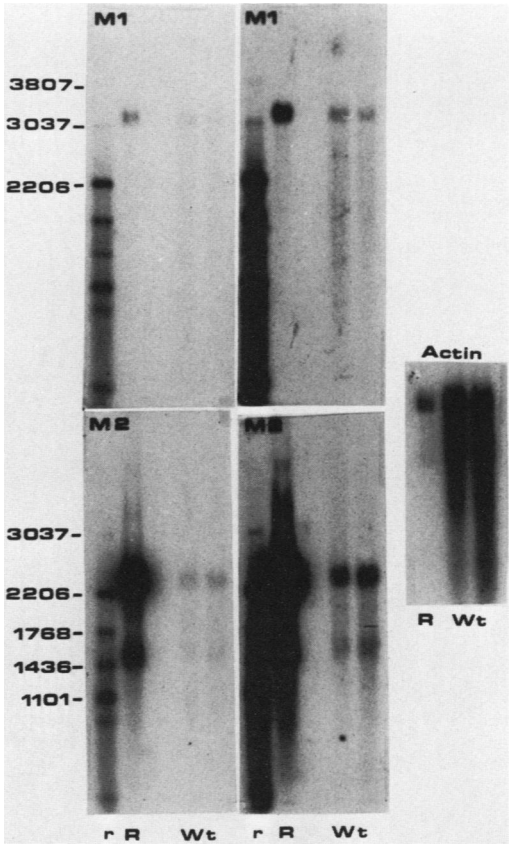

FIG. 2. RNA blots showing the sizes and relative abundances of M1 and M2 mRNAs in hydroxyurea-resistant, M2-overproducing mouse TA3 cells and the corresponding parent cells. Five micrograms of poly(A)$^+$ mRNA isolated from hydroxyurea-resistant cells (R) and 15 μg from two different preparations of poly(A)$^+$ mRNA from parent cells (Wt) were denatured with glyoxal and electrophoresed on a 1% agarose gel (32). The RNA was transferred from the gel to nitrocellulose, and the filters were hybridized for 48 h with ^{32}P-labeled *Xho*I-generated fragments containing the cDNA of clone 65 (M1 protein) or clone 10 (M2 protein) as described in Materials and Methods for the *Pst*I fragment of M2 cDNA, except that the final washing was at 50°C. The size markers (r) were a glyoxal-treated mixture of *Msp*I-*Eco*RI, *Bam*HI-*Taq*I, and *Hin*dIII endonuclease-digested SV40 DNA end labeled with [^{32}P]dATP. The autoradiograms to the left were exposed for 36 h at room temperature, while the ones to the right show the same filters exposed for 24 h at −70°C with an intensifying screen. As a control, hybridization to a ^{32}P-labeled rat β-actin cDNA probe was performed.

