RNA synthesis in regenerating mouse liver evaluated by incorporation of [methyl-¹⁴C]methionine and by determination of RNA polymerase activity

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The synthesis of RNA during mouse liver regeneration was studied by two different methods at 24 and 48 h after partial hepatectomy. Total chromatin-bound RNA polymerase activity showed an increase of 32% at 24 h after partial hepatectomy. At 48 h a slight increase in total activity was also observed in regenerating liver, but the difference was not significant. The increase in total RNA polymerase activity was due to a rise in RNA polymerase I plus III activity. This enzyme activity was increased at both 24 and 48 h. The increase was 57% at 24 h and 51% at 48 h. When [methyl-¹⁴C]methionine was used for labelling of methyl groups in rRNA, there was an increased specific radioactivity of this class of RNA at both 24 h and 48 h. The increases were 263 and 103% at 24 and 48 h respectively. Thus both methods revealed an increased synthesis of rRNA during mouse liver regeneration. The results are discussed in relation to previous results from this laboratory [Yngner, Carlberg, Lewan & Engelbrecht (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1069–1074; Yngner, Engelbrecht, Lewan & Annerfeldt (1979) *Biochem. J.* **178**, 1–8; Yngner, Bengtsson, Carlberg, Engelbrecht & Wieslander (1980) *Exp. Cell. Biol.* **48**, 393–403], which have shown that the incorporation of orotic acid or uridine into RNA is not increased in mouse liver regenerating after partial hepatectomy.

Numerous observations have been published dealing with different biochemical aspects of liver regeneration after partial hepatectomy. The regenerating rat liver is often used as a model for the study of molecular events during proliferation (Bresnick, 1971; Bucher & Malt, 1971; Lesch & Reutter, 1975; Lewan *et al.*, 1977) and as a control tissue in studies of hepatoma growth (Weber *et al.*, 1965; Lea *et al.*, 1974; Duceman & Jacob, 1980).

There is an enhanced uptake and incorporation of labelled orotic acid into rat liver RNA early after partial hepatectomy (Bresnick, 1971; Bucher & Malt, 1971). Since the specific radioactivity of RNA is increased more than that of UTP, there are strong indications of an enhanced RNA synthesis (Bucher & Swaffield, 1969; Yngner *et al.*, 1979a). This interpretation is supported by the fact that the activities of RNA polymerases I, II and III

are elevated at 18 h after partial hepatectomy (Organtini *et al.*, 1975; Yu, 1975b; Duceman & Jacob, 1980). Polymerase I is responsible for the synthesis of pre-rRNA, polymerase II produces hnRNA, and polymerase III catalyses the synthesis of pre-tRNA and 5S RNA.

In contrast with regenerating rat liver, mouse liver reveals no enhanced uptake of orotic acid or incorporation of this precursor into either UTP or RNA at 6 and 24 h after partial hepatectomy (Yngner *et al.*, 1979a, 1980), nor is there an increased incorporation of labelled uridine into UTP or RNA of regenerating mouse liver, despite an increased uptake of the precursor into the total acid-soluble fraction (Yngner *et al.*, 1979b).

An experimental system with many similarities to the regenerating liver is the kidney after unilateral nephrectomy. In this system, the labelling of RNA also seems to differ between mouse and rat. As in regenerating rat liver, there is an increased incorporation of orotic acid also in rat kidney RNA during compensatory hypertrophy (Cortes *et al.*, 1976). In mice subjected to unilateral nephrectomy, however, no increased incorporation of uridine or orotic acid into kidney RNA has

Abbreviations used: hnRNA, heterogeneous nuclear RNA; pAS, sodium 4-aminosalicylate; TIPNS, triisopropylmethylthienylsulphonate.

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been demonstrated (Hill *et al.*, 1974). If methionine is used as labelled precursor instead, there is a substantial increase in the labelling of rRNA of the hypertrophying mouse kidney (Hill *et al.*, 1974).

The fact that no increased incorporation into RNA of either orotic acid or uridine has been observed in mouse liver after partial hepatectomy may be interpreted as indicating that there is no increased RNA synthesis. However, there is obviously a rise in the RNA content during the regenerative process in mouse liver (Lewan, 1972). Thus either transcription of RNA must be increased, though it is not detected by use of labelled orotic acid or uridine, or an increased conservation of RNA must occur.

