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# Feedback Inhibition of Amidophosphoribosyltransferase Regulates the Rate of Cell Growth via Purine Nucleotide, DNA, and Protein Syntheses*

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**To clarify the contributions of amidophosphoribosyltransferase (ATase) and its feedback regulation to the rates of purine *de novo* synthesis, DNA synthesis, protein synthesis, and cell growth, mutated human ATase (mhATase) resistant to feedback inhibition by purine ribonucleotides was engineered by site-directed mutagenesis and expressed in CHO ade $^{-}$A cells (an ATase-deficient cell line of Chinese hamster ovary fibroblasts) and in transgenic mice (mhATase-Tg mice). In Chinese hamster ovary transfectants with mhATase, the following parameters were examined: ATase activity and its subunit structure, the metabolic rates of *de novo* and salvage pathways, DNA and protein synthesis rates, and the rate of cell growth. In mhATase-Tg mice, ATase activity in the liver and spleen, the metabolic rate of the *de novo* pathway in the liver, serum uric acid concentration, urinary excretion of purine derivatives, and T lymphocyte proliferation by phytohemagglutinin were examined. We concluded the following. 1) ATase and its feedback inhibition regulate not only the rate of purine *de novo* synthesis but also DNA and protein synthesis rates and the rate of cell growth in cultured fibroblasts. 2) Suppression of the *de novo* pathway by the salvage pathway is mainly due to the feedback inhibition of ATase by purine ribonucleotides produced via the salvage pathway, whereas the suppression of the salvage pathway by the *de novo* pathway is due to consumption of 5-phosphoribosyl 1-pyrophosphate by the *de novo* pathway. 3) The feedback inhibition of ATase is more important for the regulation of the *de novo* pathway than that of 5-phosphoribosyl 1-pyrophosphate synthetase. 4) ATase superactivity leads to hyperuricemia and an increased bromodeoxyuridine incorporation in T lymphocytes stimulated by phytohemagglutinin.**

Purine nucleotides are synthesized both via the *de novo* pathway and via the salvage pathway and are vital for cell functions and cell proliferation through DNA and RNA syntheses and ATP energy supply. Amidophosphoribosyltransferase (ATase)[1] is the rate-limiting enzyme in the *de novo* pathway of purine ribonucleotide synthesis (1) and is regulated by feedback inhibition by AMP and GMP. Hypoxanthine (Hx) and hypoxanthine guanine phosphoribosyltransferase (HPRT) are the most important substrate and enzyme, respectively, of the salvage pathway (1). The Lesch-Nyhan syndrome is caused by a complete deficiency of HPRT. Although patients with this syndrome show hyperuricemia with accelerated *de novo* purine synthesis, the mechanism of activation of the *de novo* pathway is not fully understood.

In a previous study, we demonstrated that the expression level of ATase limits the growth rate of cultured fibroblasts, and purine salvage strongly inhibits purine *de novo* synthesis (1). Two mechanisms by which the salvage pathway inhibits the *de novo* pathway were presumed. 1) Consumption of 5-phosphoribosyl 1-pyrophosphate (PRPP) by the salvage pathway decreases the activity of the *de novo* pathway because PRPP is the common source of both pathways. 2) Purine nucleotides synthesized via the salvage pathway inhibit ATase by feedback regulation. However, it is unclear which mechanism plays the more important role.

To resolve these questions, feedback-resistant human ATase (hATase) was engineered by site-directed mutagenesis and expressed in CHO ade $^{-}$A fibroblasts, an auxotrophic cell line deficient in ATase. In CHO ade $^{-}$A cells transfected with mutated hATase cDNA ($^{-}$A + mhATase cells), the following parameters were examined and compared with those in CHO ade $^{-}$A cells transfected with normal hATase cDNA ($^{-}$A + hATase cells): ATase activity, the subunit structure of ATase, the metabolic rates of *de novo* and salvage pathways, the rates of protein and DNA syntheses, and the rate of cell growth. For a comprehensive study, we used, in total, four cell lines: 1) CHO K1, a wild type of CHO fibroblasts, 2) CHO ade $^{-}$A cells, 3) $^{-}$A + hATase cells, and 4) $^{-}$A + mhATase cells. We also used two culture media: 1) a medium rich in purine bases and 2) a medium free of purine bases. In the purine-rich medium, both the *de novo* and salvage pathways function in CHO K1, $^{-}$A + hATase, and $^{-}$A + mhATase cells, but only the salvage pathway functions in CHO ade $^{-}$A cells. In the purine-free medium, only the *de novo* pathway functions in CHO K1, $^{-}$A + hATase, and $^{-}$A + mhATase cells, and neither of the two pathways functions in CHO ade $^{-}$A cells. Under various conditions, we

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[1] The abbreviations used are: ATase, amidophosphoribosyltransferase; hATase, human ATase; mhATase, mutated human ATase; $^{-}$A + hATase, CHO ade $^{-}$A cells transfected with normal human ATase cDNA; $^{-}$A + mhATase, CHO ade $^{-}$A cells transfected with mutated human ATase cDNA; BrdUrd, bromodeoxyuridine; CHO, Chinese hamster ovary; HamF, Ham's F-12; HPLC, high performance liquid chromatography; HPRT, hypoxanthine guanine phosphoribosyltransferase; Hx, hypoxanthine; mhATase-Tg mice, transgenic mice expressing mhATase under the control of the CAG promoter; PHA, phytohemagglutinin; PRPP, 5-phosphoribosyl 1-pyrophosphate; UA, uric acid; XO, xanthine oxidase; FCS, fetal calf serum.

examined the effects of feedback regulation of ATase on the cell growth rate and on the biosyntheses of purine nucleotides, DNA, and proteins.

