Identification of Three Hydroxysteroid Sulfotransferase Isoenzymes in the Rat Liver

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Isolation of several hydroxysteroid sulfotransferase (HS-ST) cDNAs from the rat liver cDNA library has demonstrated the possible expression of these HS-ST isoforms in rat liver. We devised a method to detect unequivocally ST-20 and ST-40 mRNAs by a reverse transcription-polymerase chain reaction, using specific primers. ST-40 mRNA was expressed only in liver, but ST-20 mRNA was present predominantly in the liver and slightly in extrahepatic tissues. On chromatofocusing, expressed ST-20 or ST-40 enzymes were eluted at approx. pH 8.2 and 5.7—4.7 or at pH 6.4—5.4, respectively. Chromatofocusing of adult female rat liver cytosols resolved HS-ST isoenzymes in a broad range of pH, and ST fractions A, B, C, D and E were eluted at approx. pH 8.2, 7.6, 7.5—6.8, 6.2 and 6.1—5.5, respectively. After PAP-agarose affinity column chromatography and SDS-polyacrylamide gel electrophoresis (PAGE), their N-terminal amino acid sequences were determined. ST isoenzymes present in fractions B and E showed identical N-terminal amino acid sequences with those of ST-21 and ST-20, respectively, whereas the ST isoenzymes present in fractions C and D had the same N-terminal amino acid sequence as those of ST-40 (and/or ST-41). The results demonstrated the presence of at least three HS-ST isoenzymes in adult female rat liver.

Key words hydroxysteroid sulfotransferase; isoenzyme; rat liver; N-terminal amino acid sequence

Hepatic sulfotransferase (ST) transfers a sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to various xenobiotics and endogenous compounds.1—3) In mammals, ST isoenzymes are classified into hydroxysteroid ST (HS-ST), phenol ST and estrogen ST, depending on their substrate specificities and the deduced amino acid sequences of ST cDNAs.4—7) Rat hepatic HS-ST catalyzes the sulfation of steroid hormones, bile acids and alcohols with overlapping substrate specificities and has been shown to comprise several isoenzymes.1—3) We purified rat hepatic androsterone-sulfating ST (AD-ST), a HS-ST isoenzyme, from adult female rat liver, which appears to comprise subunits of different pI values with the same molecular mass (30 kDa).9) Their N-terminal amino acid sequences are all identical to that of ST-40,9) a female-dominant dehydroepiandrosterone ST (DHEA-ST) reported by Ogura et al.10) So far, at least six HS-ST cDNA clones (ST-20/21, ST-40/41, ST-60 and SMP-2) have been isolated from rat liver cDNA libraries.5,11) Northern blot analysis demonstrated age- and gender-dependent alterations of the expression of these mRNAs.12) However, these HS-ST polypeptides were not isolated from rat liver cytosols except for ST-40. We isolated ST-20 and ST-40 cDNAs from a rat liver cDNA library using the antibody raised against AD-ST, and studied the site-directed mutagenesis of these cDNAs.13) These cDNAs share more than 90% identity in their amino acid sequences; however, the enzymes expressed in E. coli exhibited different substrate specificities, i.e., ST-40 exhibited high ST activity toward AD, DHEA and cortisol (CS), whereas ST-20 was mainly active toward CS.13)

In order to clarify the expression of ST-20 and ST-40 in the rat, we first devised a specific reverse transcription-polymerase chain reaction (RT-PCR) method to distinguish these highly homologous mRNAs and detect the expression of the mRNAs in various rat tissues. Secondly, these two cDNAs were expressed in E. coli, the expressed enzymes were separated by chromatofocusing and their elution pattern was compared with that of adult female rat liver cytosols. Lastly, we determined the N-terminal amino acid sequences of purified adult female HS-ST isoenzymes to know whether ST-20/21 polypeptides, as well as ST-40/41 proteins, were constitutively expressed in rat liver.