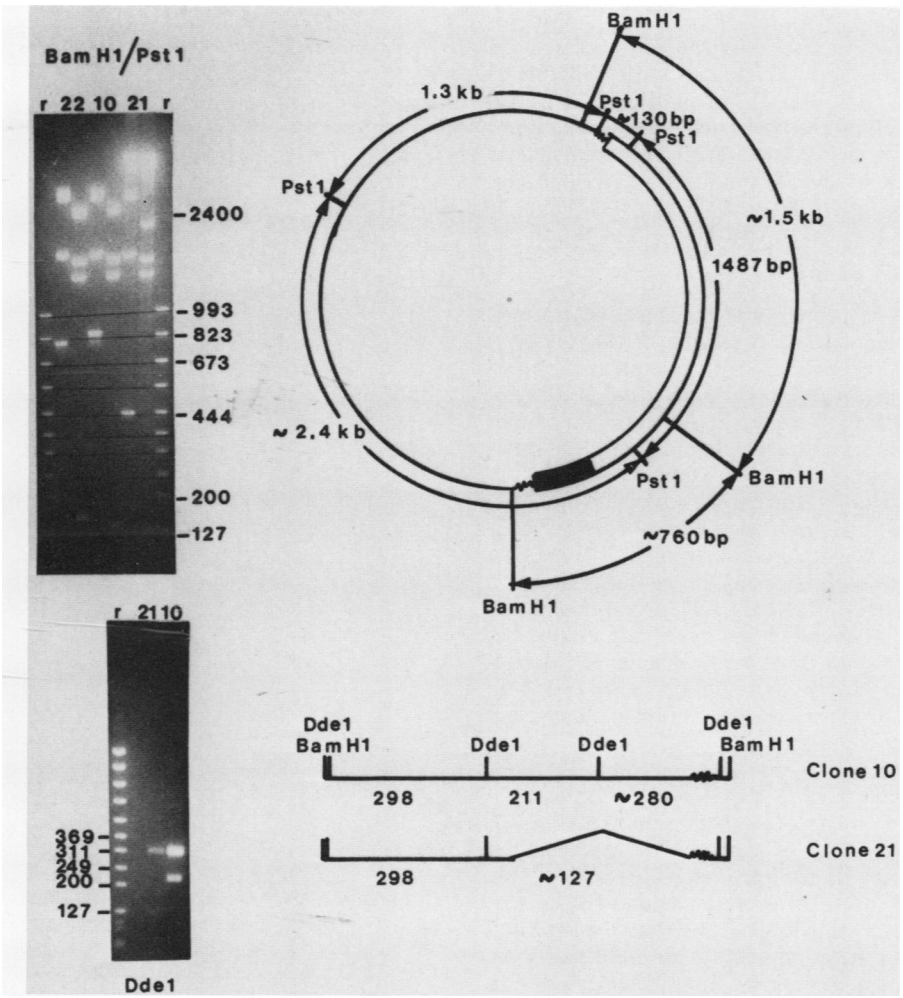

FIG. 3. Restriction endonuclease analyses of cDNA clones corresponding to the minor and major M2 protein mRNA transcripts. (Top) Plasmid DNA prepared from clone 10, 21, or 22 was digested with *Bam*HI (left lanes) or *Pst*I (right lanes) endonucleases and electrophoresed on a 2% agarose gel. The DNA size markers (lanes r) were from *Bst*NI endonuclease-digested SV40 DNA. (Bottom) The 3′-terminal *Bam*HI-generated fragments of clones 10 and 21 were isolated, digested with *Dde*I endonuclease, and electrophoresed as described above. The diagrams summarize the various enzyme cleavage sites. The black part of the cDNA insert represents the region missing in clone 21. Distances are shown in bases.

5′ end were analyzed with *Stu*I endonuclease digestion; all of the clones yielded fragments from their 5′ ends of 390 ± 30 bp. The cDNA clone with the longest 5′-terminal fragment (about 417 bp), clone 65, was selected for further use. The entire cDNA insert was isolated after *Xho*I endonuclease digestion and used as the hybridization probe for M1 RNA and DNA blotting experiments and for restriction endonuclease mapping and DNA sequencing.

Detection of M1 and M2 mRNAs from parent and hydroxyurea-resistant, M2-overproducing mouse TA3 cells. To determine the size and the relative amounts of the M1 and M2 transcripts, the cDNA segments of the M1 clone (clone 65) and M2 clone (clone 10) were used as hybridization probes to detect the poly(A)$^+$ RNAs from normal and hydroxyurea-resistant mouse TA3 cells (Fig. 2). The results show that the M1 cDNA hybridized to a single mRNA species of about 3.1 kb in both types of cells. However, by densitometric scanning, there appeared to be 5- to 10-fold more M1 mRNA in the hydroxyurea-resistant cells than in the the parent cells. The M2 cDNA probe revealed two different mRNAs in both types of cells, a major species of about 2.1 kb and a minor species of about 1.6 kb. By densitometry, both species were 50- to 100-fold more abundant in the hydroxyurea-resistant TA3 cells than in the parent cell line.

Characterization of an M2 cDNA clone corresponding to the minor M2 mRNA species. The M2 cDNA clones corresponding to the minor 1.6-kb mRNA species should be present among positive clones detected in the screening of the sublibrary with cDNA inserts of 1.1 to 2 kb. Digestion of plasmid DNA from the M2 cDNA clones 10, 21, and 22 with *Pst*I showed that all three contained an internal fragment of 1,487 bp and a 5′-terminal fragment about 130 bp long (Fig. 3). The apparent small difference in length of the 130-bp fragments was due to different lengths of the dG:dC segments, as shown later by DNA sequencing. Therefore, all three clones had the same 5′-terminal sequence. This was confirmed by the finding that the 5′ *Bam*HI fragments corresponding to the 5′ termini were the same size in the three clones. However, the *Bam*HI endonuclease digestions showed that clones 10 and 22 contained the same-sized fragment of about 760 bp corresponding to the 3′ end—the small difference in length was caused by different lengths of the dA:dT segments of the clones (cf. Fig. 1). In contrast, the

3′-terminal fragment from clone 21 (from the 1.1- to 2-kb sublibrary) was only around 440 bp.

A closer examination of the 3′ *Bam*HI fragments of clones 10 and 21 with *Dde*I endonuclease established that clone 21 lacked about 360 bp in the end of the cDNA closest to the poly(A) tail (Fig. 3). Together, the data indicate that the cDNA of clones 10 and 22 corresponds to the longer, major M2 mRNA, while the cDNA of clone 21 corresponds to the shorter, minor transcript.

Analysis of the M1 and M2 genes in chromosomal DNA from parent and hydroxyurea-resistant mouse TA3 cells. To estimate the relative number of copies of the M1 and M2 genes in the parent and hydroxyurea-resistant mouse TA3 cells, the DNA from both cell lines was digested with *Hin*dIII endonuclease, which cleaves neither the M1 nor M2 cDNAs, or with *Bam*HI, which cleaves the M1 cDNA twice and the M2 cDNA once. The Southern blots of the digestion mixtures were then hybridized with either a ^{32}P-labeled M1 cDNA insert of clone 65 or an M2 cDNA of clone 10 (Fig. 4). Both cell types yielded the same bands with the M1 probe, and the intensity of all bands was approximately the same in the two cases; however, there was clearly more DNA in the bands from the hydroxyurea-resistant cells hybridizing with the M2 cDNA. Densitometric scanning of the major bands hybridizing with the M2 cDNA showed that there was five times more hybridization in the bands from the hydroxyurea-resistant cells than in corresponding DNA bands from the parent cell line.

Both the M1 and M2 mouse cDNA probes also hybridized specifically to total genomic DNA from human KB cells, but increasing the stringency of washing from 50 to 60°C in 0.1× SSC abolished this cross-hybridization (data not shown).

