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S-Phase-Specific Expression of Mammalian Ribonucleotide Reductase R1 and R2 Subunit mRNAs†

Stefan Björklund,*,‡ Sven Skog,§ Bernard Tribukait,§ and Lars Thelander‡

Department of Medical Biochemistry and Biophysics, University of Umeå, S-901 87 Umeå, Sweden, and Department of Medical Radiobiology, Karolinska Institute, S-104 01 Stockholm, Sweden

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ABSTRACT: Ribonucleotide reductase in mammalian cells is composed of two nonidentical subunits, proteins R1 and R2, each inactive alone. The R1 protein is present in excess in proliferating cells, and its levels are constant during the cell cycle. Expression of the R2 protein, which is limiting for enzyme activity, is strictly S-phase-correlated. In this paper, we have used antisense RNA probes in a solution hybridization assay to measure the levels of R1 and R2 mRNA during the cell cycle in centrifugally elutriated cells and in cells synchronized by isoleucine or serum starvation. The levels of both transcripts were very low or undetectable in G_0/G_1-phase cells, showed a pronounced increase as cells progressed into S phase, and then declined when cells progressed into G_2+M phase. The R1 and R2 transcripts increased in parallel, starting slightly before the rise in S-phase cells, and reached the same levels. The relative lack of cell cycle dependent variation in R1 protein levels, obtained previously, may therefore simply be a consequence of the long half-life of the R1 protein. Hydroxyurea-resistant, R2-overproducing mouse TA3 cells showed the same regulation of the R1 and R2 transcripts as the parental cells, but with R2 mRNA at a 40-fold higher level.

Mammalian ribonucleotide reductase is composed of two nonidentical dimeric subunits, proteins R1 and R2, which are both essential for activity. It catalyzes the direct reduction of ribonucleotides to the corresponding deoxyribonucleotides, the first unique step in the reactions leading to DNA synthesis, to which its activity is strongly correlated (Thelander & Reichard, 1979; Reichard, 1988).

The mammalian R1 and R2 subunits have been purified to homogeneity, and the corresponding cDNAs have been cloned from mouse cells (Caras et al., 1985; Thelander & Berg, 1986). The R1 subunit binds the nucleoside triphosphate allosteric effectors and the ribonucleoside diphosphate substrates and provides redox-active sulfhydryl groups (Thelander & Reichard, 1979; Thelander et al., 1980). In analogy with the *Escherichia coli* enzyme, the R2 subunit most probably contains two non-heme binuclear iron centers, which each generate and stabilize a tyrosyl free radical essential for activity (Thelander et al., 1985; Lynch et al., 1989). Hydroxyurea specifically destroys the radical and has been used to select hydroxyurea-resistant human and rodent cells, which over-

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* To whom correspondence should be addressed.
‡ University of Umeå.
§ Karolinska Institute.

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produce protein R2 as a result of gene amplification (Thelander & Berg, 1986; Wright et al., 1987).

The genes encoding proteins R1 and R2 are located on different chromosomes in both human and mouse cells (Yang-Feng et al., 1987; Brissenden et al., 1988). Recently, the functional mouse R2 gene was cloned and sequenced (Thelander & Thelander, 1989).

Measurements in both cell extracts and whole cells have shown that the levels of protein R1 in proliferating cells are almost constant throughout the cell cycle and that the protein has a half-life of around 20 h (Engström et al., 1985; Mann et al., 1988). In quiescent or terminally differentiated cells, the level of protein R1 is virtually undetectable (Engström et al., 1984; Mann et al., 1988). On the other hand, the levels of protein R2 during the cell cycle have shown strictly S-phase-correlated expression, with a protein half-life of only 3 h (Eriksson et al., 1984). Holoenzyme activity is therefore limited during the cell cycle by the levels of R2 protein, which in turn is regulated by de novo synthesis and breakdown.

Other enzymes involved in DNA synthesis have been shown to have either cell cycle phase-dependent activity, such as thymidine kinase and thymidylate synthetase (Littlefield, 1966; Conrad, 1971) or, like DNA topoisomerase II, activity correlated to proliferation (Heck & Earnshaw, 1986; Heck et al., 1988). Ribonucleotide reductase activity depends on two nonidentical subunits, one (R1) with proliferation-specific and one (R2) with cell cycle phase-specific expression, which are located on different chromosomes. It is therefore of considerable interest to study the transcriptional control mechanisms of the R1 and R2 genes.

In this paper, we describe a solution hybridization assay using antisense RNAs specific for R1 and R2 mRNA as probes. Using this assay, we have measured the levels of R1 and R2 mRNA in cells synchronized by serum or isoleucine starvation or separated by centrifugal elutriation.

Materials and Methods

Cell Cultures. Hydroxyurea-resistant, R2-overproducing mouse mammary tumor TA3 cells, the corresponding parental cells, and BALB/3T3 cells (ATCC CCL 163) were grown as monolayer cultures in Dulbecco-modified Eagle medium plus 10% heat-inactivated horse serum (Flow) as described earlier (Thelander & Berg, 1986).

