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MMH Cells: An In Vitro Model for the Study of Retinol-Binding Protein Secretion Regulated by Retinol

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The untransformed stable cell line Met murine hepatocytes (MMH), generated from liver explants of transgenic mice expressing a constitutively active truncated form of the human hepatocyte growth factor receptor (cyto-Met), represents an innovative tool for in vitro studies of liver function. In the present report, we show that the MMH-D3 line isolated from the liver of a 3-day-old mouse is a useful model to investigate the regulation of the synthesis and secretion of retinol-binding protein (RBP) by retinol (vitamin A alcohol). Experiments with Northern blot hybridization, metabolic labeling of cellular proteins followed by immunoprecipitation, and Western blot analysis demonstrated that, similarly to the in vivo situation, in MMH-D3 cells the presence of retinol does not affect transcriptional and translational rate of the RBP gene but is essential for regulating the secretion rate of the protein. Unlike HepG2 human hepatocarcinoma cells used thus far in studies of retinoid metabolism, including the synthesis and secretion of RBP, vitamin A deficiency causes, in MMH-D3 cells, the inhibiton of RBP secretion and the protein accumulation in the cell, whereas retinol repletion promptly results in RBP secretion. This model will be very useful in future studies on vitamin A distribution in the organism. J. Cell. Physiol. 181:24–32, 1999.

Retinol (vitamin A alcohol) is a nutrient essential for fundamental processes such as vision, reproduction, and maintenance of differentiated epithelia (Blomhoff, 1994). Molecular mechanisms mediating retinol action are increasingly better understood, and much has been elucidated about the role of retinol and its metabolites in the control of gene expression (Mangelsdorf et al., 1994; Perozzi et al., 1998). Although the exact vitamin A requirement in different physiological and pathological conditions is still unknown, it is well documented that a concentration of the vitamin beyond the physiological range is harmful for both embryonic and adult cells; for this reason, elaborate mechanisms have been developed to regulate vitamin A homeostasis in the body.

In the liver, two cell types are involved in retinoid storage and metabolism: parenchimal cells (hepatocytes) and stellate cells (fat-storing cells or Ito cells). Specifically, parenchymal cells are directly involved in the uptake of retinoids introduced with the diet and circulating in chylomicron remnants, whereas stellate cells are the principal storage site for retinyl esters. The role of liver cells in hepatic retinol metabolism has been well assessed, but the way in which retinol moves among hepatocytes, stellate cells, and plasma still requires a better understanding, and therefore new models are necessary for its study. To meet retinoids systemic needs, retinol is secreted from parenchymal cells bound to its specific plasma transport protein, the retinol-binding protein (RBP), which solubilizes the hydrophobic retinol molecules and delivers them to the peripheral target tissues (Soprano and Blaner, 1994). In vitamin A deficiency, RBP secretion is blocked and, accordingly, apo-RBP accumulates in the endoplasmic reticulum (ER), even though the transcriptional and translational rates are unaltered. After retinol administration, the secretion of accumulated RBP is promptly resumed. Smith et al. (1985) postulated that retinol binding induces a conformational change of RBP that allows its exit from the ER.

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The availability of an experimental model to investigate the secretion of retinol bound to its carrier and the regulation of this process is crucial for elucidating retinol metabolism. What is known is principally based on in vivo experiments using rats and primary cultures of hepatocytes. The current models consist mainly of hepatoma cell lines, which poorly mimic in vivo conditions because crucial control mechanisms such as the influence of retinol on RBP secretion are not fully retained by transformed cell lines (Tosetti et al., 1992). We believe that this problem can be overcome by using the hepatocyte cell line Met murine hepatocyte (MMH), generated by Amicone et al. (1995) from liver explants at different developmental stages of transgenic mice harboring a truncated form of c-MET (the hepatocyte growth factor receptor). MMH cells stably grow in vitro and display characteristics typical of hepatocytes as indicated by morphological, ultrastructural, cytochemical, and functional criteria. MMHs are not transformed because they fail to grow on soft agar and are unable to give rise to tumors in nude mice; they retain epithelial cell polarity, express hepatocyte-enriched transcription factors and differentiated liver products, and are capable of reproducing complex liver functions such as hematopoiesis support (Amicone et al., 1997; Aiuti et al., 1998; Spagnoli et al., 1998).

The results in the present article show that the MMH-D3 line isolated from the liver of a 3-day-old mouse provide a suitable model to study retinol-regulated RBP secretion. Moreover, MMH-D3 cells will be useful in future studies to identify factors involved in RBP retention and secretion, which are crucial for understanding the molecular mechanisms underlying vitamin A homeostasis.

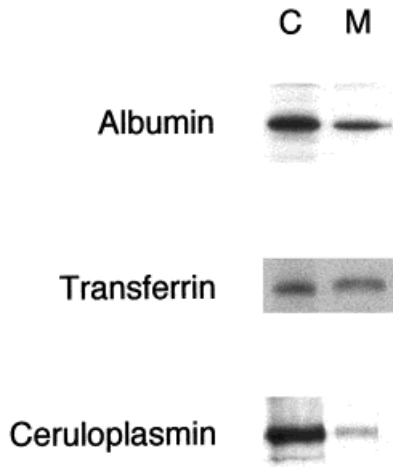

Fig. 1. Met murine hepatocyte (MMH)–D3 cells synthesize and secrete liver specific proteins. Confluent D3 cells were labeled for 90 min with ^{35}S-methionine-cysteine (100 μCi/ml). Cells (C) and culture medium (M) were subjected to immunoprecipitation with albumin, transferrin, and ceruloplasmin polyclonal antibody, analyzed on 10% SDS-PAGE, and fluorographed.

