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Detection of a Novel Sepiapterin Reductase mRNA: Assay of mRNA in Various Cells and Tissues of Various Species

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Fragments of cDNA coding for rat, murine, and human sepiapterin reductase (SR) were amplified by PCR via primer positioning close to the reported 3′-end of the coding region in the rat enzyme. They were sequenced and used as probes for mRNA detection. Northern blot analysis detected two mRNA species for SR. Their sizes were 1.3 and 2.1 kb for rat, 1.3 and 2.3 kb for mouse, and 1.6 and 2.1 kb for human cell lines. Comparison of rat cell lines and rat tissues indicated that in tissues only the 1.3-kb species is present. Washing of the Northern blots under different stringency conditions indicated a more stable interaction of the 1.3-kb mRNA species with the cDNA probe as compared to the 2.3-kb species. The 1.3-kb species corresponds to the reported 28.2-kDa molecular mass of rat SR monomer. SR mRNA expression is absent in the human NK-like cell line YT and in the murine erythroleukemia subclone B8/3, which both lack SR activity. Moreover, the relative mRNA expression correlates with the enzymatic activities of different cell lines within the same species. This indicates that SR activity is regulated by its steady state mRNA levels.

INTRODUCTION

H_4biopterin is the required cofactor for tryptophan, phenylalanine, and tyrosine hydroxylases (for review, see [1]) and for the cleavage of glyceryl ethers [2]. It is also involved in the biosynthesis of nitric oxide from arginine [3]. Tissues which are competent for neurotransmitter biosynthesis and phenylalanine degradation such as adrenal medulla, hypothalamus, and liver display high specific activities of the enzymes involved in the *de novo* pathway of H_4biopterin synthesis [4].

Recent evidence has shown that H_4biopterin is also synthesized in cells not related to the above-mentioned metabolic events. This is the case during cytokine-directed proliferation and differentiation of cells, for example, during hematopoiesis and cellular immune response (for review, see [5]). H_4biopterin, in turn, enhances the proliferation of erythroleukemic cells [6, 7] and modulates various aspects of the interleukin 2-induced clonal expansion of T cells which is directed by interleukin 2 [8, 9].

The *de novo* synthesis of H_4biopterin begins with GTP. The first step is catalyzed by GTP-cyclohydrolase I (EC 3.5.4.16) and results in the formation of dihydroneopterin triphosphate. PPH_4 synthase catalyzes the elimination of triphosphates, accompanied by an intramolecular reaction yielding 6-pyruvoyl-H_4pterin. The hydride equivalents for the final conversion of PPH_4 to H_4biopterin are provided by NADPH. Sepiapterin reductase (SR) (EC 1.1.1.153) can catalyze the reduction of both C1′ and C2′ oxo groups. In addition, the involvement of aldose reductase (EC 1.1.1.21, formerly named PPH_4 reductase) and of carbonyl reductase (EC 1.1.1.184) has been proposed [10–12]. These enzymes preferentially reduce the 2′ oxo group to yield 6-lactoyl H_4pterin which can be further reduced to H_4biopterin by either SR or aldose reductase [12]. It has been suggested that the concerted action of both these reductases could replace the catalytic action of SR by an alternative pathway, e.g., in brain tissue [12].

The enzymes involved in H_4biopterin synthesis are constitutively expressed in cells which are competent for oxygenase reactions [4]. In proliferating cells, however, GTP-cyclohydrolase I is subject to control by the action of cytokines. Increases in activity of this rate-limiting enzyme to about fourfold levels are induced by stimulation of interferon-γ in monocytes/macrophages [13] and is further enhanced by interleukin 2 in activated T cells [14]. In rat thymocytes the increase in

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Abbreviations used: biopterin, 6-(L-*erythro*-1′,2′-dihydroxypropyl)-pterin; bp, base pair(s); H_2biopterin, 7,8-dihydrobiopterin; H_4biopterin, 5,6,7,8-tetrahydrobiopterin; neopterin, 6-(D-*erythro*-1′,2′,3′-trihydroxypropyl)-pterin; PCR, polymerase chain reaction; PPH_4, 6-pyruvoyl-H_4pterin; RT-PCR, reverse transcription followed by polymerase chain reaction; sepiapterin, 6-lactoyl-7,8-dihydropterin; SR, sepiapterin reductase; SSC, standard saline citrate.

