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The structure of the human thyrotropin β-subunit gene

(TSH-producing adenoma; transcription start point; promoter region; gene regulatory region; exon; conserved nucleotide sequence; recombinant DNA: phage λ vector; genomic library)

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SUMMARY

We have determined and characterized the entire structure of the human thyrotropin β-subunit (TSHβ) gene. It is 4.5 kb in size and separated into three exons. Primer extension analysis showed one transcription start point, and S1 mapping analysis showed two blocks of polyadenylation sites in normal pituitaries. The same transcripts were observed in TSH-producing adenomas. The 5′-flanking region contains two 'TATA' boxes, two 'CAAT' boxes, and two sequences similar to the cyclic AMP-responsive elements.

INTRODUCTION

TSH is a pituitary hormone which acts in the regulatory chain of the hypothalamus-pituitary-thyroid axis, and stimulates production and secretion of thyroid hormones from the thyroid gland. TSH is a member of the glycoprotein hormone family which includes pituitary hormones, such as follitropin and lutropin, as well as the placental chorionic gonadotropin. Each of these hormones consists of two non-covalently associated subunits, α and β. These hormones share a common α-subunit, whereas the β-subunits differ, and provide the biological specificity for each hormone (Pierce and Parsons, 1981).

The production and secretion of TSH are regulated by several hormones, including thyroid hormones and those that are secreted from the hypothalamus, such as TRH, dopamine, and somatostatin (Morley, 1981). While the thyroid hormones and dopamine lower the transcriptions of both the α-subunit gene and the *TSHβ* gene, TRH acts in the opposite way (Schupnik et al., 1985, 1986). The suppressive effect of dopamine is reversed by cAMP (Schupnik et al., 1986). Recently, the

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Abbreviations: bp, base pair(s); cAMP, cyclic adenosine 3′,5′-monophosphate; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PA, polyacrylamide; TRH, TSH-releasing hormone; TSH, thyrotropin; *TSH*, gene (DNA) coding for TSH; TSHβ, TSH β-subunit.

responsive sequence elements for cAMP have been defined in the 5′-flanking region of several human genes (Silver et al., 1987). The structure of the target genes may help reveal the mechanism of regulation by these chemical signals, which may be related to the mechanism of overproduction of TSH in some adenomas and of lowered expression in some patients (Smallridge and Smith, 1983; Miyai et al., 1971; Sato et al., 1975; Kohno et al., 1980; Okada et al., 1980; Labbe et al., 1984).

The *TSHβ* genes of rat and mouse have recently been elucidated. The *TSHβ* gene of the rat has three exons (Croyle et al., 1986; Carr et al., 1987), whereas that of the mouse has five exons which are alternatively spliced (Wolf et al., 1987; Gordon et al., 1988). In both rat and mouse, two different transcription start points, about 40 bp apart, have been identified for the *TSHβ* gene, and the thyroidal status has been proposed to separately regulate the two transcription start points (Carr et al., 1987).

We have previously reported the partial sequence of the human *TSHβ* gene (Hayashizaki et al., 1985). We describe here the entire structure of the human *TSHβ* gene. It has three exons, and in contrast to the rodent genes, has one transcription start point.

MATERIALS AND METHODS

(a) Oligodeoxyribonucleotides

Three oligos, probe U (see Fig. 1), primer A, and primer B (see Fig. 3) were synthesized using the Applied Biosystem Model 381A instrument.

(b) Construction of the human genomic library and its screening

Genomic DNA was prepared from human lymphocytes, partially digested with *Sau*3AI, and sedimented through a 10–38% sucrose density gradient. Fractions carrying 10–20-kb DNA fragments were pooled, and ligated with the *Bam*HI arms of λL-47.1 (Loenen and Brammer, 1980). After in vitro packaging, 8×10^5 phages were screened for clones carrying the *TSHβ* gene by plaque hybridization assay (Benton and Davis, 1977) using an artificial human *TSHβ* cDNA probe (Hayashizaki et al., 1988) radiolabeled by the random priming method (Feinberg and Vogelstein, 1983).

