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Up-Regulation of Telomerase in Primary Cultured Rat Hepatocytes[1]

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Telomerase is a unique reverse transcriptase involved in the maintenance of telomeric DNA, which is generally undetectable in normal human somatic cells. However, it has been found in organs of normal adult rodents including the liver. In order to elucidate relevant control mechanisms operating in normal somatic cells, we examined telomerase activity in primary cultured rat hepatocytes. During culture under serum-free conditions, rat hepatocytes rapidly lose the ability of organ-specific expression of serum albumin, apolipoprotein A-I, and hepatocyte nuclear factor 4, and the capacity for cytochrome P-450 induction by xenobiotics. The telomerase activity was found to be concomitantly increased about 2.5-fold at 48 h and 3-fold at 72 h. Northern blot and RT-PCR analyses with primary cultured hepatocytes revealed the associated accumulation of rat telomerase RNA subunits (TR), and the mRNAs for a telomerase reverse transcriptase (TERT) and a telomerase-associated protein (TEP1). The activity of hepatocyte telomerase, which was elevated during the primary culture, increased further when the cells were stimulated with hepatocyte growth factor. In this case, however, the levels of TR, TERT, and TEP1 mRNA did not show any detectable changes.

Key words: de-differentiation, proliferation, rat liver, primary cultured rat hepatocytes, telomerase.

Telomeres are specialized structures at the ends of chromosomes composed of DNA and proteins that are essential for maintaining the stability of the eukaryote genome. In vertebrates, they consist of tandem hexanucleotide repeats, $(TTAGGG)_n$, (*1, 2*) maintained by a specialized ribonucleoprotein enzyme, called telomerase, which adds motif-specific nucleotides using its RNA subunit as a template.

In normal tissues of adult humans, telomerase activity is almost undetectable except in germ line cells (*3*), although very small amounts are present in normal bone marrow, peripheral blood leukocytes, lymphoid cells, and the epidermis of the skin (*4-6*). In malignant cells, however, a high level of telomerase activity is usually found and a decrease occurs when malignant cells are induced to differentiate (*7-9*). An increase has been observed in association with cellular immortalization (*10*). Telomerase activity also decreases with cellular senescence (*11, 12*).

In contrast to human tissues, normal rodent tissues express moderate levels of telomerase even in adult animals. In normal rats, for example, the liver, thymus, spleen, and testes exhibit activity (*13*). This remarkable difference between animal species may reflect different regulation mechanisms for telomerase expression, which may cause species-specific features of carcinogenesis and aging. Actually, cells in rodents become spontaneously immortalized and undergo malignant transformation at much higher rates than human cells (*14, 15*).

We have observed that the adult rat liver exhibits a substantial level of telomerase activity throughout life (*13*), changes being apparent during development and with regeneration after partial hepatectomy (*13*). Hepatocytes are multifunctional epithelial cells, having an array of different functions that are crucial to the overall physiology of the organism. They are largely in the G_0 phase but can enter the proliferation cycle in response to a metabolic overload that, for example, is readily induced by partial hepatectomy (*16, 17*).

Rat hepatocytes in primary culture have been used for the analysis of hepatocyte differentiation (*18, 19*). In serum-free medium containing insulin, they lose liver-specific functions, such as the induction of cytochrome P-450 by xenobiotics (*20*), and transcription of the serum albumin (*21, 22*) and α1-antitrypsin genes (*22*). On the other hand, growth can be stimulated by treatment of primary cultured hepatocytes with hepatocyte growth factor (HGF) and epidermal cell growth factor (EGF) (*23*).

Thus, rat hepatocytes provide a suitable system for investigating telomerase control in normal cells in a quies-

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Abbreviations: TR, telomerase RNA subunits; TERT, telomerase reverse transcriptase; TEP1, telomerase-associated protein; HNF4, hepatocyte nuclear factor 4.

cent state or in the proliferation cycle. We have examined telomerase activity in primary cultured rat hepatocytes undergoing either growth or de-differentiation, and found an increase associated with elevated expression of rat telomerase reverse transcriptase (TERT), telomerase RNA subunits (TR), and telomerase-associated protein (TEP1). When HGF was added to the primary cultured hepatocytes, the telomerase activity increased further, although without detectable changes in the levels of TERT, TEP1 mRNA, and TR.

