Isolation and Characterization of the Human Tyrosine Hydroxylase Gene: Identification of 5′ Alternative Splice Sites Responsible for Multiple mRNAs†

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ABSTRACT: A full-length genomic clone for human tyrosine hydroxylase (L-tyrosine,tetrahydropteridine:oxygen oxidoreductase, EC 1.14.16.2) has been isolated. A human brain genomic library constructed in EMBL3 was screened by using a rat cDNA for tyrosine hydroxylase as a probe [Brown, E. R., Coker, G. T., III, & O'Malley, K. L. (1987) *Biochemistry 26*, 5208–5212]. Out of one million recombinant phage, one clone was identified that hybridized to both 5′ and 3′ rat cDNA probes. Restriction endonuclease mapping, Southern blotting, and sequence analysis revealed that, like its rodent counterpart, the human gene is single copy, contains 13 primary exons, and spans approximately 8 kilobases (kb). In contrast to the rat gene, human tyrosine hydroxylase undergoes alternative RNA processing within intron 1, generating at least three distinct mRNAs. A comparison of the human tyrosine hydroxylase and phenylalanine hydroxylase [DiLella, A. G., Kwok, S. C. M., Ledley, F. D., Marvit, J., & Woo, S. L. C. (1986) *Biochemistry 25*, 743–749] genes indicates that although both probably evolved from a common ancestral gene, major changes in the size of introns have occurred since their divergence.

Tyrosine hydroxylase (TH,[1] EC 1.14.16.2), which catalyzes the hydroxylation of L-tyrosine to L-DOPA, is an iron-containing mixed function oxidase requiring a reduced pterin cofactor and molecular oxygen. This enzyme is the initial and rate-limiting enzyme in catecholamine biosynthesis and is found in abundance in certain neurons of the locus coerulus, ventral tegmental area, and substantia nigra, as well as in the adrenal medulla and sympathetic ganglia. Many studies have demonstrated that the regulation of TH is a complex process that can be affected by glucocorticoids and cyclic AMP (Lewis et al., 1987), growth factors (Goodman & Herschman, 1978; Acheson et al., 1984; Wagner & O'Malley, 1987), and protein kinases (McTigue et al., 1985; Campbell et al., 1986), as well as stress (Zigmond et al., 1986) increased neuronal activity (Thoenen, 1980; Cahill & Perlman, 1984), pharmacologic agents such as reserpine (Berod et al., 1987), and genetic mechanisms (Vadasz et al., 1985).

Recently, clones for human TH have been isolated from both pheochromocytoma and neuroblastoma cDNA libraries (Ginns et al., 1987; Grima et al., 1987). In contrast to the previously reported rodent TH cDNA, sequence analysis of the human cDNAs demonstrated heterogeneity at the 5′ end of their coding regions (Ginns et al., 1987; Grima et al., 1987). The expression of these mRNAs appears to be variable in different neuronal and neural crest derived cell populations (Grima et al., 1987), suggesting tissue-specific factors may be involved.

The TH gene has been localized near human chromosome band 11p15, close to the insulin and Harvey-ras-1 genes (Moss et al., 1986). Recently, Egeland et al. (1987) reported genetic linkage between bipolar affective disorder in the Amish and a dominant gene near the insulin and Harvey-ras-1 loci. The close proximity of TH to the putative region of linkage, together with a large body of pharmacological data implicating atypical catecholamine function in affective disorders, prompted us to isolate and characterize the human TH gene. Like the rat gene, the human gene is present in a single copy spanning 8 kb of genome. In contrast to the rodent gene, alternative splice sites are utilized in the human gene to generate multiple mRNA species.

Experimental Procedures

Materials. Enzymes were purchased from International Biotechnologies, Inc., New England Biolabs, Inc., Bethesda Research Labs., Inc., Boehringer Mannheim Biochemicals, and Promega Biotec. Deoxynucleotide triphosphates were obtained from Promega Biotec. Radionucleotides were purchased from either Amersham Corp. or New England Nuclear. Nitrocellulose sheets were from Schleicher and Schuell. The plasmid bluescript, EMBL3 λ arms, and Giga Pack packaging system were obtained from Stratagene. PrepGel was from Bethesda Research Labs, Inc.