MATERIALS AND METHODS

Chemicals

Nitrocellulose filter (Protran BA85) and nylon membranes (Nytran Plus) were purchased from Schleicker & Schuell (Keene, NH). Radioimmunoassay-grade bovine serum albumin (BSA), collagen type III insulin (from bovine pancreas), retinol, and delipidized fetal calf serum were obtained from Sigma Aldrich (Milan, Italy). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium, and penicillin-streptomycin solution were obtained from Eurobio (Les Ulis, France). Recombinant human insulin growth factor (IGF-II) and epithelial growth factor (EGF) (tissue culture grade) were obtained from Chemicon International Inc. (Temecula, CA). Rabbit polyclonal antibodies against human RBP were obtained from Dako (Glostrup, Denmark). [^{35}S]methionine-cysteine (Expre^{35}S^{35}S, specific activity $>$ 1,000 Ci/mmol) was purchased from DuPont-deNemours Italiana (Cologno Monzese, Italy). Goat antibody against rabbit IgG and SuperSignal Chemiluminescent Substrate were obtained from Pierce (Rockford, IL). Hyperfilm™ ECL™ was purchased from Amersham Italia (Milan, Italy).

Cell culture

Human hepatocarcinoma HepG2 cells (ATCC HB 8065) were grown in DMEM supplemented with 4 mM glutamine, 100 U/ml penicillin-streptomycin, 1% non-essential amino acids, and 10% FCS. D3 cells were grown in RPMI 1640 supplemented with 4 mM glutamine, 100 U/ml penicillin-streptomycin, 10% FCS, 30 ng/ml IGF II, 50 ng/ml EGF, and 0.2 U/ml insulin on plates treated with collagen type III, 40 ng/ml, in glacial acetic acid.

To induce retinol deficiency, semiconfluent cells were grown for 72 h in medium containing delipidized FCS; control semiconfluent cells were grown for 72 h in medium containing delipidized FCS supplemented with 3 μM retinol dissolved in ethanol.

RNA analysis

For Northern blot analysis, total RNA was extracted, as described by Chirgwin et al. (1979), from D3 cells grown for 72 h in the following three conditions: medium containing delipidized serum, medium containing delipidized serum supplemented with 3 μM retinol (complete medium), and medium containing delipidized serum and then supplemented with 3 μM retinol for 3 h.

RNA samples (20 μg/lane) were resolved by electrophoresis in agarose–1.85 M formaldehyde gel and blotted onto nylon membranes (Amersham Italia). Blots were hybridized with α^{32}P probes specific for RBP (Colantuoni et al., 1983) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization at 37°C overnight in 50% formamide hybridization buffer (Hybrisol-Oncor, Gaithersburg, MD). The filter was washed for 15 min at 60°C in 2× standard saline citrate (SSC)–0.1% sodium dodecylsulfate (SDS), twice for 15 min at 60°C in 2× SSC–0.1% SDS, and once more for 15 min at 60°C in 0.5× SSC–0.1% SDS (Sambrook et al., 1989). The abundance of specific transcripts in each lane was directly quantified on the filter in the Phosphoimager Analyzer (Canberra Packard, Meriden, CT).

