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Immunocytochemical evidence for the cytoplasmic localization and differential expression during the cell cycle of the M1 and M2 subunits of mammalian ribonucleotide reductase

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Mammalian ribonucleotide reductase consists of two non-identical subunits, proteins M1 and M2. We have produced and characterized rat polyclonal and monoclonal antibodies directed against protein M2 of mouse ribonucleotide reductase. Using these antibodies for immunocytochemical studies, an exclusively cytoplasmic localization of protein M2 was demonstrated both in cultured parent and hydroxyurea-resistant, M2-overproducing mouse TA3 cells, and in cells from various mouse tissues. These data, together with the previously demonstrated cytoplasmic localization of the M1 subunit, clearly show that ribonucleotide reductase is a cytoplasmic enzyme. Combining the anti-M2 antibodies with a monoclonal anti-M1 antibody allowed for double-labelling immunofluorescence studies of the two subunits in individual cells. Only ~50% of the cells in a logarithmically growing culture contained immunodetectable protein M2, while the M1-specific staining was present in all cells. The M2 staining correlates well with the proportion of cells in the S-phase of the cell cycle. In tissues, only actively dividing cells stained with either antibody and there were always fewer cells stained with the M2-antibodies than with the M1-antibody. Our data therefore present independent evidence for the earlier proposed model of a differential regulation during the cell cycle of the M1 and M2 subunits of ribonucleotide reductase.

Key words: cytoplasmic localization/differential cell cycle expression/double immunocytochemistry/rat hybridoma/ribonucleotide reductase

Introduction

Ribonucleotide reductase is present in all dividing cells, furnishing the cell with a balanced supply of the four deoxyribonucleotides needed for DNA synthesis (Thelander and Reichard, 1979). The mammalian enzyme consists of two non-identical subunits, proteins M1 and M2. Both subunits have been purified to homogeneity and characterized in detail (Engström *et al.*, 1979; Thelander *et al.*, 1980, 1985). Recently, cDNA clones encoding the M1 and M2 proteins of mouse ribonucleotide reductase were isolated and sequenced (Caras *et al.*, 1985; Thelander and Berg, 1986). Protein M1 is a dimer of mol. wt 170 000 which contains the binding sites for nucleoside triphosphates and which is responsible for allosteric regulation of the enzyme (Eriksson *et al.*, 1979; Thelander *et al.*, 1980). Protein M2 is a dimer of mol. wt 90 000, containing stoichiometric amounts of non-heme iron and a unique tyrosyl free radical essential for activity (Gräslund *et al.*, 1982; Thelander *et al.*, 1985).

The activity of ribonucleotide reductase is cell-cycle dependent, reaching its maximum during S-phase (Thelander and Reichard, 1979). Using synchronized cell populations, it was demonstrated that the variation in holoenzyme activity is regulated by *de novo* synthesis and breakdown of the M2 subunit, with a half-life of 3 h (Eriksson *et al.*, 1984). In contrast, the level of the M1 subunit is constant and in excess throughout the cell cycle and the half-life is at least 15 h (Engström *et al.*, 1985; Mann *et al.*, 1987). Previous results using monoclonal antibodies directed against the M1 subunit demonstrated a cytoplasmic localization of this protein (Engström *et al.*, 1984). This is in contrast to the 'replitase' model of Prem veer Reddy and Pardee (1979, 1982) which suggested a nuclear localization of ribonucleotide reductase in S-phase cells. The immunocytochemical results also showed that protein M1 is only present in actively dividing cells and is absent in terminally differentiated cells that have stopped synthesizing DNA.

In this paper we describe the production of polyclonal and monoclonal antibodies against the M2 subunit of mouse ribonucleotide reductase and their use in experiments which demonstrate an exclusive cytoplasmic localization of this protein as well. Furthermore, the differential regulation of the levels of M1 and M2 subunits during the cell cycle is demonstrated in individual cells.

Results

Isolation and characterization of rat polyclonal and monoclonal antibodies directed against subunit M2 of mouse ribonucleotide reductase

A highly purified preparation of protein M2 from hydroxyurea-resistant, M2-overproducing mouse mammary tumor TA3 cells was used for the immunization of LOU-rats. Polyclonal antibodies were prepared from the rat serum and monoclonal anti-M2 antibodies were obtained by fusing spleen cells from an immunized LOU-rat with rat myeloma cells by the hybridoma technique (see Materials and methods).

