

Identification and Functional Characterization of a Novel, Tissue-specific NAD⁺-dependent Isocitrate Dehydrogenase β Subunit Isoform*

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To understand the interactions and functional role of each of the three mitochondrial NAD⁺-dependent isocitrate dehydrogenase (IDH) subunits (α , β , and γ), we have characterized human cDNAs encoding two β isoforms (β_1 and β_2) and the γ subunit. Analysis of deduced amino acid sequences revealed that β_1 and β_2 encode 349 and 354 amino acids, respectively, and the two isoforms only differ in the most carboxyl 28 amino acids. The γ cDNA encodes 354 amino acids and is almost identical to monkey IDH γ . Northern analyses revealed that the smaller β_2 transcript (1.3 kilobases) is primarily expressed in heart and skeletal muscle, whereas the larger β_1 mRNA (1.6 kilobases) is prevalent in nonmuscle tissues. Sequence analysis of the IDH β gene indicates that the difference in the C-terminal 28 amino acids between β_1 and β_2 proteins results from alternative splicing of a single transcript. Among the various combinations of human IDH subunits co-expressed in bacteria, $\alpha\beta\gamma$, $\alpha\beta$, and $\alpha\gamma$ combinations exhibited significant amounts of IDH activity, whereas subunits produced alone and $\beta\gamma$ showed no detectable activity. These data suggest that the α is the catalytic subunit and that at least one of the other two subunits plays an essential supporting role for activity. Substitution of β_1 with β_2 in the co-expression system lowered the pH optimum for IDH activity from 8.0 to 7.6. This difference in optimal pH was analogous to what was observed in mouse kidney and brain (β_1 prevalent; optimal pH 8.0) versus heart (β_2 prevalent; pH 7.6) mitochondria. Experiments with a specially designed splicing reporter construct stably transfected into HT1080 cells indicate that acidic conditions favor a splicing pattern responsible for the muscle- and heart-specific β_2 isoform. Taken together, these data indicate a regulatory role of IDH β isoforms in determining the pH optimum for IDH activity through the tissue-specific alternative splicing.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)U49283, AF023265, and U40272 (for human IDH β_1 , IDH β_2 and IDH γ cDNA, respectively). The partial genomic nucleotide sequence for the human IDH β gene was deposited to the GenBank™/EMBL data bases under the accession number AF046929.

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Isocitrate dehydrogenases (ICDHs¹; EC 1.1.1.41 and EC 1.1.1.42) catalyze the oxidative decarboxylation of isocitrate into α -ketoglutarate, producing either NADH or NADPH (1). In mammals, three classes of ICDH isoenzymes exist: mitochondrial NAD⁺-dependent ICDH (IDH), mitochondrial NADP⁺-dependent ICDH, and cytosolic NADP⁺-dependent ICDH (2–4). In addition, recent data suggest that a peroxisomal NADP⁺-dependent ICDH exists as the fourth ICDH isoenzyme in yeast. This putative protein probably provides NADPH, which is required for the peroxisomal oxidation of unsaturated fatty acids (5, 6). In contrast to eukaryotes, only one type of ICDH (NADP⁺-dependent enzyme) is present in *Escherichia coli* (7).

Among the eukaryotic ICDH isoenzymes, IDH has been assumed to play a major role in the oxidative decarboxylation of isocitrate in the tricarboxylic acid cycle (8, 9). Its key role is underscored by the fact that its activity is regulated by numerous allosteric regulators. For example, it is positively regulated by ADP in mammals and AMP in yeast, yet inhibited by ATP, NADH, or NADPH. Calcium ions have also been known to enhance IDH activity in the presence of isocitrate and adenine nucleotide (10).

Recent functional studies on the yeast ICDH isoenzymes revealed the catalytic and regulatory roles of IDH2 and IDH1, respectively (11–13). In addition to its catalytic role in the tricarboxylic acid cycle, the yeast IDH protein was shown to specifically bind to the 5'-untranslated region of mRNAs of the mitochondrial cytochrome c oxidase subunits I, II, and III as well as cytochrome *b*, thus suggesting another important regulatory role of the IDH protein in controlling mitochondrial biogenesis and energy metabolism (14).

In mammals, the IDH enzyme exists as a heterotetramer consisting of 2 α , 1 β , and 1 γ subunits. All the subunits have comparable molecular masses (39–41 kDa) and highly similar amino acid sequences (15, 16). Despite numerous biochemical and kinetic studies of IDH isolated from animal tissues, the precise function of each subunit and the nature of their interactions in the catalytically active IDH protein have not been elucidated in mammals. The nucleotide and deduced protein sequences of a cDNA clone encoding the human IDH α were previously reported, and the possibility that IDH α serves as a catalytic subunit was suggested (17).