Nucleotide sequence of mouse protein M2 cDNA. A sequence of 2,111 nucleotides was obtained between the dG:dC tails at the 5′ end and the dA:dT tails at the 3′ end. That sequence contained one open reading frame starting with the first ATG at position 63 and extending a total of 390 amino acid residues (Fig. 5). The molecular weight of the corresponding polypeptide was 45,100. The 3′ untranslated part of the mRNA contained two AATAAA polyadenylation signals, one at position 1633 and one at position 2074.

From the analysis shown in Fig. 3, the cDNA segment in clone 22 seemed to have a slightly longer 5′-terminal *Pst*I fragment than clone 10. However, the DNA sequence of this fragment in both clones was exactly the same, and therefore the difference in lengths must depend on differences in the dG:dC segments at their 5′ ends.

Comparison of amino acid sequence homologies. With the Bionet IFIND program, the homology between the mouse M2 protein and the published clam sequence (28), where the amino-terminal part is missing, was strikingly similar, giving a score of 224, while the homologies to the Epstein-Barr virus (13) and herpes simplex virus type 2 (21) ribonucleotide reductases and *E. coli* B2 protein (4) amino acid sequences were less significant, giving scores of 18, 11, and 8, respectively.

The nucleic acid sequence of the mouse M2 cDNA was also quite homologous to the nucleic acid sequence of the smaller subunit of clam ribonucleotide reductase (28) (score of 497 with the IFIND program). However, a lot of third base exchanges exist, explaining the relatively poor hybridization between the two sequences.

Nucleotide sequence of the mouse M1 protein cDNA. A 2.9-kb composite nucleotide sequence from three overlapping cDNAs encoding mouse M1 protein was recently reported by Caras et al. (3). The sequence contained an

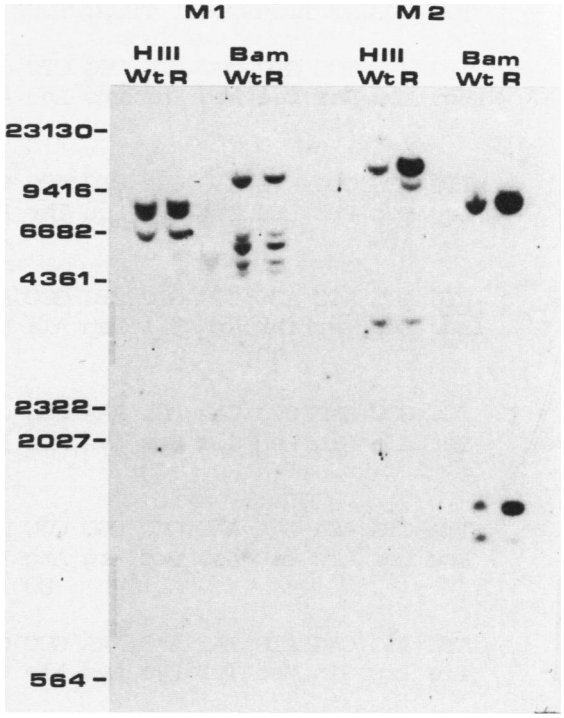

FIG. 4. Southern blot analysis of M1 and M2 protein genes in chromosomal DNA from parent and hydroxyurea-resistant mouse TA3 cells. High-molecular-weight DNA (approximately 10 μg) was digested to completion with *Hin*dIII (HIII) or *Bam*HI (Bam) endonucleases and electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized as described in the legend to Fig. 5, except that salmon sperm DNA (0.1 mg/ml) was used instead of tRNA in the hybridization solutions and the final washing was at 60°C. The DNA size markers were from *Hin*dIII-digested λ DNA. Wt, DNA from the parent TA3 cells; R, DNA from the hydroxyurea-resistant cells.

81-bp 5′ untranslated sequence, a single open reading frame of 2,376 nucleotides, and a 446-bp 3′ untranslated region. Their restriction map completely agrees with the restriction map of our clone 65 cDNA, except that clone 65 contains an additional 161 bp of untranslated sequence at the 5′ end (Fig. 6). The rest of the sequence of the 5′-terminal *Eco*RI-*Stu*I endonuclease DNA fragment of clone 65 (about 200 bp) was identical to the 5′-terminal sequence published by Caras et al. Clone 65 therefore contains a cDNA insert of 3,064 bp.

Expression of M2 protein after transfection of monkey cells. To test that our M2 cDNA clones contained the entire M2 protein coding sequence and that the plasmid contained all the information needed for transcription and processing of biologically active M2 protein in mammalian cells, we transfected mouse 3T6 cells with the clone 10 M2 cDNA plasmid by the calcium phosphate precipitation technique or electroporation. This approach did not yield hydroxyurea-resistant transformants, the expected phenotype of cells containing increased levels of M2 protein (31), although parallel and concurrent transfections with pSV2-neo (27) gave the expected frequency of G418-resistant cells (data not shown).