The rat polyclonal anti-M2 antibodies very efficiently inhibited ribonucleotide reductase activity as shown in Figure 1a. In contrast, not even a large excess of any of the monoclonal antibodies could directly inhibit enzyme activity (data not shown). Therefore, an indirect inhibition assay was used in which the monoclonal anti-M2 antibodies were coupled to Sepharose, and then incubated with partially

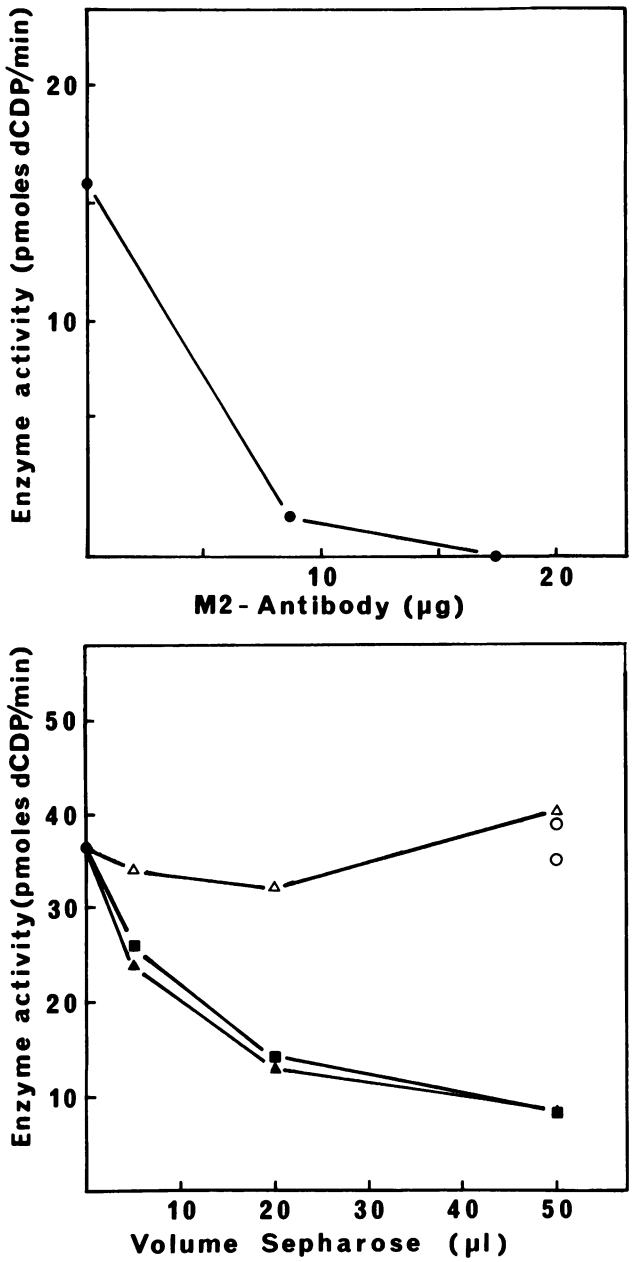

Fig. 1. (**a**) Inhibition of ribonucleotide reductase activity by the polyclonal anti-M2 antibody. Increasing amounts of antibody were pre-incubated with 10 µg of pure protein M1 and 2.5 µg of partially purified protein M2 (enzyme preparations described in Materials and methods) for 60 min at 0°C. The [^{3}H]cytidine diphosphate ([^{3}H]CDP) assay mixture was then added and the amount of [^{3}H]deoxycytidine diphosphate ([^{3}H]CDP) formed/min was measured as described previously (Engström *et al.*, 1979). (**b**) Indirect inhibition of ribonucleotide reductase activity by Sepharose-linked monoclonal anti-M2 antibodies. The antibodies were first coupled to CNBr-activated Sepharose (Pharmacia, Sweden), according to the instructions of the manufacturer (10 mg of antibody was coupled per gram of dry gel). Increasing amounts of a 50% suspension of Sepharose-coupled antibody in 0.1 M KCl, 50 mM Tris–HCl, pH 7.6, 1 mg/ml BSA were mixed with a 50% suspension of uncoupled Sepharose in the same buffer to a constant volume of 50 µ. Partially purified protein M2 (20 µl, 0.4 mg/ml) was then added to all tubes and incubated with the Sepharose mixtures for 120 min at 0°C. Control experiments were made in tubes containing protein M2 and only uncoupled Sepharose. After centrifugation, 35 µl of each supernatant were mixed with 12 µg of pure protein M1 and with the [^{3}H]CDP assay mixture. Finally, ribonucleotide reductase activity was measured as described (Engström *et al.*, 1979). Uncoupled Sepharose (○), Sepharose coupled with antibody JB 4(▲), JC 4 (■) and with an unrelated rat monoclonal antibody (△).