Accordingly, to further elucidate the functional roles of the

¹ The abbreviations used are: ICDH, isocitrate dehydrogenase; IDH, NAD⁺-dependent isocitrate dehydrogenase; IDH α , - β , and - γ , IDH α , β , and γ subunit, respectively; H-IDH α , - β , and - γ , human IDH α , β , and γ subunit, respectively; PCR, polymerase chain reaction; GFP, green fluorescent protein; PIPES, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction; MOPS, 4-morpholinopropanesulfonic acid; GFP, green fluorescent protein; kb, kilobase pair(s).

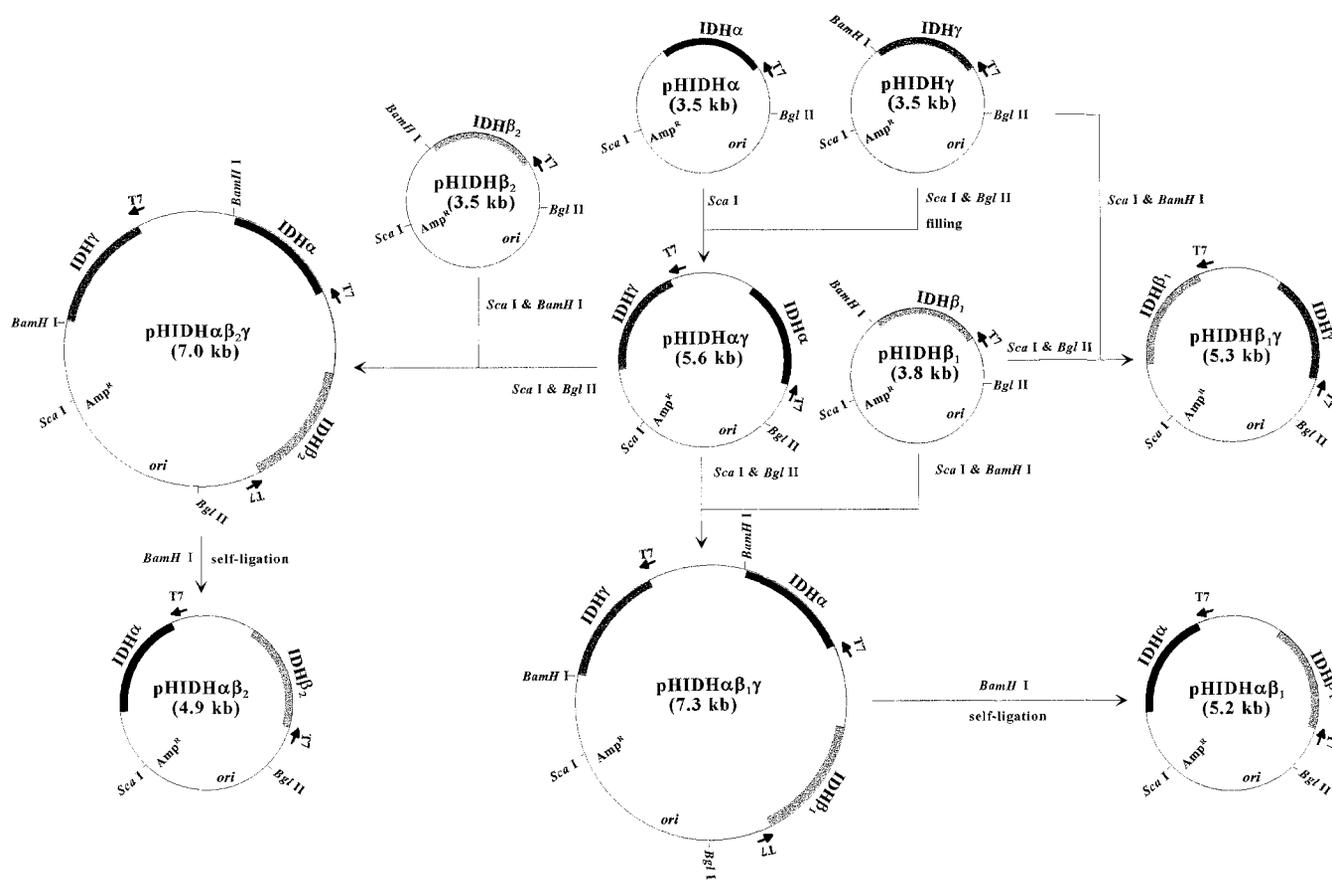


FIG. 1. The schematic diagram for construction of various recombinant IDH plasmids.