Centrifugal Elutriation. Separation of logarithmically growing cells on the basis of cell size was performed in a Beckman JE-G centrifugal elutriator as described earlier (Eriksson et al., 1984). The cells in each fraction from the elutriator were collected by centrifugation, resuspended in isotonic Tris/saline, and counted. Aliquots of $(0.2–1.0) \times 10^6$ cells were removed for fixation in 96% ethanol and subsequent flow cytometric analysis of DNA content (Eriksson et al., 1984). The proportion of cells in the different cell cycle phases (G_1, S, and G_2+M) was determined from the area of the histograms by assuming a Gaussian distribution of cells around the G_1 and G_2+M maxima and assigning the remaining area to S-phase cells. The remaining cells $[(0.5–5) \times 10^7]$ were washed twice in Tris/saline, resuspended in 1 × SET (1% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.5, and 10 mM EDTA), and immediately used to prepare total nucleic acid.

Isoleucine Starvation. Cells were grown in normal medium to a density of $(0.5–1.0) \times 10^7$ cells/14-cm dish. After being rinsed with isoleucine-free medium (The National Veterinary Institute, Uppsala, Sweden), the cells were grown for 48 h in isoleucine-free medium containing 5% heat-inactivated horse serum as described earlier (Eriksson et al., 1984). The medium was then changed to complete medium containing 10% heat-inactivated horse serum, and, at various times subsequently, cells from two plates were harvested by trypsinization, resuspended in Tris/saline, and counted. Aliquots were analyzed by DNA flow cytometry while the remaining cells were washed twice in Tris/saline, frozen as a pellet in liquid nitrogen, and stored at −70 °C. When cells from all time points have been harvested, the frozen pellets were thawed and resuspended in 1 × SET, immediately followed by preparation of total nucleic acid.

Serum Starvation. BALB/3T3 cells were plated at 2×10^6 cells per 14-cm dish (Coppock & Pardee, 1987). After 24 h, the cells were washed once with serum-free medium, and 25 mL of medium containing 0.6% heat-inactivated horse serum was added. After 42 h, this medium was replaced by normal medium containing 10% heat-inactivated horse serum, and cells were subsequently harvested and handled as described under isoleucine starvation.

Preparation of Total Nucleic Acid. Total nucleic acid samples were extracted and purified essentially as described by Durnam and Palmiter (1983). Cells, resuspended at room temperature in 1 × SET at a concentration of $10^6–10^7$ cells/mL, were immediately sonicated by using a Branson B30 sonifier cell disruptor with a microtip (10 pulses of 1 s each) to reduce viscosity. After incubation with Proteinase K (Sigma), 0.2 mg/mL, 45 °C for 45 min, sodium chloride was added to a final concentration of 0.15 M, and the samples were extracted sequentially with an equal volume of phenol/chloroform and chloroform. After precipitation in 2 volumes of ethanol for 1 h at −20 °C and centrifugation (Beckman JS 13.1-rotor, 16000*g*) for 30 min at 4 °C, the pellets were rinsed with ice-cold 70% ethanol, containing 0.15 M sodium chloride, dried, and dissolved in 0.2 × SET. The concentration of DNA in each sample was fluorometrically measured with Hoechst 33258 (Labarca & Paigen, 1980).

Mouse R1 and R2 mRNA and Antisense RNA Probes. A 384 bp *Kpn*I to *Bgl*II mouse R1 cDNA fragment corresponding to nucleotides 1447–1831 (Thelander & Berg, 1986) was subcloned into the polylinker of the vector pGEM-3 (Promega Biotec). Similarly, a 404 bp *Cla*I to *Eco*RI mouse R2 cDNA fragment corresponding to nucleotides 790–1194 (Thelander & Berg, 1986) was subcloned into the same vector. Both fragments include only translated sequences. Mouse R1 mRNA was synthesized using T7 RNA polymerase (Pharmacia) after the plasmid was linearized with *Sal*I. SP6 RNA polymerase (Promega Biotec) was used to synthesize the mouse R1 antisense RNA probe, after cleavage of the plasmid with *Eco*RI. Similarly, mouse R2 mRNA was synthesized by using *Eco*RI and SP6 RNA polymerase, and finally, the mouse R2 antisense RNA probe was synthesized with *Hin*dIII and T7 RNA polymerase.

Transcription reactions were performed as described in the Promega Biotec manual, with the following modifications: Bovine serum albumin (0.1 μg) was added to the transcription reactions, and after the DNase I (Promega Biotec) treatment and the phenol/chloroform extraction, unincorporated nucleoside triphosphates were removed by chromatography on a Sephadex G-50 column, equilibrated with 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 100 mM sodium acetate. In the synthesis of antisense RNA, 100 μCi of uridine 5′-α-[^{35}S]-thiotriphosphate with a specific activity of >400 Ci/mmol (Amersham) was used instead of UTP. The labeled probes were kept frozen at −70 °C in small aliquots and used within 3 months.