Furthermore, we generated transgenic mice expressing mhATase under the control of the CAG promoter (mhATase-Tg mice). Because this promoter functions in most cells, overexpression of mhATase was expected in all tissues of the transgenic mice. In mhATase-Tg mice, the following data were examined: ATase activity in the liver and spleen, the metabolic rate of the *de novo* pathway in the liver, urinary excretion of allantoin and uric acid (UA), serum UA concentration, and T lymphocyte proliferation by phytohemagglutinin (PHA).

## EXPERIMENTAL PROCEDURES

*Site-directed Mutagenesis*—In *Escherichia coli* ATase, amino acid replacements of K326Q and P410W result in decreased binding affinity for AMP and GMP and lead to corresponding reductions in feedback inhibition (2). Because the amino acid sequences of these two regions are conserved in *E. coli* ATase and hATase, these mutations were expected to make hATase, as well as *E. coli* ATase, feedback-resistant. Using a polymerase chain reaction-based method, a K338Q/P422W double mutation, which corresponds to K326Q/P410W of *E. coli* ATase, was generated in hATase cDNA, resulting in mhATase cDNA.

*Cell Culture*—CHO K1 and CHO ade $^{-}$A cells (3) were kind gifts from Dr. David Patterson (Eleanor Roosevelt Institute for Cancer Research, Denver, CO). The CHO fibroblasts were cultured at 37 °C in an atmosphere of humidified air:$CO_2$ (95:5) in Ham's F-12 (HamF) purine-rich medium containing 30 $\mu$M Hx + 10% fetal calf serum (FCS) or HamF with 10% FCS treated with 2.5 mg (1.8 unit)/liter xanthine oxidase (XO) from buttermilk (Sigma) at 37 °C overnight, serving as a Hx-free medium (HamF + XO). The complete removal of Hx in HamF + XO was confirmed first by the disappearance of the Hx peak in the reversed-phase high performance liquid chromatography (HPLC) analysis through a C18 column (1) and second by the absence of growth of CHO ade $^{-}$A cells in HamF + XO. Although RPMI 1640 + 10% dialyzed FCS was also used as a completely purine-free medium, the results obtained from CHO cell culture in this medium were essentially identical to those in HamF + XO containing 10% nondialyzed FCS (data not shown). Therefore, adenine and guanine carry-over from nondialyzed FCS was considered to be negligible, and HamF + XO containing 10% nondialyzed FCS was used as an essentially purine-free medium in this study. In HamF, both the *de novo* and salvage pathways function in CHO K1, $^{-}$A + hATase, and $^{-}$A + mhATase cells, but only the salvage pathway functions in CHO ade $^{-}$A cells. In HamF + XO, only the *de novo* pathway functions in CHO K1, $^{-}$A + hATase, and $^{-}$A + mhATase cells, and neither of the two pathways functions in CHO ade $^{-}$A cells.

*Transfection of CHO ade $^{-}$A Cells*—hATase or mhATase cDNA (2.2 kilobase pairs) was inserted into the cloning site downstream of the cytomegalovirus promoter in pBCMGSNeo (14.5 kilobase pairs), which includes the replication origin of bovine papillomavirus leading to 10–500 copies/cell in mammalian cells (4, 5). Each plasmid vector (5 $\mu$g) was mixed with Lipofectin reagent (Life Technologies, Inc.) according to the manufacturer's protocol and overlaid onto CHO ade $^{-}$A cells ($2 \times 10^6$ cells/90-mm tissue-culture plate) in 5 ml of Opti-MEM (Life Technologies, Inc.). After incubation at 37 °C for 6 h in a $CO_2$ incubator, the DNA-containing medium was replaced with 10 ml of HamF containing 1 mg/ml G418 (Life Technologies, Inc.). Several clones of $^{-}$A + hATase and $^{-}$A + mhATase cells were isolated after G418 selection for 7 days. To select subclones with high ATase activity, these transfectants were further cultured in HamF + XO for several weeks. Long term culture in this purine-free medium positively selected the subclones with high ATase activity associated with a high rate of cell growth.

*Measurement of Cell Growth Rate*—Cultured cells during the logarithmic growth phase were counted by an improved Neubauer hemocytometer (1). The doubling time (h) was determined from cell counts, and its reciprocal was defined as the cell growth rate.