three IDH subunits, human cDNA clones for both IDH β and IDH γ were isolated and characterized and then co-expressed with IDH α in *E. coli* using a co-expression system that we developed. We demonstrate that IDH α is critical for the catalytic activity of IDH, whereas the IDH β or γ subunit has a supporting role in constituting the IDH activity. We also report, for the first time, the tissue-specific expression of an IDH β isoform (IDH β_2), which is probably derived from a pH-dependent exon-splitting alternative splicing from a single IDH β gene caused by an internal acceptor within an intron (18). The substitution of IDH β_1 (non-muscle-specific) with IDH β_2 (muscle-specific) in the recombinant IDH $\alpha\beta\gamma$ protein lowered the optimal pH values for IDH activity from 8.0 to 7.6.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 DNA ligase were purchased from POSCOHEM (Sungnam, Korea). GeneScreen membrane, [α - 32 P]dCTP (specific radioactivity 3000 Ci/mmol), and [α - 35 S]dATP (specific radioactivity 500 Ci/mmol) were from NEN Life Science Products. Bovine and human heart λ gt11 cDNA libraries were from CLONTECH (Palo Alto, CA). Nitrocellulose membrane was obtained from Schleicher & Schuell. Terminal deoxyribonucleotide transferase and random primer labeling kit were the products of Roche Molecular Biochemicals. Sephacryl S-300HR, expression vector pT7-7, and protein standards for gel filtration were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) or Bio-Rad. DL-Isocitrate, EGTA, and other chemicals were purchased from Sigma.

Oligonucleotide Probes for Screening IDH Clones by Plaque Hybridization—Based on the amino acid sequences of the internal tryptic peptides of pig heart IDH β and IDH γ (16), oligonucleotide probes were synthesized using the best codon usage (19) with a DNA synthesizer (Applied Biosystems, Foster City, CA): oligonucleotide probe 1 (sense direction for IDH β), 5'-GCCTCCCGGTCCAGGCTGAGGAC-3', corresponding to the peptide sequence NH $_2$ -ASRSQAED-COOH; oligonucleotide probe 2 (sense direction for IDH β), 5'-GTGCGGGTGGAGGGCGCCTTCCCTGTGACCATGCT-3', corresponding to the peptide sequence

NH $_2$ -VRVEGAFVPTML-COOH; oligonucleotide probe 3 (sense direction for IDH γ), 5'-TATGCCAATGTCATTCCTGTAAG-3', corresponding to the peptide sequence NH $_2$ -YANVIHC-COOH; and oligonucleotide probe 4 (antisense direction for IDH γ), 5'-CTTGAGGTGGTCCAGCATCATGCA-3', corresponding to peptide sequence NH $_2$ -CMMLDHLK-COOH. Oligonucleotides were radiolabeled at the 3'-end with [α - 32 P]dCTP using terminal deoxyribonucleotide transferase as described by Rosenberg *et al.* (20) and then used as probes for plaque hybridization.

Isolation of cDNA Clones Encoding IDH β and IDH γ —Bovine heart λ gt11 cDNA library was screened with 32 P-labeled oligonucleotide probes (oligo 1 and 2 for IDH β and oligo 3 and 4 for IDH γ). Conditions for hybridization and washing were as described (20). The positive cDNA clones from plaque hybridization were subcloned into plasmid pGEM7(+), and their nucleotide sequences were determined. The cDNA inserts containing a deduced amino acid sequence matching the amino acid sequences of the partial tryptic peptide sequences (16) of the pig heart IDH β and IDH γ were isolated, labeled with [α - 32 P]dCTP by random primer labeling, and then used to screen a λ gt11 cDNA library from human heart. Conditions for hybridization and washing were as described previously (21). The cDNA inserts for human IDH β_1 , IDH β_2 , and IDH γ were isolated and subcloned into plasmid pGEM7(+) for subsequent characterization as described below.

Isolation of Genomic Clones for Human IDH β Gene—Genomic clones for human IDH β were isolated from a λ EMBL3 human lymphocyte genomic DNA library (Dr. Frank J. Gonzalez, NCI, National Institutes of Health) by plaque hybridization with [α - 32 P]dCTP-labeled IDH β_1 cDNA as a probe. The conditions for labeling, hybridization, and washing were the same as described above.

Nucleotide Sequence Analyses—Complete nucleotide sequences of the largest cDNA clones encoding human IDH β_1 , IDH β_2 , and IDH γ were determined by the dideoxynucleotide chain termination method (22) with a Sequenase version 2.0 kit (U.S. Biochemical Corp.). To accomplish double-stranded DNA sequencing, cDNA subclones were gradually deleted from both ends using a kit (Erase-a-Base from Promega (Madison, WI)) with the protocol recommended by the manufacturer. Nucleotide sequence data were assembled and analyzed using the computer software PC/GENE (IntelliGenetics, Mountain View, CA).

sity). To introduce T7 RNA polymerase gene into the chromosome of *E. coli* EB106, the recombinant phage λ DE3 was infected to *E. coli* EB106 by λ DE3 Lysogenization kit (Novagen, Madison, WI) according to the manufacturer's instructions. The λ DE3 lysogen of *E. coli* EB106 was designated as *E. coli* EB106 (DE3) and used as a host to overproduce the recombinant IDH proteins of various combinations, as described above.