Solution Hybridization Assay. Aliquots of total nucleic acid extracts, corresponding to 0.2–50 μg of DNA, were hybridized

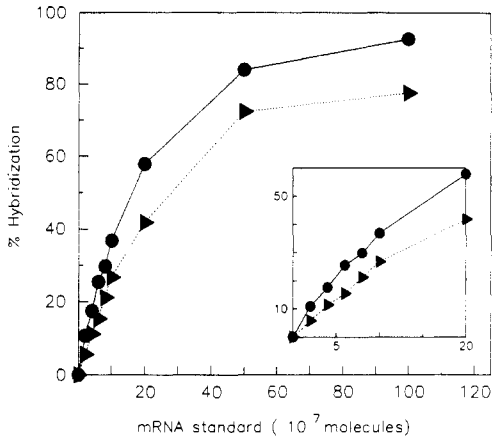

FIGURE 1: Hybridization of an excess of antisense [α-^{35}S]UTPαS-labeled R1 and R2 RNA probes to varying amounts of synthetic R1 and R2 mRNAs, as described under Materials and Methods. The ordinate gives the amounts of RNase-stable, acid-insoluble radioactivity in percent hybridization, where 100% corresponds to 10^4 cpm. Background radioactivity (50–100 cpm), present in samples lacking added mRNA, has been subtracted. Inset: the linear part of the standard curve. (●) R1 standard curve; (▲) R2 standard curve.

to 10^4 cpm of either R1 or R2 [α-^{35}S]UTPαS-labeled antisense RNA probes essentially as described (Durnam & Palmiter, 1983; Idzerda et al., 1986). Hybridization was performed at 70 °C, for at least 16 h under paraffin oil, in a final volume of 40 μL containing 0.6 M NaCl, 22 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.75 mM dithiothreitol, 25% formamide, and 0.1% sodium dodecyl sulfate. RNase digestion (RNase A from Sigma and RNase T_1 from Boehringer) of nonhybridized RNAs, trichloroacetic acid precipitation of hybridized fragments, filtration through Whatman GF/C glass fiber filters, and scintillation counting were performed as described (Idzerda et al., 1986).

In each assay, standard curves were generated by hybridization of various known amounts of mRNA, and amounts of test extract were chosen to yield values within the linear range. The concentration of the mRNA solutions was determined spectrophotometrically assuming that 1 μg/μL gives an A_{260} of 40 (Maniatis et al., 1982).

Results

Solution Hybridization Assay Using R1 and R2 Antisense RNA Probes. Preliminary attempts using a dot blot technique and cDNA probes to measure R1 and R2 mRNA in extracts from cell cultures indicated that the levels were too low to be measured in this way. Accordingly, a more sensitive solution hybridization technique using labeled antisense RNA probes was adopted (Idzerda et al., 1986; Yang-Feng et al., 1987). In this assay, total nucleic acid preparations from cells were hybridized in solution under stringent conditions to an excess of [α-^{35}S]UTPαS-labeled antisense R1 and R2 RNA probes. Nonhybridized RNA was digested with RNase, and double-stranded hybridized RNA was quantitated by comparison with standard curves generated with pure unlabeled mRNA. As shown in Figure 1, the standard curves were linear up to 10 × 10^7 molecules of mRNA. The amounts of total nucleic acid in each sample were therefore chosen to give hybridization values corresponding to (1–10) × 10^7 molecules of mRNA. The following calculation illustrates the sensitivity of the assay. A single assay could include up to 20 μL of total nucleic acid extract which could contain up to 50 μg of DNA and therefore mRNA from 7 × 10^6 cells, assuming 7 pg of DNA/diploid mouse cell. Since the lower limit of the standard curve is 1 × 10^7 molecules of mRNA, as little as 1 molecule of mRNA per cell could be detected.

To test the validity of the assay, known amounts of pure R1 or R2 mRNA were added to aliquots of total nucleic acid preparations prior to hybridization. In both R1 and R2 assays, this procedure repeatedly yielded hybridization values equal to the sum of the values obtained for added mRNA and the total nucleic acid extracts when measured separately. Labeled antisense R1 RNA was also hybridized with pure unlabeled R2 mRNA and vice versa, and both tests resulted in background values. Furthermore, RNase-treated hybrids were separated on 8 M urea-denaturing polyacrylamide gels to determine the size of the protected double-stranded RNA hybrids. Autoradiography showed that the radioactive fragments had the predicted sizes (data not shown).

Levels of R1 and R2 mRNA in Elutriated Mouse TA3 Cells. Fractions enriched for cells from different phases of the cell cycle were obtained from logarithmically growing TA3 cultures by centrifugal elutriation, a method that separates cells according to differences in cell size. The distribution of cells in different cell cycle phases in each fraction from the elutriator was analyzed by DNA flow cytometry. Total nucleic acid was prepared, the DNA content was determined, and the amounts of R1 and R2 mRnA were measured by solution hybridization.