DNA Preparation. After informed consent was obtained, blood or surgical biopsy from temporal cortex was obtained. High-molecular-weight DNA was prepared according to standard protocols (Maniatis et al., 1982).

Isolation of the TH Gene. A partial *Sau*3A digest of genomic DNA (500 μg) from normal human temporal cortex biopsy tissue was size-fractionated on PrepGel. The fractions containing DNA fragments of 15–20-kb size were pooled, ligated to *Bam*HI EMBL3 arms, and packaged according to manufacturers' protocols. Recombinant bacteriophage were screened by standard methods using a radiolabeled rat TH

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[1] Abbreviations: TH, tyrosine hydroxylase; PH, phenylalanine hydroxylase; bp, base pair(s); kb, kilobase(s).

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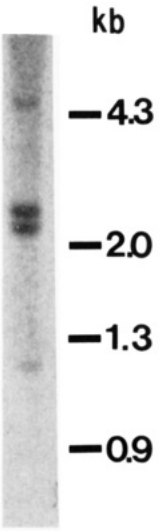

FIGURE 1: Determination of the number of *Bam*HI fragments in the human genome homologous to a full-length TH cDNA (Ginns et al., 1987). DNA, blots, and the radiolabeled human TH cDNA probe were prepared as described in the text. All of the hybridizing fragments could be accounted for in the restriction map of the human TH gene. Increased hybridization signals for the 2300- and 2150-bp bands reflect the larger number of exons in these fragments.

cDNA as a probe. Prehybridizations, hybridizations, and washes were carried out at 42 °C to allow for species variation (Wahl et al., 1979). Out of one million recombinant phage, one clone was identified that hybridized to probes derived from the 5′ and 3′ ends of the rat cDNA.

DNA Sequence Analysis. Subclones spanning the entire insert were prepared in plasmids pBR322, pGEM, or bluescript for further restriction mapping (Smith & Birnstiel, 1975). Exons and splice junctions were sequenced by the dideoxynucleotide chain-terminator method using either strand of denatured double-stranded DNA as a template for primer-extended synthesis (Chen & Seeburg, 1985). Commercially available primers and/or synthetic oligodeoxynucleotides complementary to sequences of either the human or rat TH cDNA were used as sequencing primers. All exons and intron/exon boundaries were sequenced on both strands. Oligodeoxynucleotides were synthesized by the solid-phase phosphoramidite method with an automatic DNA synthesizer (Model 380A, Applied Biosystems, or Beckman System I). DNA sequence analysis was performed on commercially available software (PCGENE and Bionet programs, Intelligenetics).

Southern Blot Analysis. Five micrograms of leukocyte high-molecular-weight DNA was digested to completion with *Bam*HI endonuclease, fractionated electrophoretically in 1.2% agarose gels, and transferred to nitrocellulose filters. Prehybridization of the filters, hybridization to a radiolabeled full-length human TH cDNA (Ginns et al., 1987), and posthybridization washes followed the protocol of Wahl et al. (1979). Exon-specific probes were generated at appropriate restriction fragments of the human TH cDNA (Ginns et al., 1987) as determined by the intron positions in the rat TH gene (Brown et al., 1987).