*Determination of Synthesis Rates of Purine Nucleotides and Proteins*—The metabolic rates of the *de novo* and salvage pathways were determined, respectively, by the incorporation of [$^{14}$C]glycine (Amersham Pharmacia Biotech) or [$^{3}$H]Hx (Amersham Pharmacia Biotech) in acid-soluble purines (1, 6). In a 90-mm culture dish, $2 \times 10^6$ cells were plated. After the recovery of cell function from plating by 18 h of culture, [$^{14}$C]glycine or [$^{3}$H]Hx was added to the medium at the final concentration of 150 $\mu$M (0.3 MBq/ml) and 40 $\mu$M (0.15 MBq/ml), respectively. The cells were cultured in radioactive medium for 30 min, washed three times with 10 ml of ice-cold phosphate-buffered saline, and harvested with a rubber policeman. Purine nucleotides were extracted from the cells in 1 ml of 0.4 N perchloric acid at 100 °C for 60 min. After centrifugation at 12,000 $\times$ *g* at 4 °C for 5 min, the supernatant was applied to a column (0.5 $\times$ 3 cm) of AG-50W-X8 (Bio-Rad) equilibrated with 0.1 N HCl. After washing with 5 ml of 1 N HCl, the acid-soluble purines were eluted with 5 ml of 6 N HCl and counted with Aquasol-2 (Packard Instrument Co.) in a scintillation counter. The amount of purines was determined by the absorbance of the eluate at 260 nm. The rates of both purine *de novo* synthesis and protein synthesis were simultaneously determined by the incorporation of [$^{14}$C]glycine (6). To measure the incorporation of [$^{14}$C]glycine in protein, the acid-insoluble precipitate from the last centrifugation was washed three times in 10% trichloroacetic acid and dissolved in 200 $\mu$l of 0.3 N NaOH, and the radioactivity was counted with Aquasol-2 in a scintillation counter. The protein concentrations were assayed by Bradford's method (7).

*Determination of Rate of DNA Synthesis*—The rate of DNA synthesis was determined by bromodeoxyuridine (BrdUrd) incorporation. In a 90-mm culture dish, $2 \times 10^6$ cells were plated. After the recovery of cell function from plating by 18 h of culture, the cells were cultured with 10 $\mu$M BrdUrd for 30 min. Incorporated BrdUrd was quantified with an enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer's protocol.

*Assay of ATase Activity*—Cell lysate for enzyme assay was prepared by sonication and centrifugation (1). To assay ATase activity, the cell lysate was incubated in 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM PRPP (Sigma), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 5 mM [$^{14}$C]glutamine (Amersham Pharmacia Biotech; 5.55 kBq/mmol) at 37 °C for 1 h. Formed [$^{14}$C]glutamate was separated from [$^{14}$C]glutamine by high voltage paper electrophoresis at 800 W for 15 min and counted with toluene scintillation mixture in a scintillation counter. The PRPP-dependent hydrolysis of glutamine to glutamate was regarded as representing ATase activity (8). In the assay solution, ATase was activated by PRPP with the conformational change from tetramer to dimer. Therefore, ATase activity in this study indicates the amount of ATase protein, but not real enzyme activity, in cells.

*Subunit Structure of hATase*—The small active form of hATase is a dimer of subunits, whereas the large inactive form of hATase is a tetramer (9). These two molecular forms of hATase were identified by the retention time of gel filtration by HPLC. The cell lysate prepared for enzyme assay was separated through a TSK-G3000SWXL column (Tosoh, Tokyo, Japan) in 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 M NaCl, 1 mM dithiothreitol, and 10 mM AMP at a flow rate of 0.4 ml/min. The retention time of hATase was determined by ATase assay of eluate fractions.

*Generation of mhATase Transgenic Mice*—An expression vector containing the CAG promoter (chicken $\beta$-actin promoter with cytomegalovirus enhancer) was a kind gift from Dr. Jun-ich Miyazaki (Department of Nutrition and Physiological Chemistry, Osaka University, Japan). mhATase cDNA was inserted into the cloning site of the expression vector, and a *Sal*I fragment excluding plasmid-derived sequences was microinjected into the male pronuclei of fertilized eggs obtained from superovulated BDF1 (C57BL/6 $\times$ DBA/2 F1) female mice crossed with males of the same strain. Injected embryos were implanted into the oviducts of pseudopregnant female mice and allowed to develop (10). DNA was extracted from tail snips of live offspring by the proteinase K/SDS method. The integration of the transgene into the mouse genome was detected by polymerase chain reaction and Southern blot analysis. The copy numbers of integrated transgenes were determined from the intensity of each radioactive band in Southern blot analysis compared with indicator bands of 1, 10, and 100 copies of the transgene. F1 transgenic progeny were bred by crossing transgenic founder mice with BDF1 mice and used for experiments with sex-matched nontransgenic littermates (wild-type mice) at the age of 15–25 weeks. Body weight was determined every week.

*Measurement of Purine Derivatives*—Serum UA concentrations of mice were determined by a uricase method with a UA-L kit (Serotec, Sapporo, Japan). Urinary excretions of allantoin, UA, Hx + xanthine, and creatinine were measured by HPLC through two $\mu$Bondapak C18 columns (Waters Corp., Milford, MA) in 20 mM potassium phosphate buffer (pH 4.0) at a flow rate of 0.5 ml/min. The effluent was monitored at 205 nm, and peak areas of purine derivatives were compared with those of the corresponding standard solutions.

*T Lymphocyte Proliferation by PHA*—Splenocytes ($5 \times 10^5$ cells/well) obtained from mice of 15–20 weeks of age were cultured in RPMI 1640 medium containing 10% FCS and 6 $\mu$g/ml PHA-L (Roche Molecular Biochemicals) in a 96-well plate. After 24, 48, and 72 h, BrdUrd incorporation for 6 h was measured with an enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer's protocol.