MATERIALS AND METHODS

Materials [9,11-3H]AD (2.22 TGBq/mmol), [4-14C]DHEA (2.0 GBq/mmol), and [4-14C]CS were purchased from New England Nuclear (Boston, MA, U.S.A.). BL21 (DE3) and reagents for RT-PCR were obtained from Stratagene (La Jolla, CA, U.S.A.). Restriction enzymes and Taq polymerase were purchased from Takara (Shiga, Japan). The expression vector pKK223-3 and reagents for DNA sequencing were obtained from Pharmacia Japan (Tokyo, Japan). A thioreredoxin-expressing vector, pTXr, was kindly provided by Dr. A. Miyawaki. PAPS and PAP (adenosine 3',5'-bisphosphate)-agarase were prepared by the methods of Singer14) and Brodelius et al.,15) respectively.

Isolation of Total RNA and RT-PCR Tissues were excised from male and female Wistar rats (21, 38, 59 or 70 d of age), and total RNA was isolated using the guanidinium thiocyanate phenol–chloroform extraction method according to the manufacturer's protocol (Isogen RNA isolation kit, Nippon Gene, Tokyo, Japan). Total RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrometrically. RNA quality was evaluated by the integrity of ribosomal RNA bands of the denatured agarose gel electrophoresis of the total RNA. The first strand of cDNA was synthesized from 2.5 µg total RNA by 1 unit MLTV reverse transcriptase with oligo(dT) primers according to the manufacturer's protocol (Stratagene). PCR was performed, using the cDNA as a template, by Taq polymerase. Primers used for PCR were as follows: the forward primer (primer 1) was 5' AGGC-CAAGGTGATCTATCCTC3' (numbered by taking A of the initiation codon ATG as 1). Specific reverse primers

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were 5'-TGATTTTTTCATCAGGAGGC692-3' for ST-20 (primer 20) and 5'-CAGTTTCTTCTCCCATGAGAT692-3' for ST-40 (primer 40), respectively (different nucleotides between ST-20 and ST-40 cDNAs were underlined). Cycling conditions were programmed per cycle to 1 min at 91°C, 1 min at 54°C and 1 min at 72°C. 25—45 cycles were used depending on the experiments. The PCR products (377 bp) were subjected to restriction endonuclease digestion with MvaI to monitor the specificity. A pair of 20 nucleotide primers to amplify β-actin cDNA as a control was selected from the sequence of exon 2 of the rat β-actin gene to produce a 550 bp product.16

**Tissue Specific Expression of ST-20 and ST-40 mRNAs**

Total RNA was prepared from several tissues of rats at 70 d of age, and cDNA was synthesized as described above. The cDNA was subjected to specific PCR (30 cycles) for ST-20 and ST-40, separately. In order to detect minor transcripts, the number of cycles of PCR was increased to 45.

**Expression of HS-ST cDNAs**

cDNA clones of ST-20 and ST-40, ligated to expression vector pKK223-3, were transformed into E. coli BL21(DE3) harboring thioredoxin-expressing vector, pT- Trx, and the transformed E. coli cells were then cultured in 200 ml of LB broth containing 25 μg/ml ampicillin for 3 h. Incubation continued for 4 h after the addition of 1 mm isopropyl-β-D-thiogalactopyranoside. The cells were then harvested and washed by centrifugation at 6500 g for 10 min and resuspended in 6 ml buffer A (10 mm Tris–HCl (pH 7.4), 250 mm sucrose, 0.1 mm EDTA and 3 mm 2-mercaptoethanol). After sonication, the lysates were prepared by centrifugation at 105000 g for 60 min, then used for ST assays and separation by chromatofocusing.

**Preparation of Liver Cytosols**

Adult female Wistar rats (100 d of age) had free access to food and water and were decapitated. Cytosolic fractions were prepared as described previously,95 and were used for ST assays and separation by chromatofocusing.

**Assays of ST Activities**

ST activities toward AD, DHEA, CS and 4-nitrophenol (NP) were determined as described previously,95 Protein concentrations were measured by the method of Bradford71 with bovine serum albumin as a standard.