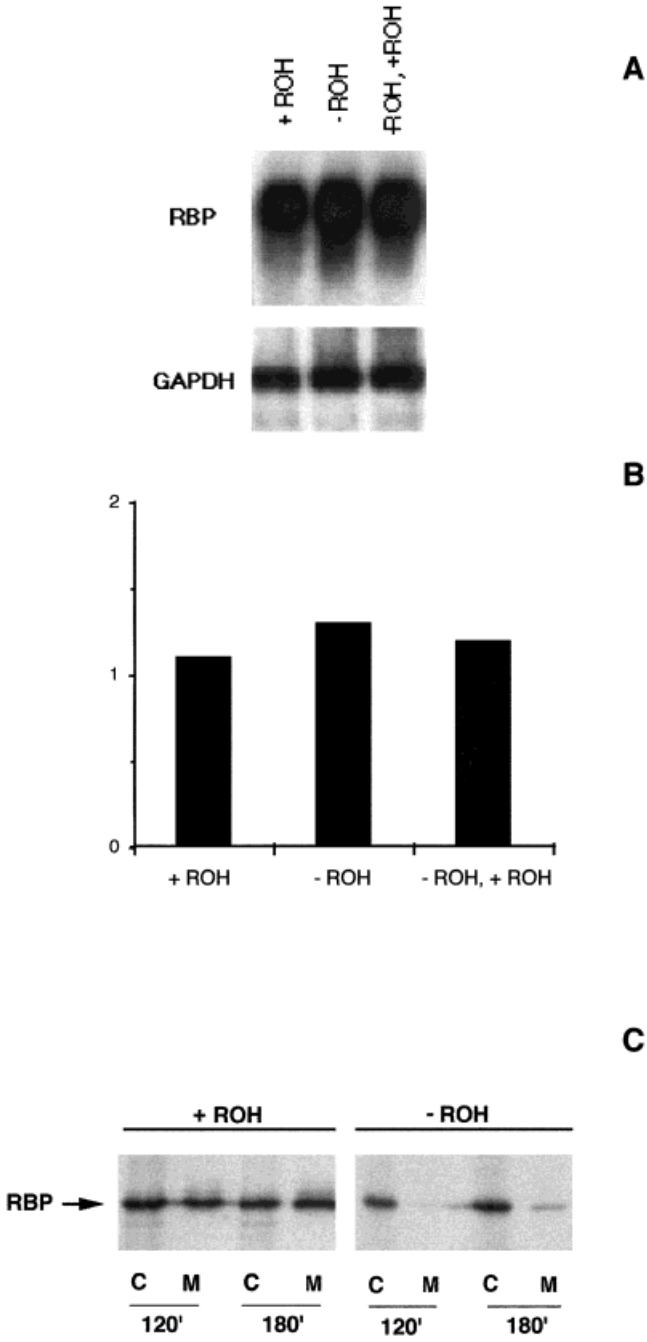

Fig. 2. The expression of the retinol-binding protein (RBP) gene is not regulated by vitamin A. **A,B:** Transcript analysis. A: Met murine hepatocyte (MMH)–D3 cells were grown for 72 h with delipidized serum with the addition of 3 μM retinol (+ROH), without retinol addition (−ROH), and with ROH added 3 h before RNA extraction (−ROH/+ROH). RNA was fractionated and blotted as described in Materials and Methods, and blots were hybridized with ^{32}P-labeled probe for RBP. Filters were then rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe to allow normalization. B: The quantitation of the abundance of RBP transcripts corrected for GAPDH in +ROH, −ROH, and −ROH/+ROH cells. The abundance of specific transcripts in each lane was quantified directly on the filters in the Phosphoimager Analyzer (see Materials and Methods). **C:** RBP secretion analysis of an immunoprecipitation experiment with RBP-specific antibody. Cells were grown for 72 h with (+ROH) or without (−ROH) retinol addition (3 μM). At the end of treatment, cells were radiolabeled with ^{35}S-methionine-cysteine (100 μCi/ml) for the indicated times. Cells (C) and culture medium (M) were subjected to immunoprecipitation, analyzed by 13% SDS-PAGE, and followed by fluorography, as described in Materials and Methods.

Metabolic labeling

Cells grown in 35-mm culture plates were washed with phosphate buffered saline (PBS), supplied with methionine-cysteine-free medium, and incubated at 37°C for 30 min. After this period, ^{35}S-methionine-cysteine (100 μCi/ml) was added to fresh methionine-cysteine-free medium, and the cells were incubated for the times indicated in the figure captions.

In the pulse-chase experiments, the ^{35}S-methionine-cysteine medium was removed after 30 min of labeling (pulse), and the cells were incubated with fresh medium supplemented with cold methionine-cysteine (chase) for the times indicated in the figure captions.

Immunoprecipitation

Cells were harvested in 1 ml of cold radioimmunoprotein assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Na deoxycholate, 1% Triton X-100), and samples were sheared by repeated cycles of suck and blow by using a syringe with a 23-gauge needle. The lysates were centrifuged in Eppendorf tubes at 15,000*g* for 10 min at 4°C (Ole-Dich 154 microcentrifuge) to remove debris. Antibody was added at a final dilution of 1:200, and the mixture was rotated for 1 h at room temperature. This first incubation was followed by another incubation for 1 h at room temperature with 30 μl of 1:1 protein A:agarose suspension in RIPA buffer. At the end of incubation, the samples were centrifuged for 30 s at 6,000*g*, and the pellet was washed three times by repeated cycles of suspending the pellet gently in 1 ml of RIPA buffer and centrifuging for 30 s.

SDS–polyacrylamide gel electrophoresis (PAGE)

Immunoprecipitates were dissolved in 10 μl of sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 μg/ml bromophenol blue, 10 mM β-mercaptoethanol), incubated in boiling water for 5 min, and then analyzed on 13% SDS-PAGE. Na-salicylate fluorography was performed as described by Chamberlain (1979).

Western blotting

Proteins fractionated on SDS-PAGE were transferred onto a nitrocellulose filter by using 25 mM Tris-HCl, 192 mM glycine (pH 8.3) buffer containing 10% methanol, for 60 min at 90 V at 4°C. The blot was stained with Ponceau S to check the extent of transfer, destained with PBS, and blocked for 60 min at room temperature with PBS containing 5% BSA (blocking solution). The filter was then incubated for 60 min at room temperature with PBS containing 3% BSA and specific antibodies at a final dilution 1:1000, washed three times with a washing solution (PBS containing 0.1% Triton X-100), and incubated for 60 min at room temperature with PBS containing 5% defatted milk and secondary antibody at a final dilution of 1:10,000. The blot was washed twice with the washing solution,