Preparation of the Recombinant IDH Proteins in *E. coli*—The recombinant plasmids were transferred to *E. coli* EB106 (DE3) by the CaCl₂ method (24), and bacterial colonies with positive inserts were grown overnight at 37 °C in LB media supplemented with ampicillin (50 μ g/ml). The overnight cultures of transformants were diluted 100-fold into 1 liter of the same media and allowed to grow at 37 °C until A₆₀₀ reached 0.6. The cultures containing transformants were cooled in an ice-water bath and treated with 0.4 mM isopropyl β -D-thiogalactopyranoside to induce gene expression. During the induction period, bacterial cells were grown at 25 °C for 24 h with poor aeration. *E. coli* cells were harvested by centrifugation, washed with 1 \times phosphate-buffered saline, and resuspended in a lysis buffer (10 ml of phosphate-buffered saline containing 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride). Resuspended cells were disrupted by sonication with five 20-s treatments at half-maximum power of a sonicator (Bronson model 350) on ice. The soluble lysates obtained by centrifugation at 9000 \times g for 20 min were used to measure the IDH activity. All procedures for the soluble lysate preparation were conducted at 4 °C.

Measurement of IDH Activity—IDH activity was determined by monitoring the production of NADH at 340 nm, the same as the method described by Rutter and Denton (25) with slight modification. IDH activity was measured at 25 °C in reaction mixture (50 mM MOPS, pH 7.4, containing 5 mM three-D_s-ISOCITRATE, 35.5 mM triethanolamine, 2 mM NAD⁺, 1 mM ADP, 2 mM MgCl₂, and 1 μ g/ml rotenone). Enzyme reaction was initiated by the addition of the *E. coli* soluble lysate containing the recombinant IDH proteins into the reaction mixture (final volume of 1 ml), and optical density was measured using a Beckman DU-65 spectrophotometer. To determine the optimal pH value for each IDH isoform, the pH of the reaction mixture was gradually changed in 0.2-unit increments starting from 6.2 and ending with 8.8. The rate of NADH production was linear for at least 1 min. One unit of IDH activity is defined as the amount catalyzing the conversion of 1 μ mol of substrate/min at 25 °C. Kinetic constants (K_m and V_{max}) for the recombinant IDH $\alpha\beta_1\gamma$ and IDH $\alpha\beta_2\gamma$ were obtained by nonlinear regression analysis and fitted to $v = V_{max}S^n/(K_m + S^n)$. Enzyme reactions were performed at pH 7.8 and 25 °C. Protein concentration was measured by the Bradford method (26) with bovine serum albumin as a standard. To determine the optimal pH value for IDH activity in different tissues, mitochondrial fractions from mouse brain, heart, and kidney were prepared by the method of Loverde and Lehrer (27).

Construction of an Alternative Splicing Reporter Construct—To study the effect of pH on the level of splicing responsible for H-IDH β_2 , the DNA fragment containing part of the human IDH β gene (nucleotide positions 539–1888 in Fig. 6A) was cloned by PCR using the following set of oligomers. A modified sense primer (5'-TAGCCGTC-GACTCTCTCTCCAACAGGG-3') and an antisense primer (5'-TGGGTGGGCCAGGCAGCAATGAC-3') were used to contain the respective *Sal*I and *Apa*I restriction sequences, as marked by boldface letters. This PCR fragment was double-digested with *Sal*I and *Apa*I and then ligated to the *Sal*I- and *Apa*I-cleaved plasmid pEGFP-N1 (CLONTECH) containing the green fluorescent protein (GFP) gene as a marker. The resulting plasmid, designated as pIDH β GFP, was subsequently used to start the translation of GFP from AUG codon (nucleotide positions 607–609 in Fig. 6A) present in the exon of the H-IDH β gene.

Stable Transfection and Cell Cultures—To prepare cells expressing the IDH β -GFP construct, 10 μ g of pIDH β GFP DNA was transfected into HT1080 fibrosarcoma cells by the calcium phosphate co-precipitation method. Transfected cells were grown for 48 h in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO₂. To obtain cells that were stably transfected, HT1080 cells were selected for 2 weeks in culture media containing G418 (500 μ g/ml) with media changed every 2 days. HT1080 cells that were both resistant to G418 and fluorescent (from GFP) under a fluorescence microscope were collected and used for subsequent experiments. The cells expressing GFP were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 15 mM sodium bicarbonate (approximately pH 7.4). At 70% confluence, HT1080 cells were rinsed once with Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, pH 7.4, and further allowed to grow for 16 h in fresh media at the following pH: 6.2, 6.6, 7.0, 7.4, and 7.8 (0.9, 2.3, 5.9, 15.0, and 37.0 mM sodium bicarbonate). The condition for pH adjustment was as previously re-