**Separation of HS-ST Isoenzymes by Chromatofocusing**

HS-ST isoenzymes of the lysates of the expressed ST-20 cDNA (631 mg protein), the expressed ST-40 (135 mg protein) or adult female rat liver cytosols (378 mg protein) were mixed with an equal volume of a starting buffer (25 mm imidazole (pH 7.4) and 250 mm sucrose) and applied on a PBE 94 chromatofocusing column (1×40 cm). HS-ST isoenzymes were eluted at a flow rate of approx. 9 ml/h with an elution buffer (11.1% Polybuffer 74 (pH 4.0), 250 mm sucrose and 10 mm dithiothreitol). Each fraction (6 ml) was collected and assayed for HS-ST activities toward AD and CS.

**PAP-Agarose Chromatography**

ST fractions pooled after the chromatofocusing of rat liver cytosols were dialyzed against an affinity buffer (10 mm Tris–HCl (pH 7.4), 250 mm sucrose and 3 mm 2-mercaptoethanol) and applied on a PAP-agarose column (1.5×7 cm). After sequential washings with 200 ml of the affinity buffer and 150 ml of 1 mM 5'-AMP in the affinity buffer, HS-ST was specifically eluted with 125 ml of 0.3 mM PAP or 0.4 mM PAPS in the affinity buffer as described previously.95

**Electrophoresis**

SDS-polyacrylamide gel electrophoresis (PAGE) was done as described previously.95 Proteins were stained with Coomassie Brilliant blue or immunoblotted with anti-AD-ST antiserum as described previously.95 The anti-AD-ST antiserum used in this study recognizes similarly the expressed ST-20 and ST-40 polypeptides.13

**N-Terminal Amino Acid Sequencing**

Proteins separated by electrophoresis were electrophoretically transferred to a ProBlott PVDF (polyvinylidene fluoride) membrane filter. After Amido Black 10B staining, HS-ST polypeptide spots were excised and applied to a protein sequencer (Applied Biosystems Japan, model 473A or Shimadzu model PSQ-2) by the method previously reported.95

**RESULTS**

**Specific Detection of ST-20 and ST-40 Expression by RT-PCR**

Since 94% of the nucleotide sequences of ST-20 and ST-40 are identical, it is difficult to distinguish between these two transcripts by conventional hybridization-directed methods such as Northern or dot blot hybridization. Consequently, we devised a method to detect a distinct PCR product (377 bp length) derived from either ST-20 cDNA or ST-40 cDNA by RT-PCR using specific primers for the respective cDNAs, allowing unequivocal discrimination between

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**Fig. 1. Unequivocal Determination of ST-20 and ST-40 mRNAs by RT-PCR**

(A) Strategy for the detection of ST-20 and ST-40 transcripts by a specific PCR. Primer 1 indicates a 20-mer forward primer for both ST-20 and ST-40, and primer Z or primer 40 indicates a 20-mer reverse primer specific for ST-20 or ST-40, respectively, as described in Materials and Methods. The PCR product (377 bp) from ST-20 has two MvaI sites, whereas that of ST-40 shows one site. (B) RT-PCR was performed with female rat liver RNAs at 59 d of age. Lane 1, PCR product using ST-20 specific primers; lane 2, its MvaI digest; lane 3, PCR product using ST-40 specific primers; lane 4, its MvaI digest; lane M, DNA size markers.
ST-20 and ST-40 mRNAs (Fig. 1A). Digestion of the PCR product derived from ST-20 cDNA or ST-40 cDNA with MvaI yields three fragments (97, 110 and 170 bps) or two fragments (170 and 207 bps), respectively (Fig. 1A). To confirm the validity of this method, RT-PCR was performed using RNAs obtained from female rat liver at 59 d of age and two pairs of specific primers. Each PCR product was digested with MvaI and subjected to electrophoresis. As indicated in Fig. 1B, only the expected bands were observed, indicating that this method is highly specific to ST-20 and ST-40 mRNAs.