(c) Mapping and sequencing analysis

After cloning of the 4.2-kb *Bam*HI DNA fragment (see Fig. 2) into pBS (Stratagene, USA), two deletion sets from both directions were prepared by exonuclease III digestion (Yanisch-Perron et al., 1985). Nucleotide sequencing analyses were done by the dideoxy method (Hattori and Sakaki, 1986).

(d) Preparation of RNA

Human normal pituitaries were obtained from either autopsy or surgical operations. Human TSH-producing pituitary adenomas were obtained from surgical operations. Total RNAs were extracted from the tissues essentially as described (Chirgwin et al., 1979).

(e) Northern blot analysis

The 0.2-kb *Eco*RI-*Afl*II fragment (Northern probe, shown in Fig. 2) was subcloned into M13mp18. The single-stranded DNA complementary to the TSHβ-coding strand was labeled with [α-^{32}P]dCTP (Burke, 1984) and used as a probe. Northern blot analysis was done using total RNA (20 μg) and either a Northern or artificial human *TSHβ* cDNA probe (Lehrach et al., 1977).

(f) Primer extension analysis

Total RNA (5 μg) was hybridized with 5′-^{32}P-end-labeled oligo primer at 37°C for 12 h. The hybridized primer was extended using reverse transcriptase as described by Krainer et al. (1984). Newly synthesized DNA was subjected to 6% PA-8 M urea sequencing gel followed by autoradiography.

(g) S1 nuclease mapping

The 236-bp *Rsa*I fragment (S1 probe, shown in Figs. 2 and 3) was subcloned into M13mp18. The single-stranded DNA complementary to the TSHβ-coding strand was labeled with [α-^{32}P]dCTP (Burke, 1984) and used as a probe. Total RNA (5 μg) was hybridized with this probe at 30°C for

12 h, and treated with S1 nuclease (Berk and Sharp, 1977). The S1-resistant hybrid was subjected to 6% PA-8 M urea sequencing gel followed by autoradiography.

RESULTS AND DISCUSSION

(a) Screening of the human genomic library for clones that carry 'exon 1' of *TSHβ* gene

As noted in the introduction, we have previously identified and sequenced the coding exons of the human *TSHβ* gene (Hayashizaki et al., 1985). However, identification of the 5'-noncoding exon(s), 'exon 1', could not be done, because mRNA for the human *TSHβ* gene was not available. As shown in Fig. 1, the sequence around the initiation codon of the bovine *TSHβ* cDNA (Maurer et al., 1984) is homologous with exon 1 and exon 2 of the rat *TSHβ* gene (Croyle et al., 1986). Also the coding region of the human *TSHβ* gene (Hayashizaki et al., 1985) is homologous with that of bovine and rat sequences, and the 3' splicing acceptor site of the rat gene is conserved in the human gene. Thus, it was likely that the human *TSHβ* gene had a 5'-noncoding exon(s), 'exon 1', that might be homologous to the exon 1 of rat. We, therefore, attempted to screen a clone carrying the *TSHβ* gene extending upstream from the two coding exons that we had determined previously.