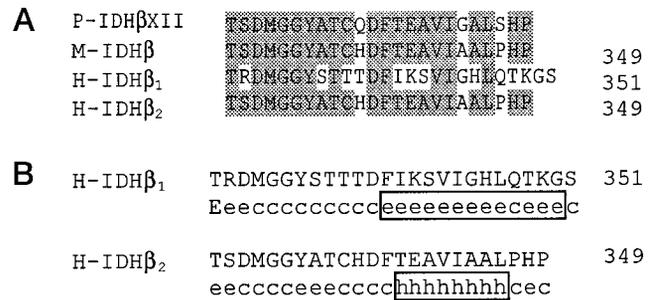


FIG. 3. Comparison of the amino acid sequences and predicted secondary structures of the C-terminal amino acid sequences of IDH β isoforms. A, the partial tryptic amino acid sequence for pig heart IDH β subunit (P-IDH β XII) (16), monkey IDH β (M-IDH β) (29), H-IDH β_1 , and H-IDH β_2 are compared. B, the secondary structures of the C-terminal regions of H-IDH β_1 and H-IDH β_2 are predicted by the GOR method (31). *h*, helix structure; *e*, extended or β -sheet; *c*, coil structure. Amino acid positions are designated on the right, and the identical amino acid residues are shaded. Helix-rich region in H-IDH β_2 (*h*), and extended (*e*) or β -sheet-rich region in H-IDH β_1 are marked by the respective boxes.

ported (28).

Microscopic Observation and Measurement of GFP Fluorescence—GFP fluorescence in HT1080 cells was observed by an inverted microscope (Olympus BX50) with a GFP filter. To measure the relative intensity of GFP fluorescence, transfected HT1080 cells grown under different pH values were homogenized with a Dounce homogenizer with a Teflon pestle in ice-cold phosphate-buffered saline with 0.1% Triton X-100. Clear cell lysates were obtained by centrifugation at 15,000 \times g for 20 min at 4 °C, and protein concentration was determined by the Bradford method (26) with bovine serum albumin as standard. The intensity of GFP fluorescence was determined for each cell lysate (0.3 mg of protein in 3 ml) at least three times per sample by a fluorescence spectrometer (Kontron SFM25) with excitation and emission at 488 and 510 nm, respectively.

RESULTS

Isolation and Partial Characterization of cDNA Clones for Bovine IDH β and IDH γ —Mixed oligonucleotide probes as described under "Experimental Procedures" were used to screen a bovine heart *lgt11* cDNA library in order to isolate cDNA clones encoding IDH β and IDH γ subunits. Six and five positive cDNA clones for IDH β and IDH γ , respectively, were isolated from about 2 million plaques screened. The size of DNA inserts ranged from 0.7 to 1.6 kb. Independent cDNA inserts for IDH β and IDH γ were purified and subcloned into plasmid pGEM7(+), and their nucleotide sequences were determined as described under "Experimental Procedures." Among the cDNA clones isolated for IDH β , one clone (1.4 kb) showed a deduced amino acid sequence of SSRTQGEDVRVE, which is very similar to the partial tryptic peptide sequence (ASRSQAEDVRVE) of pig heart IDH β (16). Another cDNA clone (1.6 kb) showed a deduced amino acid sequence (FSQQTIPPSAKYGGRRHTVT-MIPGDGIGP), which is highly homologous to the partial tryptic peptide sequence (FSQQTIPPSAKYGGILTVTMSPGDG-DGP) of pig heart IDH γ (16).

Isolation and Characterization of cDNA Clones for Human IDH β and IDH γ —The two partially characterized bovine cDNA clones for bovine IDH β (1.4 kb) and IDH γ (1.6 kb) were then used as DNA probes to isolate their human counterpart cDNAs. Eighteen positive clones (insert size ranging from 0.6 to 1.6 kb) for IDH β and 20 positive clones (from 0.4 to 1.5 kb) for IDH γ were isolated from about 1 million phage plaques from a human heart *lgt11* cDNA library. The positive phage plaques were further purified. The entire nucleotide sequences of the two largest cDNA inserts for IDH β (1.6 kb) and IDH γ (1.5 kb) were subsequently determined from both ends.