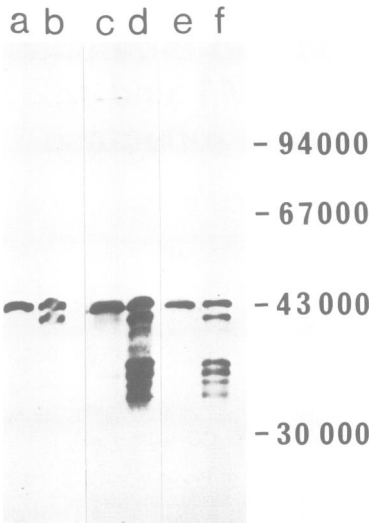

Fig. 2. Immunoblot assay of anti-M2 antibodies. Immunoblots of a crude extract from M2-overproducing TA3 cells (**lanes a, c** and **e**) or a highly purified protein M2 (**lanes b, d** and **f**) were prepared as described under Materials and methods. After electrophoresis and transfer, the membrane was cut into three identical strips and incubated with antibody in the following order. The strip containing **lanes a** and **b** was incubated with the monoclonal antibody JB 4 (0.5 µg/ml), **lanes c** and **d** with the monoclonal antibody JC 4 (0.5 µg/ml) and **lanes e** and **f** with the polyclonal antibody (5 µg/ml).

purified mouse protein M2. Following centrifugation to remove protein M2 bound to the Sepharose-linked antibodies, the supernatants were assayed for enzyme activity in the presence of protein M1 (Figure 1b). Using this method, two monoclonal antibodies, called JB 4 and JC 4, were shown to inhibit ~80% of the enzyme activity. No inhibition was observed using uncoupled Sepharose or Sepharose linked to an unrelated rat monoclonal antibody.

To study further the specificity of the protein M2 antibodies, a crude extract from M2-overproducing TA3 cells was analyzed by an immunoblot assay. As shown in Figure 2 lanes a, c and e all antibodies reacted with one single band which had a mol. wt of ~43 kd. No cross-reactivity was observed with protein M1, which has an approximate mol. wt of 84 kd, in this immunoblot or in other blots using purified M1 preparations (data not shown). During purification, protein M2 is often proteolytically degraded, and therefore our highly purified preparations contain variable amounts of partially cleaved protein M2 (Thelander *et al.*, 1985). As shown in the immunoblot analysis of highly purified protein M2, antibody JB 4 bound only to the full-length M2 polypeptide chain and to a slightly degraded polypeptide (Figure 2, lane b). Antibody JC 4 and the polyclonal antibodies also reacted with a set of shorter polypeptide chains (Figure 2, lanes d and f), reflecting their ability to bind to an epitope still present in proteolytically degraded protein M2. This shows that the antibodies JB 4 and JC 4 are directed against different determinants on protein M2.

Localization and differential expression of subunits M1 and M2 of ribonucleotide reductase in cultured cells

The results of immunofluorescence studies on cultured parent or M2-overproducing mouse TA3 cells, using the polyclonal antibodies or the monoclonal JB 4 antibody directed against