The present investigation was undertaken to elucidate the question of a possible increase in transcription during mouse liver regeneration. Consequently, RNA synthesis was determined by two different methods. (1) The synthesis of rRNA was studied by use of *methyl*- ^{14}C -labelled methionine. One advantage of using methionine instead of a pyrimidine precursor is that its incorporation into RNA is not influenced by possible changes in size of the pyrimidine pools. (2) The rate of RNA transcription was also evaluated by determination of RNA polymerase activity.

Materials and methods

Materials

L-[*methyl*- ^{14}C]Methionine (60.2 mCi/mmol) and [5,6- ^3H]uridine 5'-triphosphate (45 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. ATP, GTP, CTP, UTP, phosphocreatine, creatine kinase (EC 2.7.3.2), dithiothreitol, Tris base and Tris hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; α -amanitin was from Serva Entwicklungslabor, Heidelberg, Germany, and glass-fibre filters were from Arthur H. Thomas Co., Philadelphia, PA, U.S.A. The chemicals used were of analytical grade whenever possible.

Animals

Male NMRI mice (SPF quality; Anticimex, Stockholm, Sweden) weighing 26–28 g were used for the experiments. The animals were kept under constant conditions of light and temperature, and they had free access to food and water.

Partial hepatectomy

Partial hepatectomy was performed by the method of Higgins & Anderson (1931). In sham-operated animals the peritoneal cavity was opened

and the liver lobes exposed. Operations were carried out under ether anaesthesia and took place between 9:00 and 11:00 h. The animals were killed at 24 and 48 h after partial hepatectomy or sham-operation respectively.

Experiments in vivo

Radioactive labelling. The animals received an intraperitoneal injection of 6 μCi of L-[*methyl*- ^{14}C]methionine dissolved in 90 μl of 0.9% NaCl. Determinations of radioactivity were carried out with Insta-Gel scintillation fluid (Packard) in a Packard liquid-scintillation counter.

Tissue sampling. Animals were killed 180 min after injection of [*methyl*- ^{14}C]methionine. At 90 s before liver excision, the animals were anaesthetized in a diethyl ether/O₂ atmosphere (Bucher & Swaffield, 1966). Blood was collected from the brachial vessels, whereafter the liver was immediately excised and clamp-frozen in metal tongs precooled in liquid N₂. Both blood and liver were rapidly immersed into liquid N₂. Tissues were kept frozen until analysis.

Extraction of rRNA. Ribosomal RNA was extracted by the method of Parish & Kirby as modified by Axelsson *et al.* (1978). Liver tissue (1 g), was homogenized with 20 ml of a solution containing 6% *p*AS, 6% (v/v) butanol, 1% NaCl and 1% TIPNS. Then 13 ml of a 'phenol solution' (500 g of phenol, 70 ml of *m*-cresol, 0.5 g of 8-hydroxyquinoline and 55 ml of water) was added. The mixture was stirred for 30 min at room temperature before centrifugation at 10000 *g* for 10 min. The upper aqueous phase was removed, and to the phenol phase 20 ml of the *p*AS/butanol/NaCl/TIPNS solution was added. After the mixture had been stirred for 15 min the phases were separated by centrifugation at 10000 *g* for 10 min. The aqueous phases were combined, and the solution was made 3% in NaCl. A 0.5 vol. of the phenol solution was added and the mixture was stirred for 15 min. After centrifugation for 10 min at 10000 *g* the aqueous phase was collected and 2 vol. of ethanol containing 10% (v/v) *m*-cresol were added. RNA and DNA were allowed to precipitate at -20°C for 1 h. The precipitate was washed at $+4^\circ\text{C}$ with 2% (w/v) sodium acetate in 75% (v/v) ethanol and was then dissolved in 0.1 M-sodium acetate, pH 6.0. The solution was made 4 M in NaCl, and RNA was allowed to precipitate overnight at $+4^\circ\text{C}$. After centrifugation the RNA pellet was washed with 4 M-NaCl in 0.1 M-sodium acetate and dissolved in 0.5 ml of 0.1 M-Tris/HCl buffer, pH 7.0. For determination of RNA content and radioactivity the isolated rRNA was further analysed by the Schmidt-Tannhauser method as modified by Munro & Fleck (1966).

Experiments in vitro

Preparation of nuclei. Liver nuclei were prepared as described by Yu (1975a) in 2.3M-sucrose/1 mM-CaCl₂. Isolated nuclei were suspended in chromatin medium [1 mM-Tris/HCl, pH 8 at 25°C, 1 mM-dithiothreitol and 4% (v/v) glycerol] to give an A_{260} of 20 units/ml. Approx. 19 A units were obtained per g of liver wet weight after partial hepatectomy and 27 A units/g in sham-operated and unoperated controls.