RESULTS

The Human TH Gene Is Single Copy. To assess the number of TH gene fragments contained in the human genome, a radiolabeled full-length human TH cDNA (Ginns et al., 1987) was hybridized to nitrocellulose blots of *Bam*HI digested human leukocyte DNA. As seen in Figure 1, the human cDNA probe identifies a discrete set of bands in the human

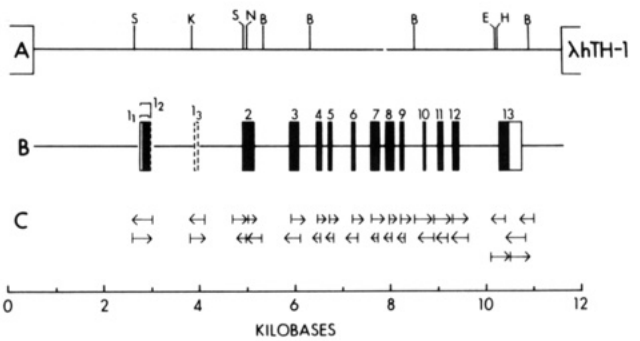

FIGURE 2: Structure of the human TH gene. (A) Restriction map of the human TH locus. Restriction enzyme abbreviations: B, *Bam*HI; K, *Kpn*I; E, *Eco*RI; S, *Sac*II; N, *Not*I. (B) Constitutively expressed exons are depicted by solid boxes and untranslated regions by clear boxes, while the alternatively spliced exons are depicted by dotted boxes. Introns and flanking regions are represented by the thin line. (C) DNA sequencing strategy. Arrows indicate the direction of sequencing for each exon. Each intron/exon boundary was sequenced several times.

chromosomal DNA. Subsequent analyses using exon-specific human probes allowed us to assign the 4.6-kb fragment to exons 1 and 2, and 1.0-kb band to exon 3, and the 2.1- and 2.3-kb restriction fragments to exons 4–13. From these results it appears that the human TH gene is single copy and, based on the sum of the restriction fragments, its size is no greater than 10 kb.

Characterization of the Human TH Gene. We have used detailed restriction endonuclease mapping (Figure 2A), Southern blotting, and DNA sequencing to characterize the bacteriophage clone containing the human TH gene (λ hTH-1). This 11-kb clone hybridized with restriction fragments corresponding to the 5′ and 3′ ends of the rat cDNA (O'Malley, 1986; Brown et al., 1987), suggesting that the entire TH coding region was contained in this isolate. We subsequently confirmed this by direct sequence comparison with full-length human cDNAs (Ginns et al., 1987; Grima et al., 1987). The human TH gene spans 8.0 kb, which in λ hTH-1 is bounded by about 1.5 kb of 5′ and 3′ flanking sequence.

Organization of Expressed Sequences. All exons and splice junctions were sequenced and aligned with the human TH cDNA sequence (Ginns et al., unpublished results; Grima et al., 1987). Exon positions are depicted in Figure 2B, and the sequencing strategy is outlined in Figure 2C. The human TH gene contains a minimum of 13 exons ranging from 51 to 479 bp in length. In contrast to the rodent gene, the human gene also contains at least two alternative splice sites (exons 1-2 and 1-3) that generate heterogeneous 5′ mRNAs (Ginns et al., 1987; Grima et al., 1987). Precise characterization of the transcription start site is in progress although we anticipate it to be in a position analogous to that in the rat (Grima et al., 1987; Harrington et al., 1987; G. T. Coker and K. L. O'Malley, unpublished results).

All splice sites contain consensus sequences (Mount, 1982; Ohshima & Gotoh, 1987) (Figure 3). Most splice donors had one to two mismatches with the established consensus sequence (Mount, 1982; Ohshima & Gotoh, 1987) although two sites had three mismatches and one site had four mismatches out of nine with the consensus. Three of the acceptor sequences matched the consensus perfectly. Nine sites had one to two mismatches out of eleven for the consensus; one site had four mismatches. Additionally, all splice sites have been conserved between the rat and human genes (Brown et al., 1987) with the exception of the alternative splice sites within the first human intron. Although the coding regions of both species