Transfection of COS-7 monkey cells with clone 10 M2 cDNA plasmid was tested for the transient production of M2 protein. COS-7 cells produce the SV40 large T antigen, which promotes plasmid replication at the SV40 origin, thereby copying the transfecting pcD DNA. Amplification of

```
TGCACCGGAT TCCAGCTGTT TTCGCCTGCT CCTCGCCGTC TCCGCCGCTG CCCTCGTTCG CC    62

ATG CTC TCC GTC CGC ACC CCG CTC GCC ACC ATC GCT GAC CAG CAG CAG CTG CAG 116
Met Leu Ser Val Arg Thr Pro Leu Ala Thr Ile Ala Asp Gln Gln Gln Leu Gln
 1

TTG TCG CCG CTG AAG CGA CTC ACC CTG GCT GAC AAG GAG AAC ACG CCC CCG ACT 170
Leu Ser Pro Leu Lys Arg Leu Thr Leu Ala Asp Lys Glu Asn Thr Pro Pro Thr
     20

CTC AGC AGC ACC CGC GTC CTG GCC AGC AAA GCT GCG AGG AGA ATC TTC CAG GAC 224
Leu Ser Ser Thr Arg Val Leu Ala Ser Lys Ala Ala Arg Arg Ile Phe Gln Asp
            40

TCC GCC GAG CTG GAA AGT AAA GCG CCT ACT AAC CCC AGC GTT GAG GAT GAG CCG 278
Ser Ala Glu Leu Glu Ser Lys Ala Pro Thr Asn Pro Ser Val Glu Asp Glu Pro
                    60

TTA CTG AGA GAA AAC CCC CGC CGC TTC GTT GTC TTT CCC ATC GAG TAC CAT GAT 332
Leu Leu Arg Glu Asn Pro Arg Arg Phe Val Val Phe Pro Ile Glu Tyr His Asp
                            80

ATC TGG CAG ATG TAC AAG AAA GCC GAG GCC TCC TTT TGG ACT GCC GAG GAG GTG 386
Ile Trp Gln Met Tyr Lys Lys Ala Glu Ala Ser Phe Trp Thr Ala Glu Glu Val
                                    100

GAC CTT TCC AAG GAT ATT CAG CAC TGG GAA GCT CTG AAA CCC GAT GAG AGA CAT 440
Asp Leu Ser Lys Asp Ile Gln His Trp Glu Ala Leu Lys Pro Asp Glu Arg His
                                        120

TTT ATA TCT CAC GTT CTG GCT TTC TTT GCA GCG AGT GAT GGC ATA GTC AAT GAG 494
Phe Ile Ser His Val Leu Ala Phe Phe Ala Ala Ser Asp Gly Ile Val Asn Glu
                                            140

AAC TTG GTG GAG CGA TTT AGC CAA GAA GTT CAA GTT ACA GAG GCC CGC TGT TTC 548
Asn Leu Val Glu Arg Phe Ser Gln Glu Val Gln Val Thr Glu Ala Arg Cys Phe
                                                160

TAT GGC TTC CAA ATT GCC ATG GAA AAC ATA CAC TCT GAA ATG TAC AGT CTC CTT 602
Tyr Gly Phe Gln Ile Ala Met Glu Asn Ile His Ser Glu Met Tyr Ser Leu Leu
                                                    180

ATT GAC ACT TAC ATT AAA GAT CCC AAG GAA AGA GAA TAT CTC TTC AAT GCT ATT 656
Ile Asp Thr Tyr Ile Lys Asp Pro Lys Glu Arg Glu Tyr Leu Phe Asn Ala Ile


GAA ACT ATG CCT TGT GTG AAG AAG AAG GCT GAC TGG GCC TTG CGC TGG ATT GGG 710
Glu Thr Met Pro Cys Val Lys Lys Lys Ala Asp Trp Ala Leu Arg Trp Ile Gly
    200

GAC AAA GAG GCT ACG TAT GGA GAA CGC GTT GTG GCC TTT GCC GCC GTA GAA GGA 764
Asp Lys Glu Ala Thr Tyr Gly Glu Arg Val Val Ala Phe Ala Ala Val Glu Gly
            220

ATC TTC TTT TCC GGT TCT TTT GCA TCG ATA TTC TGG CTC AAG AAA CGG GGG CTG 818