The levels of both R1 and R2 transcripts, expressed as molecules mRNA per microgram of DNA, were 2–2.5-fold higher in the S-phase-enriched fraction, containing 44% cells in G_1 and 56% cells in S, than in the G_1-phase-enriched fraction, containing 86% cells in G1 and 14% in S (Figure 2). Since the DNA content doubles during S phase, this increase in mRNA content actually represents a 3–4-fold increase per cell (assuming that the average S-phase cell contains 1.5 times the G_1 DNA content). G_2+M-enriched fractions had intermediate values.

Levels of R1 and R2 mRNA in Parental and Hydroxyurea-Resistant, R2-Overproducing Mouse TA3 Cells Synchronized by Isoleucine Starvation. Cultures of parental (Figure 3) and hydroxyurea-resistant (Figure 4) TA3 cells were exposed to isoleucine-deficient medium to generate predominantly G_0/G_1-phase cells (Ley & Tobey, 1970); a delayed, synchronous wave through S phase was then induced by repletion of isoleucine, as observed earlier using these cells (Eriksson et al., 1984).

Levels of R1 and R2 mRNA in parental cells were very low or undetectable after 48-h isoleucine starvation, by which time virtually all cells had accumulated in G_0/G_1 phase. Note that only diploid G_0/G_1 cells are recorded in Figure 3C. As evident from the flow cytometry histograms (Figure 3, histograms number 3–5 from the left), the cell culture contained a sizable fraction of tetraploid cells arrested in G_1 and indistinguishable from diploid G_2+M-phase cells. After 4–8 h of isoleucine repletion, both R1 and R2 mRNA levels began to rise, slightly preceding the rise in S-phase cells, which appeared between 8 and 12 h. The levels of both transcripts recorded during the peak S-phase response correlated well with those recorded from S-phase-enriched elutriated fractions shown in Figure 2.

The levels of R1 mRNA were the same in logarithmically growing, R2-overproducing TA3 cells as in the parental cells, whereas R2 mRNA were 32 times more abundant in the mutant (see legends to Figures 2 and 4). These values agreed with previous estimates (Thelander & Berg, 1986) from Northern blots, and with EPR measurements of the relative levels of tyrosyl free radical containing R2 protein in parental and mutant cells (Thelander et al., 1985).

However, the R2-overproducing TA3 cells were more difficult to synchronize than the parental cells, and in the ex-

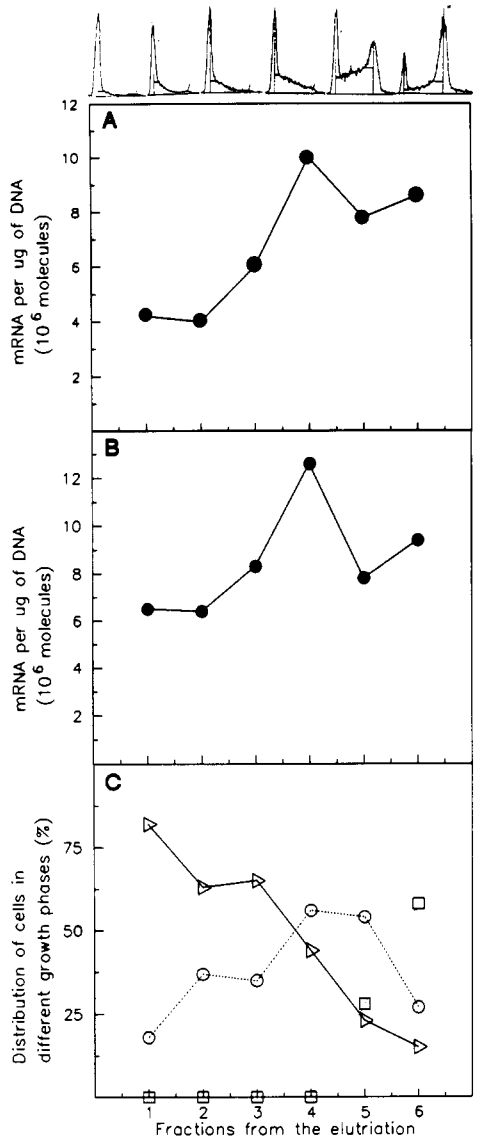

FIGURE 2: Correlation between R1 and R2 mRNA levels and cell cycle phase composition in elutriated cells. Total nucleic acid was prepared from fractions of elutriated parental TA3 cells and assayed for R1 and R2 mRNA. (A) R1 mRNA, (B) R2 mRNA, (C) cell cycle phase composition, determined by DNA flow cytometry. (△) G_1-phase cells; (○) S-phase cells; (□) G2+M-phase cells. In the DNA histograms, the *ordinate* gives the number of cells and the *abscissa* the fluorescence proportional to the DNA content, with the peak on the left corresponding to G_1 cells. Values for unseparated, logarithmically growing parental TA3 cells (G_1 = 45%, S = 32%, G_2+M = 23%): R1 mRNA, 5.1×10^6 molecules/μg of DNA; R2 mRNA, 5.9×10^6 molecules/μg of DNA. To get an approximate number of molecules of mRNA per cell, the ordinate figures should be multiplied by 7. This assumes a mean DNA content of 7 pg per diploid mouse cell.