As shown in Fig. 2, one cDNA clone for human IDH β (H-IDH β) was 1,597 bp long with an open reading frame (1,155 bp)

clones coding IDH γ subunit (Fig. 4). Our cDNA clone (1.5 kb) for IDH γ (H-IDH γ) contained 181 bases of 5'-untranslated region followed by 1,179 bases of an open reading frame and 108 bases of 3'-untranslated region prior to a poly (A) tail. The structural analysis of H-IDH γ cDNA revealed that it possesses the entire IDH γ protein coding region, comprising the leader sequence for the precursor protein and the mature protein coding sequences. The canonical polyadenylation signal AATAAA in the 3'-untranslated region was observed 15 base pairs upstream of the poly(A) tail. The N terminus of the deduced human IDH γ protein sequence (Phe¹-Val³⁴) is identical with the N terminus 34-amino acid sequence of the pig IDH γ (16). Therefore, the precursor protein for human IDH γ contains 393 amino acids (42,794 Da) with the 39 amino acids as a mitochondrial signal peptide. Thus, the mature protein consists of 354 amino acids (38,814 Da). The deduced H-IDH γ protein sequence shares less amino acid homology (53%) with that of H-IDH β_1 (44%).

Tissue-specific Expression of IDH Transcripts in Human and Mouse Tissues—The expression patterns of the IDH β and IDH γ transcripts in human and mouse tissues were analyzed by mRNA Northern blot analyses. As shown in Fig. 5A (top), more than two species of IDH β mRNA transcripts exist in various human tissues. The smallest mRNA transcript (1.3 kb) is predominantly expressed in heart and skeletal muscle, whereas a larger transcript (1.6 kb), at a lower level, is present in all nonmuscle tissues examined. A larger transcript (2.4 kb), detected in human heart, skeletal muscle, and pancreas may be the unprocessed IDH β mRNA. To further characterize the two major IDH β mRNA transcripts (1.3 and 1.6 kb), we performed Northern blot analysis with a H-IDH β_1 -specific probe (175 bp of *XhoI*-*NcoI* fragment from the 317-bp intron-like sequence in H-IDH β_1 but absent in H-IDH β_2 , as in Fig. 2). The H-IDH β_1 -specific probe (H-IDH β XN) only recognized the larger mRNA transcript (1.6 kb) (Fig. 5A, middle). These data indicate that the smaller transcript (1.3 kb) present in heart and skeletal muscle represents the H-IDH β_2 transcript. A similar pattern of expression of the two IDH β transcripts seen in human tissues was also observed in mouse tissues. The smaller IDH β mRNA transcript (1.2 kb) is only detected in heart and skeletal muscle, whereas the larger IDH β mRNA transcript (1.5 kb) is expressed in all other tissues examined. (Fig. 5B, top). In contrast, only a single species of IDH γ mRNA transcript was detected in the various human (1.5 kb, Fig. 5A, bottom) and mouse tissues studied (1.3 kb, Fig. 5B, middle). The pattern and level of expression of the major IDH γ transcript are very similar in both human and mouse tissues.

Alternative Splicing of H-IDH β_1 and H-IDH β_2 mRNA Transcripts from a Single Human IDH β Gene—In order to determine whether the two species of H-IDH β mRNAs were transcribed from two distinct but structurally similar genes or from a single gene and then produced by an alternative splicing mechanism, the region of the IDH β gene coding for the amino terminus of IDH β was isolated. Several genomic clones (average size about 20 kb) for H-IDH β were isolated from a λ EMBL3 human lymphocyte genomic DNA library by plaque hybridization (data not shown). A single DNA fragment (1.9 kb) was amplified from these genomic clones by PCR analyses using a sense primer C (5'-CTGCAGTGTGAGGAAGTT-3') and an antisense primer B. The amplified genomic DNA fragment was subcloned into plasmid pGEM7(+), and its entire nucleotide sequence was determined. The nucleotide sequence of the amplified genomic DNA verified that the 317-bp intron-like sequence, present in H-IDH β_1 and absent in H-IDH β_2 , exists in the genomic sequence (Fig. 6A). Therefore, the two human IDH β mRNA transcripts corresponding to H-IDH β_1 and H-