Ile Phe Phe Ser Gly Ser Phe Ala Ser Ile Phe Trp Leu Lys Lys Arg Gly Leu
                    240

ATG CCG GGC CTT ACA TTT TCC AAT GAG CTT ATT AGC AGA GAC GAG GGT TTA CAC 872
Met Pro Gly Leu Thr Phe Ser Asn Glu Leu Ile Ser Arg Asp Glu Gly Leu His
                            260
```

```
TGT GAC TTT GCC TGC CTG ATG TTC AAG CAC CTG GTA CAC AAG CCA GCG GAG CAG 926
Cys Asp Phe Ala Cys Leu Met Phe Lys His Leu Val His Lys Pro Ala Glu Gln
                                    280

AGG GTC CGA GAG ATA ATC ACC AAC GCC GTT AGG ATA GAG CAG GAG TTC CTC ACG 980
Arg Val Arg Glu Ile Ile Thr Asn Ala Val Arg Ile Glu Gln Glu Phe Leu Thr
                                        300

GAG GCC TTG CCC GTG AAG CTC ATC GGG ATG AAC TGC ACT TTG ATG AAG CAG TAC 1034
Glu Ala Leu Pro Val Lys Leu Ile Glu Met Asn Cys Thr Leu Met Lys Gln Tyr
                                            320

ATT GAG TTT GTG GCC GAC AGG CTT ATG CTG GAG CTG GGT TTT AAC AAG ATT TTC 1088
Ile Glu Phe Val Ala Asp Arg Leu Met Leu Glu Leu Gly Phe Asn Lys Ile Phe
                                                340

AGA GTA GAA AAT CCG TTT GAC TTC ATG GAA AAT ATC TCA CTA GAA GGA AAG ACA 1142
Arg Val Glu Asn Pro Phe Asp Phe Met Glu Asn Ile Ser Leu Glu Gly Lys Thr
                                                    360

AAC TTC TTT GAG AAG CGA GTA GGC GAG TAT CAG AGG ATG GGA GTC ATG TCG AAT 1196
Asn Phe Phe Glu Lys Arg Val Gly Glu Tyr Gln Arg Met Gly Val Met Ser Asn


TCG ACA GAG AAC TCT TTT ACC TTG GAT GCT GAC TTC   TAAGTAACTG ATCGTGTGCT 1252
Ser Thr Glu Asn Ser Phe Thr Leu Asp Ala Asp Phe
    380

CTTCGCTGAT TTTTGTCCCC TTGCCATTAA AAGAAACCAG CAAAAACAAC CAACTGGCTA CACCA 1317

TGAATTGTCA TTAAATTTGC TAAACAGGTG TCTAAAAAGC TGTGTAGCTA CCTCAGTCCT GTTTG 1382

CCAGGCTGGT CACTAGAAGA AAGTATACTT CAAACAATGG GTACTTGGAT CCTTAGGGAG ATCCT 1447

GTCCTTGGCT TTTACAAGTA GTGTGGTCAC CTTTGACCTC ATCAAAGTAC TAACAGCACT GGGCC 1512

AGGTTTTAGG AGCAGTGACC ATCAAGCAAG CAGGTTTAAA CATTTAGATG CTGTTTAGGG CTGTT 1577

TAAAGATGTC GGACTGCTTC CTGCAGGCAT GCAGAGTCTA CTTAACAAGT TTGTAAATAA AATTG 1642

GCACTTTGCA CACACACACA TAGTACTGTC AGGCGATTAA ACTATACATT TTATGAGGTA GTACC 1707

TCTATGCTTT TTTTTATTTT AATGCTCAGT ATTATCTTGA AGTTTGCAAA TGCTATGATG GTACA 1772

GTAAATTCTG ACATTTGCCC TAATAAGAGT GTCACTTTTT TTTTTTTTCT TCGAGACAGA GTTTC 1837

TCTGTATAGC CCTGGCTGTA CGGAATTCAC AAGTGAGTTT GAGCCCAGTG GTGGGTACAC CCGTG 1902

GGACTCTCTT ACAAACCAAA ACAGGAAAAG CAAGTGTTCC CTGAGGTAGT TTACTGTGAT CTAGC 1967

TTCCTCATGA ACTGACATAA CCCTGATCAG TTTCCTTGAT TATTGTATAA ATGTTTTTGT AATAT 2032

GAAAAGCCTT TGTACCTTTT AAATTATTGT TACTTAAAAT TAATAAACTC TTGAATTAAC AGTCT 2097

TGAACTTTCA TGGC                                                        2111
```