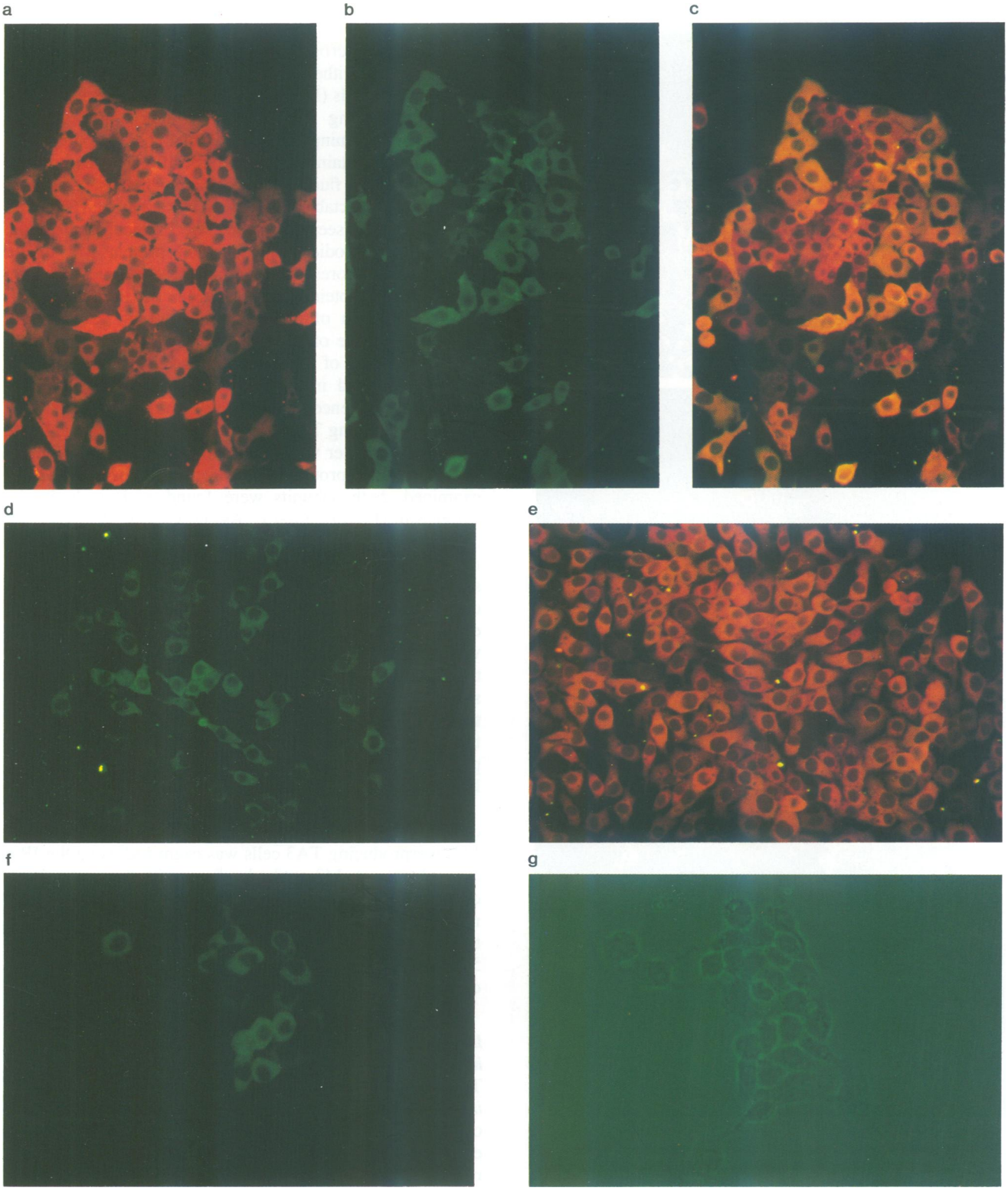

Fig. 3. Localization and differential expression of proteins M1 and M2 in parental and M2-overproducing mouse TA3 cells. The fluorescence micrographs were obtained using single or double labelling as described in Materials and methods. Bars: 50 μm (**a**–**c**) M2-overproducing cells stained with (**a**) mouse monoclonal anti-M1 antibody AD 203, (**b**) rat polyclonal anti-M2 antibodies and (**c**) double exposure of (a) and (b); cells containing only protein M1 fluoresce in red, cells containing only protein M2 fluoresce in green and cells containing both proteins fluoresce in yellow. (**d** and **e**) Parental TA3 cells stained with (**d**) rat polyclonal anti-M2 antibodies as in (b) but using a longer exposure time, (**e**) both M1- and M2-antibodies as in (c) but using a longer exposure time. (**f** and **g**) M2-overproducing cells (**f**) stained with the rat monoclonal anti-M2 antibody JB 4 at a concentration of 10 μg/ml, (**g**) phase-contrast micrograph of the same visual field as in (f).

protein M2, are illustrated in Figure 3b, d and f. In all experiments, using both types of antibodies and cells, protein M2 showed cytoplasmic localization without any detectable nuclear staining. The M2-overproducing TA3 cells were earlier shown to have a 40-fold increased M2-specific tyrosyl free radical concentration compared with the parental