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GTP-cyclohydrolase I activity during the G_1 phase of the cell cycle correlates with increasing steady state mRNA levels specific for this enzyme [15]. The decline of GTP-cyclohydrolase I activity during the DNA synthesizing phase, however, does not correlate with a decrease in mRNA levels but, rather, appears to be due to post-translational modification of this enzyme [15; Schott *et al.*, submitted for publication].

The specific activities of SR are generally 5- to 20-fold higher than those of GTP-cyclohydrolase I. In mammalian tissues which are competent for the oxygenase reaction such as liver, SR activities average 2200 pmol mg^{-1} min^{-1} [16]; they vary between 500 and 1400 pmol mg^{-1} min^{-1} in different regions of the brain [17]. In cells which undergo cytokine-directed proliferation the activity of SR is in the range of 100 pmol mg^{-1} min^{-1} [14, 18]. Lectin stimulation of resting T cells causes a slowly progressing increase, starting from levels below the detection limit [19] and a transient enhancement of SR activity is found after treatment of activated T cells by interferon-γ plus interleukin 2 [14]. The NK-like human cell line YT [20] and the murine erythroleukemic cell line B8/3 [7] lack SR activity. Thus far, a deficiency of SR activity in human organs has not been reported. It has been speculated that the absence of activity is compensated by the concerted action of both aldose and carbonyl reductase [12]. The molecular basis of SR regulation has not been explained in any of these cases.

Based on the published sequences of rat [21] and human [22] SR cDNA, we constructed cDNA probes which specifically hybridize with rat, murine, or human SR mRNA, respectively. They were used for Northern blot analysis of mRNA expression in different tissues and cell lines of these mammalian species. This study characterizes two mRNA species specific for SR and investigates the regulation of SR activity at the level of mRNA expression.

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials. The origins of the following materials are given in parentheses: T7 sequencing kit and Nick columns (Pharmacia, Freiburg, FRG); PCR TA cloning kit (Invitrogen, ITC Biotechnology, Heidelberg, FRG); DNA molecular weight marker V (8–587 bp), Taq DNA polymerase and primer $p(dT)_{15}$ (Boehringer-Mannheim, FRG); RNAsin and PolyATtract mRNA isolation kit (Promega, Serva, Heidelberg, FRG); Moloney-murine leukemia virus reverse transcriptase (GIBCO-BRL, Eggenstein, FRG); Microgrado 12% homogeneous polyacrylamide gel HST-120 and 3–30% gradient polyacrylamid gel GST-330 (Gradipore, Pyrmont, Australia); DEAE-paper NA-45 (Schleicher & Schüll, Dassel, FRG); and HPLC column 4000-5 DEAE 125 × 6 mm (Macherey & Nagel, Düren, FRG). Other chemicals and materials used are listed in [15] or were of high-quality commercial grade. The cDNA probe for human β-tubulin (264 bp) was a gift of Dr. Thomas Krieg (University of Munich).

Cell cultures and tissues. The rat liver cell line HTC, the murine liver cell line BW1-J [23], the human liver cell line Hep G2, the human T cell line HuT 102, the human natural killer-like cell line YT, and the subclones F4N and B8/3 of the murine Friend erythroleukemia cell line were grown under the conditions described by Ziegler *et al.* [14] and Schott *et al.* [15]. For cultivation of the murine cytotoxic T cell clone CTLL-2, interleukin 2 (10 U ml^{-1}) was added. The adherent liver cell lines were grown to confluence, the suspension cultures to a density of 1×10^6 cells ml^{-1}. The thymocytes, synchronized at the G_1 stage, were obtained from K. Brand (Institut für Biochemie, Erlangen) [15]. The organs and tissues of rats (*Rattus norvegicus*) were dissected immediately after death and kept frozen in liquid nitrogen.

Enzyme assay. Aliquots of 5×10^7 cells were harvested by trypsinization of the adherent cells (50 μg ml^{-1} trypsin, 2 m*M* EDTA in 5 m*M* phosphate-buffered saline, pH 7.5), or by centrifugation (500*g*, 10 min) of the suspension cultures. The samples were extracted and desalted as described [24]. SR activity was determined in triplicate by NADPH-dependent reduction of sepiapterin to H_2biopterin. The assay conditions have been described by Kerler *et al.* [25]. The detection limit was ~0.2 pmol biopterin mg^{-1} min^{-1}. Protein concentrations were estimated by the Coomassie blue dye assay [26] with serum albumin as a standard.