FIG. 5. Nucleotide sequence of the cDNA corresponding to the mRNA for mouse M2 protein and the predicted amino acid sequence of the protein. The nucleotide sequence begins with position 1 at the first nucleotide following the oligo(dG:dC) segment and nucleotides are numbered to the right of each line. The amino acid sequence begins with the first in-phase ATG codon for the single long open reading frame, and amino acid residues are numbered below each line. The position of the active-site free radical tyrosyl residue is underlined (cf. reference 26) as well as two potential polyadenylation signals in the 3′ untranslated region. Computer-assisted sequence analyses were accomplished with the programs of IntelliGenetics, Inc.

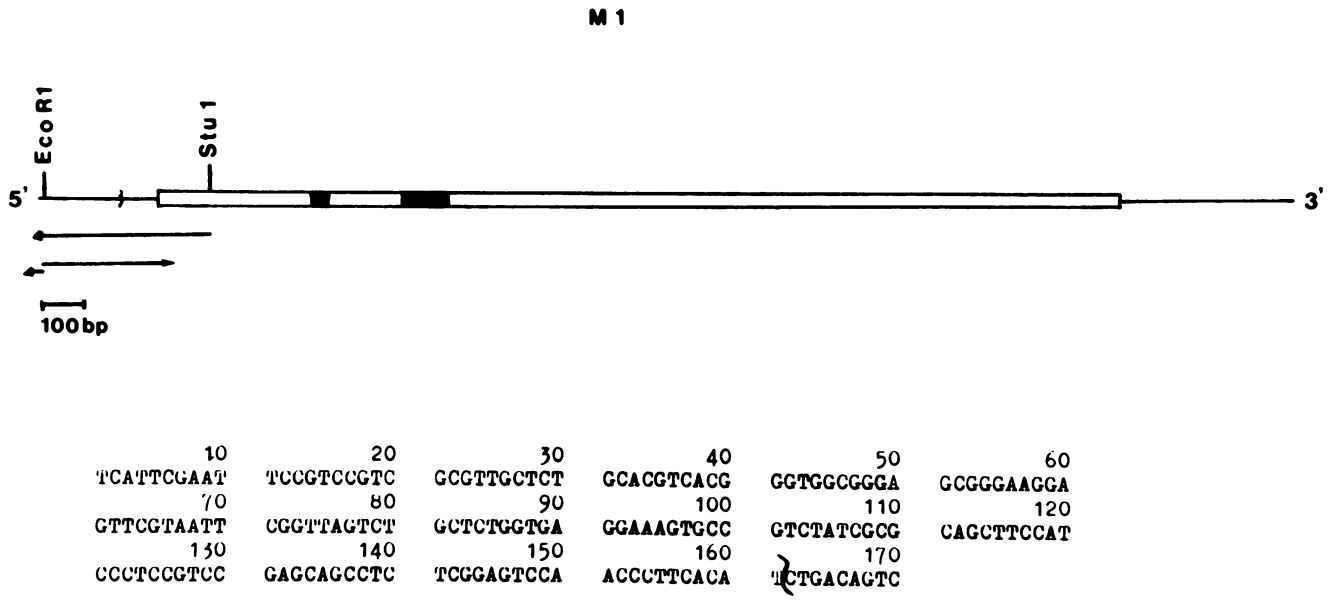

FIG. 6. Restriction map of the 5'-terminal part of mouse M1 cDNA and sequencing strategy. The protein-coding region is indicated by double lines. The arrows indicate the direction and extent of sequence determination for each fragment analyzed. The sequence to the right of the wavy line was published by Caras et al. (3). The sequence to the left of the wavy line constituting the 5'-terminal end of the M1 cDNA is shown. The deduced amino acid sequence of the filled-in parts of the protein-coding region (residues 99–110 and 174–210) was confirmed by amino acid sequence analyses (see Materials and Methods).

the pcD plasmid copy number frequently increases the transient expression of genes on the plasmid (33). We detected the expression of M2 protein in the transfected COS cells by measuring the EPR signal characteristic of the tyrosyl free radical present in functional M2 protein (31). Accordingly, we examined packed, frozen M2 cDNA-transfected COS-7 cells by EPR spectroscopy (see Materials and Methods).

Figure 7 shows the EPR signals recorded from the hydroxyurea-resistant, M2-overproducing TA3 cells, COS-7 cells transfected with the clone 10 plasmid, and COS-7 cells transfected with salmon sperm DNA. The M2-specific tyrosyl free radical signal was clearly seen in both the TA3 cells and in the clone 10-transfected COS cells (upper two curves of Fig. 7), while the cells transfected with salmon sperm DNA only showed a nonspecific background signal (the lower curve of Fig. 7). The concentration of radical in the TA3 cells was 1.4 μM, and since the concentration of radical is proportional to the amplitude of the EPR signal, we estimate that the concentration of the tyrosyl radical in the clone 10-transfected cells is 0.2 to 0.4 μM, the exact figure depending on the background used for correction.

DISCUSSION

Nearly full-length cDNA clones have been obtained which encode the two nonidentical subunits of mouse ribonucleotide reductase, proteins M1 and M2. This is evident from the uniformity of the size of the cDNAs in the different clones corresponding to each gene and from the size comparisons with the corresponding mRNAs. Caras et al. (3) reported that the M1 mRNA was 4.4 kb in length, but this differs significantly from our value of about 3.1 kb. However, a parallel RNA blot analysis of glyoxal-denatured poly(A)$^+$ RNA from normal and hydroxyurea-resistant mouse TA3 cells and of an identically treated poly(A)$^+$ RNA sample from mouse S49 cells by B. Levinson, Genentech, showed that all contained only the 3.1-kb M1 mRNA (data not shown). This estimate was obtained by using glyoxylated DNA size markers as internal size standards, a practice that has been well documented (5); moreover, the β-actin mRNA in our RNA preparations was about 2 kb, in agreement with published data (8). Therefore, we conclude that the M1 mRNA is 3.1 kb in length.

The appearance of an M2 protein-specific tyrosyl free radical EPR signal in COS-7 cells after transfection with the clone 10 M2 cDNA plasmid indicates that the cDNA contains the information necessary for specifying biologically active M2 protein. As observed earlier (11), the tyrosyl radical could not be detected in normal, untransfected, or salmon sperm DNA-transfected cells. The failure to detect stable hydroxyurea-resistant transformants of 3T6 cells after transfection with the cloned M2 cDNA may be a consequence of too low an expression of the M2 protein from the few integrated copies or may indicate that constant expression of M2 protein from the SV40 early promotor was lethal to cells.

The Southern blot analyses of genomic DNA from the hydroxyurea-resistant, M2-overproducing mouse TA3 cells showed that the M2 gene was amplified approximately fivefold compared with the parent TA3 cells. Interestingly, the same cells contained 50- to 100-fold more M2-specific mRNA and, from earlier studies, about 40-fold more of the M2 protein-specific tyrosyl free radical (11). Thus, unlike the findings with the dihydrofolate reductase gene (1), the relationship between the level of M2 protein, M2 mRNA, and M2 gene copies is not linear. The situation is more reminiscent of the levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase in compactin-resistant UT-1 cells; in that case there was a 15-fold increase in the number of gene copies and a 100- to 1,000-fold increase in the amount of reductase protein (18). Since the Southern blot analysis indicates that the structure and organization of the M2 gene has not been grossly changed, we tend towards the explanation that the rate of transcription of each amplified M2 gene copy in the hybroxyurea-resistant TA3 cells is increased. Nevertheless,