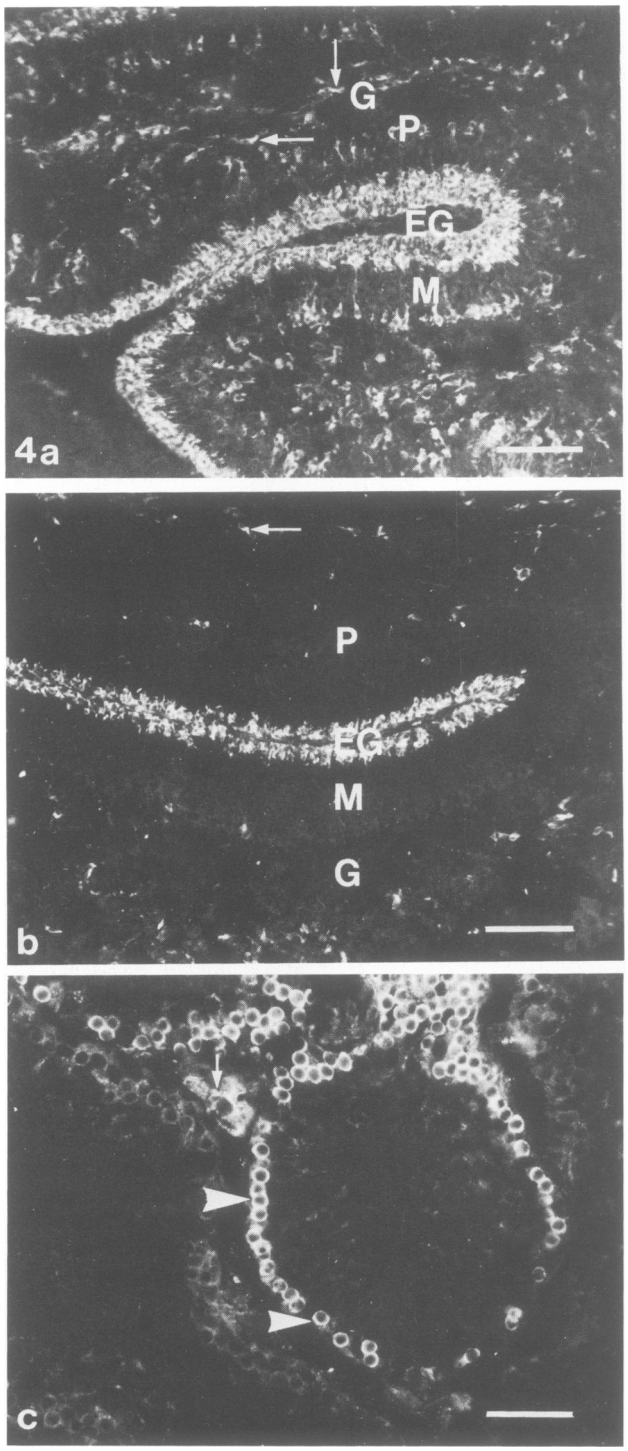

Fig. 4. Fluorescence micrographs of mouse tissues stained with polyclonal anti-M2 antibodies as described in Materials and methods. (**a**) Cerebellum 3 days postnatally. Strongly positive staining of proliferating, immature cells are seen in the broad external granular (EG) layer. Numerous M2-positive glial cells (arrows) are found in the Purkinje (P) cell layer, the granular (G) layer and the molecular (M) layer. Bar: 100 μm. (**b**) Cerebellum 9 days postnatally. At this stage of development the external granular layer is reduced to a few cells in width. The arrow shows one of the few positively stained glial cells. Bar: 100 μm. (**c**) Seminiferous tubules of testis. A tubule is seen in the center with a ring of positively stained spermatogonia (arrow). Note the weak M2-immunoreactivity of the spermatogonia in the adjacent tubules. In general, no staining was seen in the absence of the primary antibody, although a non-specific reaction due to the fluorescein-streptavidin conjugate was seen in the Leydig interstitial cells (small arrow). Bar: 50 μm.

cells (Thelander *et al.*, 1985). In Figure 3b and d, the fluoroscence micrographs from parallel immunostaining experiments on either M2-overproducing (Figure 3b) or on parental TA3 cells (Figure 3d) are shown. As expected, the M2-overproducing cells showed a much more intense fluorescence staining than the parental cells, strongly indicating that the staining was M2 specific. However, upon longer exposure, fluorescence staining which was otherwise only poorly detectable was visible in the parental TA3 cell line, as can be seen in Figure 3d. Preincubation of the polyclonal antibodies with purified protein M2 totally abolished the fluorescence staining, whereas preincubation with purified protein M1 had no effect (data not shown). Finally omission of the primary antibody resulted in a complete absence of staining.