MATERIALS AND METHODS

Primary Culture of Hepatocytes—Parenchymal hepatocytes were isolated from a 175-g male Donryu rat by perfusing its liver *in situ* with collagenase as described previously (*24*). Their viability was over 85%, as determined by means of the trypan blue exclusion assay. Non-parenchymal cells were separated by differential centrifugation according to Tateno and Yoshizato (*25*). Hepatocyte suspensions (2.5×10^6 cells) were plated on 100-mm plastic dishes coated with type I collagen (Iwaki, Funabashi), and aliquots were frozen immediately after isolation as the 0 time samples. The plated cells were cultured in serum-free Waymouth's MB752 culture medium (GIBCO-BRL, NY, USA) containing penicillin (50 IU/ml) and streptomycin (50 μg/ml). After 4, 24, 48, and 72 h, the medium was changed and the non-attached dead cells were removed. The number of hepatocytes at each time was determined as described previously (*26*).

HGF Treatment of Primary Cultured Hepatocytes—Isolated hepatocytes were cultured for 24 h in medium containing 0.1 μM insulin. HGF (recombinant human HGF; Institute of Immunology, Tokyo) was then added at the concentration of 1 ng/ml, together with EGF (2 ng/ml; Wako). Cells were further incubated for 24 or 48 h before harvesting.

Extraction of Telomerase—Cultured hepatocytes were washed twice with ice-cold phosphate-buffered saline (PBS), scraped off and placed in 1.5 ml microtubes. After centrifugation for 3 min at $300 \times g$ at 4°C, the pellets were suspended in 500 μl of the wash buffer (pH 7.5) comprising 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM dithiothreitol (DTT), followed by centrifugation again for 3 min at $300 \times g$ at 4°C. Whole cell extracts were prepared by suspending cells in 30 μl of 3{(3-cholamidopropyl)-dimethyl-ammonio}-1-propane-sulfonate (CHAPS) lysis buffer (*9*), comprising 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenyl methylsulfonyl fluoride (PMSF), 5 mM 2-mercaptoethanol, 0.5% CHAPS, and 10% glycerol. Cell suspensions were subjected to 3 cycles of freezing and thawing using liquid nitrogen. After being placed on ice for 30 min, they were centrifuged for 30 min at $10{,}000 \times g$ and the supernatants were collected. The protein concentrations of the supernatants were determined by the method of Bradford (*27*) and adjusted to 2.5 μg/μl by adding CHAPS lysis buffer, followed by aliquoting and storage at −80°C until used (CHAPS extract). Five independent experiments were performed to measure the telomerase activity of the cells at each time point.