Exon #	Size(bp)	Intron #	Size(bp)	Exon	Intron	Exon
1_1	125			ATC ATG ILE MET 29,30	gtaagagggcag.....	
1_2	137	1a	1200	GGG CAG GLY GLN	gtaggtgccc..... ccctccccag	GGC GCC GLY ALA
1_3	82	1b	900	CCA AGG PRO ARG	gtaagtaagtg..... gtccccacag	TCC CCC SER PRO 31,32
2	220	2	900	TTT GAG PHE GLU 103,104	gtgagctggtg..... gctccctaag	ACG TTT THR PHE 105,106
3	175	3	350	AAG G LYS VAL 162,163	gtgaggcgg..... acccataaag	TC CCC VAL PRO 163,164
4	90	4	140	CAC CCG HIS PRO 191,192	gtgagtggtgc..... ctgccctcag	GGC TTC GLY PHE 193,194
5	68	5	440	AGG CA ARG HIS 214,215	gtgaggggccc..... ccctccccag	C GGC HIS GLY 215,216
6	51	6	310	ACC TG THR TRP 231,232	gtgagacctcc..... ctgcccgcag	G AAG TRP LYS 232,233
7	146	7	110	AAG G LYS GLU 280,281	gtgtgcccaga..... cccccttcag	AG CGC GLU ARG 281,282
8	136	8	120	GAG CC GLU PRO 325,326	gtgagtgcg..... tacgcgcag	G GAC PRO ASP 326,327
9	70	9	400	TCG CAG SER GLN 348,349	gtacgccgc..... agctccacag	GAC ATT ASP ILE 350,351
10	57	10	240	TCC ACG SER THR 367,368	gtqggttga..... gtccccgcag	CTG TAC LEU TYR 369,370
11	96	11	220	CTC CTG LEU LEU 399,400	gtgaqagtc..... ctcctgccag	CAC CTG HIS CYS 401,402
12	134	12	890	CTC AG LEU ARG 444,445	gtgggctag..... tcctccccag	G AGC ARG SER 445,446
13	459			GGC TAG GLY STOP	..aataaaaggcactgtggtctctacacc	

FIGURE 3: Exon/intron junctions of the human TH gene. All exons and splice sites were sequenced as depicted in Figure 1C. Exon sequences are in upper case and the intron sequences are in lower case. Corresponding amino acids are below the exon sequences. Exon 1-1 refers to the human cDNA analogous to the rat comprising amino acids 1–30 (Grima et al., 1985; Brown et al., 1987). Exon 1-2 signifies the additional 4 amino acids that may be expressed in some human TH messages. Exon 1-3 is used to designate the additional 27 amino acids expressed in certain human messages derived from the adrenal gland (Grima et al., 1987). Consensus polyadenylation signals are underlined.