The human genomic library carrying partially digested DNAs with *Sau*3AI was screened for this purpose, using an artificial human *TSHβ* cDNA probe (Hayashizaki et al., 1988). From 8×10^5 plaques, three positive clones (λTβ1, λTβ2, and λTβ3) were isolated. Restriction maps of the DNA of these isolated recombinant phage clones revealed that they comprise three overlapping clones (Fig. 2). The extensive overlap of restriction sites between the three λ clones suggests strongly that sequence rearrangements did not occur during construction of the genomic library.

```
bovine cDNA  GCTCACCAATGCAAAATAAG ------------------------- C ATG ACTGCT
rat    gene  ACTCGCCAGTGCAAAGTAAG gtagg···(intron 1)···tttag C ATG AATGCT
human  gene                                                TTTAG C ATG ACTGCT
probe  U       CTCACCAATGCAAAATAAG GT
                  G   G       G
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Fig. 1. Comparison of *TSHβ* cDNA and genes. Sequences of the region around the start codon in the bovine *TSHβ* cDNA (Maurer et al., 1984), the rat *TSHβ* gene (Croyle et al., 1986), and the human *TSHβ* gene (Hayashizaki et al., 1985) are aligned. The nucleotide sequence of intron 1, shown in part, of the rat *TSHβ* gene is represented by lower case letters, and exons in upper case letters. The ATG start codons are boxed. Gaps which have been introduced for maximum alignment are indicated by dashes. Oligo-mixed probe U was designed to detect the 5'-noncoding exon(s), 'exon 1', of the human *TSHβ* gene (see RESULTS AND DISCUSSION, section b).

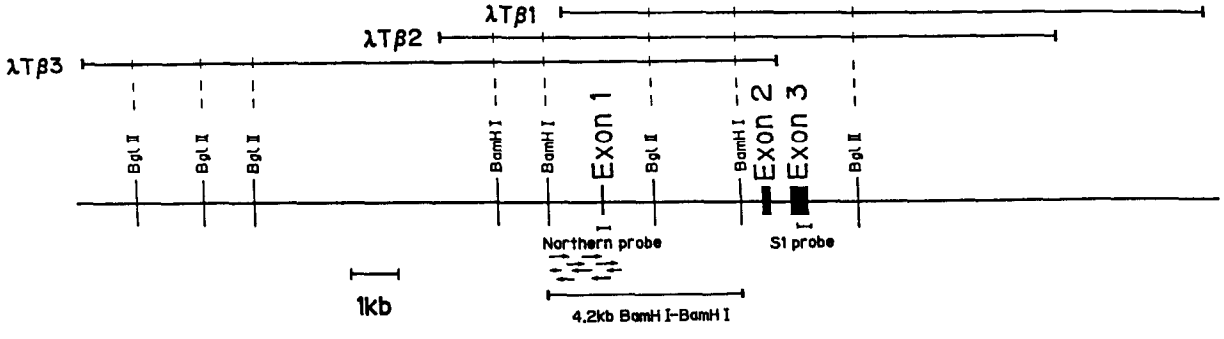

Fig. 2. Three λ clones and the contiguous restriction enzyme map of the human *TSHβ* gene. Three recombinant phage clones containing fragments of *TSHβ* genomic DNA are shown above the map. The relative positions of exons are indicated by black boxes. The bars shown below the map represent the 4.2-kb *Bam*HI DNA fragment, and the regions used as Northern probe and S1 probe. Only relevant restriction sites are displayed. The arrows indicate the sequencing strategy.

Fig. 3. Nucleotide sequence and the deduced amino acid sequence of the human *TSHβ* gene. The sequence expressed in mature mRNA is underlined. The nucleotides are numbered in relation to the major transcriptional start point that has been designated + 1. The sequences homologous to the core sequence of cAMP-responsive elements are double underlined. The 'TATA' boxes, the 'CAAT' boxes, and the AATAAA hexamers are boxed. The nucleotide sequence complementary to the synthetic oligo primers are shown by wavy lines. The dashed line as the 'S1 probe' represents the region used as a probe for S1 nuclease mapping. The major and minor transcription start points are indicated by thick and thin downward arrows, respectively. The polyadenylation sites are indicated by horizontal brackets. The deduced amino acid sequence of the TSHβ is shown. The region between + 340 and + 341 (shown as 3.0-kb intron 1) has not been sequenced. Sequences from + 341 to + 810 and + 1200 to + 1598 are taken from the literature (Hayashizaki et al., 1985). Sequences from + 811 to + 1199 and + 1599 to + 1777 were determined in the present study.