Assay for chromatin-bound RNA polymerase activity

Approx. 2 A units of liver nuclei were incubated in a total volume of 140 μ l. The mixture contained 25 mM-Tris/HCl buffer (pH 7.8 at 25°C), 2 mM-MgCl₂, 2 mM-MnCl₂, 5 mM-dithiothreitol, 70 mM-(NH₄)₂SO₄, 4% glycerol, 0.3 mM each of ATP, GTP and CTP, 0.3 mM-[³H]UTP (125 mCi/mmol), 3 mM-phosphocreatine and 0.25 unit of creatine kinase. For inhibition of RNA polymerase II, 1.4 μ g of α -amanitin/ml was included.

After incubation at 35°C for 20 min, 100 μ l from each incubation was transferred to glass-fibre filters prepared with trichloroacetic acid. The filters were transferred to individual 25 ml Erlenmeyer flasks. Ice-cold 10% (w/v) trichloroacetic acid containing 1 mM-AMP (10 ml) was added. After 15 min the solution was changed to 10 ml of 5% (w/v) trichloroacetic acid containing 0.05 M-Na₄P₂O₇. After gentle vibration for 15 min this step was repeated, followed by one extraction in 10 ml of 5% trichloroacetic acid for 15 min and then 3 $\times$ 10 ml of ethanol for 5 min each. The glass-fibre filters were dried and counted for radioactivity with Insta-Gel scintillation fluid (Packard) in a Packard liquid-scintillation counter.

Evaluation of results

Results of RNA polymerase activity were expressed as pmol of UMP incorporated per mg of nuclear DNA. It was assumed that 21 A units

corresponded to 1 mg of chromatin DNA. For each of the liver treatments care was taken to ensure that there was a linear relationship between the incorporation of UMP into RNA and the concentration of DNA up to the concentration used in the experiments.

Statistical analysis of the data was performed by Student's *t* test.

Results and discussion

When, in the present study, the synthesis of rRNA in regenerating mouse liver was examined by use of [*methyl*-¹⁴C]methionine, an increased incorporation into this class of RNA was revealed at both 24 and 48 h after partial hepatectomy (Table 1). The increase in specific radioactivity was 263 and 103% of the values of sham-operated controls at 24 and 48 h respectively. Compared with that in unoperated controls the increase was even more pronounced. Thus, when labelled methionine was utilized as precursor, an increased RNA synthesis was indicated in regenerating mouse liver. The results are in agreement with the increased incorporation into rRNA demonstrated in mouse kidney after unilateral nephrectomy (Hill *et al.*, 1974).

Furthermore, determination of RNA polymerase activities supported the interpretation of increased transcription of rRNA (Table 2). RNA polymerase I plus III was increased both 24 and 48 h after partial hepatectomy. The increase was 57 and 51% of the value of sham-operated controls at 24 and 48 h respectively. Compared with that in unoperated controls the rise in RNA polymerase I plus III activity was 35% (*P* < 0.05) and 53% (*P* < 0.001) at 24 and 48 h respectively. Since RNA polymerase III makes up only approx. 17% of the RNA polymerase I plus III activity (Tata & Baker, 1978), the results most certainly reflect an increase in the synthesis of rRNA.

Table 1. *Specific radioactivity of rRNA after labelling with [*methyl*-¹⁴C]methionine*

Mice received an intraperitoneal injection of 6 μ Ci of L-[*methyl*-¹⁴C]methionine 180 min before liver dissection. Then rRNA was isolated and the specific radioactivity was determined. For details, see the Materials and methods section. The values are means ($\pm$ S.E.M.) for the number of animals (*n*) indicated in parenthesis. Incorporation after partial hepatectomy was compared with the values from sham-operated controls. Significance levels: ***P* < 0.01; ****P* < 0.001.

Animal treatment (<i>n</i>)	Time after operation (h)	Specific radioactivity of rRNA	
		(c.p.m./mg of RNA)	(% of unoperated-control value)
Unoperated control (5)	—	154 $\pm$ 7	100
Sham-operated control (5)	24	207 $\pm$ 38	134
Partial hepatectomy (6)	24	752 $\pm$ 70***	488
Sham-operated control (4)	48	191 $\pm$ 13	124
Partial hepatectomy (5)	48	387 $\pm$ 45**	251

Table 2. *RNA polymerase activities in mouse liver after partial hepatectomy*

Liver nuclei were isolated 24 and 48 h after partial hepatectomy or sham operation and from unoperated controls. After incubation with [3 H]UTP at 35°C for 20 min, the rate of incorporation of [3 H]UMP into RNA by these nuclei was determined as described in the Materials and methods section. Total RNA polymerase activity was measured, and RNA polymerase I plus III activity was determined in the presence of α -amanitin (1.4 μ g/ml). The results are means ($\pm$ s.e.m.) for twelve separate experiments, with at least two determinations for each preparation. The RNA polymerase activity after partial hepatectomy was compared with the values from sham-operated controls. Significance levels: * P < 0.05; *** P < 0.001.