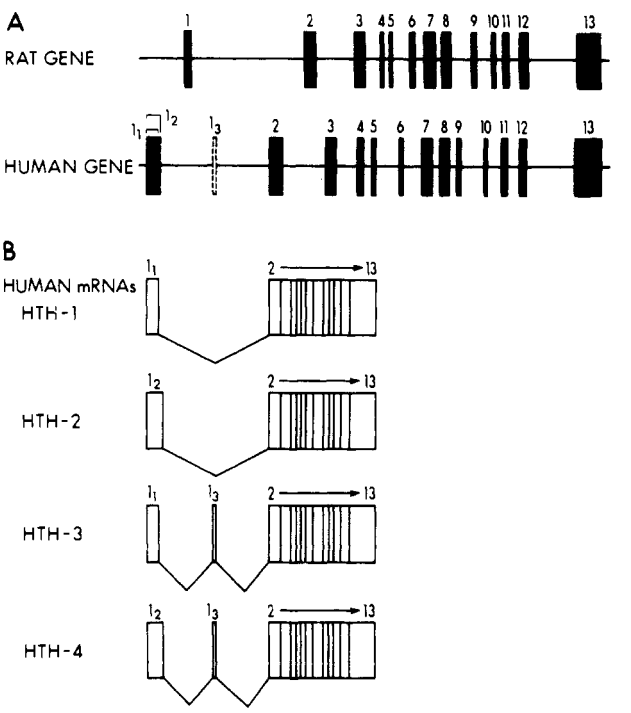

FIGURE 4: Comparison of the rat TH transcription unit versus the more complex transcriptional pattern of the human TH gene. (A) Relative size and spacing of the rat TH exons versus those in the analogous human loci. (B) Single initiation site but variable splicing generating multiple human TH mRNAs. The various forms of human TH mRNA are schematically depicted below the genomic arrangement from which they are derived. HTH-1 and HTH-2 have been isolated by ourselves (Ginns et al., 1987) and Grima et al. (1987); a portion of HTH-3 has been isolated by Grima et al. (1987), and HTH-4 is predicted from the splicing pattern of HTH-2 and HTH-3.

share 89% identity (Grima et al., 1987), there is only limited homology between the portions of introns that were sequenced. However, the relative sizes and positioning of the introns have been conserved (Figure 4A) (Brown et al., 1987).

Exon 13, the most 3′ exon, contains 157 bp of coding and 305 bp of noncoding nucleotides. Conserved sequences determining the site of 3′ cleavage including the canonical -AATAAA-, located just upstream from a -CACTG- motif (Birnstiel et al., 1985), followed by a poly(A) addition site (TA) 8 bp downstream. The order of these consensus sequences is the reverse of that found in either the rat TH gene or in the human PH gene.

Having sequenced all exons using both strands, we confirmed the cDNA sequence of Grima et al. (1987) with the following exception. We have determined amino acid 370 to be a tyrosine (-TAC-) versus the serine (-TCA-) reported by Grima et al. (1987). The assignment of tyrosine agrees with the analogous amino acid in the rat in a region that has 91% identity between the two species (Grima et al., 1985; Brown et al., 1987).

Identification of 5′ Alternative Splice Sites Responsible for Multiple mRNAs. Sequence analysis of genomic DNA between exon 1 and exon 2 allowed us to identify regions responsible for the heterogeneity observed in the human mRNAs (Ginns et al., 1987; Grima et al., 1987). Among the various genomic arrangements for the possible generation of multiple mRNAs (Leff et al., 1986), the gene for human TH falls in the "single initiation site, single poly(A) site, variable splicing" category. This is illustrated in Figure 4.

A comparison of the variable human sequences with analogous positions in the rat is shown in Figure 5. The human exon 1 and rat exon 1 share 82% identity. The additional 12-bp extension in human exon 1-2 may have arisen from a point mutation creating a new splice site within intron 1. These 12 bp share 50% identity with the rat splice site junction. However, since the splice donor for exon 1-2 is an eight out of nine match with the consensus donor (Mount, 1982; Ohshima & Gotoh, 1987) and human bases 100–108 share only 22% identity with rat, this site probably arose by multiple mutations. To determine whether human exon 1-3 was present in the rat TH gene, we used a 52-mer oligonucleotide complementary to the human sequence as a hybridization probe. Weak hybridization was detected within the rat intron 1 approximately 400 bp upstream of exon 2. However, sequence analysis of this region revealed no significant homologies as determined by alignment using the IFIND program from Bionet (Figure 5). Of the 81 nucleotides, 40 matched, 24 mismatched, and 8 were unmatched with two gaps. There were no acceptor or donor sequences in this region. This evidence suggests that the rat TH gene is a simple transcriptional unit; at least the analogous human forms are not expressed in the rat.

Discussion

In this paper we report the organization of the human TH gene and demonstrate the origin of the heterogeneity in the different human mRNAs. Like the rat gene, the human TH locus spans 8 kb and contains 13 primary exons. In contrast to rodent TH, the human gene appears to undergo alternative splicing, creating at least three different messages (Ginns et al., 1987; Grima et al., 1987). Analysis of the sequence at the end of exon 1 reveals two donor dinucleotides (-GT-), one adjacent to the -ATG- (+90) and the other 12 nucleotides downstream creating a 3′ extension (Figure 5). Clearly, both