Expression of ST-20 and ST-40 mRNAs in Rat Liver and Other Tissues Total RNAs were isolated from male and female rat livers at 21, 38 and 59 d of age and were subjected to the specific RT-PCR. Figure 2A shows PCR products amplified from ST-20, ST-40 and β-actin (control) cDNAs at various ages. The levels of ST-20 mRNA were very low at 21 d of age in both sexes, whereas ST-40 mRNA was moderately expressed. In females, the levels of ST-20 and ST-40 mRNAs increased from 21 to 59 d of age, respectively. In males, ST-20 mRNA increased from 21 to 38 d of age and subsequently declined at 59 d of age, while the levels of ST-40 mRNA were continuously reduced from 21 to 59 d of age. As a result, the amounts of ST-20 and ST-40 mRNAs were much higher in females than in males at 59 d of age. These results were reproducible in three separate experiments.

As indicated in Fig. 2B, the expression of ST-40 was strictly restricted to the liver. Even after increasing the cycles (from 30 to 45 times) of ST-40 specific PCR, no bands were detected in other tissues (data not shown). Though ST-20 mRNA was mainly expressed in liver, it was present slightly in kidney, heart, lung, spleen and nasal tissues examined so far (Fig. 2B).

Separation of HS-STs by Chromatofocusing ST-20 and ST-40 cDNAs were expressed in E. coli and their lysates were subjected to chromatofocusing. As shown in Fig. 3a, the expressed ST-20 enzyme provided two ST fractions which were eluted at approx. pH 8.2 and 5.7–4.7 and were active toward CS (Table 1). At present we have no explanation why the ST-20 enzyme was eluted into two fractions. The expressed ST-40 enzyme gave a single ST fraction eluted at pH 6.4–5.4 (Fig. 3b) and had high ST activities toward AD and CS (Table 1). To determine whether rat liver cytosolic ST-20 and ST-40 enzymes can be separated in a similar manner to those of the expressed cDNAs, we separated adult female rat liver cytosols by chromatofocusing. As indicated in Fig. 3c, cytosolic STs were separated into five ST frac-
tions, A, B, C, D and E, which were eluted at approx. pH 8.2, 7.6, 7.5–6.8, 6.2 and 6.1–5.5, respectively, and showed different ST activities toward AD, CS and NP. The pooled fraction A had comparatively high ST activity toward NP but not toward AD and CS (Table 1), so we did not further study this fraction. The pooled fraction B showed high ST activity only toward CS, while the pooled fraction C had comparatively high ST activities toward three substrates. The pooled fraction D provided high ST activities toward AD and CS, while the pooled fraction E showed comparatively low ST activity only toward CS (Table 1).

**PAP-Agarose Chromatography and SDS-PAGE** The pooled fractions B, C, D, and E, which had comparable HS-ST activities, were purified by PAP-agarose affinity column chromatography. Subsequent SDS-PAGE revealed the presence of a major band of HS-ST polypeptide (31 kDa) in these fractions by Coomassie Blue staining (Fig. 4A), and these polypeptides were immunoreactive with anti-AD-ST antiserum as shown in Fig. 4B.

**N-Terminal Amino Acid Sequencing** After SDS-PAGE and transfer onto PVDF membranes, HS-ST polypeptides with a molecular mass of 31 kDa were subjected to Edman degradation. The N-terminal amino acid sequence of fraction B was identical to that of ST-21 within the area determined (Table 2). Fractions C and D gave an identical amino acid sequence to that of ST-40, while the N-terminal amino acid sequence of fraction E was identical with that of ST-20 (Table 2).