Both subunits of ribonucleotide reductase were simultaneously studied in individual cells by double-labelling immunofluorescence experiments both on parental and M2-overproducing TA3 cells using the polyclonal anti-M2 antibodies together with the monoclonal antibody AD 203 directed against protein M1 (Engström, 1982). In all cells examined, both subunits were found exclusively in the cytoplasm (Figure 3a–c). As previously demonstrated (Engström *et al.*, 1984), protein M1 was present in all cells in a logarithmically growing culture (Figure 3a). When the same cells were examined for protein M2-specific fluorescence, there was a great variation in intensity between different cells (Figure 3b). By double exposure at two different wavelengths we could visualize the presence of either protein M1 or M2 alone or the presence of both proteins in every single cell (Figure 3c). Again, we found that all cells contained protein M1 while only ~50% of the cells also contained protein M2. The same result was obtained using parental TA3 cells, although these cells contained much lower levels of protein M2 (Figure 3e).

Finally, the variation in M2-specific staining between different cells in a logarithmically growing culture of M2-overproducing TA3 cells was examined using the JB 4 monoclonal anti-M2 antibody. Figure 3f shows the fluorescence micrograph and Figure 3g the phase-contrast micrograph and Figure 3g the phase-contrast micrograph of the same visual field from this experiment. Clearly, only 50% of the cells showed M2-specific immunoreactivity, confirming the results obtained with polyclonal antibodies.

Localization of subunit M2 of ribonucleotide reductase in developing mouse tissues

To confirm our *in vitro* results using cells in tissue culture, *in vivo* studies of protein M2 in normal tissues were carried out. The neonatal mouse cerebellum is characterized by an external granular layer containing immature and proliferating neurons. This layer gradually decreases in width during development and has completely disappeared 21 days postnatally (Altman, 1982). Immunofluorescence studies of cerebellar section of mice pups using the polyclonal anti-M2 antibodies were therefore performed at various times post-partum and the results are shown in Figure 4a and b. At day 3, strongly M2-positive stained cells were seen in the broad external granular layer. In the Purkinje cell layer and internal granular layer a number of proliferating glial cells were also positively stained (Figure 4a). At day 9, many cells in the external granular layer contained immunoreactive protein M2 but the layer was reduced to a few cells in width.

In the surrounding cell layers only a few positively stained cells were seen at this stage of development (Figure 4b). In conclusion, protein M2 immunoreactivity was preferentially found in the cell layers known to have a large proportion of actively dividing, immature cells (Altman, 1982). Furthermore, the proportion of cells which showed protein M2-positive staining was always smaller than the proportion of protein M1-immunoreactive cells in the same tissue (data not shown). Again the M2-immunoreactivity was exclusively present in the cytoplasm of the cells.

Similar immunofluorescence studies of testicular sections from adult mice demonstrated that protein M2 was present in the proliferating spermatogonia of the seminiferous tubules (Figure 4c). Within each tubule the M2-positive fluorescence was relatively constant in all spermatogonia but a great variation in the staining intensity was observed when different tubules were compared. In this case as well, M2-specific fluorescence was only detected in the cytoplasm of the spermatogonia.

Discussion

We have produced monoclonal and polyclonal antibodies, specifically directed against the M2 subunit of mouse ribonucleotide reductase. The M2-antibodies were used together with monoclonal M1-antibodies in immunocytochemical studies of the intracellular localization and of the cell-cycle-dependent expression of ribonucleotide reductase. The advantage of using this immunocytochemical approach was that we could simultaneously study the localization and expression of each of the two enzyme subunits in individual cells.

Our results demonstrate that the M1 and M2 subunits of ribonucleotide reductase are differentially regulated during the cell cycle. According to flow cytometry, ~50% of the cells utilized in the experiments shown in Figure 3 were in the S-phase of the cell cycle. The same proportion of cells showed M2-specific fluorescence. These data are consistent with the results of earlier studies which showed that the tyrosyl radical of protein M2 is specifically expressed during S-phase, while protein M1 is expressed constitutively and at constant levels throughout the cell cycle (Eriksson *et al.*, 1984; Engström *et al.*, 1985). These earlier results were obtained studying cell populations; however, the studies presented above provide evidence for a differential cell cycle regulation of the M1 and M2 subunits of ribonucleotide reductase in individual cells.

In spite of the greatly enhanced level of M2-specific staining intensity in M2-overproducing, hydroxyurea-resistant mouse TA3 cells, the strict cell cycle regulation of the expression of protein M2 was retained in these cells (Figure 3b and f). It has been shown that the M2 gene is amplified 5-fold in this drug-resistant TA3 cell line and that the cells contain a 50- to 100-fold excess of M2 mRNA compared with the parental TA3 cells (Thelander and Berg, 1986). It was found in the same study that the level of M1-specific mRNA was also enhanced ~10-fold although in M1-gene was not amplified. Indeed, we also observed a stronger M1-specific immunostaining in the hydroxyurea-resistant TA3 cells compared with the parent TA3 cells (comparative data not shown).