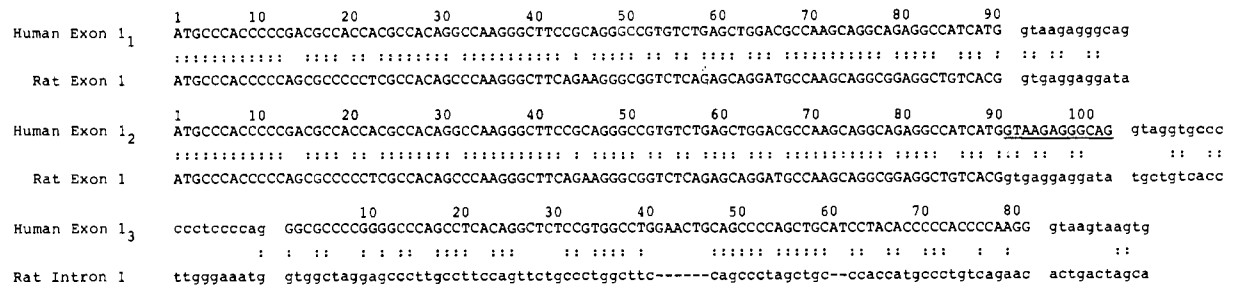

FIGURE 5: Comparison of the rat intron 1 sequence with the analogous human splice sites responsible for generation of the 5′ heterogeneity in human TH messages. (Top) Human and rat exon 1-1: the first 30 amino acids share 82% identity and are spliced in an logous positions. Exon sequences are in upper case and the intron sequences are in lower case. (Middle) Human exon 1-2 is generated by a second splice site 12 bp downstream from that for exon 1-1. These additional bases are underlined. The donor dinucleotide found at base 103 of the human gene is missing in the rat gene. (Bottom) An oligonucleotide probe corresponding to the first 52 bases of human exon 1-3 weakly hybridized to a region of rat intron 1 located 400 bp upstream from exon 2 (results not shown). Sequence analysis of this region in the rat TH gene revealed 50% identity with human exon 1-3 (with the addition of two gaps). However, in the rat sequence there are no consensus donor or acceptor sequences in this region.

sites are spliced as we and others have isolated full-length cDNAs corresponding to both forms of the message (Ginns et al., 1987; Grima et al., 1987). Comparison of the analogous sequences in the rat shows no consensus donor splice sequences at this junction, confirming that these alternative forms of TH are not expressed in the rat (Figure 5).

Grima et al. (1987) have reported that the third form of the human TH message beings with an -ATG- codon followed by 27 amino acids that are different than those found in either the human or rat exon 1. These investigators suggest either that this messenger is transcribed from a different promoter or that the last 3 bp of the first exon are utilized to generate the -ATG- located at the 5′ end of this variant message. Our data demonstrate that the third form of human TH messenger RNA is created by alternative splicing of exon 1-3. Therefore, this messenger initiates at the normal -ATG- codon in exon 1-1 and includes an additional 27 amino acids (exon 1-3) before joining exon 2. We would predict that a cDNA corresponding to this model will be isolated. It is also conceivable that a fourth form of TH mRNA is expressed, one that would include exons 1-2 and 1-3. We are currently screening various tissues to investigate these possibilities.

The functional significance of the multiple mRNAs for the human TH gene remains to be determined. Grima et al. (1987) have presented evidence for the tissue-specific expression of HTH-3 (Figure 5). This mRNA appears to be expressed only in pheochromocytoma and in adrenal glands, while HTH-1 and HTH-2 are expressed in central and peripheral neurons (Grima et al., 1987). Recently we have isolated another variant form of a human TH cDNA that corresponds to a 5′ extension of exon 2 into intron 1 (Ginns et al., unpublished observations). Whether this variant is expressed or is derived from an unprocessed intron remains to be determined.

The multiple human TH mRNAs generate different polypeptides that may have altered functions. We are using baculovirus expression vectors (Smith et al., 1986) to explore the significance of the various forms of human TH. The availability of large quantities of the different forms of TH will permit kinetic and structural analyses, as well as production of specific antiserum for immunocytochemical and pulse–chase studies. These types of approaches should clarify the physiological significance of the various forms of TH.