Preparation of mRNA. Frozen tissue (1–2 g) or cells (5×10^7–1×10^8) were homogenized under liquid nitrogen with lysis buffer (10 ml) of 5 *M* guanidinium isothiocyanate, 5 m*M* β-mercaptoethanol, 25 m*M* sodium citrate, pH 7.0, and processed according to the method described by Chirgwin *et al.* [27]. Poly(A)$^+$ RNA was obtained from 0.7–1.2 mg of total RNA with the magnet bead method of the PolyATtract kit according to the manufacturer's instructions.

Primer design for RT-PCR and oligonucleotide synthesis. The selection of the primers basically followed the guidelines provided by Innis and Gelfand [28], Saiki [29], and Kawasaki [30], using the cDNA sequence of rat SR [21]. The primers were synthesized on an Applied Biosystems DNA synthesizer (Model 381 A) and purified by gel filtration with Sephadex G-50.

Polymerase chain reaction (PCR). Reverse transcriptase PCR (RT-PCR) was performed according to Kawasaki [30] with an annealing temperature of 55°C. For amplification of murine and human cDNA with the nonhomologous rat primers, the annealing temperature was lowered to 50°C and the primer concentration was doubled. One microliter of the resulting mixture was reamplified for 20 cycles with an annealing temperature of 55°C. Reamplification of purified products was performed by 20 cycles of PCR using an annealing temperature of 55°C with 1 ng of template DNA. The PCR products were analyzed by polyacrylamide gel electrophoresis and visualized with ethidium bromide.

Purification of PCR products. Products of RT-PCR and reamplification mixtures were separated on a 2% agarose gel and bands were collected by electrophoretic transfer to DEAE-paper NA-45 [31]. They were eluted, precipitated with ethanol, and used for reamplification. The resulting PCR products were purified by HPLC anion-exchange chromatography on a Nucleogen 4000-7 DEAE column with a gradient of 0–1.2 *M* NaCl in 30 m*M* phosphate buffer, pH 6.7, containing 6 *M* urea (flow rate 1 ml min^{-1}).

Cloning of PCR fragments. The PCR fragment generated by primers SR-8 and SR-10 from human template cDNA was purified by agarose gel electrophoresis and HPLC procedure. Ligation with the T-tailed pCR 1000 vector of the TA cloning kit from Invitrogen and transformation was performed according to the manufacturer's instructions.

DNA sequencing. The purified PCR products were directly sequenced by the chain termination method of Sanger *et al.* [32], as modified by Bachmann *et al.* [33]. The T7 DNA polymerase sequencing kit was used according to the manufacturer's instructions. The reaction products were labeled with [α-^{32}P]dCTP. The plasmid containing cloned PCR product was sequenced according the manufacturer's instructions for double-strand plasmid sequencing.

Probe labeling. Random priming was performed with the multiprime labeling kit according to the manufacturer's instructions. Probe synthesis by PCR was performed as described by Verbeek and

TABLE 1

Sequences of Primers Used in PCR Amplification

Primers used			Sequence spanned
Primer	Direction	Bases	5′ to 3′
SR-1	Forward	145–162	AAGGAGGAGCTCTGTACG
SR-2	Forward	176–194	TGCAAGTGGTGCTGGCAGC
SR-3	Reverse	282–299	CCTGCATTGTTGATGAGC
SR-4	Reverse	349–356	CCAGTAGTTGTTCACCTC
SR-5	Forward	481–497	CCTTTCAAGGGCTGGGG
SR-6	Reverse	546–562	TGGGTTCCTCAACAGCC
SR-7	Reverse	597–613	GCTGCATGTTGGTGTCC
SR-8	Forward	597–613	GGACACCAACATGCAGC
SR-9	Reverse	706–724	AGCTCAGCAGTTTCTGGGC
SR-10	Reverse	756–773	TCATAGAAGTCCACGTGG

Note. The positions are indicated according to the published cDNA sequence of rat SR [21].

Tijssen [34] for double-strand DNA amplification systems and was modified by using 5 μl of [α-^{32}P]dCTP and an annealing temperature of 55°C. Both labeling methods yielded identical results in Northern blot analysis. Radiolabeling of human β-tubulin cDNA was performed by random priming.