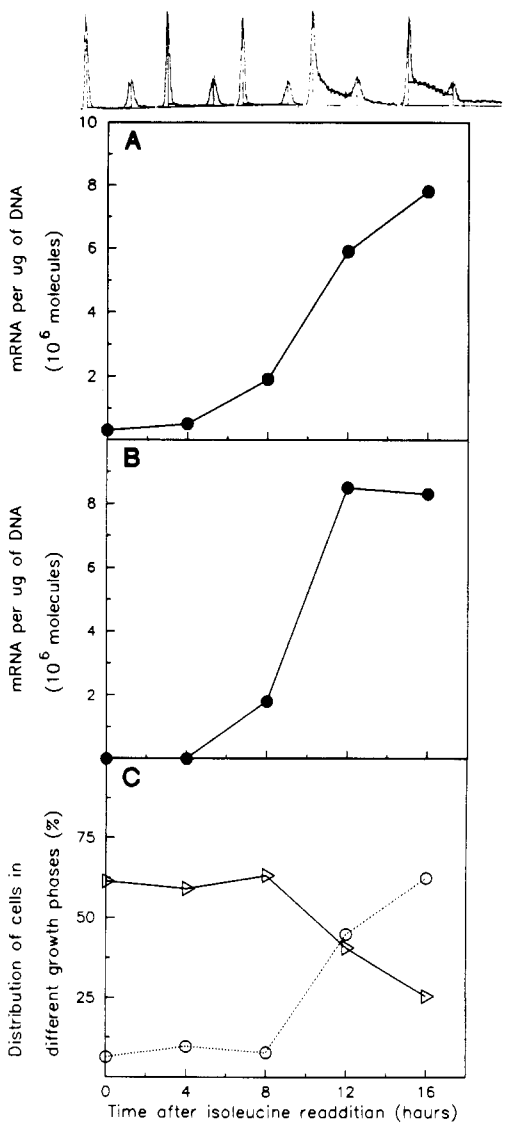

FIGURE 3: Levels of R1 and R2 mRNAs and cell cycle phase composition in parental TA3 cells synchronized by isoleucine starvation. (A) R1 mRNA, (B) R2 mRNA, (C) cell cycle phase composition. (△) G_1-phase cells; (○) S-phase cells. No value for G_2+M cells is given because the cultures contained some tetraploid cells (see DNA histograms, labeled as described in the legend to Figure 2, and text). In the first two points, total nucleic acid corresponding to 5.7×10^6 cells were used. See legend to Figure 2 concerning conversion to molecules of mRNA per cell.

periment shown in Figure 4, substantial numbers of S-phase cells were still present after 48-h isoleucine starvation. This presumably accounted for the continued detectability of both R1 and R2 transcripts at this time, albeit at levels less than half those obtained in logarithmically growing cells. Otherwise, the time course of R1 and R2 mRNA expression after isoleucine repletion was similar in the mutant R2-overproducing cells to that in parental cells although, in the case of R2 mRNA, at a 40-fold higher level.

Expression of R1 and R2 mRNAs in BALB/3T3 Cells Synchronized by Serum Starvation. The serum dependence of BALB/3T3 murine fibroblasts has been used to characterize the expression of several growth-related and cell cycle related gene products, including c-*fos*, c-*myc* (Lau & Nathans, 1987), and c-*jun* (Ryseck et al., 1988), and the DNA precursor enzyme thymidine kinase (Coppock & Pardee, 1987).

At the time of serum readdition, about 80% of the cells were in G_0/G_1, and 20 h later, 80% had progressed into S phase (Figure 5). The general pattern of expression of R1 and R2 mRNAs was the same as in the isoleucine starvation experiment, although more distinct due to the higher degree of synchronization. Both R1 and R2 mRNAs were undetectable in G_0/G_1 cells but increased dramatically from about 8 h after serum repletion to a peak corresponding to the peak S-phase response at 20 h. There was also a distinct fall in R1 and R2 mRNA levels when cells progressed from S phase to G_2+M phase at the 24-h time point.