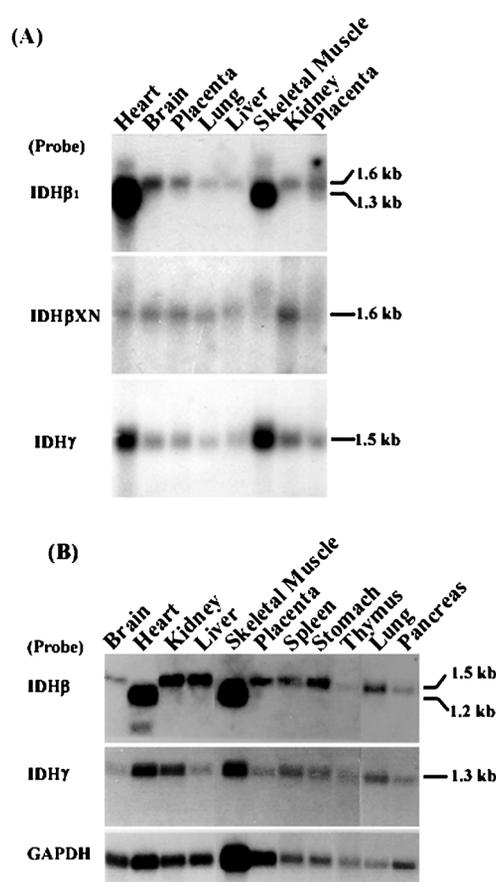


FIG. 5. Northern analyses of IDH β and γ subunits. A, tissue-specific expression of IDH β_1 , IDH β_2 , and IDH γ mRNA transcripts in various human tissues. Human multiple tissue Northern blot (MTNTM from CLONTECH) was hybridized with ³²P-labeled human IDH β_1 cDNA (upper panel), IDH β_1 -specific probe (IDH β XN; *XhoI*- and *NcoI*-digested 175-bp fragment from human IDH β_1 cDNA in Fig. 2) (middle panel), and human IDH γ cDNA (bottom panel). B, tissue-specific expression of IDH β isoforms and IDH γ transcript in various mouse tissues. Polyadenylated mRNA (4 μ g/lane) from various mouse tissues were separated on 1% agarose gel containing 0.66 M formaldehyde. The RNAs transferred to membrane were hybridized with ³²P-labeled human IDH β_1 cDNA (upper panel), IDH γ cDNA (middle panel), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (bottom panel) probe, respectively.

IDH β_2 are most likely produced by an alternative splicing from a single IDH β gene (Fig. 6B). Thus, the GT dinucleotide (nucleotide positions 819 and 820 in Fig. 6A) is used as the common 5'-splicing site in all tissues, while two alternative AG dinucleotides (nucleotide positions 1503 and 1504 for H-IDH β_1 or 1820 and 1821 for H-IDH β_2 in Fig. 6A) are utilized as the 3'-splicing sites, depending on the tissues (Fig. 6B). This type of alternative splicing pattern has been characterized previously in the fibronectin, G_{α_s} signal transducer, histocompatibility antigen H2K, and prolactin transcripts (18).

Production and Characterization of Recombinant IDH Subunit Proteins in *E. coli*—Among the subunit combinations produced in *E. coli* EB106 (DE3) (Fig. 1), IDH $\alpha\beta_2\gamma$, IDH $\alpha\beta_1\gamma$, IDH $\alpha\gamma$, IDH $\alpha\beta_1$, and IDH $\alpha\beta_2$ exhibited the IDH activity of 235.9 ± 32 , 197.8 ± 2.5 , 25.5 ± 0.7 , 22.7 ± 0.7 , and 4.6 ± 0.2 milliunit/mg soluble protein, respectively. None of the subunits produced alone or pHIDH $\beta_1\gamma$ showed any detectable IDH activity. These data strongly suggest that IDH α serves as an essential subunit for the catalytic activity of IDH enzyme and that at least one of the other two subunits plays a necessary supporting role for activity. Furthermore, the catalytically active recombinant H-IDH $\alpha\beta_1\gamma$ protein produced in *E. coli*

TABLE I
Oligonucleotide PCR primers used to modify the restriction enzyme sites for the expression of IDH subunits in *E. coli*

For production of the mature IDH proteins, NdeI sites containing translation initiation codon ATG were introduced to the sense primers.

Subunit	Primers	Sequence (5' → 3')	Nucleotide positions
IDH α	AM1 (sense)	AGACATATGACTGGTGGTGTTCAGACA <i>Nde</i> I	82–99 ^a
	AM2 (antisense)	GTGGGATCCATTAGAGTGACTGATGTAATGC <i>Bam</i> HI	1138–1116 ^a
IDH β	BM1 (sense)	GCGCATATGGCATCGCGGACGACGGCCGAG <i>Nde</i> I	103–123 ^b
	BM2 (antisense)	TATGGATCCGGCCTATGGGTGGGGCAA <i>Bam</i> HI	1491–1474 ^b
IDH γ	GM1 (sense)	CGAGGCATATGTTTTTCAGAACAAACAATT <i>Nde</i> I	117–135 ^c
	GM2 (antisense)	AGGGGATCCTAGGGCCAGCCCTAGGCCTCCA <i>Bam</i> HI	1191–1172 ^c

^a Nucleotide positions in human IDH α cDNA sequence (17).

^b Nucleotide positions in human IDH β cDNA sequence as shown in Fig. 2.

^c Nucleotide positions in human IDH γ cDNA sequence as shown in Fig. 4.