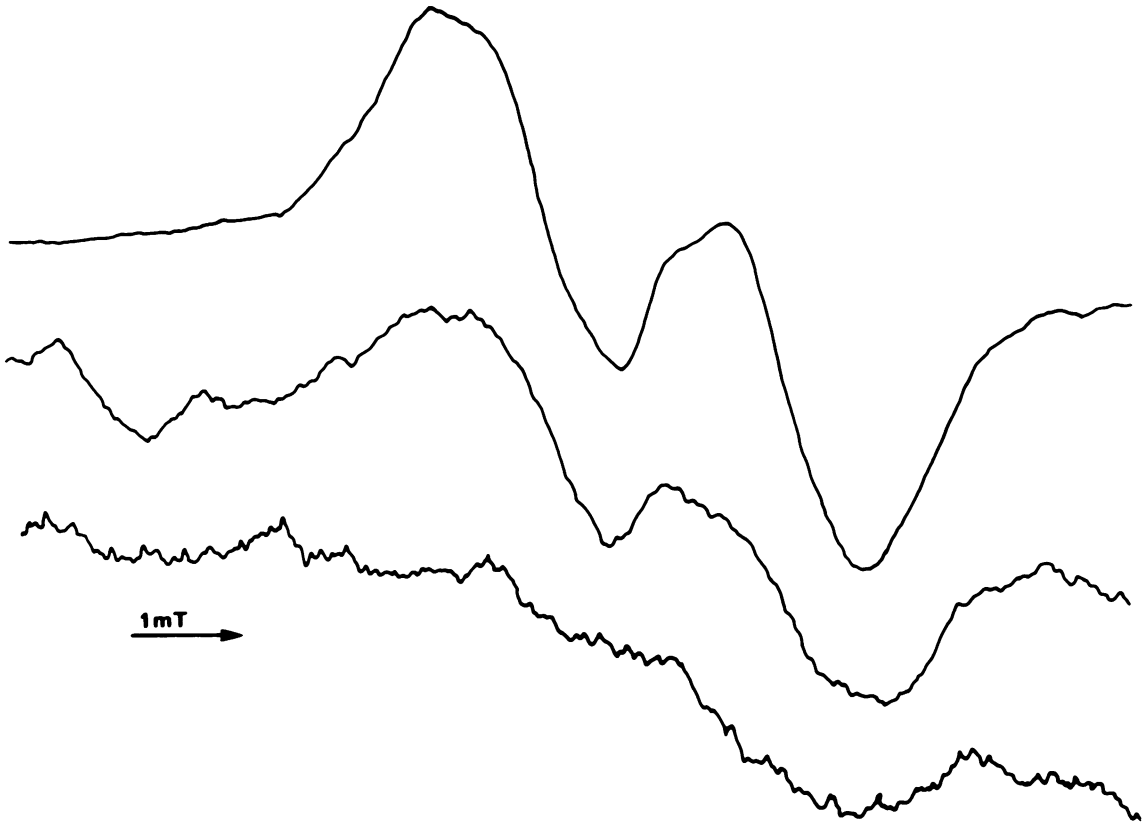

FIG. 7. EPR spectra at 77 K of packed samples of M2 protein-overproducing, hydroxyurea-resistant TA3 cells (upper curve), COS-7 cells transfected with the clone 10 M2 cDNA plasmid (middle curve), and COS-7 cells transfected with sonicated salmon sperm DNA (lower curve). In the EPR measurements, the microwave power was 91.5 mW and the modulation was 0.63 mT. For the upper curve the spectrometer amplification was 1/2 that of the two lower curves.

the expression of M2 protein appears to be regulated normally with respect to the cell cycle occurring at S phase with an increase in the concentration of protein M2 (11).

The level of M1 mRNA is also strikingly increased in hydroxyurea-resistant TA3 cells even though the number of M1 gene copies remains unchanged. This suggests that the expression of the two genes may be coordinated, but because they are located on different chromosomes (L. Thelander and U. Francke, unpublished results), it must be via a *trans*-acting regulator. Alternatively, the M1 mRNA may be stabilized by high levels of M2 mRNA.

The 3′ untranslated regions of M1 and M2 mRNAs are 14 and 41% of their entire length, respectively. Perhaps this is related to differences in their regulation of expression: the level of M1 protein is constant during the cell cycle, while there is an S-phase-correlated increase in the level of M2 protein. Sequences in the 3′-terminal part of the mouse histone H4 gene have been implicated in the regulation of gene expression during the cell cycle, presumably at the posttranscriptional level (19). The existence of two distinct M2 mRNAs in both the normal and hydroxyurea-resistant mouse TA3 cells, differing in sequence in the 3′ ends but identical in their 5′ ends, suggests that different polyadenylation sites are used to end the mRNA. There are indeed two AATAAA sites 441 bp apart in the 3′ untranslated part of the M2 mRNA. Therefore, it may be that alternate utilization of polyadenylation signals is involved in the regulation of M2 gene expression, as was suggested for dihydrofolate reductase (16). Moreover, different polyadenylation sites might be utilized in growing and stationary-phase cells. The mRNA we used was extracted from a culture containing cells at various stages of their cell cycle.

The comparison between the predicted amino acid sequences of the smaller subunits of ribonucleotide reductases of different species shows a striking homology between the mouse and the clam (*Spisula solidissima*) proteins. There is much weaker homology between the corresponding proteins from Epstein-Barr virus, herpes simplex type 2 virus, and *E. coli*. It is interesting that the most highly conserved sequences are not located close to the tyrosine residue that carries the free radical but further downstream in the sequence. Still, the active-site tyrosine and a histidine residue, situated four residues upstream in the sequence and assumed to participate in iron binding (26), are present in all five proteins.

The carboxy-terminal sequence of -Ala-Asp-Phe in the M2 protein is also consistent with the ability of the M2 protein to bind to the known antigen binding site of the monoclonal antitubulin antibody YL1/2 used in the purification of the protein (31).