Northern blot analysis. Five micrograms mRNA of each sample was electrophoresed and subsequently transferred to nylon membranes as described in Schott *et al.* [15]. Relative positions of 18 S and 28 S rRNAs were used for determination of molecular size. Nylon membranes were prehybridized for 6 h and hybridization was performed for 18 h at 42°C, then the membranes were finally washed at 42°C for SR mRNA detection and at 56°C for β-tubulin mRNA detection [15]. Signals from the autoradiograms were estimated by laser densitometry (2222-020 Ultroscan XL, 2D program, Pharmacia) and the sum of the signal intensities of both visible bands was used. The range of linear response was determined by calibration curves. Calibration of SR mRNA expression relative to the amount of mRNA loaded on the gels was performed by hybridization with a β-tubulin cDNA probe.

RESULTS

Construction of cDNA fragments by RT-PCR. The oligonucleotides for RT-PCR were selected according to the guidelines summarized in the methods section and are listed in Table 1. For amplification experiments, each of the four forward primers was alternatively combined with each of the six reverse primers. mRNA was isolated from the rat liver cell line HTC, the murine liver cell line BW1-J, and the human liver cell line Hep G2 and the products obtained with these templates were analyzed by polyacrylamide gel electrophoresis. Among the forward primers two different types were found. SR-1 and SR-2, but not SR-5, in combination with the reverse primers SR-3, SR-4, SR-6, and SR-10, yielded amplificates of expected molecular weights and without unspecific bands only when mRNA from rat was used as a template (data not shown). In contrast, SR-8, in combination with the reverse primers SR-9 or SR-10, yielded amplificates showing the expected molecular weight with rat, murine, and human liver cDNA as well

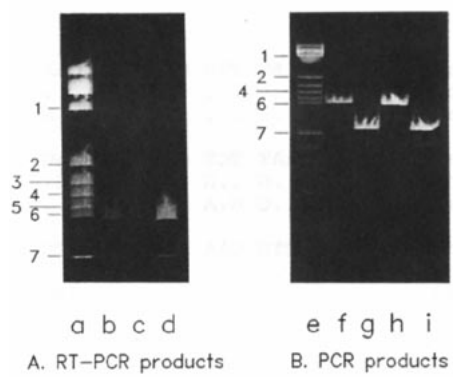

FIG. 1. SR cDNA fragments obtained by RT-PCR from rat, murine, and human template mRNA by combination of the primers SR-8 with SR-9 or SR-10. (A) Separation of the RT-PCR products H4, M4, and R4 (using primers SR-8 and SR-10) with 12% polyacrylamide gel electrophoresis. Lane a, DNA molecular weight markers with 434 bp (1), 267 bp (2), 234 bp (3), 213 bp (4), 192 bp (5), 184 bp (6), and 124 bp (7); lane b, mRNA from human liver cell line Hep G2; lane c, mRNA from the murine liver cell line BW1-J; lane d, mRNA from the rat liver cell line HTC. (B) Separation of the cDNA fragments M4, M5, H4, and H5 by 3–33% gradient polyacrylamide gel electrophoresis after HPLC purification of RT-PCR product M4 followed by PCR amplification (generating PCR product M4 using primers SR-8 and SR-10 and M5 using primers SR-9 and SR-10) or cloning of RT-PCR product H4 and amplification (generating PCR product H4 using primers SR-8 and SR-10 and H5 using primers SR-9 and SR-10). Lane e, DNA molecular weight markers with 434 bp (1), 267 bp (2), 213 bp (4), 184 bp (6), and 124 bp (7); lane f, M4; lane g, M5; lane h, H4; lane i, H5.

(Fig. 1A). The identity of the amplificates was confirmed by direct sequencing. The product M4 obtained from murine mRNA template was reamplified and purified by HPLC; the product H4 obtained from human mRNA template was cloned to a T-tailed vector (Fig. 1B). Figure 2 summarizes the position of all cDNA fragments which were further used for Northern blot analysis (see below).

The fragments R4, M4, and H4 were enriched by reamplification and purified by HPLC. Their sequences and the deduced amino acid sequences are shown in Fig. 3. The rat and murine cDNA fragments are 92% identical. This value drops to 77 and 74% when human cDNA

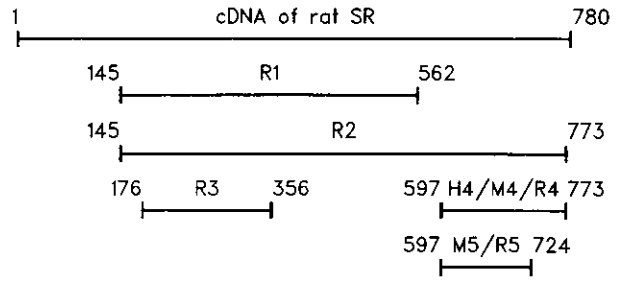

FIG. 2. Map of PCR products which were used as hybridization probes. Their relative positions in the protein coding region of the rat cDNA are indicated according to [21].