It has been previously shown that TH and PH evolved from a common ancestral gene (Ledley et al., 1985; Dahl & Mercer, 1986). The human PH gene spans over 90 kb with introns as large as 23 kb (DiLella et al., 1986). Our characterization of both rat (Brown et al., 1987) and human TH genes demonstrates that this size difference between TH and PH is primarily a result of changes in intervening sequences. Additionally, we have demonstrated that 10 out of 12 splice sites present in the human PH gene are in homologous positions in the rat TH gene (Brown et al., 1987) and, by extension, in the human TH gene as well. On the basis of these results we have predicted that the ancestral hydro ylase gene would have encoded exons similar to those of TH p or to evolutionary drift to other members of this gene family. More information from different species for all of these enzymes, TH, PH, and tryptophan hydroxylase as well as dopamine β ydroxylase, could elucidate the evolutionary history of this ene family.

The loca ization of the human TH gene with the region of chromosome 11 linked to bipolar-affective illness in the Amish (Egeland et al., 1987) suggests that the TH gene might be involved in the pathogenesis of this disorder. With the availability of full-length human cDNAs, as well as a detailed description of the "normal" TH gene locus, we have the tools with which to begin a detailed examination of putative genetic disorders, mutant alleles, and genetic polymorphisms in man that may be associated with this gene.

ADDED IN PROOF

While this paper was in press, Kaneda et al. (1987) reported the isolation of a human TH cDNA corresponding to our hypothesized HTH-4. Their result confirms our prediction.

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REFERENCES

Acheson, A. L., Naujoks, K., & Thoenen, H. (1984) *J. Neurosci. 4*, 1771–1780.

Berod, A., Faucon-Biguet, N., Dumas, S., Bloch, B., & Mallet, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1699–1703.

Birnstiel, M. L., Busslinger, M., & Strub, K. (1985) *Cell (Cambridge, Mass.) 41*, 349–359.

Brown, E. R., Coker, G. T., III, & O'Malley, K. L. (1987) *Biochemistry 26*, 5208–5212.

Cahill, A. C., & Perlman, R. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7243–7247.

Campbell, D. G., Grahame, D., Hardie, D. G., & Vulliet, P. R. (1986) *J. Biol. Chem. 261*, 10489–10492.