Assaying of Telomerase—The TRAP (telomere repeat amplification protocol) assay (*28*) was employed with minor modifications. It was performed in two steps, *i.e.*, telomerase-mediated extension of the first primer, followed by hot start PCR amplification of the products. The cell extract (5 μg protein) was incubated for 30 min at 37°C in a mixture comprising 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM each of dATP, dGTP, and dTTP, 0.1 μg/μl bovine serum albumin (BSA), and 0.1 mg TS primer (5′-AATCC-GTCGAGCAGAGTT-3′) (*9*). The telomerase-mediated extension products were heated for 10 min at 85°C, and a 4 μl aliquot was mixed with a 46 μl PCR mixture comprising 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM each of dATP, dCTP, and dTTP, 10 μM dGTP, 2 μCi [α-^{32}P]dGTP, 0.1 μg/μl BSA, 2 units of *Taq* DNA polymerase, 0.1 μg TS primer, and 0.1 μg ACX primer (5′-GCGCGGCTTACCCT-TACCCTTACCCTAACC-3′) (*28*). To examine endogenous inhibitors of the PCR reaction in CHAPS extracts, an internal PCR standard (ITAS) was routinely added to the mixture. ITAS consists of 0.1 μg of NT primer (5′-AT-CGCTTCTCGGCCTTTT-3′) and 0.01 amol of TSNT primer (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGA-GAAGCGAT-3′), as described by Kim and Wu (*28*). After the mixtures had been heated at 94°C for 90 s, they were subjected to 30 cycles of PCR amplification (one cycle consisting of 94°C for 30 s and 58°C for 45 s). After the reaction, the PCR products (45 μl aliquots) were analyzed by 12.5% non-denaturing polyacrylamide gel electrophoresis (PAGE, 1 mm thick). The gels were autoradiographed with X-ray film (Hyperfilm™ MP; Amersham Pharmacia Biotech, Buckinghamshire, UK) at −80°C for 2 h, and telomerase activity was assessed on incorporated radioactive substrate in ladders of product DNA, multiples of 6 bases corresponding to the telomere repeat unit. Signal intensity was quantified with a BAS 2000 (Fuji Film, Tokyo). This assay method was shown to be linear with increasing amounts of extract up to 10 μg protein of CHAPS extract. Since the TRAP method involved a PCR protocol, we also measured the dose-dependency of the PCR with various amounts of extension products by telomerase in the first reaction. The signal intensity increased in a linear manner with increasing amounts of the products. We verified this system was able to detect an approximately 1.2-fold increase in telomerase activity per fixed amount of protein (Hayakawa *et al.*, manuscript submitted).

Lysis buffer without an extract and RNase-treated samples were used as negative controls. For RNase treatment, the cell extract (5 μg protein) was incubated with 0.5 μg RNase during the extension reaction.

Northern Blot Analysis—Hepatocytes were homogenized in RNA extraction buffer [4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, 0.1 M β-mercaptoethanol], and the RNA was extracted with acidic phenol-chloroform and precipitated with 2 volumes of isopropanol, as described previously (*29*). Each RNA sample (20 μg) was subjected to Northern blot analysis. Complementary DNAs were labeled with [α-^{32}P]dGTP by random priming to a specific radioactivity of $> 10^6$ cpm/ng of DNA using the Klenow fragment of *Escherichia coli* DNA polymerase I (Megaprime™ DNA labelling system; Amersham Pharmacia Biotech). Probe cDNAs were cloned by PCR on the basis of the reported DNA sequences of the genes for rat TEP1 (*30*), mouse TR (*31*), mouse albumin (GenBank; AA276126), rat hepatocyte nuclear factor 4

(*32*), apolipoprotein A-I (*33*), and β-actin (*34*). Autoradiography and signal intensity measurements were carried out as described above.

RT-PCR Amplification of a Rat TERT cDNA Fragment—Since the rat TERT gene has not been cloned, guess-PCR was performed to obtain rat TERT cDNA. The guess-PCR primers were 5′-AGACTSCGCTTCATCCCCA-AG-3′ (sense) and 5′-GTCTGGAGGCTGTTCACCTGC-3′ (antisense), constructed according to the conserved sequence of human (*35*) and mouse (*36*) TERT cDNAs. Rat TERT cDNA fragments were amplified by RT-PCR from 100 ng rat liver mRNA and then subjected to DNA sequencing. The base sequence was 85% identical to the corresponding sequence of mouse TERT cDNA (nucleotides 1857-2975, data not shown).

RT-PCR of Rat TERT—Total RNA was isolated from rat hepatocytes as described (*29*), and PCR primers for TERT were constructed according to the rat TERT cDNA sequence. They were 5′-GACATGGAGAACAAGCTGTT-TGC-3′ (sense) and 5′-ACAGGGAAGTTCACCACTGTC-