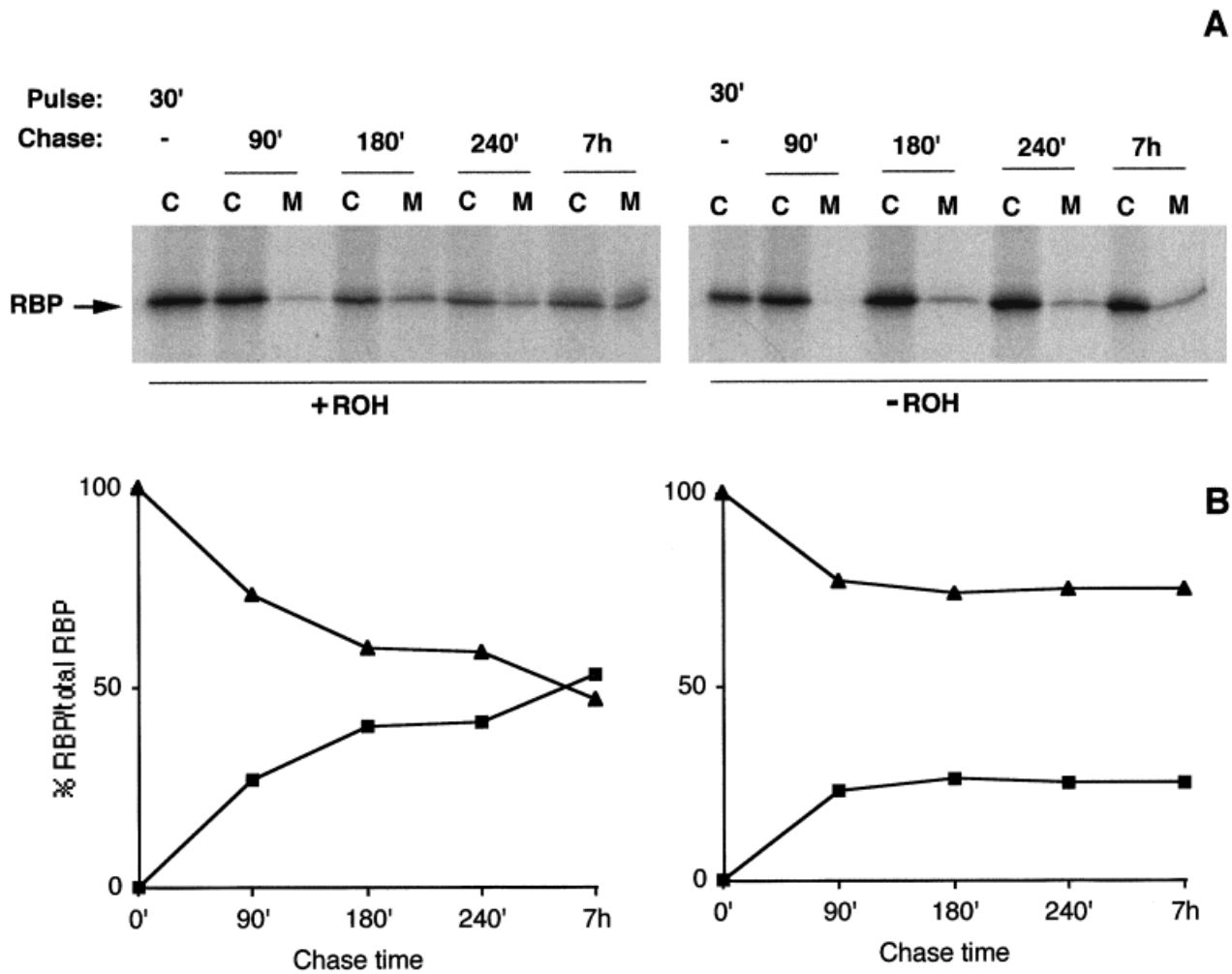

Fig. 3. Secretion of retinol-binding protein (RBP) is regulated by vitamin A in Met murine hepatocyte (MMH)–D3 cells. **A:** MMH-D3 cells grown for 72 h in the presence (+ROH) or absence (−ROH) of 3 μM retinol were pulsed for 30 min with ^{35}S-methionine-cysteine (100 μCi/ml) and then chased for the indicated times in the presence (+ROH) or absence (−ROH) of 3 μM retinol. RBP was then immunoprecipitated from cells (C) and from culture medium (M) with specific antibody, analyzed by 13% SDS-PAGE, followed by fluorography. **B:** Results shown in A are reported as a percentage of RBP in the cell or in the medium/total RBP (intracellular + secreted). The values were calculated by densitometry of the bands shown in A and plotted against the chase time to visualize the effect of retinol on RBP secretion. ▲, cell; ■, medium.

incubated for 3 min with enhanced chemiluminescence reagents, and exposed for appropriate time.

RESULTS

D3 cells synthesize and secrete liver specific secretory proteins

To characterize MMH-D3 cells, we monitored the synthesis and secretion of additional liver-specific secretory proteins by metabolic labeling of the cells with ^{35}S-methionine-cysteine, followed by immunoprecipitation with specific antibodies.

Figure 1 shows that albumin, transferrin, and ceruloplasmin are synthesized by D3 cells and are secreted into the culture medium.

Vitamin A modulates RBP secretion but not its expression in D3 cells

We next analyzed the retinol-regulated RBP expression and secretion. To this end, cells were grown to subconfluence, as described in Materials and Methods, and incubated for 72 h with delipidized serum in the presence (3 μM), in the absence, or in the absence and then addition of retinol 3 h before RNA extraction. The viability of cells in the different growth conditions was completely unaffected (data not shown).

The expression of RBP was studied by Northern blotting. Figure 2A shows the Northern blot of RNA extracted from cells in the different experimental conditions (ROH+, ROH−, and ROH−/ROH+), hybridized with a ^{32}P-RBP probe, and then rehybridized with a GAPDH probe to normalize for the amount of RNA. In Figure 2B, the amount of RBP-mRNA has been normalized for that of GAPDH, demonstrating that in MMH-D3 cells the RBP gene expression level is unaffected by retinol concentration.

The synthesis and secretion of RBP were studied by metabolic labeling experiments in which cells were radiolabeled with ^{35}S-methionine-cysteine for different

periods of time. As shown in Figure 2C, in MMH-D3 cells RBP is synthesized and secreted efficiently and, more interestingly, the secretion of the protein is strongly inhibited by the absence of retinol: only minor amounts of protein are detectable in the medium after 180 min of labeling, wheras a significant accumulation can be detected inside the cell.

With vitamin A deficiency, RBP secretion is strongly inhibited, and the protein accumulates in the cell

For a better understanding of the retinol modulated RBP secretion in MMH-D3 cells, we next analyzed the kinetics of protein secretion in the presence or absence of retinol.

Pulse-chase experiments demonstrated that, under control conditions, the percentage of radiolabeled RBP secreted in the medium increased progressively, and after 7 h of chase the amount of protein was roughly equally distributed between the cell and the medium. By contrast, vitamin A deficiency led to RBP retention in MMH-D3 cells; only a small amount of protein could be found in the medium after 180 min of chase, and this amount did not increase after a longer chase period. Figure 3A illustrates these experiments. The quantitation of results shown in Figure 3A is shown in Figure 3B.

The results of the pulse-chase experiment demonstrate that RBP secretion is regulated in MMH-D3 cells but do not show whether and at which extent protein accumulates within the cell when its secretion is blocked by vitamin A deprivation. An evaluation of the amount of RBP retained in retinol-deficient D3 cells was performed by Western blot analysis (Fig. 4A).

RBP present in FCS is detected by the anti-human RBP antibody; to avoid possible interference with the results, the cells, grown in delipidized serum for 72 h with or without retinol, were washed, incubated for 180 min with serum-free medium, and harvested for RBP immunoprecipitation and Western blot analysis.

The results of this experiment clearly demonstrated that, when MMH-D3 cells are grown in the absence of retinol, RBP accumulates in the cell so that the amount found in the medium is extremely low compared with that of the control. The quantitation shows that the amount of RBP found within deficient cells is roughly three times higher than that in cells grown in complete medium (Fig. 4A,B).