The role of protein M2 in cell cycle control is not known, nor are the effects of out-of-phase production of protein M2 clearly defined. The work of Thelander and Berg (1986) suggests that constant expression of protein M2 throughout the cell cycle is lethal to cells. In this case, attempts to express protein M2 constitutively from a mouse M2 cDNA, which was cloned into the pcDV1 expression vector, did not yield any stable M2-producing transformants in mouse 3T6 cells. In contrast, the same construct could be expressed at significant levels using a transient assay in COS-7 monkey cells. Furthermore, Standart *et al.* (1986) have investigated the expression of ribonucleotide reductase in clam oocytes. Although the M1-like component is stored as protein, only stored message encoding the equivalent of the M2 protein, and not the protein itself, is found in these cells. In the light of both these and our results, we suggest that it is necessary for cell viability that protein M2 is not present throughout the cell cycle, but that its function is restricted to the S-phase.

Our observation that proteins M1 and M2 were differentially expressed in cultured cells was extended by studying the expression of the two proteins in normal mouse tissues. Both proteins M1 and M2 were preferentially found in developing tissues, known to contain a high proportion of actively dividing cells. The fraction of M2-immunoreactive cells, in mouse tissue, was always smaller than that of M1-positive cells. In mouse testis, we observed that protein M2 was expressed at an almost constant level in all spermatogonia within each segment of a single seminiferous tubule and that there was a pronounced variation in staining intensity between adjacent tubules. This observation also supports a cell-cycle-dependent expression of protein M2 since the spermatogonia in the same segment of each tubule divide in a synchronous manner (Clermont, 1972).

A further aim of this study was to ascertain the intracellular localization of ribonucleotide reductase. We have previously shown that the M1 subunit is present in excess over the M2 subunit and that ribonucleotide reductase activity during the cell cycle is controlled by the amount of protein M2 (Engström *et al.*, 1985). Therefore our previous demonstration of an exclusively cytoplasmic localization of the M1 subunit did not exclude the replitase model of Prem veer Reddy and Pardee (1980, 1982), which proposes a nuclear localization of ribonucleotide reductase in S-phase cells. However, our demonstration of an exclusively cytoplasmic localization of the M2 subunit as well, both in cultured cells and in mouse tissues, strongly argues against the replitase model. Rather, our results indicate that ribonucleotide reduction takes place in the cytoplasm and that the deoxyribonucleotides are transported into the nucleus to participate in DNA synthesis.

Materials and methods

Cell cultures

The rat myeloma cell in Y3/Ag 1.2.3. (Galfré *et al.*, 1979) and the rat hybridoma cells were grown as suspension cultures in Dulbecco's modified Eagle's medium plus 10% heat-inactivated fetal calf serum. The atmosphere was 10% CO_2 – air. Monolayer cultures of the parental and hydroxyurea-resistant, M2-overproducing mouse mammary tumor TA3 cell lines were grown as described by Thelander *et al.* (1985).

Enzyme preparations

Protein M2 was purified from M2-overproducing, hydroxyurea-resistant mouse mammary tumor TA3 cells according to Thelander *et al.* (1985). Partially purified protein M2 refers to material after DEAE-chromatography and highly purified protein M2 refers to material after affinity chromatography on antibody YL 1/2-Sepharose. Protein M1 was purified from calf thymus as described previously (Thelander *et al.*, 1980).

Immunization

LOU-rats (originally obtained from the Centre de Selection et d'Élèvage d'Animaux de Laboratoire, Orléans) were immunized with the highly purified preparation of protein M2 of mouse ribonucleotide reductase. Protein (60–80 μg) was emulsified in Freund's complete adjuvant and injected s.c. The rats were boosted i.p. with 20 μg of the same protein in incomplete Freund's adjuvant after 4 weeks. One rat was then boosted in the same way four times at 4-week intervals. At day 10 after every boost, the rat was bled and polyclonal anti-M2 serum was obtained. Another rat was injected i.v. 4 weeks after the first boost with 20 μg of protein M2 without adjuvant and then used for production of rat hybridoma cell lines.

Fusion and derivation of hybridoma cell lines

Four days after the i.v. injection the rat was killed and the spleen was removed. Fusion was made with 3×10^8 spleen cells and 10^8 rat myeloma cells, strain Y3/Ag-1.2.3., as described elsewhere (Fazekas de St Groth and Scheidegger, 1980). All supernatants were tested for the presence of anti-M2 antibodies by ELISA and positive hybridoma lines were cloned by limiting dilution and the monoclonal antibodies were further tested on immunoblots.