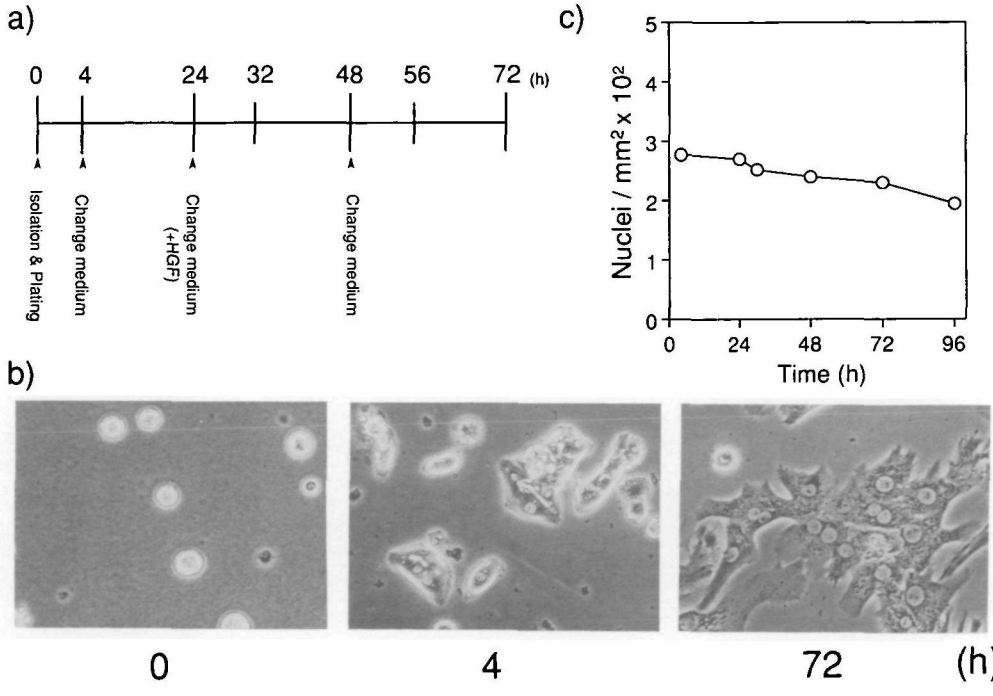

Fig. 1. **Experimental design involving primary cultured rat hepatocytes.** (a) Experimental schedule. Isolated hepatocytes were plated at 2.5×10^6 cells on 100-mm-diameter plastic dishes coated with collagen. The culture medium was changed at the times indicated by arrows. In some experiments, HGF and EGF were added to serum-free Waymouth's MB752 culture medium containing insulin as indicated. For details, see under "MATERIALS AND METHODS." (b) Light microscopy of adult rat hepatocytes cultured for 0 h (left), 4 h (middle), and 72 h (right). (c) Number of nuclei, reflecting viable hepatocytes, at the indicated times.