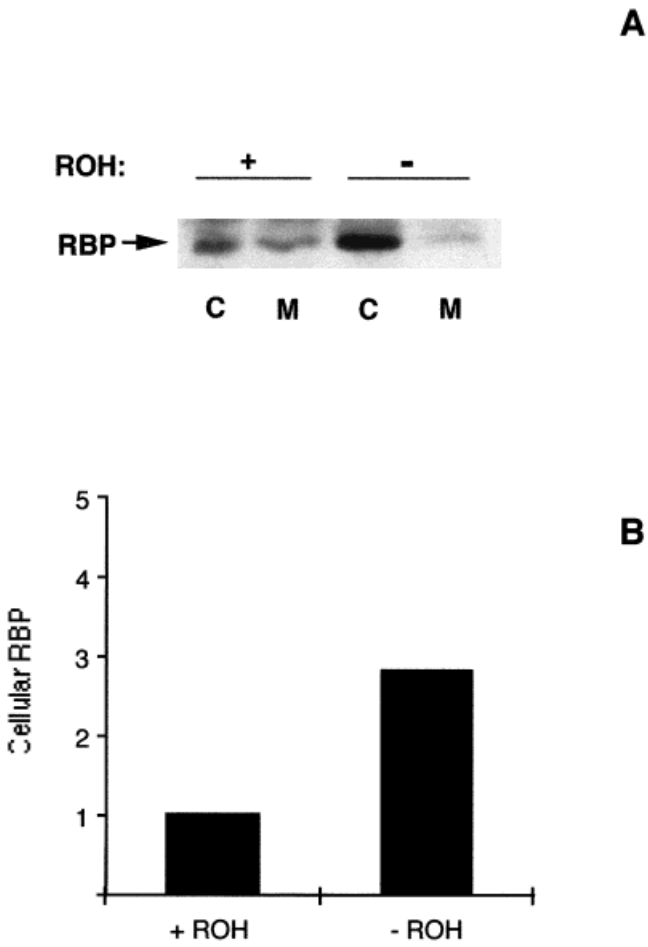

Fig. 4. Retinol-binding protein (RBP) accumulation in ROH-depleted MMH-D3 cells is the result of the inhibition of secretion. **A:** Met murine hepatocyte (MMH)–D3 cells were grown for 72 h in the presence or absence of 3 μM retinol. RBP was immunoprecipitated with specific antibody from the cell extract (C) and from the culture medium (M) after 3 h of incubation with serum-free medium, separated on a 13% SDS-PAGE, and analyzed by Western blot. **B:** Quantitation of cellular RBP in A was performed by densitometry. The amount of RBP, expressed in arbitrary units, is referred to as +ROH = 1.

Retinol administration promptly restores RBP secretion in vitamin A–deficient MMH-D3 cells

In vivo, the block of RBP secretion caused by vitamin A deficiency is rapidly released by retinol administration. Our results demonstrate that this complex mechanism is also conserved in the MMH-D3 cells. As shown in Figure 5, the rate of RBP secretion in cells grown and labeled in vitamin A deficiency and then repleted with retinol during the chase time is comparable to that of control cells grown in complete medium.

Vitamin A deficiency does not block RBP secretion in HepG2 cells

The behavior of MMH-D3 cells was compared with that of a different cell line, the HepG2 cells derived from a human hepatocarcinoma. These cells have been widely used as a model for studies of hepatic functions because they express most of the liver proteins and their secretory apparatus is well developed. However, HepG2 cells are transformed tumor cells, and for this reason they may lack some regulatory paths typical of hepatocytes.

Figure 6A shows the results of a pulse-chase experiment performed under the same conditions as those used for MMH-D3 cells. As is evident by comparing the results obtained with cells grown in complete medium with those of cells grown under retinol deprivation, the rate of RBP secretion is slower when retinol is not available; this result is emphasized by the graphic representing the quantitation of RBP in the cell and in the medium during the different chase times (Fig. 6B).

An important piece of evidence confirming these data comes from the Western blot analysis of the amount of RBP present in HepG2 cells grown in the two different conditions. The amount of protein detected with spe-

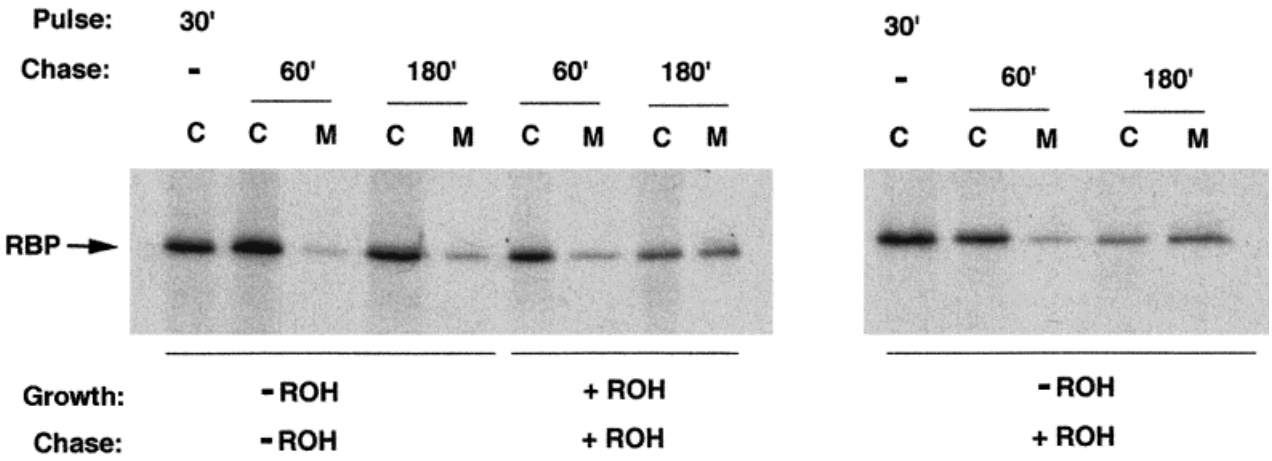

Fig. 5. Repletion of vitamin A–depleted Met murine hepatocyte (MMH)–D3 cells with retinol restores retinol-binding protein (RBP) secretion. MMH-D3 cells were grown for 72 h in the presence (+ROH) or absence (−ROH) of 3 μM retinol and pulsed for 30 min with ^{35}S-methionine-cysteine; cells were then chased for 60 min and 180 min in the absence (−ROH) or presence of 3 μM retinol (+ROH, −ROH/+ROH). RBP was immunoprecipitated from cell extracts (C) and culture medium (M) and separated by 13% SDS-PAGE, followed by fluorography.

cific antibodies inside retinol-deficient cells and the amount secreted is very similar to that seen in cells grown in complete medium, thus demonstrating that, in HepG2 cells, RBP secretion is not regulated by retinol (Fig. 7A,B).