Discussion

Using an antisense RNA solution hybridization assay, we have demonstrated that the messenger RNAs encoding the R1 and R2 subunits of ribonucleotide reductase are present in similar amounts in murine cells and that their abundance

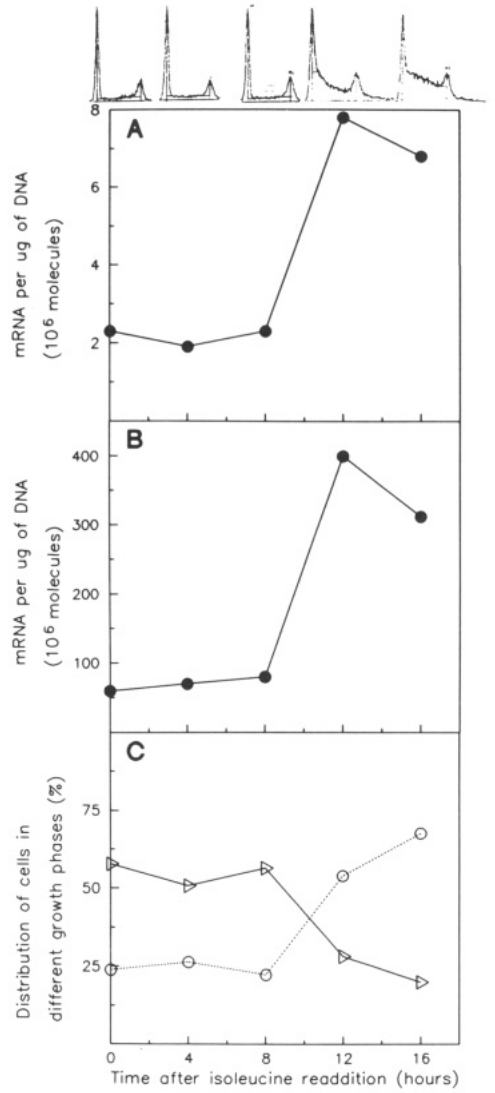

FIGURE 4: Levels of R1 and R2 mRNAs and cell cycle phase composition in hydroxyurea-resistant, R2-overproducing TA3 cells synchronized by isoleucine starvation. (A) R1 mRNA, (B) R2 mRNA, (C) cell cycle phase composition. (△) G_1-phase cells; (○) S-phase cells. No value for G_2+M cells is given because the culture contained some tetraploid cells. Values for logarithmically growing hydroxyurea-resistant, R2-overproducing cells (G_1 = 38%, S = 41%, G_2+M = 21%): R1 mRNA, 5.3×10^6 molecules/μg of DNA; R2 mRNA, 186×10^6 molecules/μg of DNA. The DNA histograms are labeled as described in the legend to Figure 2. See legend to Figure 2 concerning conversion to molecules of mRNA per cell.

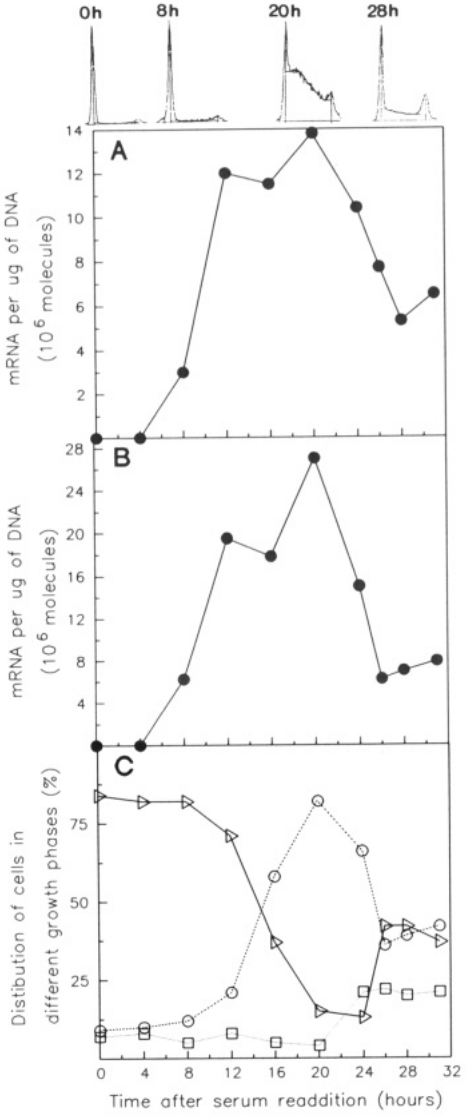

FIGURE 5: Levels of R1 and R2 mRNAs and cell cycle phase composition in BALB/3T3 cells synchronized by serum starvation. (A) R1 mRNA, (B) R2 mRNA, (C) cell cycle phase composition. (△) G_1-phase cells; (○) S-phase cells; (□) G2+M-phase cells. In the first two points, total nucleic acid corresponding to 2.3×10^6 cells was used. The DNA histograms are labeled as described in the legend to Figure 2. Note that only the indicated time points are represented. See legend to Figure 2 concerning conversion to molecules of mRNA per cell.

is regulated in a coordinate, S-phase-correlated manner. Three different methods for isolating cell cycle phase enriched populations, namely, centrifugal elutriation, isoleucine starvation, and serum starvation, showed that the R1 and R2 transcripts increased in parallel when cells progressed from either G_0 or G_1 into S phase. It should be noted that the S-phase-related increases have been underestimated by the expression of mRNA levels in this paper as molecules of mRNA per microgram of DNA; because cellular DNA content increases during S phase, values for molecules of mRNA per cell would have been even higher.