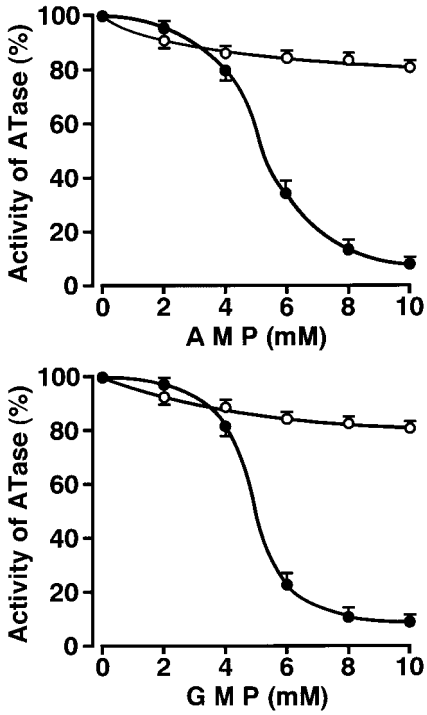


FIG. 1. **Feedback inhibition of ATase by AMP and GMP.** Cell lysates of $^{-}$A + hATase (●——●; $n$ = 4) and $^{-}$A + mhATase (○——○; $n$ = 4) cells were used for the ATase assay. mhATase is resistant to inhibition by AMP and GMP.

*Statistical Analysis*—The data are presented as means ± S.E. For a comparison of two means, Student's paired or unpaired $t$ test was used. A probability value ($p$) of less than 0.05 was considered statistically significant.

## RESULTS

*Characterization of mhATase*—Using cell lysates of $^{-}$A + hATase and $^{-}$A + mhATase cells, the effects of AMP and GMP on ATase activity were examined. hATase was allosterically inhibited by AMP and GMP, whereas mhATase was resistant to inhibition by AMP and GMP (Fig. 1).

hATase has two molecular forms: a homodimer and a homotetramer of subunits. hATase activated by PRPP forms the homodimer, whereas hATase inactivated by purine ribonucleotides forms the homotetramer (9). In the presence of 10 mM AMP, the molecular form of ATase was analyzed by gel filtration. The retention time of ATase showed that all hATase forms were inactive tetramers, whereas most mhATase forms were active dimers even in the presence of 10 mM AMP (Fig. 2).

*Long Term Culture of Transfectants*—Several clones of $^{-}$A + hATase and $^{-}$A + mhATase cells were further cultured in purine-free medium, HamF + XO. Because subclones with high ATase activity proliferate more rapidly than those with low ATase activity, subclones with high ATase activity were gradually selected in the time course of cell culture. As the ATase activity of $^{-}$A + hATase cells increased, the metabolic rate of the *de novo* pathway, the synthesis rates of DNA and proteins, and the growth rate also increased in parallel (Fig. 3). $^{-}$A + mhATase cells cultured in HamF + XO also showed similar results (data not shown). These results showed strong links among ATase activity, the metabolic rate of the *de novo* pathway, the synthesis rates of DNA and proteins, and the cell growth rate.

After a 2-week culture, the ATase activities of $^{-}$A + hATase and $^{-}$A + mhATase cells increased to the same level as ATase in CHO K1 cells (450–550 cpm/$10^6$ cells). When the ATase activity is measured *in vitro*, ATase is activated by PRPP at the maximal level. Therefore, the ATase activity indicates the amount of ATase protein in cells but not the actual ATase activity *in vivo*. Using $^{-}$A + hATase, $^{-}$A + mhATase, and CHO K1 cells with the same ATase levels, the mutual regulation of the *de novo* and salvage pathways was analyzed.

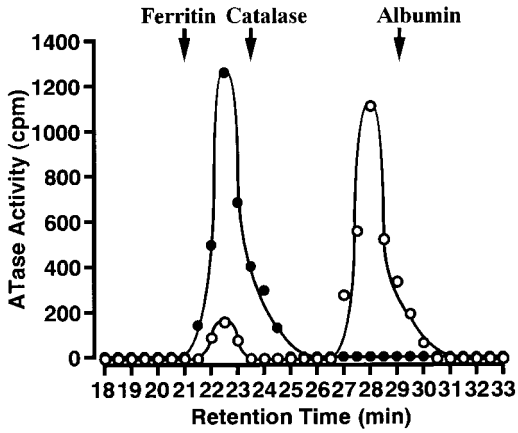


FIG. 2. **Subunit structure of ATase.** Cell lysates of $^{-}$A + hATase (●——●) and $^{-}$A + mhATase (○——○) cells were separated by HPLC through a TSK-G3000SWXL column in 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 M NaCl, 1 mM dithiothreitol, and 10 mM AMP at a flow rate of 0.4 ml/min. ATase activity with a peak retention time of 22.5 min represents the inactive tetrameric form, whereas the enzyme activity with a peak retention time of 28.0 min represents the active dimeric form. In the presence of 10 mM AMP, all hATase formed tetramers, and most mhATase formed dimers. Molecular markers: ferritin, 440 kDa; catalase, 232 kDa; albumin, 67 kDa.

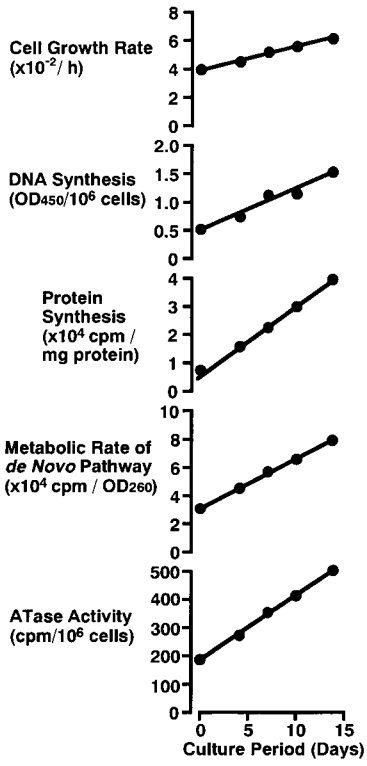


FIG. 3. **Long term culture of $^{-}$A + hATase cells.** $^{-}$A + hATase cells were cultured in HamF + XO. Because subclones with high ATase activity proliferate more rapidly than those with low ATase activity, subclones with high ATase activity were positively selected. As intracellular ATase activity gradually increased, the metabolic rate of the *de novo* pathway, the rates of DNA and protein syntheses, and the cell growth rate also increased in parallel.