DISCUSSION

The several MMH lines that Amicone et al. (1997) established may represent a powerful tool in the comprehension of mechanisms controlling liver function and liver development. Aiuti et al. (1998) characterized some MMH lines, derived from embryonic and newborn livers, for the capacity to express a wide repertoire of hematopoietic growth factors and to sustain long-term differentiation of hematopoietic cells. Moreover, studies by Spagnoli et al. (1998), who performed the clonal analysis of two MMH lines, have identified a bipotential precursor designated the palmate cell. Palmate cells do not yet express the liver-enriched transcription factors, but they differentiate into hepatocytes capable of expressing liver functions and into bile ductular cells in response to extracellular matrix and soluble factors.

In the present study, we have demonstrated that the MMH-D3 cells, previously characterized by the retention of epithelial cell polarity and the expression of hepatocyte-enriched transcription factors and hepatic products (aldolase, albumin, and β-fibrinogen), secrete albumin, transferrin, ceruloplasmin, and RBP. We have also demonstrated that in MMH-D3 cells RBP secretion is regulated by retinol concentration in the medium.

RBP is responsible for the transport of vitamin A (retinol) from its storage site in the liver to the different vitamin A–dependent tissues (Soprano and Blaner, 1994); its secretion from the hepatocyte is controlled by vitamin A. Several reports by different investigators (Dixon and Goodman, 1987a,b; Perozzi et al., 1991), with experiments in vivo and in primary hepatocytes, have shown that RBP accumulates under vitamin A deficiency because the secretion but not the synthesis is impaired. Much is still unknown about the mechanisms through which this process is regulated. RBP is a soluble protein synthesized by membrane-bound polysomes and is translocated into the lumen of the ER, where it accumulates in the absence of its ligand, in spite of the fact that it does not possess an ER retention signal. In this condition, its amount in the ER increases three to six times compared with the amount present in the liver of control animals, but then stays stable even if vitamin A deficiency becomes more serious. Bellovino et al. (1996) postulated that proteins with the function of chaperone may be involved in the process. Proteases may attack and digest the protein not bound to chaperones, but RBP bound to them would be protected from digestion. This or other hypotheses can never be demonstrated because of the lack of suitable model systems. Experiments in vivo are hampered by the impossibility of obtaining a complete vitamin A depletion because of its action in many sites of the body, with the consequence that a severe deficiency alters the general metabolism and renders the results ambiguous.

Studies by several investigators (Ronne et al., 1983; Dixon and Goodman, 1987a,b) using animals' primary hepatocytes have provided important contributions to the understanding of retinol metabolism. However, the preparation of hepatocytes from the liver of rats under different nutritional conditions (vitamin A deficiency or hypervitaminosis A) is time consuming, and the results are not always reproducible.

Recently, human RBP has been expressed in the yeast *Saccharomices cerevisiae* (Reppe et al., 1998). Those investigators reported that RBP is efficiently secreted and that the addition of retinol to the culture medium induces an increase in the rate of its secretion. Thus, the yeast seems to be a useful model to study the mechanisms involved in RBP secretion, given the availability of numerous secretion mutants and powerful