Ribonucleotide reductase activity in secondary cultures of mouse embryo cells synchronized by serum starvation was previously shown to increase about 12 h after serum readdition, reached a peak value at approximately 18 h, the time of maximal DNA synthesis, and then declined (Nordenskjöld et al., 1970). This pattern closely resembled the variations in R1 and R2 mRNA which we describe, with the important difference that the increase in mRNA levels started about 4 h before the previously reported increase in enzyme activity. This was much later than the appearance of the proliferation-related c-*fos*, c-*myc* (Lau & Nathans, 1987), or c-*jun* (Ryseck et al., 1988) transcripts in the same system, but was similar to the increase of thymidine kinase mRNA (Coppock & Pardee, 1987).

Ribonucleotide reductase activity is dependent upon the presence of both the R1 and R2 subunits. In earlier measurements of the protein levels of the individual subunits during the cell cycle, the expression of the two subunits seemed to be differentially regulated. The levels of the R1 subunit were constant and in excess during the cell cycle while the R2 subunit increased 3–7-fold as cells progressed from G_0/G_1 to S phase (Eriksson et al., 1984; Engström et al., 1985). Unexpectedly, our present data show that the R1 and R2 transcripts are regulated in parallel. We conclude that the constant level of R1 subunit protein during the cell cycle reflects the long half-life (more than 20 h) of the R1 protein.

This is also indirectly supported by data obtained from terminally differentiating HL 60 human promyelocytic leukemia cells, where growth arrest was seen 48 h after the addition of the differentiation-inducing agent, but R1 protein could be detected for up to 8 days (Mann et al., 1988).

In the case of the genes for histones (Heintz et al., 1983; Sittman et al., 1983; Alterman et al., 1984; Schumperli, 1986), thymidine kinase (Stewart et al., 1987), and thymidylate synthase (Jenh et al., 1985), there is evidence that both transcriptional and posttranscriptional controls occur. However, there are significant levels of both thymidine kinase (Lieberman et al., 1988) and thymidylate synthase (Ayusawa et al., 1986) mRNA in serum-starved cells, whereas the levels of R1 and R2 mRNA in isoleucine-starved TA3 cells or in serum-starved BALB/3T3 cells were virtually undetectable. The threshold of quantitation was 2 and 4 molecules of mRNA/cell, respectively, in those experiments. Consequently, the rapid increase in R1 and R2 transcripts observed when isoleucine- or serum-repleted cells progressed into S phase most likely resulted from increased transcription and not from stabilization of existing transcripts. The results obtained after serum refeeding of mouse 3T3 cells indicate a minimum half-life of both the R1 and R2 transcripts of about 2 h between 20 and 26 h after addition of serum (Figure 5).

It can be argued that the isoleucine and serum starvation methods perturb normal cellular processes. The values obtained with these methods may be more representative of quiescent cells starting to proliferate than of normal cell cycle events. However, in continuously cycling cells separated by centrifugal elutriation, R1 and R2 transcript levels *per cell* in the S-phase-enriched fraction, which contained 56% S-phase cells, were 3–4-fold higher than in the G_1-phase-enriched fraction, which contained 4 times fewer (14%) S-phase cells. The simplest interpretation of these data is that S-phase cells on average contain manyfold higher levels of the R1 and R2 mRNA than do G_1-phase cells. Taken together, these data indicate that expression of the R1 and R2 genes is primarily cell cycle regulated. This contrasts with the proliferation-specific expression of DNA polymerase α (Wahl et al., 1988) and primase p49 (Tseng et al., 1989) genes.

The R1 and R2 transcripts were regulated the same way in hydroxyurea-resistant, R2-overproducing TA 3 cells as in the parental cells, with the exception of the 40-fold higher level of the R2 transcript. It has previously been shown that, at the R2 protein level as well, the hydroxyurea-resistant cells behave the same way during the cell cycle as the parental cells (Eriksson et al., 1984). These mutant, hydroxyurea-resistant cells are therefore a valid model with which to study R1 and R2 gene regulation. The increased R2 mRNA level in the resistant cells probably results from an elevated transcription rate, as has been suggested for another hydroxyurea-resistant mouse cell line on the basis of nuclear runoff transcription analyses (McClarty et al., 1987). Furthermore, R2 gene transcription was recently shown in yeast to be inducible by DNA damage (Elledge & Davis, 1987).

Our data are consistent with a model in which the cell cycle dependent expression of both the ribonucleotide reductase R1 and R2 genes is mainly regulated at the transcriptional level.