*Mutual Regulation of de Novo and Salvage Pathways*—The metabolic rate of the *de novo* pathway in CHO K1 cells under

TABLE I
*Metabolic rates of de novo and salvage pathways of four cell lines*

| Cultured Cells | *De Novo* Pathway (x10$^5$ cpm/OD$_{260}$) | | Salvage Pathway (x10$^5$ cpm/OD$_{260}$) |
|---|---|---|---|
| | Culture Medium | | |
| | HamF | HamF+XO | HamF |
| CHO K1 | 1.6 ± 0.1 | 7.9 ± 0.3 | 12.4 ± 0.5 |
| | [-80%] | | [-13%] |
| $^-$A+hATase | 1.9 ± 0.1 | 8.2 ± 0.4 | 12.0 ± 0.3 |
| | [x 16] [-77%] | [x 3.8] | |
| CHO ade $^-$A | not detectable | not detectable | 14.3 ± 0.5 |
| | | | [-52%] |
| $^-$A+mhATase | 25.0 ± 1.0 | 29.8 ± 0.9 | 6.9 ± 0.1 |
| | [-16%] | | |

conditions where only the *de novo* pathway was functioning in HamF + XO was 5 times higher than that under conditions where both purine biosynthetic pathways were functioning in HamF, *i.e.* the salvage pathway strongly suppresses the *de novo* pathway down to 20% of the initial value (Table I). $^-$A + hATase cells also showed a similar result. In contrast, in $^-$A + mhATase cells, the suppression of the *de novo* pathway by the salvage pathway was only 16%, and the metabolic rate of the *de novo* pathway in HamF was 16 times higher than that in CHO K1 cells. These findings indicate that the suppression of the *de novo* pathway by the salvage pathway was primarily due to feedback inhibition of ATase by purine ribonucleotides produced through the salvage pathway. Moreover, even in HamF + XO, the metabolic rate of the *de novo* pathway in $^-$A + mhATase cells was 3.8 times higher than that in CHO K1 cells, indicating that AMP and GMP produced through the *de novo* pathway also inhibit ATase in a negative feedback loop *in vivo*.

The metabolic rate of the salvage pathway in CHO K1 cells under the conditions where both pathways were functioning in HamF was only 13% less than that under the conditions where only the salvage pathway in CHO ade $^-$A cells was functioning in HamF (Table I). A similar result was obtained in $^-$A + hATase cells. In $^-$A + mhATase cells, however, the metabolic rate of the salvage pathway was strongly suppressed by the *de novo* pathway in HamF.

*Contributions of ATase and Its Feedback Regulation to Biosyntheses*—The contributions of ATase to various biosyntheses are represented as the rates of biosyntheses relative to the ATase level (Table II). The contributions of ATase to biosyntheses in $^-$A + hATase and $^-$A + mhATase cells were compared, and the contributions of feedback regulation of ATase to these biosyntheses are represented as the ratio of the contributions of hATase and mhATase to the biosyntheses (mhATase/hATase ratio). The contributions of hamster ATase in CHO K1 cells to the biosyntheses were similar to those of hATase in $^-$A + hATase cells (Table II).

The contribution of hATase to purine *de novo* synthesis in HamF was smaller than that in Ham F + XO (37.1 *versus* 163.1) because the salvage pathway inhibits the *de novo* pathway in HamF (Table II). Therefore, the contribution of the feedback regulation to purine *de novo* synthesis in HamF was greater than that in HamF + XO (13.5 *versus* 3.7).

In hamster ATase, hATase, and mhATase, the contributions of ATase to protein and DNA syntheses in HamF were modestly larger than those in HamF + XO, although the differences are not statistically significant (Table II). These findings indicate that the *de novo* pathway alone in HamF + XO can supply almost all purine nucleotides necessary for protein and DNA syntheses, and the additional effect of the salvage pathway in HamF is trivial. The contributions of mhATase to protein and DNA syntheses were, respectively, 1.6–1.8 and 3.5–3.9 times higher than those of hATase, suggesting that the feedback inhibition of ATase also regulates protein and DNA syntheses in cultured fibroblasts.

*Contributions of ATase and Its Feedback Regulation to Cell Growth Rate*—$^-$A + hATase and $^-$A + mhATase cells were cultured in HamF + XO for several weeks. When their ATase levels reached maximum plateau values (about 3 times the ATase level of CHO K1 cells), their maximal growth rates were determined in HamF and compared with the growth rates of CHO K1 and CHO ade $^-$A cells (Table III). The maximal growth rates of $^-$A + hATase and $^-$A + mhATase cells were higher than those of CHO K1 cells. In particular, the doubling time of $^-$A + mhATase cells was shortened to 6.0 h by long term culture for more than 8 weeks. To our knowledge, $^-$A + mhATase cells grow at the highest rate of all cultured mammalian cells investigated. These results show that ATase and its feedback inhibition regulate the growth rate of cultured fibroblasts.