(b) Identification of 'exon 1'

From comparisons of the nucleotide sequences shown in Fig. 1, we assumed that the homologous sequences between exon 1 of the rat *TSHβ* gene (Croyle et al., 1986) and the bovine *TSHβ* cDNA (Maurer et al., 1984) may also be conserved in the human *TSHβ* gene. To identify a region carrying this sequence, we designed 'probe U' (Fig. 1) and used it as a probe in Southern blot analysis of the recombinant phage clone, λTβ3 (Southern, 1975). The 4.2-kb *Bam*HI DNA fragment shown in Fig. 2 was found to carry the sequence that hybridizes with this probe. We subcloned this fragment into pBS and created two deletion sets by exonuclease III digestion from both ends of the insert (Yanisch-Perron et al., 1985). Sequencing analyses were done with those subclones that hybridized with probe U. The subclones that carry the sequence upstream of them were also sequenced. The sequencing strategy and the results are shown in Figs. 2 and 3, respectively.

Fig. 4 shows the results of comparison of the nucleotide sequences of the *TSHβ* genes between human and rat (Croyle et al., 1986) or mouse (Gordon et al., 1988). The human sequence has a region homologous to that of exon 1 and its vicinity of the rodent sequences. Exon 1 and the 5′-flanking region of the rodent sequences and the corresponding region of the human sequences are aligned in Fig. 5A. We assumed that 'exon 1' of the human gene lies within this region (see below).

To see that this region is really transcribed, Northern hybridization was done with total RNA (20 μg) from human normal pituitary using either this homologous region (Northern probe, shown in Fig. 2) or an artificial human *TSHβ* cDNA probe. Both probes reacted with a single component, approx. 650 nt in size (Fig. 6). Thus, the 'exon 1' transcript is joined to the downstream transcript from the human *TSHβ* gene.

(c) Identification of the 5′ and 3′ borders of the human *TSHβ* gene

To determine the transcription start point, primer extension analysis was done, using total RNAs from four human normal pituitary specimens as templates and oligo primer A as a primer that is complementary to the region between nt +652 to +672 (Fig. 3). A major fragment of 92 nt and a minor fragment of 89 nt were detected (Fig. 7, lanes N1–N4). When primer B, that is complementary to the region

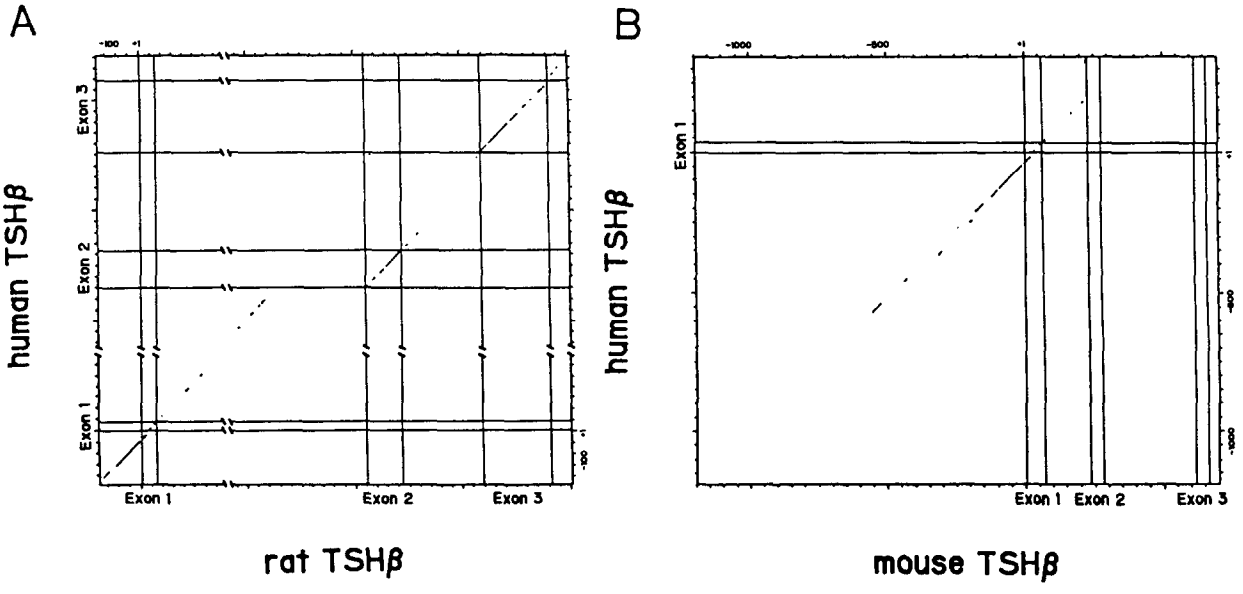

Fig. 4. Dot matrix analyses comparing the nucleotide sequences of the *TSHβ* genes between human and rat (Croyle et al., 1986) or mouse (Gordon et al., 1988). Each dot represents the region where more than 15 out of 20 nt match between the two sequences. The boundaries of the exons are indicated. Position + 1 for each gene represents the site of the uppermost transcription start point. Note that the mouse *TSHβ* gene carries two extra exons, viz., exon 2 and exon 3.