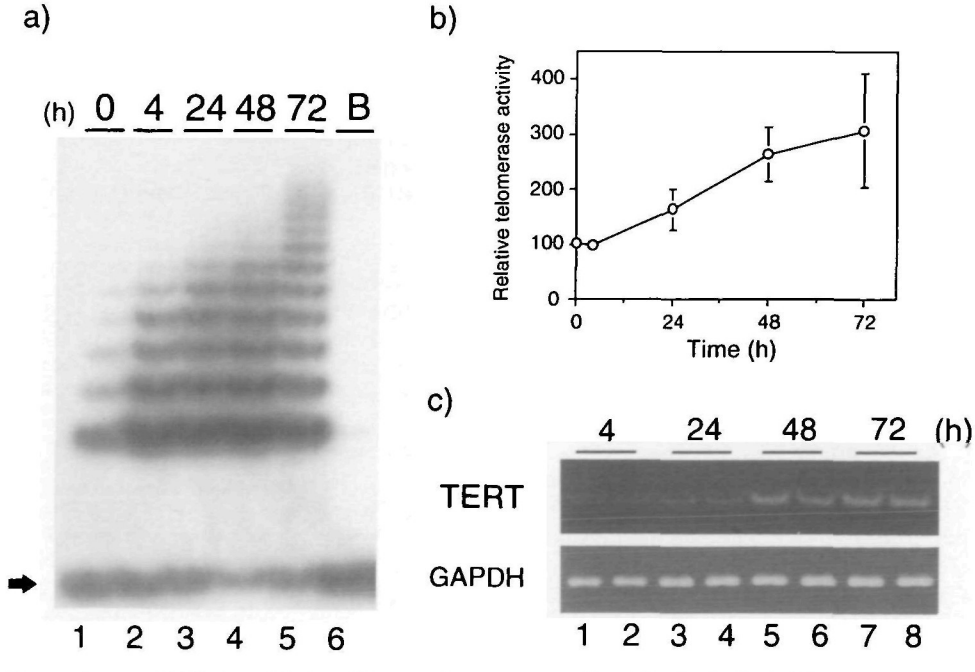

Fig. 2. **Telomerase activity in primary cultured rat hepatocytes.** (a) CHAPS extracts of hepatocytes that had been cultured for 0, 4, 24, 48, and 72 h (lanes 1 to 5, respectively) were prepared and assayed for telomerase activity using aliquots containing 5 μg protein by the TRAP method as described under "MATERIALS AND METHODS." As a negative control, CHAPS lysis buffer was used (lane 6, B). Five independent experiments were performed and typical results are shown. The position of the ITAS band is indicated by the arrow. (b) Quantification of telomerase activity was performed with a Fuji Bas 2000. The activities shown are the means of five independent experiments ± SEM. The telomerase activities (means ± SEM), relative to that at 4 h taken as 100, were: 163.7 ± 37.4% at 24 h; 264.1 ± 49.3% at 48 h; 307.1 ± 103.6% at 72 h. (c) RT-PCR for rat TERT (top) and GAPDH (bottom) was performed in duplicate using total RNA (1 μg) isolated from rat hepatocytes cultured for the indicated times (lanes 1 and 2, 4 h; lanes 3 and 4, 24 h; lanes 5 and 6, 48 h; lanes 7 and 8, 72 h).

3′ (antisense), and the size of the target was 185 bp. First strand cDNA was synthesized with 1 μg of total RNA using a First Strand cDNA Synthesis Kit (Boehringer Mannheim, Germany) in the presence of 1.6 μg oligo-$(dT)_{15}$ primer in a final volume of 25 μl. After denaturation for 5 min at 94°C, 4 μl of reaction product was amplified by PCR for 29 cycles (94°C, 30 s/55°C, 30 s/72°C, 1 min). The amplified products were separated by electrophoresis on either a 2% agarose gel or by 8% PAGE, and visualized with SYBR™ Green I (Molecular Probes, Rockland ME, USA). The identities of RT-PCR products were confirmed at least once by DNA sequencing. Each RT-PCR was performed three times with independent preparations of RNA, and typical results are shown in Figs. 2 and 4. As an internal control, RT-PCR of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed for all RNA samples, using the PCR primers, 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense) (*37*). The linearity of RT-PCR with respect to the RNA amount was determined and the estimation of mRNA for rat TERT was performed within a linear range.

RESULTS

Telomerase Activity Increases in Rat Primary Cultured Hepatocytes—Figure 1a shows the experimental design involving primary cultured rat hepatocytes. In the course of culture on collagen-coated plastic dishes in serum-free medium, hepatocytes dramatically change in shape, from round to flattened epithelial cell-like (Fig. 1b), as observed previously (*38*). Under the standard culture conditions when HGF was absent, the cells did not proliferate. Approximately 70% of the inoculated cells were viable after 72 h (Fig. 1c) (*26*).

The isolated rat hepatocytes (0 h) showed low but substantial levels of telomerase activity (Fig. 2a, lane 1). The level in non-parenchymal cells was comparable with that in hepatocytes (data not shown). During the culture, telomerase activity in the hepatocytes gradually increased (Fig. 2a). Densitometric quantification showed the up-regulation was approximately 2.5-fold at 48 h, and 3-fold at 72 h (Fig. 2b). Throughout the time course, the densities of the ITAS bands were essentially identical. The reaction products were sensitive to RNase treatment (data not shown), indicating a dependence on the intrinsic template RNA subunit.