The growth rate of CHO K1 cells in HamF + XO was higher (6.1 *versus* 4.2) than that of CHO ade $^-$A cells in HamF, *i.e.* the contribution of the *de novo* pathway to the growth rate was greater than that of the salvage pathway (Table III). The additional contribution of the salvage pathway to the growth rate was also small (from 6.1 to 6.6).

*Generation of mhATase Transgenic Mice*—We obtained nine founders of mhATase-Tg mice. The number of transgene copies in these transgenic mice ranged from 5 to 100. In expectation of high level mhATase expression, two founder mice with high copy numbers of more than 50 were bred, and their F1 mice were used in this study. Body weights measured weekly were similar in both lines of transgenic mice and wild-type mice at least up to 25 weeks of age.

*Purine Nucleotide Metabolism in mhATase-Tg Mice*—In mhATase-Tg mice, liver ATase activity (1.8-fold), spleen ATase activity (1.6-fold), the metabolic rate of the *de novo* pathway in the liver (1.8-fold), urinary excretion of allantoin + UA normalized to creatinine excretion (1.5-fold), and serum UA concentration (1.5-fold) were significantly higher than these values in wild-type mice (Figs. 4 and 5).

*T Lymphocyte Proliferation by PHA*—T lymphocytes in mouse splenocytes were stimulated by PHA. After 48 and 72 h, BrdUrd incorporation by T lymphocytes in mhATase-Tg mice was significantly greater than that in wild-type mice (Fig. 6).

## DISCUSSION

*Subunit Structure of ATase*—In the presence of 10 mM AMP, hATase formed inactive tetramers, whereas most mhATase remained in the active dimeric form, *i.e.* mhATase was resistant to a conformational change induced by AMP. A minor part of the mhATase was in tetrameric form. This tetramer may be a complex of mhATase subunits and hamster ATase subunits inactivated by a mutation in CHO ade $^-$A cells because hamster ATase was always found in the form of tetramers, even in the presence of PRPP, and because the less common tetrameric forms of hATase and mhATase were detected along with the major dimeric forms in the presence of PRPP (data not shown).[2]

*Mutual Regulation of de Novo and Salvage Pathways*—Purine nucleotides are synthesized preferentially by the salvage pathway as long as hypoxanthine is available, with concomitant inhibition of the *de novo* pathway for sparing the energy expenditure required for *de novo* synthesis (1, 11). In $^-$A + mhATase cells, the suppression of the *de novo* pathway by the salvage pathway was strongly inhibited even in the presence of

[2] T. Yamaoka and M. Itakura, unpublished data.

TABLE II
*Contributions of ATase and its feedback regulation to various biosyntheses*

| Contribution to purine *de novo* synthesis | | | | |
|---|---|---|---|---|
| Culture medium | *De novo* synthesis/ hamster ATase | *De novo* synthesis/ hATase | *De novo* synthesis/ mhATase | mhATase/hATase ratio |
| HamF | 32.4 ± 3.8 | 37.1 ± 4.1 | 500.9 ± 42.5 | 13.5 |
| HamF + XO | 157.3 ± 13.9 | 163.1 ± 20.9 | 596.5 ± 67.5 | 3.7 |
| **Contribution to protein synthesis** | | | | |
| Culture medium | Protein synthesis/ hamster ATase | Protein synthesis/ hATase | Protein synthesis/ mhATase | mhATase/hATase ratio |
| HamF | 43.4 ± 3.9 | 48.6 ± 4.1 | 88.6 ± 5.9 | 1.8 |
| HamF + XO | 39.8 ± 5.1 | 45.7 ± 2.9 | 73.0 ± 7.5 | 1.6 |
| **Contribution to DNA synthesis** | | | | |
| Culture medium | DNA synthesis/ hamster ATase | DNA synthesis/ hATase | DNA synthesis/mhATase | mhATase/hATase ratio |
| HamF | 0.017 ± 0.002 | 0.019 ± 0.002 | 0.075 ± 0.010 | 3.9 |
| HamF + XO | 0.016 ± 0.002 | 0.015 ± 0.004 | 0.053 ± 0.009 | 3.5 |

TABLE III
*Growth of CHO fibroblasts*

| Cultured cells | Culture medium | Doubling time | Growth rate |
|---|---|---|---|
| | | *h* | $10^{-2}/h$ |
| CHO ade $^{-}$A | HamF | 23.8 ± 0.2 | 4.2 |
| CHO K1 | HamF + XO | 16.5 ± 0.1 | 6.1 |
| CHO K1 | HamF | 15.1 ± 0.2 | 6.6 |
| $^{-}$A + hATase | HamF | 10.1 ± 0.1 | 9.9 |
| $^{-}$A + mhATase | HamF | 6.0 ± 0.1 | 16.7 |

sufficient Hx in HamF. Therefore, the mechanism by which the salvage pathway suppresses the *de novo* pathway was shown to be mainly due to the feedback inhibition of ATase rather than to the consumption of PRPP, the common substrate of both pathways. From these results, the mechanism of the accelerated *de novo* pathway in patients with Lesch-Nyhan syndrome can also be explained as follows: AMP and GMP produced through the salvage pathway decrease because of complete HPRT deficiency; decreased AMP and GMP reduce the feedback inhibition of ATase, and activated ATase increases the *de novo* synthesis of purine nucleotides. Indeed, primary cultured astroglia from HPRT-deficient mice showed reduced levels of intracellular purine ribonucleotides with a 9.4-fold acceleration of *de novo* purine synthesis (12). Even under conditions where only the *de novo* pathway functions without the suppression by the salvage pathway, mhATase activated the *de novo* pathway by a factor of 3.7–3.8. Therefore, it was first demonstrated in cultured cells that purine ribonucleotides produced through the *de novo* pathway inhibit ATase in a negative feedback loop.