Fig. 5. Comparison of homologous sequences of the *TSHβ* genes among human, rat (Croyle et al., 1986; Carr et al., 1987), and mouse (Wolf et al., 1987; Gordon et al., 1988). Homologous bases are indicated with stars. Gaps which have been introduced for maximum alignment are indicated by dots. Sequences expressed in mature mRNAs are underlined. The uppermost and the downstream transcription start points are indicated by open and closed downward arrowheads, respectively. The 'TATA' boxes, and the 'CAAT' boxes are boxed. The sequences homologous to the core sequence of cAMP-responsive elements are double-underlined. Position +1 for each gene represents the site of the uppermost transcription start site. **A**, Exon 1 and its 5′-flanking region of the *TSHβ* genes. **B**, Further upstream locus carrying the cAMP-responsive element.

between nt +14 to +37 (Fig. 3), was used, a major fragment of 37 nt and a minor fragment of 34 nt were detected (data not shown). In both experiments, the minor fragments that are 3 nt shorter than the major fragments may represent the minor transcription start point. Alternatively, they might have resulted from premature termination of cDNA synthesis by reverse transcriptase (Williams and Mason, 1985).

With the major transcription start point at 92 nt upstream from the end of primer A (+672 in Fig. 3), the 5′ border of the uppermost coding exon should lie between nt +580 (= 672 − 92) and +619. In this region, the conserved AG dinucleotides for the 3′ splicing acceptor site (Green, 1986) are found at nt +590 to +591 or +616 to +617. The remaining 11 nt [= 92 − (672 − 591)] or 37 nt [= 92 − (672 − 617)] will represent the size of 'exon 1'. As 13 nt were extended using primer B, we defined it as 37 nt, and the 5′ border of the uppermost coding exon at nt +618. The size of 'exon 1' was the same as that produced in an experiment using primer B, and too small for multiple exons. Thus, we defined this exon as exon 1, and its 3′ and 5′ borders as +37 and +1, respectively. The two nt GT next to the 3′

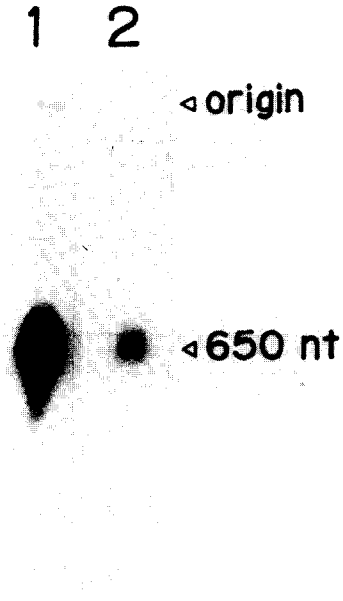

Fig. 6. Northern blot analysis of RNAs from human pituitaries. Total RNAs (20 μg) from human normal pituitary were electrophoresed in a 2% agarose formaldehyde gel, transferred to a nylon membrane (Gene Screen Plus, NEN, USA) and hybridized to an artificial human *TSHβ* cDNA probe (lane 1) or a Northern probe (lane 2).

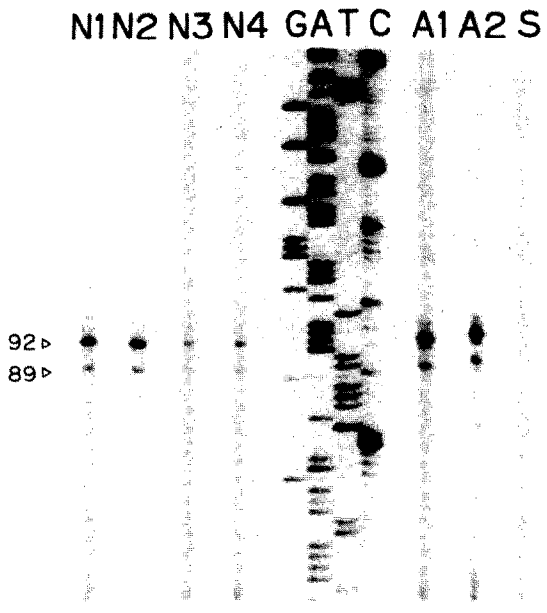

Fig. 7. Primer extension analysis for defining mRNA start point of the human *TSHβ* gene. The ^{32}P-labeled primer A (see Fig. 3) was hybridized to four mRNAs (5 μg each) from human normal pituitaries (NI-N4) at 37°C for 12 h and extended with reverse transcriptase. Newly synthesized DNA was subjected to 6% PA-8 M urea sequencing gel. Two mRNAs (5 μg each) from human TSH-producing adenomas (A1, A2) were also used. S is an mRNA sample (5 μg) from rat spleen. Lanes GATC show the dideoxy sequencing ladders used as a size marker. The sizes of the fragments are indicated on the left margin (in nt).