**DISCUSSION**

Several HS-ST isoenzymes such as HS-STs 1, 2 and 3, glucocorticoid STs I, II and III, or bile acid ST I have been purified from rat liver. However, it is difficult to characterize these isoforms on a molecular basis because of their similar catalytic and physicochemical properties. Recent cloning studies on HS-STs have provided the basis for the characterization of this family. Five HS-ST cDNA clones (ST-20/21, ST-40/41 and ST-60) have been isolated from the rat liver cDNA library. These cDNAs share high sequence homologies. The amino acid sequences between ST-20 and ST-21 are 98% identical, while ST-40 and ST-41 show only one amino acid substitution out of 284 amino acid residues. ST-20 and ST-60 have 90 and 86% identities with ST-40, respectively. Since we are unable to distinguish between ST-20 and ST-21 or between ST-40 and ST-41 by either our RT-PCR method or our immunoblot analysis, ST-20 and ST-40 in our discussion inevitably include ST-21 and ST-41, respectively.

<table>
<thead>
<tr>
<th>Source</th>
<th>Fraction</th>
<th>Elution pH</th>
<th>ST activity (nmol/min/mg)</th>
<th>CS</th>
<th>AD</th>
<th>NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>0.12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.7–4.7</td>
<td>0.32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ST-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.4–5.4</td>
<td>0.80</td>
<td>10.19</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female rat liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>8.2</td>
<td>0.02</td>
<td>0.02</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>7.6</td>
<td>1.08</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>7.5–6.8</td>
<td>0.25</td>
<td>1.46</td>
<td>1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>6.2</td>
<td>0.07</td>
<td>1.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>6.1–5.5</td>
<td>0.04</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not detectable. ST activities toward DHEA were comparable with those toward AD and were 12.94, 2.24 and 1.77 nmol/min/mg in ST-40 (pH 6.4–5.4) and fractions C and D, respectively.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>N-Terminal amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-20</td>
<td>M-...-W-F-E-G-P</td>
</tr>
<tr>
<td>ST-21</td>
<td>M-...-W-F-E-G-P</td>
</tr>
<tr>
<td>ST-40</td>
<td>M-...-W-F-E-G-P</td>
</tr>
<tr>
<td>ST-41</td>
<td>M-...-W-F-E-G-P</td>
</tr>
<tr>
<td>ST-60</td>
<td>M-M-S-...-W-F-E-G-P</td>
</tr>
</tbody>
</table>

Dashes in the sequences represent amino acid residues identical to those deduced from ST-40/41. The deduced amino acid sequences of ST-20, ST-21 and ST-60 were reported by Watabe et al.75.

![Fig. 4](https://example.com/fig4.png)

(a) Coomassie Brilliant blue staining and (b) immunoblot staining. Lanes B, C, D and E correspond to fractions B, C, D and E shown in Fig. 3c, respectively. M, Marker proteins.
High sequence homology between rat liver senescence marker protein, SMP-2\(^{15}\) and rat HS-ST cDNAs, suggests that SMP-2 is an isoform of HS-ST.\(^{10}\) Rat hepatic STas\(^{9}\) and AD-ST\(^{9}\) have been identified as the expressed ST-40, based on their physicochemical properties and N-terminal amino acid sequences. However, the identities among the purified enzymes as well as between the purified STs and isolated cDNAs, except for ST-40, have not yet been made, and the numbers of HS-ST isoenzymes present in rat liver cytosols are still unclear.