Expression of the Telomerase-Related Genes Is Enhanced during Primary Culture—RT-PCR was performed with respect to mRNA for TERT using total RNA obtained from primary cultured hepatocytes. Northern blot analyses were performed with respect to the telomerase RNA subunit (TR) and mRNA for the telomerase-associated protein, TEP1.

As shown in Fig. 2c, the level of TERT mRNA was increased at 24 h and reached a maximum at 48-72 h. The TEP1 mRNA was elevated markedly at 24 h, reached a maximum at 48 h and remained at a high level thereafter (Fig. 3a). The level of TR was increased at 48 h and was further elevated at 72 h. These patterns were consistent with the increase in telomerase activity (Fig. 2a).

Liver-Specific Genes Are Down-Regulated during Primary Culture—Possible correlations between the increased telomerase activity and loss of differentiation markers were also investigated. The culture of freshly isolated hepatocytes on collagen-coated dishes in a serum-free medium resulted in extensive down-regulation of the liver-specific genes for serum albumin, apolipoprotein A-I, and hepatocyte nuclear factor 4 (Fig. 3d), in agreement with previous reports (*18, 21*). On the other hand, the level of mRNA of β-actin, a major cytoskeletal protein, increased (Fig. 3d), in line with the change in morphology during the culture period (Fig. 1b). Thus, activation of the hepatocyte telomerase, together with the increased amounts of mRNAs for TERT, TEP1 and TR, was negatively correlated with the expression of liver-specific genes.