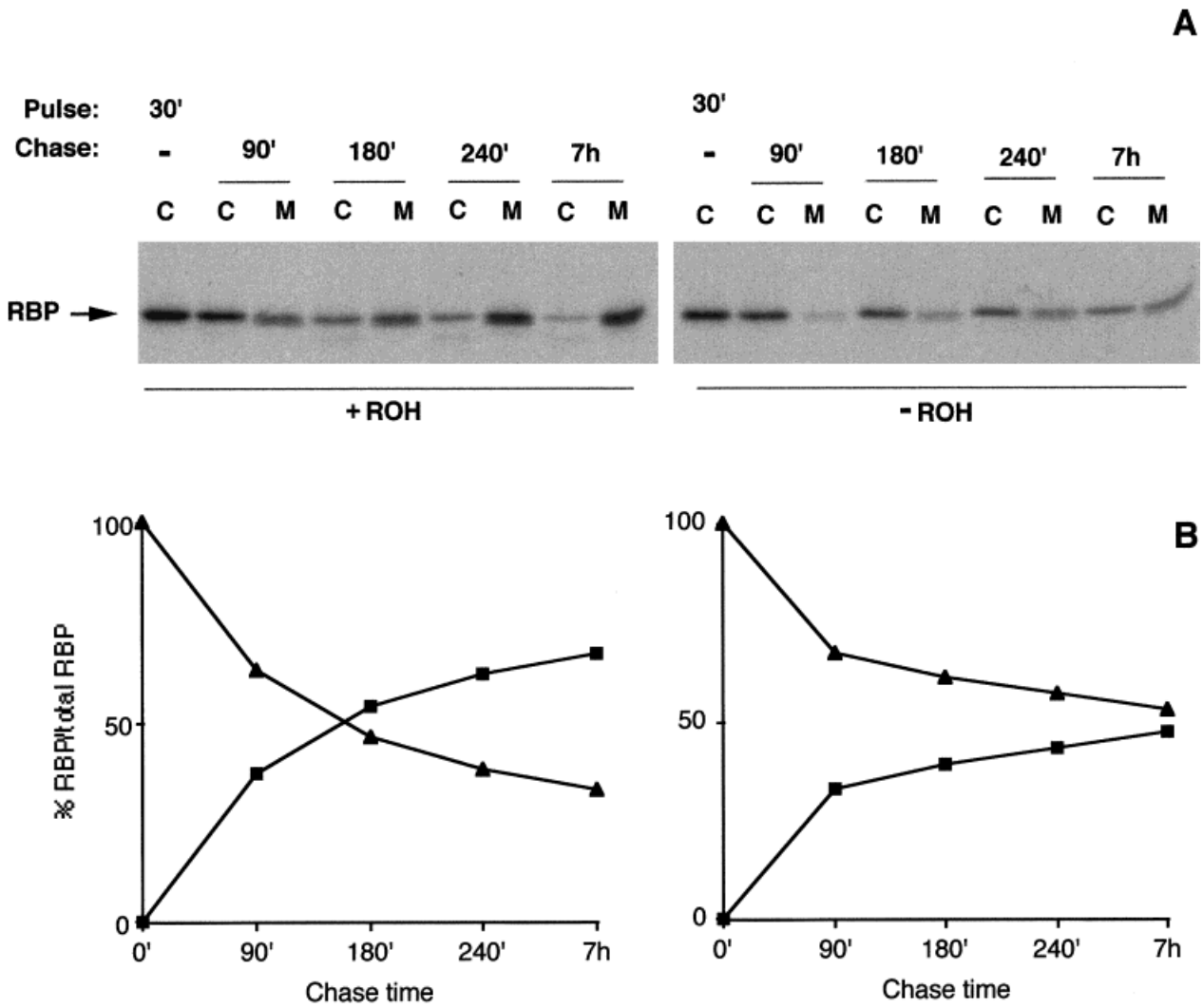

Fig. 6. In retinol-depleted HepG2 cells, retinol-binding protein (RBP) secretion is slowed down but not blocked. **A:** HepG2 cells grown for 72 h in the presence (+ROH) or absence (−ROH) of 3 μM retinol were pulsed for 30 min with ^{35}S-methionine-cysteine (100 μCi/ml) and chased for the indicated times. RBP was then immunoprecipitated from cells (C) and from the culture medium (M), analyzed on a 13% SDS-PAGE, followed by fluorography. **B:** The amount of RBP in the cell or in the medium (relative to total amount) was calculated with the same method as described in Figure 3B. ▲, cell; ■, medium.

genetic properties. However, the limits of this model are that yeast does not use retinol physiologically and the secretion of transfected RBP also takes place in the absence of retinol.

The present results demonstrate that MMH-D3 cells represent a suitable model for the study of retinol-dependent secretion of RBP. In retinol-depleted cells, secretion is only 20% of the amount of the protein secreted by control cells. This amount of RBP not increase with time, possibliy resulting from the secretion of residual RBP that is balanced by continuous uptake of the protein. In agreement with this hypothesis, Tosetti et al. (1994) showed in HepG2 and in CAKI-1 kidney cells that ^{125}I-RBP is taken up by the culture medium. Those investigators' interpretation of the result is that the coordinated process of RBP secretion and endocytosis may be involved in retinol recycling between plasma and tissue and may regulate retinol availability to the cells. However, further experiments are needed to clarify the significance of this result.

The results obtained by continuous labeling of MMH-D3 cells for 120 and 180 min illustrate very clearly how RBP is retained inside the cell and is not secreted in the absence of the ligand.

The comparison with the results obtained in the same conditions with HepG2 cells, in which it is evident that there is only a delay in the exit of RBP, confirm that RBP secretion is not well regulated in these cells.

The D3 cell line, in the culture condition we used, does not express the transthyretin (TTR) gene (Spag noli et al., 1998). TTR is a homotetrameric protein synthesized abundantly by the hepatocytes that circulate in plasma as a complex with holoRBP (Soprano and Blaner, 1994). It has been demonstrated

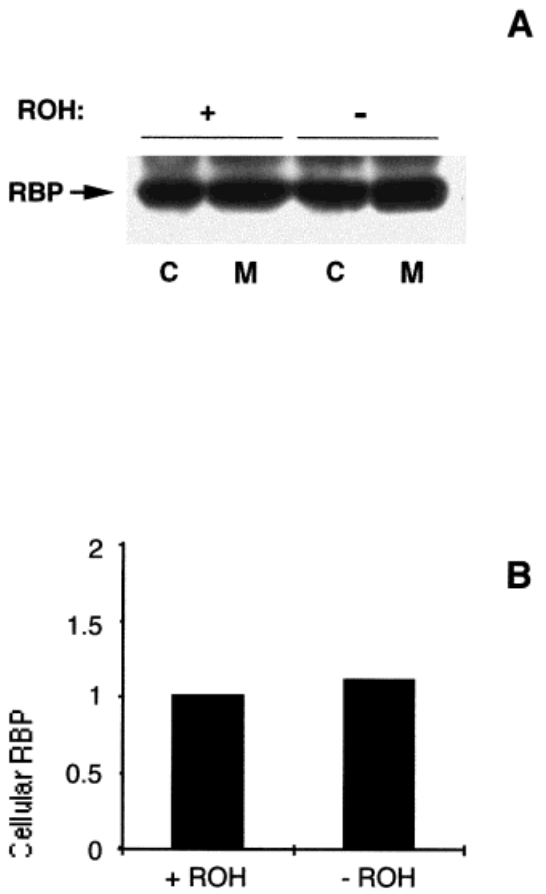

Fig. 7. Retinol-binding protein (RBP) does not accumulate in retinol depleted HepG2 cells because the secretion is not blocked. **A:** HepG2 cells were grown for 72 h in the presence or absence of 3 μM retinol. RBP was immunoprecipitated with specific antibody from the cell extracts (C) and from the culture medium (M) after 3 h of incubation in serum-free medium, separated by 13% SDS-PAGE, and analyzed by Western blot. **B:** Quantification of cellular RBP in A was performed by densitometry. The amount of RBP, expressed in arbitrary units, is referred as to +ROH = 1.

that the complex is made in the ER before secretion (Bellovino et al., 1996; Mehlus et al., 1991, 1992). The formation of the retinol–RBP–TTR complex takes place presumably in several steps co- or post-translationally, and one or more proteins with the function of chaperone are probably involved in the process. To unravel the mechanism by which the holoRBP–TTR protein complex is formed and secreted, we decided to study independently the single steps of the formation. In a previous study (Bellovino et al., 1998), we investigated the conditions and requirements of TTR tetramerization. In this paper, we have studied how the synthesis and the secretion of RBP are regulated in a system in which there is no interference with TTR. Spagnoli et al. (1998) demonstrated that the growth of the MMH cells (E14 and D3; data not shown) on gelatin- rather than on collagen-coated dishes leads to a more differentiated hepatocyte phenotype, with abundant expression of transthyretin. We are now able to study D3 cells expressing both RBP and TTR and in which the RBP–TTR complex secretion is regulated by retinol. This may allow the identification of the factors involved and the mechanism of the entire process.