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A

rat     614    AG TTG GCC CGG GAA ACC TCC ATG GAC CCA GAG TTG AGG AGC AGA CTG CAG AAG TTG
mouse          .. ... ..T ... ... ... ... .A. ... ... ... ..A ... ... .A. ... ... ... ...
man            .. ... ... ... ..G ... ... G.. ... ... ..C A.. C.A .AA G.G ... ... G.. C..

rat     670    AAT TCT GAG GGG GAG CTG GTG GAC TGT GGG ACT TCA GCC CAG AAA CTG CTG AGC TTG
mouse          ..G ..G ..T ... .C. ... ... ... ... ... ... ... ... ... ... ... ... G.. ...
man            ..G G.A A.. ... A.. ... ... ..T ..C AA. GTG ... ... ... ... ... ... ... ..A

rat     727    CTG CAA AGG GAC ACC TTC CAA TCT GGA GC   755
mouse          ... ... .A. ... ... ... ..G ... ... ..
man            ... G.. .A. ... GAG ... A.G ... ... ..

B

rat     209    LARETSMDPELRSRLQKLNSEGELVDCGTSAQKLLSLLQRDTFQSGA   255
mouse          ......K......K....K.D.A.............G...K.......
man            ......V..DM.KG..E.KAK.K....KV.........EK.E.K...
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FIG. 3. Sequence alignment of the cDNA fragments R4, M4, and H4 of rat, murine, and human SR. Identical primary structure is indicated by points. (A) cDNA sequence; (B) corresponding amino acid sequence (one-letter code).

fragment is compared with rat and murine fragments, respectively.

Northern blot analysis of SR mRNA. The cDNA probes R3, R5, M5, and H4 (see Fig. 2) were constructed to specifically hybridize with SR mRNA from rat, murine, and human sources, respectively. For Northern blot analysis of the liver cell lines HTC, BW1-J, and Hep G2, as much as 20 μg of total RNA had to be loaded on the gel to obtain a clear signal. Analysis of brain tissue, spleen, kidney, and thymocytes from rat and of all other cell lines (Figs. 4 and 7) essentially depended on prior isolation of poly(A)$^+$ RNA. This shows that only low steady state mRNA levels are expressed in cells.

Hybridization with mRNA from rat, murine, and human cell lines yielded two species (Fig. 4A). In rat and mouse they have a size of 1.3 and 2.1 kb or 2.3 kb, respectively, whereas human mRNA results in a 1.6- and a 2.1-kb species. All of the rat-specific cDNA probes R1–R5 (see Fig. 2) detected both mRNA species in the rat cell line HTC (data not shown). In contrast to the liver cell line, liver tissue and all other rat tissues lack the 2.1-kb species. Only the 1.3-kb band remains (Fig. 4B).

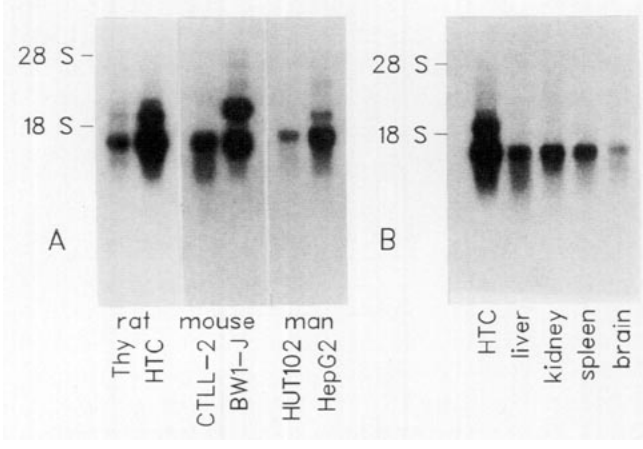

FIG. 4. Northern blot analysis of mRNA (5 μg) specific for SR in rat, murine, and human cell lines and tissues. For hybridization with rat mRNA, probe R5 was used, with murine mRNA, probe M5, and with human mRNA, probe H4. The positions of 18 S and 28 S rRNA are indicated. (A) mRNA from cell lines, (B) mRNA from rat tissues. Thy, thymocytes 48 h after activation.