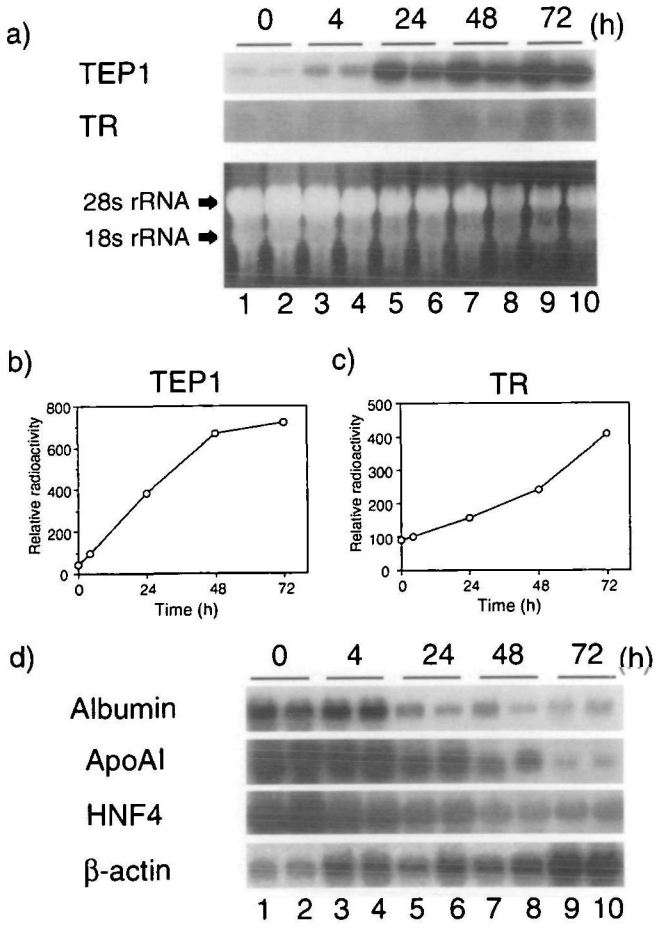

Fig. 3. **Expression of rat TR and TEP1 mRNA in comparison with that of liver specific genes.** (a) Northern blot analysis was carried out using total RNA (20 μg per lane) isolated from hepatocytes cultured for the indicated times (lanes 1 and 2, 0 h; lanes 3 and 4, 4 h; lanes 5 and 6, 24 h; lanes 7 and 8, 48 h; lanes 9 and 10, 72 h). The blots were probed with rat TEP1 (top) and mouse TR (middle) cDNAs labeled with ^{32}P. Ethidium bromide-staining of the RNA samples is shown as a control (bottom). These results were reproducible in three independent experiments. (b and c) Quantification of Northern blots for rat TEP1 and TR, respectively, with a BAS 2000. Each point represents the mean of duplicate experiments, relative to the value at 4 h taken as 100. (d) Expression of liver-specific differentiation markers assessed by Northern blot analysis using antisense DNA probes for serum albumin, ApoAI (apolipoprotein A-I), and HNF4 (hepatocyte nuclear factor 4). Total RNA (20 μg per lane) samples were isolated from hepatocytes that had been cultured for the indicated times (lanes 1 and 2, 0 h; lanes 3 and 4, 4 h; lanes 5 and 6, 24 h; lanes 7 and 8, 48 h; lanes 9 and 10, 72 h). Blots for β-actin mRNA are shown as a nonspecific control.

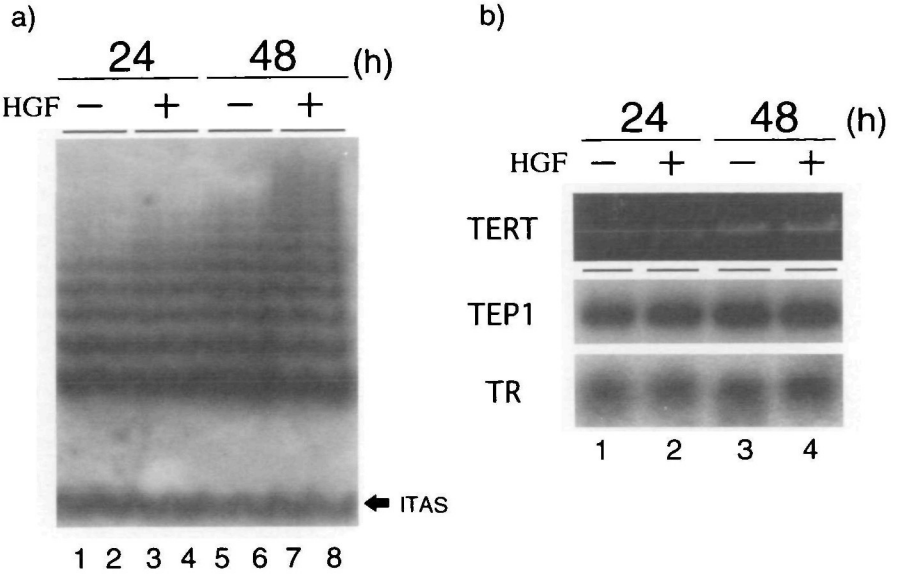

Fig. 4. **Change in telomerase activity in primary cultured hepatocytes treated with HGF.** (a) CHAPS extracts were prepared with hepatocytes that had been cultured for 24 h (lanes 3 and 4) and 48 h (lanes 7 and 8) in the presence of HGF, EGF and insulin, and control cells incubated for the same periods in the absence of these growth factors (lanes 1, 2, 5, and 6). Aliquots containing 5 μg protein were used for the TRAP assay as described under "MATERIALS AND METHODS." Five independent experiments were performed and typical results are shown. The position of the ITAS band is indicated by the arrow. (b) Analyses of mRNAs for telomerase-related genes. Expression of TERT was examined by RT-PCR (top). Northern blot analysis was carried out using total RNA (20 μg per lane), probed with rat TEP1 cDNA (middle) and the mouse telomerase RNA subunit (mTR) (bottom). Rat hepatocytes were treated with (lanes 2 and 4) and without (lanes 1 and 3) HGF for 24 and 48 h. Other conditions were as given under "MATERIALS AND METHODS."