border of exon 1 agree with the 5′ splicing donor site (Green, 1986), and 'TATA' box is observed at −28 to −22.

The previously defined coding exons (Hayashizaki et al., 1985) should be exon 2 and exon 3, the same structure as for the rat (Croyle et al., 1986; Carr et al., 1987). In contrast, the mouse carries two extra 5′-noncoding exons (exon 2 and exon 3) between exon 1 and the coding exons (exon 4 and exon 5) (Wolf et al., 1987; Gordon et al., 1988).

(d) Comparison of exon 1 and the 5′-flanking region of the *TSHβ* genes among different species

Sequences around exon 1 and its 5′-flanking region, as well as those around the human TSHβ coding exons (exon 2 and exon 3) are highly conserved among the three species. This can be seen in the dot matrix analyses in Fig. 4. The homologous sequences around exon 1 and its 5′-flanking region (−221 to +48 in Fig. 3) are aligned in Fig. 5A. The homology of this region is 85.0% between human and rat (Croyle et al., 1986) and 85.4% between human and mouse (Gordon et al., 1988), equally conserved with the homology of the protein-coding region (+619 to +780 and +1230 to +1483 in Fig. 3), being 82.3% between human and rat and 83.9% between human and mouse.

(e) Comparison of the transcription start points among different species

In the 5′-flanking region, two sets of promoter sequences, 'TATA' boxes and 'CAAT' boxes are for the most part conserved among the three mammalian species (Fig. 5A). Rodents use both of these two transcription start points (Carr et al., 1987; Wolf et al., 1987; Gordon et al., 1988). In pituitaries of hypothyroid mouse or rat or in mouse TSH-producing adenoma, the downstream transcription start point is mainly used. On the other hand, Carr et al. (1987) reported that in rat normal pituitary, the two transcription start points are used equally. Although the single transcription start point in the human *TSHβ* gene is located 10 bp upstream from the downstream ones in the rodents, these are equivalent transcription start points preceded by the downstream 'TATA' boxes, because 9 bp are deleted in the rodent sequences between 'TATA' boxes and the transcription start points.

To see whether a different transcription start point might be used in human TSH-producing adenomas as observed in rodents, primer extension analysis was performed as described above, using total RNAs from two human TSH-producing adenomas. Primer A was used as a primer for this purpose. Fragments identical to those obtained from normal samples (Fig. 7 lanes A1 and A2) were observed. Thus, just one transcription start point acting in human normal pituitaries is used also in human TSH-producing adenomas.

(f) Transcriptional regulatory elements

Cyclic AMP-responsive elements have been described in the 5′-flanking region of several human and rat genes (Silver et al., 1987). These elements contain a highly conserved core sequence,