In addition to the activation of the *de novo* pathway, mhATase also strongly suppressed the salvage pathway. The suppression of the salvage pathway by the *de novo* pathway is probably due to consumption of PRPP by the *de novo* pathway because the activity of the *de novo* pathway has no effect on HPRT expression (1) and HPRT is not regulated by the feedback inhibition. Indeed, a large consumption of PRPP by *de novo* synthesis is suggested by the 16-fold increase in PRPP concentration in human leukemia cells treated with 6-methylmercaptopurine riboside to inhibit *de novo* synthesis (13), whereas the primary cultured astroglia from HPRT-deficient mice showed only a 27.8% increase in intracellular PRPP level (12).

*Contributions of ATase and Its Feedback Regulation to Various Biosyntheses and Cell Growth Rate*—In addition to the acceleration of purine *de novo* synthesis, mhATase also increased the rates of DNA and protein syntheses and of cell growth, suggesting that ATase and its feedback inhibition regulate the growth rate via the rates of DNA and protein syntheses. We previously reported that purine *de novo* synthesis is a determinant of intracellular ATP concentration and promotes $G_1$ to S phase transition in the cell cycle, *i.e.* the initiation of DNA synthesis (11). Therefore, ATase and its feedback inhibition probably regulate DNA and protein syntheses through ATP production.

Surprisingly, the minimum doubling time of $^{-}$A + mhATase cells was as short as only 6.0 h, thus representing one of the most rapidly growing cell types. This result also indicates the great contribution of ATase and its feedback inhibition to regulating the growth rate of cultured fibroblasts.

*Purine Nucleotide Metabolism in mhATase-Tg Mice*—In transgenic mice expressing normal hATase, the acceleration of purine *de novo* synthesis cannot be expected because of the feedback inhibition of hATase. Indeed, $^{-}$A + hATase cells with an ATase level twice that of CHO K1 cells showed only a 1.2-fold increase in the rate of purine *de novo* synthesis (1). Therefore, mhATase was used to generate transgenic mice in this study. In mhATase-Tg mice, increases in ATase levels in the liver (1.8-fold) and spleen (1.6-fold) were comparable to the increase in the metabolic rate of the *de novo* pathway (1.8-fold). Furthermore, ATase activities in the liver and spleen of mhATase-Tg mice were resistant to inhibition by AMP (data not shown). These results suggest that mhATase is primarily expressed in mhATase-Tg mice, and the endogenous expression of mouse ATase is suppressed. Normal ATase protein may have a short half-life in the presence of excessive mhATase, whereas mhATase may also be resistant to protein degradation. mhATase-Tg mice also showed increases in serum UA concentration and urinary excretion of allantoin + UA. These findings suggest that the superactivity of ATase leads to hyperuricemia in humans, although the germline mutation of the human ATase gene has not been reported. Purine *de novo* synthesis is also regulated by the feedback inhibition of PRPP synthetase, and the superactivity of this enzyme by point mutations results in hyperuricemia and gout (14). Both in $^{-}$A + mhATase cells and in mhATase-Tg mice, the feedback regulation of PRPP synthetase by purine ribonucleotides was intact, but the acceleration of the *de novo* pathway was not suppressed by the feedback regulation of PRPP synthetase. This indicates that the feedback inhibition of ATase is more important for the regulation of the *de novo* pathway than that of PRPP synthetase and that PRPP synthetase indirectly regulates the *de novo* pathway via ATase activation by producing PRPP. Indeed, the studies using fibroblasts from patients with Lesch-Nyhan syndrome and with PRPP synthetase superactivity supported the following concepts. 1) The rate of purine *de novo* synthesis is regulated at both PRPP synthetase and ATase reactions, and the latter reaction is more sensitive to small changes in purine ribonucleotide concentrations. 2) PRPP is a major regulator of the purine synthesis rate

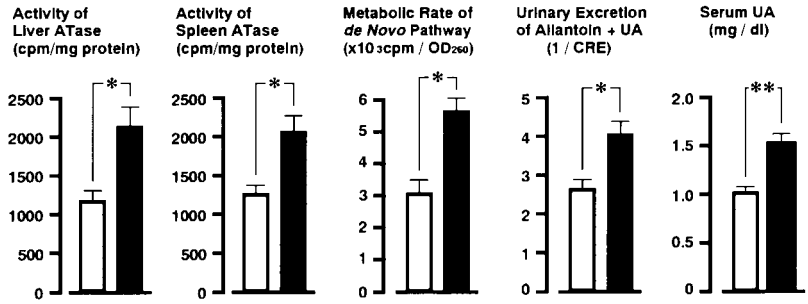


FIG. 4. **Purine nucleotide metabolism in mhATase-Tg mice.** Liver and spleen homogenates in 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM dithiothreitol, 100 $\mu$g/ml phenylmethylsulfonyl fluoride, and 2 $\mu$g/ml aprotinin were centrifuged at 12,000 $\times$ *g* at 4 °C for 20 min. The supernatants were used for the ATase assay. For determination of the metabolic rate of the *de novo* pathway, 200 $\mu$l of saline containing 50 mM glycine with [$^{14}$C]glycine (1.85 MBq) were administered intraperitoneally. After 60 min, the mouse liver was removed, and a liver homogenate in 0.4 N perchloric acid was prepared for the extraction of radiolabeled purines. The numbers of samples ranged from seven to nine for each bar. *White bars*, wild-type mice; *black bars*, mhATase-Tg mice. *, $p < 0.05$; **, $p < 0.01$.