Using anti-AD-ST antisera, we isolated highly homologous ST-20 and ST-40 cDNAs from a rat liver cDNA library, but the enzymes expressed in E. coli exhibit different substrate specificities.\(^{12,13}\) ST-40 enzyme has high ST activities toward AD, DHEA and CS, whereas ST-20 enzyme is predominantly active for CS. In the present study, we devised a specific RT-PCR method to distinguish these highly homologous mRNAs and detected the expression of these mRNAs in male and female rat livers at 21, 38 and 59 d of age (Fig. 2A). The levels of liver ST-20 mRNA were very low at 21 d of age and increased at 38 d of age, irrespective of sex. However, at 59 d of age, they increased in females, but declined in males. This developmental pattern is compatible with the Northern blot study on the ontogeny of rat ST-20 mRNA, using a specific oligonucleotide probe.\(^{12}\) In contrast to ST-20, ST-40 mRNA was moderately expressed at 21 d of age in both sexes and increased at 38 and 59 d of age in females, while it was continuously diminished in males. This developmental pattern is similar to the Northern blot study of rat ST-40 mRNA\(^{22}\) and is compatible with that of AD-ST activity in the rat liver.\(^{22}\) These results are partly inconsistent with the Northern blot study reported by Runge-Morris and Wilusz,\(^{23}\) probably due to the specificity of their oligonucleotide probe, which appears to recognize both ST-20 and ST-40 mRNAs. These discrepancies do not appear to be due to strain difference, because the coding regions of ST-20 and ST-40 cDNAs are identical between the Sprague-Dawley strain\(^{21}\) and Wistar strain\(^{31}\) of rats, which were used in these studies.

Immunoblot analysis with anti-AD-ST antisera reveals that the ST-40 polypeptide appears to be restricted to the liver.\(^{24}\) In the present study, we demonstrated that the expression of ST-40 mRNA was detected only in the liver, whereas ST-20 mRNA was present predominantly in the liver, but also slightly in the kidney, heart, lung, spleen and nasal tissues examined so far (Fig. 2B). These results suggest that the expression of two highly homologous HS-ST genes is regulated differently in extrahepatic tissues.

By chromatofocusing, the expressed ST-20 enzyme was eluted as two ST fractions at approx. pH 8.2 and 5.7—4.7, whereas the expressed ST-40 enzyme was eluted as a single ST fraction at pH 6.4—5.4 (Fig. 3a). Subsequently, we separated adult female rat liver cytosols by chromatofocusing to clarify whether the ST-20 enzyme is constitutively expressed, because the level of ST-20 mRNA was much higher in adult females than in adult males, similarly to that of ST-40 mRNA. ST activities were eluted in five fractions, A—E, in the range of pH 8.2—5.5. These fractions were further purified by PAP-agarose affinity chromatography, followed by SDS-PAGE. Subsequently, their N-terminal amino acid sequences corresponding to the immunoreactive 31 kDa polypeptides were determined. Fraction B showed ST activity toward CS, and its N-terminal amino acid sequence was identical with that of ST-21. Fractions C and D displayed high ST activity toward AD, and their N-terminal amino acid sequences were the same as that of ST-40 (or ST-41). Fraction E showed relatively low ST activity only toward CS, and its N-terminal amino acid sequence was identical with that of ST-20. The elution profiles of these ST isoenzymes were compatible with those of the expressed ST-20 and ST-40, though the ST-21 isoenzyme was not available. Some differences in their elution profiles might be ascribable to different oligomeric structures and/or different interactions by coexistent proteins and lipids. Thus, the present study demonstrated the presence of at least three HS-ST isoenzymes in female rat liver cytosols.

Liu and Klaassen\(^{22}\) have studied the ontogeny and hormonal regulation of female-dominant rat hepatic ST mRNAs (ST-20/21, ST-40/41 and ST-60) by Northern blot analyses using specific nucleotide probes. They demonstrated that all the mRNAs showed different developmental patterns. These data also suggest the possible expression of an ST-60 polypeptide in adult females, but it is not known whether the expressed ST-60 enzyme can sulfate DHEA, bile acids or glucocorticoids. It is the same with SMP-2 protein.\(^{11}\) Further studies on expressed ST-60 and SMP-2 enzymes are needed in order to isolate and characterize these proteins from rat liver cytosols.

The present study provides evidence that ST-20 and ST-40 enzymes are regulated differently in extrahepatic tissues. Recently, we assigned ST-20 and ST-40 genes to the same chromosomal region of 1q21.3—22.1 by fluorescence in situ hybridization.\(^{25}\) It is of interest to speculate that these genes might be located in tandem on chromosome 1 but are regulated in a different manner. To define the molecular basis of the regulatory elements, it is necessary to elucidate their regulatory gene structures.

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