ACKNOWLEDGMENTS

We are indebted to J. Ruderman for supplying us with the clam clone 2 cDNA, to Robert Margolskee and Michael McPaul for their invaluable aid and advice in the cloning and sequencing of the M1 and M2 cDNAs and for many helpful discussions throughout the course of this work, and to H. Jörnvall for performing the amino acid sequence determinations.

This work was supported by Public Health Service grants GM-13235 and CA-31928 from the National Institutes of Health and by grant 1806 from the Swedish Cancer Society. L.T. was the recipient of a European Molecular Biology Organization long-term fellowship and a fellowship from the Swedish Medical Research Council and was also supported by the Swedish Natural Science Research Council.

LITERATURE CITED

1. **Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke.** 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. **253**:1357–1370.
2. **Aviv, H., and P. Leder.** 1972. Purification of biological active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
3. **Caras, I. W., B. B. Levinson, M. Fabry, S. R. Williams, and D. W. Martin, Jr.** 1985. Cloned mouse ribonucleotide reductase subunit M1 cDNA reveals amino acid sequence homology with Escherichia coli and herpesvirus ribonucleotide reductases. J. Biol. Chem. **260**:7015–7022.
4. **Carlson, J., J. A. Fuchs, and J. Messing.** 1984. Primary structure of the Escherichia coli ribonucleoside diphosphate reductase operon. Proc. Natl. Acad. Sci. USA **81**:4294–4297.
5. **Carmichael, G. G., and G. K. McMaster.** 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enzymol. **65**:380–391.
6. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. J. Mol. Biol. **138**:179–207.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
8. **Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner.** 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell **20**:95–105.
9. **Engström, Y., B. Rozell, H.-A. Hansson, S. Stemme, and L. Thelander.** 1984. Localization of ribonucleotide reductase in mammalian cells. EMBO J. **3**:863–867.
10. **Engström, Y., S. Eriksson, I. Jildevik, S. Skog, L. Thelander, and B. Tribukait.** 1985. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. J. Biol. Chem. **260**:9114–9116.
11. **Eriksson, S., A. Gräslund, S. Skog, L. Thelander, and B. Tribukait.** 1984. Cell cycle-dependent regulation of mammalian ribonucleotide reductase. The S phase-correlated increase in subunit M2 is regulated by de novo protein synthesis. J. Biol. Chem. **259**:11695–11700.
12. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
13. **Gibson, T., P. Stockwell, M. Ginsberg, and B. Barrell.** 1984. Homology between two EBV early genes and HSV ribonucleotide reductase and 38 K genes. Nucleic Acids Res. **12**: 5087–5099.
14. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
15. **Hanke, P. D., and J. A. Fuchs.** 1983. Characterization of the mRNA coding for ribonucleoside diphosphate reductase in *Escherichia coli*. J. Bacteriol. **156**:1192–1197.
16. **Kaufman, R. J., and P. A. Sharp.** 1983. Growth-dependent expression of dihydrofolate reductase mRNA from modular cDNA genes. Mol. Cell. Biol. **3**:1598–1608.
17. **Lammers, M., and H. Follmann.** 1983. The ribonucleotide reductases—a unique group of metalloenzymes essential for cell proliferation. Struct. Bonding **54**:27–91.
18. **Luskey, K. L., J. R. Faust, D. J. Chin, M. S. Brown, and J. L. Goldstein.** 1983. Amplification of the gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not for the 53 kDa protein, in UT-1 cells. J. Biol. Chem. **258**:8462–8469.
19. **Lüscher, B., C. Stauber, R. Schindler, and D. Schümperli.** 1985. Faithful cell-cycle regulation of a recombinant mouse histone H4 gene is controlled by sequences in the 3′-terminal part of the gene. Proc. Natl. Acad. Sci. USA **82**:4389–4393.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. In Molecular cloning: a laboratory manual, p. 368–369. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **McLaughlan, J., and J. B. Clements.** 1983. DNA sequence homology between two colinear loci on the HSV genome which have different transforming activities. EMBO J. **2**:1953–1961.
22. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
23. **Okayama, H., and P. Berg.** 1982. High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. **2**:161–170.
24. **Okayama, H., and P. Berg.** 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol. **3**:280–289.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26. **Sjöberg, B.-M., H. Eklund, J. A. Fuchs, J. Carlson, N. M. Standart, J. V. Ruderman, S. J. Bray, and T. Hunt.** 1985. Identification of the stable free radical tyrosine residue in ribonucleotide reductase. FEBS Lett. **183**:99–102.
27. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
28. **Standart, N. M., S. J. Bray, E. L. George, T. Hunt, and J. V. Ruderman.** 1985. The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal RNAs in clam and sea urchin eggs. J. Cell. Biol. **100**:1968–1976.
29. **Thelander, L., and P. Reichard.** 1979. Reduction of ribonucleotides. Annu. Rev. Biochem. **48**:133–158.
30. **Thelander, L., S. Eriksson, and M. Åkerman.** 1980. Ribonucleotide reductase from calf thymus. J. Biol. Chem. **255**:7426–7432.
31. **Thelander, M., A. Gräslund, and L. Thelander.** 1985. Subunit M2 of mammalian ribonucleotide reductase. J. Biol. Chem. **260**:2737–2741.
32. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
33. **Yokota, T., N. Arai, F. Lee, D. Rennick, T. Mosmann, and K.-I. Arai.** 1985. Use of a cDNA expression vector for isolation of mouse interleukin 2 cDNA clones: expression of T-cell growth-factor activity after transfection of monkey cells. Proc. Natl. Acad. Sci. USA **82**:68–72.