Preparation of polyclonal and monoclonal anti-M2 antibodies

Polyclonal anti-M2 antibodies were prepared from the rat anti-M2 serum and monoclonal antibodies were produced either by incubating the hybridoma cells (0.5×10^6 cells/ml) in serum-free tissue culture medium for 7 days or by induction of ascites tumors. All types of antibody preparations were purified by ammonium sulfate precipitation followed by dialysis against 0.1 M KCl in 50 mM Tris–HCl, pH 7.6, as described earlier (Engström, 1982). The polyclonal antibodies were further purified by affinity chromatography on Staphylococcus Protein A Sepharose (Pharmacia, Sweden) according to the instructions of the manufacturer.

ELISA

This assay was performed as described previously except that highly purified protein M2 was used as antigen (4 μg/ml, 50 μl/well) (Engström, 1982).

Immunoblot assay

Crude extract (5 μg) from M2-overproducing, hydroxyurea-resistant mouse mammary tumor TA3 cells or 0.5 μg of highly purified protein M2 was analyzed on 10% linear sodium dodecyl sulfate–polyacrylamide gels, as described previously (Engström *et al.*, 1979). The separated proteins were then transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). The transfer was performed for 12–16 h at 250 mA at 20°C. The membranes were blocked in 50 mM Tris–HCl, pH 7.6, in saline (TBS) containing 0.5% Tween 20 and 1% bovine serum albumin for 1h. Incubation with alkaline phosphatase-conjugated rabbit anti-mouse anitbodies (Sigma), washing and development of the alkaline phosphatase reaction were carried out according to Blake *et al.* (1984), except that the veronal acetate buffer was replaced by 0.1 M ethanolamine–HCl, pH 9.6.

Preparation of cells for immunocytochemistry

Cells were grown as monolayers on glass coverslips as described earlier (Engström *et al.*, 1984). The cells were rinsed with serum-free medium for 2×5 min at 37°C, fixed in 4% (w/v) freshly prepared paraformaldehyde solution in 10 mM sodium phosphate-buffered saline (PBS), pH 7.3, for 30 min at 20°C, rinsed in PBS 3×20 min and permeabilized with 0.2% Triton X-100 in PBS for 2 min followed by several changes of PBS.

Preparation of tissues for immunofluorescence microscopy

Adult BALB/c mice and pups ranging in postnatal age from 3 to 9 days were anaesthetized with Nembutal and fixed by transcardial perfusion with 4% (w/v) freshly prepared paraformaldehyde in PBS as previously described (Engström *et al.*, 1984). Sections at a nominal thickness of 7 μm were obtained on a cryostat.

Immunocytochemistry

TBS containing 0.04% Triton X-100 was used for all antibody dilutions and rinsing steps. Non-specific antibody adsorption was blocked by preincubating the samples in 2% normal swine serum for 30 min at 20°C. Swine serum (2%) was also included in all dilutions and incubations with the specific antibodies. Mouse monoclonal anti-M1 antibody AD 203 was used at a concentration of 50 μg/ml, rat polyclonal anti-M2 antibodies were diluted to 15 μg/ml and rat monoclonal anti-M2 antibody JB 4 was used at concentrations ranging from 1 to 50 μg/ml. Double-labelling experiments with rat polyclonal anti-M2 antibodies and mouse monoclonal anti-M1 antibody AD 203 were performed by the simultaneous incubation with both antibodies at the concentrations indicated above. All incubations with primary antibodies were performed overnight at 4°C. After several rinses in TBS, the cells were processed for visualization of the primary antibody reaction. Double-labelled cells were first incubated with swine anti-mouse antibodies labelled with rhodamine (DAKO, Denmark) (diluted 1:30), rinsed and then incubated with biotinylated donkey anti-rat antibodies (diluted 1:100) followed by streptavidin–fluorescein isothiocyanate (FITC) (Amersham International, UK, 5 μg/ml). For single labelled cells, the biotin–streptavidin system was used for all rat antibodies, while a donkey anti-mouse antibody FITC conjugate (Amersham) was used for the detection of the mouse monoclonal antibodies. The slides were mounted as described before (Engström *et al.*, 1984) and observed in a microscope using epi-illumination and appropriate filters for fluorescein and rhodamine.

Tissue sections were processed as described above, except that no double-labelling experiments were performed.

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