HGF Enhances Telomerase Activity without an Increase in Expression of Telomerase-Related Genes—Under the standard culture conditions, most hepatocytes stayed in the non-proliferating state. Upon treatment with 1 ng/ml HGF, together with EGF and insulin, the incorporation of [^{3}H]thymidine into DNA was increased 1.25-fold at 24 h and 1.65-fold at 48 h (data not shown), in agreement with results reported previously (*23, 39*). The telomerase activity in hepatocytes, which had been elevated 2- to 3-fold by the preculture, was further increased approximately 1.5-fold by treatment with HGF for 48 h (Fig. 4a). In this case, however, no increase was detected in the mRNA for either TERT, TEP1, or TR (Fig. 4b). On the other hand, the mRNA levels for serum albumin, apolipoprotein A-I, and hepatocyte nuclear factor 4 were increased by the HGF treatment (data not shown). These results imply that the mechanism controlling telomerase during de-differentiation may differ from that in HGF-induced proliferation.

DISCUSSION

Telomerase activity has been detected in 85 to 90% of human cancers (*9*), but is undetectable in almost all somatic cells in normal adults (*3*). In rodents, however, telomerase is expressed in normal organs having a self-renewal potential, such as the liver, spleen, thymus, and testes, even in adult animals (*13, 40*). Although the up-regulation of telomerase in immortal tumor cells has been described in detail (*9, 10*), the regulation of telomerase activity in non-transformed, normal mammalian cells has remained obscure. Since a primary culture of rat hepatocytes can be led into de-differentiation in serum-free culture medium, and its growth can be induced by adding HGF, this system could be used to address this problem.

In the present study, we showed that the telomerase activity in primary cultured rat hepatocytes rapidly increases (Fig. 2) under culture conditions under which the cells lose their differentiation markers (Fig. 3). In a serum-free synthetic culture medium without growth factors and hormones, the mRNA levels of liver-specific genes for serum albumin, apolipoprotein A-I, and liver-enriched transcription factors such as HNF4 and C/EBP rapidly decreased in agreement with previous observations (*18, 21*). In human leukemic cell lines, telomerase activity decreases rapidly as the cells differentiate under the influence of various inducers (*7, 8*). These findings also suggest an inverse correlation with the differentiation status not only in immortalized cells, but also in normal cells that ubiquitously express telomerase activity such as those of rodent liver, lung, and thymus.

As shown here, the culture-dependent increase in activity was associated with elevated transcription of telomerase-related genes, encoding TERT, TR, and TEP1. A parallelism between TERT mRNA and telomerase activity in normal mouse tissues was demonstrated by Greenberg *et al.* (*41*) through studies an mouse embryos, several organs of adult mice and mitogen-induced splenocytes. A positive correlation with the TERT mRNA level in malignant human tissues and cultured cells (*42, 43*) was also found under conditions of either differentiation or de-differentiation.

Although it has been reported that the TR level parallels telomerase activity in human tumor cells (*44, 45*), normal T-cells (*46*), and somatic cells of mice during postnatal development or immortalization (*31*), there are many exceptions in which TR is expressed independently (*7, 41, 47*). TEP1 expression may similarly change without increased activity, for example in human cervical cancers (*43*). Furthermore, in HL60 cells, down-regulation of telomerase is loosely associated with up-regulation of TEP1 (*48*).

The modest increase in telomerase activity on HGF stimulation found here is consistent with previous results for regenerating liver after partial hepatectomy (*13, 49, 50*). In contrast to the case of de-differentiation, however, the enhancement by growth factors was not associated with detectable increases in TR, TERT, and TEP1 mRNA (Fig. 4b). The increase might thus be due to some proliferation-

dependent alteration in post-transcriptional regulation such as phosphorylation (*51*). This might also be linked to reduced expression of several liver-specific genes, as shown for regeneratory hepatocytes [(*52*), Kurumiya *et al.*, manuscript submitted].

From the results presented here, we propose that the complex behavior of telomerase in eukaryotes may be due to combinations of two independent regulation mechanisms. The primary cultured rat hepatocytes described here may constitute a useful system for investigating up-regulation under different conditions, particularly that linked to cell differentiation.

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