ATGACGTCAG. The human *TSHβ* gene contains similar sequences at nt positions −565 to −556 and −188 to −179. Mouse *TSHβ* gene also carries such homologous sequences (Gordon et al., 1988). We compared these regions among human, rat, and mouse and found that there are regions of more than 30 nt (−573 to −544 or −200 to −171) which are highly conserved (Figs. 4 and 5). These regions are likely to be used for cAMP regulation which reverses the suppressive effect by dopamine.

(g) Identification of the polyadenylation site

To determine the polyadenylation site, S1 nuclease mapping was done with total RNA from normal pituitary using a S1 probe (+1445 to +1580 in Figs. 2 and 3). Fragments of 107 to 111 nt and 119 to 124 nt were obtained (Fig. 8, lane N). Thus, human TSHβ mRNA from normal pituitary has two blocks of polyadenylation sites (+1551 to +1555 and +1563 to +1568). Two AATAAA hexamers lie just in front of these sites (Fig. 3).

To see how these polyadenylation sites are used in tumors, S1 nuclease mapping was performed as described above using total RNA from human TSH-producing adenomas. Fragments similar to those obtained above were observed (Fig. 8, lane A). Thus, these polyadenylation sites are used both in normal human pituitaries and in TSH-producing adenomas.

(h) Comparison of the whole *TSHβ* genes between different species

Our study has shown that the human *TSHβ* gene covers about 4.5 kb of the genome and has three exons interrupted by two introns of 3.5 and 0.45 kb in size. Exon 1 (37 bp) contains only the 5′-non-coding region, whereas exon 2 (163 bp) and exon 3 (about 325 bp and 335 bp) contain the entire coding sequence for TSHβ precursor.

The location and nucleotide sequence of exon 1 and the two TSHβ-coding exons are conserved among humans and rodents (Fig. 4). The mouse *TSHβ* gene has two extra 5′-noncoding exons, exon 2 and exon 3 (see Fig. 4B), in between exon 1 and exon 2 of the human or rat *TSHβ* genes. The human and rat sequences have short homologous sequences in some parts of introns (Fig. 4A), but their role is presently unknown.

The determination of the entire structure of the human *TSHβ* gene should allow us to further elucidate mechanisms involved in the regulation of this gene.

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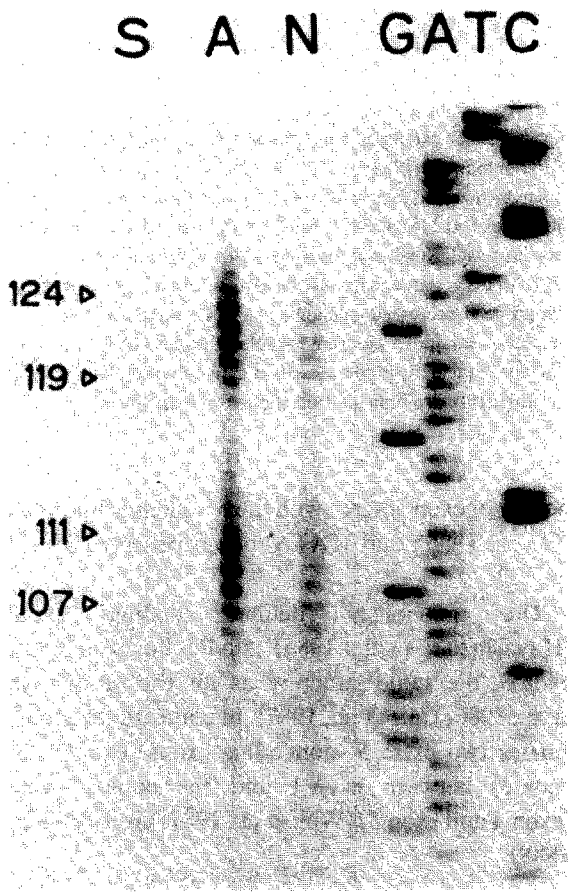

Fig. 8. S1 nuclease mapping for defining the polyadenylation site of the human *TSHβ* gene. S1 probe (see Figs. 2 and 3) was hybridized to mRNAs at 30°C for 12 h and the hybrid was digested with S1 nuclease. The S1-resistant hybrid was subjected to 6% PA-8 M urea sequencing gel. Total RNA (5 μg each) from human normal pituitary (N), human TSH-producing adenoma (A), and rat spleen (S) were used. Lanes GATC show the dideoxy sequencing ladders used as a size marker. The sizes of the fragments are shown on the left margin (in nt).

yashi, K. Koto, N. Kuzuya, A. Nagai, K. Okuno, M. Ota, Z. Ro, M. Sakurai, I. Shikata, M. Takami, T. Tamagawa, A. Teramoto, T. Uozumi, and A. Wada. We also thank Drs. T. Kobayashi and N. Tomita for their valuable advice.

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