Chen, E. Y., & Seeburg, P. H. (1985) *DNA 4*, 165–170.
Dahl, H. H. M., & Mercer, J. F. B. (1986) *J. Biol. Chem. 261*, 4148–4153.
DiLella, A. G., Kwok, S. C. M., Ledley, F. D., Marvit, J., & Woo, S. L. C. (1986) *Biochemistry 25*, 743–749.
Egeland, J. A., Gerhard, D. S., Pauls, D. L., Sussex, J. N., Kidd, K. K., Allen, C. R., Hostetter, A. M., & Housman, D. E. (1987) *Nature (London) 325*, 783–787.
Ginns, E. D., Kelsoe, J. R., Martin, B. M., Winfield, S. L., LaMarca, M. E., Luu, M. D., Paul, S. M., & O'Malley, K. L. (1987) *Soc. Neurosci. Abstr. 13*, 856.
Goodman, R., & Herschman, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4587–4590.
Grima, B., Lamouroux, A., Blanot, F., Faucon-Biquet, N., & Mallet, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 73*, 617–621.
Grima, B., Lamouroux, A., Boni, C., Julien, J.-F., Javoy-Agid, F., & Mallet, J. (1987) *Nature (London) 326*, 707–711.
Harrington, C. A., Lewis, E. J., Krzemien, D., & Chikaraishi, D. M. (1987) *Nucleic Acids Res. 15*, 2363–2387.
Kaneda, N., Kobayashi, K., Ichinose, H., Kishi, F., Nakazawa, A., Kurosawa, Y., Fujita, K., & Nagatsu, T. (1987) *Biochem. Biophys. Res. Commun. 146*, 971–975.
Ledley, F. D., DiLella, A. G., Kwok, S. C. M., & Woo, S. L. C. (1985) *Biochemistry 24*, 3394–3397.
Leff, S. L., Rosenfeld, M. G., & Evans, R. M. (1986) *Annu. Rev. Biochem. 55*, 1091–1117.
Lewis, E. J., Harrington, C. A., & Chikaraishi, D. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3550–3554.
Maniatis, T., Fritsch, E. F., & Sambrook, M. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McTigue, M., Cremins, J., & Halegoua, S. (1985) *J. Biol. Chem. 260*, 9047–9056.
Moss, P. A. H., Davies, K. R., Boni, C., Mallet, J., & Reeders, S. T. (1986) *Nucleic Acids Res. 14*, 9927–9932.
Mount, S. M. (1982) *Nucleic Acids Res. 10*, 459–472.
Ohshima, Y., & Gotoh, Y. (1987) *J. Mol. Biol. 195*, 247–259.
O'Malley, K. L. (1986) *J. Neurosci. Res. 16*, 3–12.
Smith, G. E., Ju, G., Ericson, B. L., Moschera, J., Lahm, H.-W., Chizzonite, R., & Summers, M. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8404–8408.
Smith, H. O., & Birnstiel, M. L. (1975) *Nucleic Acids Res. 3*, 2387–2395.
Thoenen, H. (1974) *Life Sci. 14*, 223–235.
Vadasz, C., Baker, H., Fink, S. J., & Reis, D. J. (1985) *J. Neurogenet. 2*, 219–230.
Wagner, C. E., & O'Malley, K. L. (1987) *Proc. Int. Catecholamine Symp., 6th* (in press).
Wahl, G. M., Stern, M., & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3683–3687.
Zigmond, R. E., Schon, F., & Iversen, L. L. (1974) *Brain Res. 70*, 547–552.

Chemical Relaxation in a Chemiosmotic-Coupled System: Driving the Calcium Adenosinetriphosphatase with Bacteriorhodopsin†

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ABSTRACT: Phase-lifetime spectroscopy has been used to measure chemical relaxation processes in a chemiosmotic-coupled system. In this experiment the calcium ATPase and bacteriorhodopsin were coreconstituted into phospholipid vesicles. Upon illumination the bacteriorhodopsin pumps protons into the vesicles and forms a membrane potential. This membrane potential alters the activity of the internal calcium and thus perturbs the equilibrium of ATP hydrolysis/synthesis coupled to calcium transport. Mechanically chopping the actinic light provides a periodic perturbation to the system, and small response signals can be observed by using phase-sensitive detection. It is shown that this periodic perturbation occurs about a steady-state membrane potential that is independent of chopping frequency. The amplitude dispersion curve for the fluorescence of a calcium indicator was observed and analyzed in terms of the relaxation time for the ATPase-catalyzed calcium transport. Thus, this technique provides a method of measuring ion transport kinetics against a constant chemiosmotic potential. The calcium ATPase showed a single relaxation time on this time scale. The dependence of this relaxation time on ADP and phosphate concentration was measured and analyzed with a random sequential mechanism. This analysis gave dissociation constants for ADP and phosphate of 3.2 mM and 1.4 mM, respectively. These binding steps are followed by slow isomerization steps with forward and reverse rate constants (in the direction of ATP synthesis) of 67 s^{-1} and 227 s^{-1}, respectively. These results demonstrate that highly accurate kinetic data can be obtained with this modulation relaxation technique.

Knowledge of the rates of the individual, elementary reaction steps is required for a detailed, molecular description of ion transport across biological membranes. Such information has proven to be unusually difficult to obtain for even the best characterized membrane-bound transport proteins. This is, in part, due to a lack of high-resolution kinetic techniques for measuring ion transport against a constant chemiosmotic potential. Large changes in the chemiosmotic

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