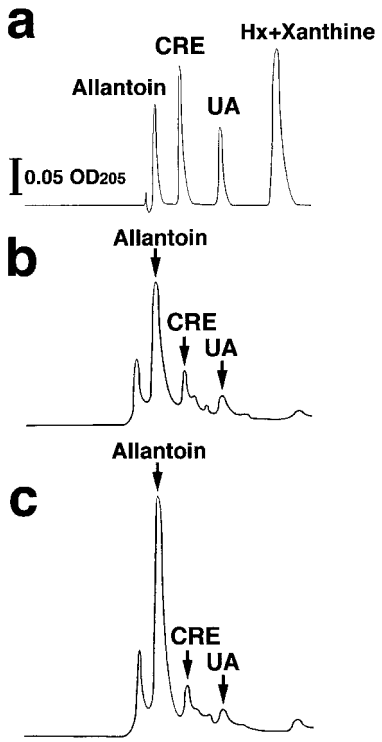


FIG. 5. **Urinary excretion of purine derivatives.** *a*, standard solution; *b*, a wild-type mouse; and *c*, an mhATase-Tg mouse. Under the chromatographic conditions in this study, Hx and xanthine were not separated, and the peak of Hx + xanthine was not detected in mouse urine. Therefore, the urinary excretion of allantoin + UA was regarded as the urinary excretion of total purine derivatives and was normalized to creatinine excretion (Fig. 4).

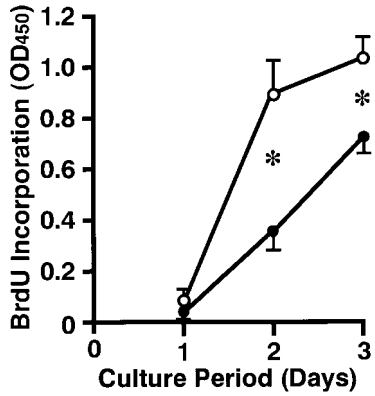


FIG. 6. **T lymphocyte proliferation by PHA.** Splenocytes obtained from mice of 15–20 weeks of age were cultured in RPMI 1640 medium containing 10% FCS and 6 $\mu$g/ml PHA in a 96-well plate, and BrdUrd incorporation was determined every 24 h. ○——○, mhATase-Tg mice ($n = 7$); ●——●, wild-type mice ($n = 7$). *, $p < 0.05$.

because it is a determinant of ATase activity. 3) Activation of ATase by PRPP is nearly maximal at baseline in fibroblasts with PRPP synthetase superactivity (15).

*T Lymphocyte Proliferation by PHA*—Resting human T lymphocytes are reported to meet their metabolic demands via the salvage pathway, whereas intact *de novo* synthesis is essential for the proliferation of PHA-stimulated T lymphocytes (16). Even in patients with Lesch-Nyhan syndrome, the proliferation of T lymphocytes in response to mitogenic and antigenic stimulation was normal (17). Moreover, in leukocytes from two gouty patients affected with a partial deficiency of HPRT, *de novo* synthesis was accelerated to more than 13 times that of normal controls (18). These reports suggest that the large capacity of purine *de novo* synthesis can compensate for the defect in the salvage pathway. In mhATase-Tg mice, T lymphocytes stimulated by PHA proliferated more rapidly than those in wild-type mice. The rate of purine *de novo* synthesis may determine the growth rate of T lymphocytes under mitogen stimulation. Indeed, severe immunological abnormalities are induced by deficiencies in enzymes of purine nucleotide metabolism such as adenosine deaminase and purine nucleoside phosphorylase. Recently, methotrexate, an immunosuppressive agent, has been viewed as the drug of choice in treating rheumatoid arthritis. Low dose methotrexate inhibits ATase and mitogen-induced expansion of ATP and GTP pools in human T lymphocytes and induces cell arrest at the $G_1$ phase, thus exerting immunosuppressive effects (19, 20).

In conclusion, the following concepts are supported by this study using CHO transfectants with mhATase and mhATase-Tg mice. First, ATase and its feedback inhibition regulate not only the rate of purine *de novo* synthesis but also DNA and protein synthesis rates and the growth rate in cultured fibroblasts. Second, the suppression of the *de novo* pathway by the salvage pathway is primarily due to the feedback inhibition of ATase by purine ribonucleotides produced through the salvage pathway, whereas the suppression of the salvage pathway by the *de novo* pathway is due to the consumption of PRPP by the *de novo* pathway. Third, the feedback inhibition of ATase is more important for the regulation of the *de novo* pathway than that of PRPP synthetase, and PRPP synthetase indirectly regulates the *de novo* pathway via ATase activation by producing PRPP. Finally, ATase superactivity leads to hyperuricemia and increases BrdUrd incorporation in T lymphocytes stimulated by PHA. Understanding the regulation of purine synthetic pathways and their relationship to DNA and protein syntheses and cell growth is useful for various medical

applications such as developing therapies for malignant neoplasms and autoimmune diseases.

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