For further characterization of both murine mRNA species the stability of their hybridization products obtained with cDNA fragment M5 under washing conditions of varying stringency was compared. In standard Northern blots the signals of both mRNA species found in the cell lines CTLL-2 and BW1-J resisted washing with 0.1× SSC at 42°C for 30 min. Increasing the stringency conditions by continued washing at 56°C did not affect the strength of both signals. After additional washing at 65°C, however, dissociation of the cDNA probe from the 2.3-kb mRNA species was increased as compared to the 1.3-kb species, causing a shift in their relative signal intensities (Fig. 5). This shows a more stable interaction of the cDNA fragment M5 with the 1.3-kb mRNA species as compared with the 2.3-kb species and suggests a more specific binding.

The similarity of cDNA fragments R4, M4, and H4 (see Fig. 2) spanning the coding region close to the 3′ terminal end was higher among both rodents than between the rodents and man (see upper section). Due to these species-specific differences cross-hybridization of a rat probe with murine mRNA yielded a markedly stronger signal than equal amounts of mRNA from human sources (Fig. 6).

SR activity and steady state mRNA expression in tissues and cell lines. In previous studies no SR activity had been detected in both the human NK-like cell line YT [20] and in the murine erythroleukemia cell line B8/3 [7]. Northern blot analysis of both cell lines demonstrated that the absence of enzyme activity correlates

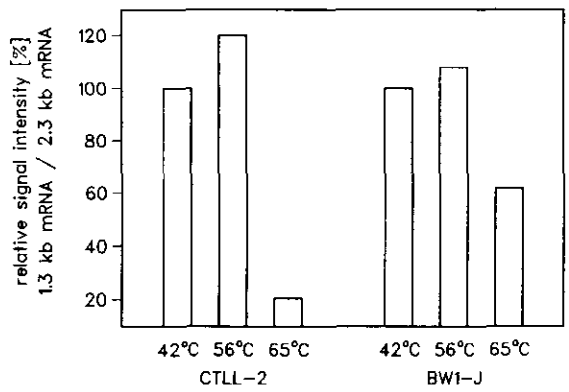

FIG. 5. Stability of specific cDNA binding (probe M5) to the 1.3- and 2.3-kb SR mRNA species of murine cell lines under different stringency conditions. Northern blots were washed with 0.1× SSC for 30 min at 42°C, then for 30 min at 56°C, and for an additional 30 min at 65°C. The relative signal intensity of the 1.3-kb mRNA species to the 2.3-kb mRNA after washing at 42°C was set to 100%.

with a lack of mRNA expression (Fig. 7). The specific activities of SR vary among different cell types and cell lines of a species. They were highest in the liver cell lines Hep G2, HTC, and BW1-J, respectively, and showed lower activities in the T-cell lines HuT 102 and CTLL-2 (Fig. 7A). Rat thymocytes or the murine erythroleukemia cells F4N have only low specific activities. A comparison among the cell lines of a species demonstrates that a decrease in specific activity correlates with a decrease in the steady state mRNA levels (Figs. 7A and 7B).

DISCUSSION

In this study we have shown that Northern blot analysis with species-specific cDNA probes detects two SR

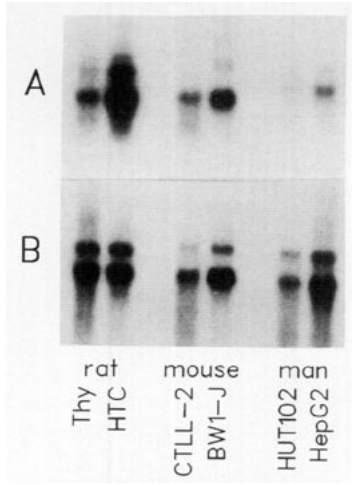

FIG. 6. Northern blot analysis of rat, murine, and human SR mRNA with the rat-specific cDNA probe R5. (A) Hybridization of the rat-specific cDNA probe R5 with SR mRNA from rat, murine, and human cell lines. Thy, thymocytes 48 h after activation. (B) Hybridization with human β-tubulin.