Animal treatment	Time after operation (h)	Total RNA polymerase activity		RNA polymerase I + III activity	
		(pmol of UMP per mg of nuclear DNA)	(% of unoperated-control value)	(pmol of UMP per mg of nuclear DNA)	(% of unoperated-control value)
Unoperated control	–	288 $\pm$ 26	100	175 $\pm$ 12	100
Sham-operated control	24	230 $\pm$ 19	80	150 $\pm$ 14	86
Partial hepatectomy	24	304 $\pm$ 22*	106	236 $\pm$ 19***	135
Sham-operated control	48	287 $\pm$ 23	100	177 $\pm$ 15	101
Partial hepatectomy	48	339 $\pm$ 20	118	268 $\pm$ 16***	153

In rat liver, all three major classes of RNA polymerase activities have been shown to be increased during liver regeneration, and an increase of 119–180% in total RNA polymerase activity has been observed (Organtini *et al.*, 1975; Yu, 1975b). In mouse liver there was an increase of 32% in total RNA polymerase activity 24 h after partial hepatectomy compared with the value for sham-operated controls (Table 2). At 48 h, an increase of 18% was found, but the difference was not significant. Thus the increase of total RNA polymerase activity in mouse liver is very low in comparison with what has been demonstrated in the rat. In a series of Morris rat hepatomas, RNA polymerase I has been shown to be elevated relative to the value of this enzyme in normal, resting liver. No significant changes in RNA polymerase II activity were found in any of the tumours examined (Duceman & Jacob, 1980). Thus the pattern of RNA polymerase activities found in some hepatomas, i.e. increased activity of RNA polymerase I but not of polymerase II, is not restricted to neoplastic growth. A proliferating tissue such as mouse liver during regeneration revealed the same pattern.

The increased synthesis of rRNA in regenerating mouse liver cannot be demonstrated by incorporation of labelled orotic acid (Yngner *et al.*, 1979a, 1980). In regenerating rat liver, however, an increased transcription has been shown with this method (Bresnick, 1971; Bucher & Malt, 1971; Lewan *et al.*, 1977). The different pictures revealed by mouse liver and rat liver when labelled orotic acid is used as precursor may depend on the fact

that rat liver is extracting orotic acid from blood far more effectively than does mouse liver (Yngner *et al.*, 1979a; Durschlag & Robinson, 1980). The 'de novo' pathway is the dominating route for pyrimidine-nucleotide synthesis in the rat (Yu & Feigelson, 1970; Hogans *et al.*, 1971; Witschi, 1972), whereas in mouse liver both the 'de novo' and the salvage pathways for synthesis of nucleotides and RNA are well utilized (Yngner *et al.*, 1977). During rat liver regeneration the incorporation of orotic acid is even more accentuated (Ord & Stocken, 1972), a fact that may be due to an activated transport of the precursor into the cells. In mouse liver, no increased transport of orotic acid into the liver after partial hepatectomy has been found (Yngner *et al.*, 1979a). In rat hepatoma it has been suggested that an impaired transport of orotic acid across the membrane may explain the low rate of incorporation of the precursor into the acid-soluble fraction and into RNA when compared with regenerating liver and with host liver (Weber *et al.*, 1965; Matsudaira *et al.*, 1968; Lea *et al.*, 1974).

Furthermore, compartmentation of the nucleotide pools may lead to different utilization of orotic acid in mouse liver compared with rat liver. It has been proposed that hnRNA and pre-rRNA are synthesized from partly independent nuclear pyrimidine pools (Genchev *et al.*, 1980). This suggestion is based on the findings that hnRNA tends rather to be labelled by orotic acid, whereas the synthesis of pre-rRNA tends rather to be supplied by UTP labelled by incorporation of uridine. Since there is a considerable increase in the synthesis of

hnRNA during rat liver regeneration but not during mouse liver regeneration at 24h and 48h, the utilization of orotic acid for RNA synthesis should not be expected to be the same during regeneration in these two species.

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