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A Hybrid Adenosinetriphosphatase Composed of F_1 of *Escherichia coli* and F_o of *Propionigenium modestum* Is a Functional Sodium Ion Pump†

Werner Laubinger,‡ Gabriele Deckers-Hebestreit,§ Karlheinz Altendorf,§ and Peter Dimroth*,‡

Institut für Physiologische Chemie der Technischen Universität München, 8000 München 40, FRG, and Fachbereich Biologie/Chemie der Universität Osnabrück, 3500 Osnabrück, FRG

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ABSTRACT: Analyses on immunoblots indicated strong binding of the α- and β-subunits of the ATPase of *Propionigenium modestum* to antibodies raised against the corresponding subunits of the F_1F_o ATPase of *Escherichia coli*. Cross-reactivities of antibodies against the other ATPase subunits were not observed. The use of Na^+ or H^+ as alternate coupling ions, observed previously for the *P. modestum* ATPase [Laubinger, W., & Dimroth, P. (1989) *Biochemistry 28*, 7194-7198], is not found for the F_1F_o ATPase of *E. coli*, which is specific for protons. However, a hybrid consisting of the F_1 moiety of the *E. coli* ATPase and F_o of that from *P. modestum* performed Na^+ or H^+ transport in a reconstituted system. As with the homologous ATPase of *P. modestum*, H^+ pumping of the hybrid was abolished at Na^+ concentrations of >1 mM. The F_o sector and not F_1, therefore, determines the cation specificity of these F_1F_o ATPases.

The strict anaerobic bacterium *Propionigenium modestum* grows from the fermentation of succinate to propionate and CO_2 (Schink & Pfennig, 1982). A unique feature of the energy metabolism of this organism if the operation of a Na^+ cycle for ATP biosynthesis [Hilpert et al., 1984; for a review, see Dimroth (1987)]. The free energy of methylmalonyl-CoA decarboxylation is used to pump Na^+ ions out of the cell, and the electrochemical Na^+ ion gradient thus established drives ATP synthesis by a Na^+-translocating ATPase.

It is remarkable that in spite of this unusual cation specificity the ATPase of *P. modestum* has properties typical of an ATPase of the F_1F_o type (Laubinger & Dimroth, 1987, 1988). The structures and functions of the ATPases of *P. modestum* and *Escherichia coli* [for a review, see Schneider and Altendorf (1987)] are in fact very similar. They both consist of a water-soluble F_1 moiety, composed of five different subunits, α, β, γ, δ, and ϵ, that catalyzes hydrolysis (or synthesis) of the terminal phosphoric acid anhydride bond of ATP. The β-subunits of these two enzymes have 69% sequence homology (Amann et al., 1988a). Each F_o moiety is a complex of three different subunits, a, b, and c, that is firmly embedded within the lipid bilayer. It provides the pathway for conduction of cations (H^+ or Na^+) across the membrane.

In the ATPase complexes (F_1F_o) the chemical events of ATP hydrolysis (or synthesis) catalyzed by F_1 are coupled to the transport of cations through F_o across the membrane. Whereas the ATPase of *E. coli* translocates protons, that of *P. modestum* uses Na^+ as the coupling ion (Laubinger & Dimroth, 1988). However, at Na^+ concentrations below 1 mM, protons are also translocated by this enzyme (Laubinger & Dimroth, 1989). The Na^+ binding site was suggested to be located on the F_o portion, because the ATPase activity of F_1F_o of *P. modestum*, but not that of F_1 alone, was specifically activated by Na^+ ions (Laubinger & Dimroth, 1987).

The two ATPases also behaved similarly with respect to the inhibitors dicyclohexylcarbodiimide, venturicidin, tributyltin chloride, and azide (Laubinger & Dimroth, 1988). This homology has been extended by the immunological studies described in this paper. In addition, we report here the formation of a hybrid ATPase consisting of F_1 from *E. coli* and F_o from *P. modestum* and its specificity with respect to Na^+ and H^+ translocation.

Experimental Procedures

Materials. Fluorescein isothiocyanate conjugated goat anti-rabbit IgG was purchased from Sigma (Munich, FRG).

Bacterial Growth. Escherichia coli ML308-225 was grown in minimal medium (Davis & Mingioli, 1950) with 0.4% glucose as the energy source. *Klebsiella pneumoniae* (ATCC 138 2) was grown on citrate medium supplemented with L-(+)-glutamic acid (3 mM) and *d*-biotin (7 μg/L) as described (Dimroth, 1986). *Propionigenium modestum* was grown on succinate under strictly anaerobic conditions (Laubinger & Dimroth, 1988). *Veillonella alcalescens* was grown anaerobically on lactate as the energy source (Hilpert & Dimroth, 1986).

Preparative Procedures. Membranes of *E. coli* wild-type ML 308-225, *K. pneumoniae*, and *V. alcalescens* were prepared as described (Vogel & Steinhart, 1976). EF_1[1] (Steffens

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* Address correspondence to this author at Mikrobiologisches Institut ETH-Zentrum, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland.
‡ Institut für Physiologische Chemie der Technischen Universität München.
§ Fachbereich Biologie/Chemie der Universität Osnabrück.

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