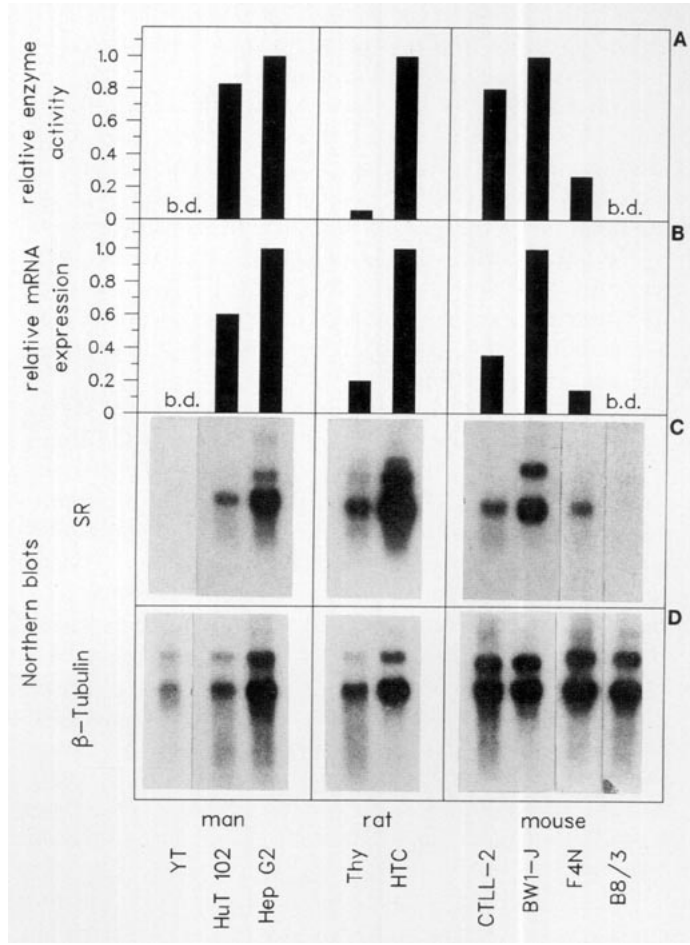

FIG. 7. Enzymatic activity of SR and steady state SR mRNA expression in rat, murine, and human cell lines. (A) Relative enzymatic activities of SR within each species. The specific activities of the liver cell lines were set to 1.0. (B) Relative SR mRNA expression within each species. The steady state mRNA levels of the liver cell lines were set to 1.0. (C) Northern blot analysis of SR mRNA expression with the species-specific probes R5 (for rat cells), M5 (for murine cells), and H4 (for human cells). Five micrograms of mRNA was applied to each lane. (D) Rehybridization with a cDNA probe for β-tubulin. b.d., below detection limit; Thy, thymocytes 48 h after activation.

mRNA species in rat, murine, and human cell lines. A closer analysis of the rat system showed that the 2.1-kb mRNA species, which was found in the liver cell line HTC and in activated rat thymocytes, was absent in all tissues investigated. In liver, kidney, brain, and spleen only the 1.3-kb species was detectable. The lengths of the 1.3-kb rat mRNA and of the 1.6-kb human mRNA both agree with the published cDNA clone lengths of 1.157 kb [21] and 1.67 kb [34, 35], respectively. The 2.3-kb signal of murine cell lines proved to be more sensitive toward increased stringency of washing conditions, indicating a less specific binding as compared to the 1.3-kb species. This may argue against the hypothesis that both mRNA species are alternatively spliced forms with

identical protein coding regions which are expressed from the same promotor and gene.

Katoh and Sueoka [37] have reported isozymes with SR activity, determined in monkey tissue extracts. Antibodies which were raised against a rat SR enzyme preparation and were used in Western blot analysis of rat liver detected a 27.5- and a 30.5-kDa protein species [38]. In both studies the existence of SR isozymes was suggested. The detection of two SR mRNA could support the existence of isozymes, but cannot yet exclude the possibility of different enzymes of similar primary structure and function.

Furthermore, the data presented above demonstrate a major difference between the regulation of GTP-cyclohydrolase I and of SR activity. Previous studies have suggested that a post-translational modification essentially contributes to the regulation of GTP-cyclohydrolase I. In contrast, protein modification does not appear to play a major role in the regulation of SR activity. The human cell line YT and the murine cell line B8/3, which are deficient in enzyme activity, correspondingly lack mRNA expression. Moreover, different activity levels within cell lines or tissues of a species correlate with relative steady state mRNA expression, thus indicating that SR activity is regulated at this level.

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