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LITERATURE CITED

Aiuti A, Cicchini C, Bernardini S, Fedele G, Amicone L, Fantoni A, Tripodi M. 1998. Hematopoietic support and cytokine expression of murine stable hepatocyte cell lines (MMH). Hepatology 28:1645–1654.

Amicone L, Galimi MA, Spagnoli FM, Tommasini C, De Luca V, Tripodi M. 1995. Temporal and tissue-specific expression of the MET ORF driven by the complete transcriptional unit of human A1AT gene in transgenic mice. Gene 162:323–328.

Amicone L, Spagnoli FM, Spath G, Giordano S, Tommasini C, Bernardini S, De Luca V, Della Rocca C, Weiss MC, Comoglio PM, Tripodi M. 1997. Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes. EMBO J 16:495–503.

Bellovino D, Morimoto T, Tosetti F, Gaetani S. 1996. Retinol-binding protein and transthyretin are secreted as a complex formed in the endoplasmic reticulum in HepG2 human hepatocarcinoma cells. Exp Cell Res 222:77–83.

Bellovino D, Morimoto T, Pisaniello A, Gaetani S. 1998. In vitro and in vivo studies on transthyretin oligomerization. Exp Cell Res 243: 101–112.

Blomhoff R. 1994. Overview of vitamin A: metabolism and function. In: Blomhoff R, editor. Vitamin A in health and disease. New York: Marcel Dekker. p 1–35.

Chamberlain JP. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water soluble fluor, sodium salicylate. Anal Biochem 268:5182–5192.

Chirgwin JM, Przybyla AE, Mac Donald RJ, Rutter WI. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299.

Colantuoni V, Romano V, Bensi G, Santoro C, Costanzo F, Raugei G, Izzo P, Pietropaolo TC, Bougueleret L, Cimino F, Salvatore F, Cortese R. 1983. Cloning and sequencing of a full-length cDNA coding for human retinol-binding protein. Nucleic Acids Res 11: 7769–7776.

Dixon JL, Goodman DS. 1987a. Effects of nutritional and hormonal factors on the metabolism of retinol-binding protein by primary cultures of rat hepatocytes. J Cell Physiol 130:6–13.

Dixon JL, Goodman DS. 1987b. Studies on the metabolism of retinol-binding protein by primary hepatocytes from retinol-deficient rats. J Cell Physiol 130:14–20.

Mangelsdorf DJ, Umesono K, Evans RM. 1994. The retinoid receptors. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids: biology, chemistry and medicine. 2nd ed. New York: Raven Press. p 319–349.

Marinari L, Lenich CM, Ross AC. 1987. Production and secretion of retinol-binding protein by a human hepatoma cell line, HepG2. J Lipid Res 28:941–948.

Mehlus H, Nilsson T, Peterson PA, Rask L. 1991. Retinol-binding protein and transthyretin expressed in Hela cells form a complex in the endoplasmic reticulum both in the absence and in the presence of retinol. Exp Cell Res 197:119–124.

Mehlus H, Brehon L, Rask L, Peterson PA. 1992. Ligand-dependent secretion of rat retinol-binding protein expressed in Hela cells. J Biol Chem 267:12036–12041.

Perozzi G, Mengheri E, Colantuoni V, Gaetani S. 1991. Vitamin A intake and in vivo expression of the genes involved in retinol transport. Eur J Biochem196:211–217.

Perozzi G, Barilà D, Sambuy Y, Nobili F, Gaetani S. 1998. Effects of retinoids on gene expression in different epithelial models in vivo and in vitro. Zeit Ernahrungswiss 37(Suppl 1):29–34.

Reppe S, Smeland S, Moskaug JO, Blomhoff R. 1998. Retinol-induced secretion of human retinol-binding protein in yeast. FEBS Lett 427:213–219.

Ronne H, Ocklind C, Wiman K, Rask L, Obrink B, Peterson PA. 1983. Ligand-dependent regulation of intracellular protein transport: effect of vitamin A on the secretion of the retinol-binding protein. J Cell Biol 96:907–910.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a lab-

oratory manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.
Smith JE, Borek C, Gawinowicz MA, Goodman DS. 1985. Structure–function relationship in their effects on retinol-binding protein metabolism in cultured H4IIEC3 liver cells. Arch Biochem Biophys 238:1–9.
Soprano DR, Blaner WS. 1994. Plasma retinol-binding protein. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids: biology, chemistry and medicine. 2nd ed. New York: Raven Press. p 257–282.
Spagnoli FM, Amicone L, Tripodi M, Weiss MC. 1998. Identification of a bipotential precursor cell in hepatic cell line derived from transgenic mice expressing cyto-Met in the liver. J Cell Biol 143:1101–1112.
Tosetti F, Ferrari N, Pfeffer U, Brigati C, Vidali G. 1992. Regulation of plasma retinol binding protein secretion in human HepG2 cells. Exp Cell Res 200:467–472.
Tosetti F, Pedemonte V, Pfeffer U, Brigati C, Ferrari N, Vidali G. 1994. Cellular fate of plasma retinol binding protein. In: Livrea MA, Vidali G, editors. Retinoids: from basic science to clinical application. Basel: Birkhauser-Verlag. p 103–113.