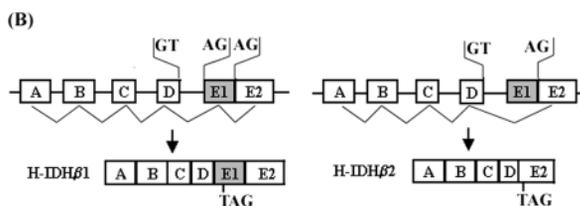
FIG. 6. Partial nucleotide sequence and organization of a genomic clone for human IDH β gene. A, a genomic DNA clone for human IDH β gene was isolated, and its partial sequence (1.9 kb) was determined. Nucleotide sequences corresponding to the exons and introns of H-IDH β_1 gene are indicated by boldface uppercase letters and lowercase letters, respectively. The tentative nucleotide positions and exon names in the partial human IDH β gene are marked on the left and right sides, respectively. The two underlined nucleotide sequences indicate the PCR primers used to amplify the 1,359-bp genomic DNA fragment linked with GFP gene for the construction of pIDH β GFP DNA plasmid. The shaded region represents the 317-bp intron-like cassette sequence that is alternatively spliced out in human IDH β_2 mRNA transcript. The 5'-splicing site common to H-IDH β_1 and H-IDH β_2 is indicated by *gt* with an underline, and the two alternatively used 3'-splicing sites for H-IDH β_1 and H-IDH β_2 are represented as *ag* and *AG*, respectively, with double underlines. The triple nucleotide ATG used as a translation initiation codon for GFP expression in the pIDH β GFP DNA construct is marked by the shadow. The two TAG translation termination codons for H-IDH β_1 and H-IDH β_2 are indicated with rectangular boxes. B, organizations of H-IDH β_1 and H-IDH β_2 mRNAs transcribed from the alternative splicing from the human IDH β gene. In H-IDH β_1 , both exon E1 and exon E2 are used as a single exon, while exon E2 is used as the only exon in the H-IDH β_2 mRNA transcript.

(A)

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1 CTGCAGTCTGTGAGGAGTTGCTGAACTGTACCCCAAAATCAAATTTGAGACAATGATC exon A
61 ATAGACAACCTGCTGATGTCAGgtgaggcctccccacatgtgtactcatggggcgaggag
121 gagggtgggagtggaatttacttcatgcctgcatctcaccctctagCTGTGTCAGAAATC
181 CTTACCAGTTTGTATGTCTGTGTATGCCCAATCTCTATGGGAACATTATTGACAATCTGG exon B
241 CTGCTGGCCTGGTTGGGGAGCTGGTGTGGTCCCTGGTGAGAGCTATAGTCAAGTATAGC
301 CAGTCTTTGAGACGgtgaggctgctttctcctcgccctctcctctgacctcaagtcccgcc
361 attcctctcccatcccaatatactccacttctcaagtgatctggcctgttcttcccccgac
421 gctgccaccatcgctcttctcagtgaccctggcctgagctccccctcccataatgtcct
481 ttccaacctaaacctctgcataccctcctgctcctcccagtgctagctgcccctc
541 tctccacagGGTGCCTGGCACCCATTGCCCCAGGACAGGAATATAGCCAATCCC exon C
601 ACGGCCATGCTGCTGTGCGGCTTCCAACATGCTGCGGCATCTTAAgtaggtcatgggagggc
661 tgtcggcagaggttgggtgtcgtgaggaggagcaggtggggaattagcgggaggtgtct
721 tgggggagtcacagtttctcctgtccatataccacagTCTTGAGTATCACTCCAGCATGA exon D
781 TCCGAGATGCGGTGAAGAAGGTGATCAAAGTTGGCAAGgtgagttgagaagagtagtgct
841 ggcttcaggatttagagtagttctggtgtcagcaccctcgccactaccctctgggaacctg
901 aatgggggtctccttccccccaggtccccagaacatctgctccctgacatccctgcatc
961 cctctcaagggtgttcttcatctccttctcttggctgttccatcctcttgatctctttt
1021 gcctcttagatcccatctgctgttccctcttctcctgctccatccactgccccttttcag
1081 gccatctgaatgcacaacttgacagctctccttgggggtggaacagcaaggatatacagga
1141 ggctgaagcatcagcttcccttccagcccatctccttctcgtgtgattcttccctc
1201 cacctgtctctgtctctatttcttccaaggaaggtagccacatcctgctctgactgtgca
1261 tttcccatcatttggcctgctcagcctcctgctcctgcccctgagcagcagcagtaata
1321 gtggcttaagaggagtgccaggtccatgtgcttccatcctggctcctccttccattcct
1381 gtgcattgccttttctggtgtcagcatttctggcttccatttccatcaatcacattg
1441 ccctacctccttctcagggctctcttcttctcctccccacggctgtgtccgggtggct
1501 gtagGTGCGGACTCGAGACATGGGCGGTACAGCACCACAACCGACTTTCATCAAGTCTGT
1561 CATCGGTCACCTCGACATAAAGGGAGCTAGAGCCCTTTATTTCTTCCAACCTTGCAAGG
1621 ACCACACTCCCCATACCTTCAGTGCAGTGTACCCAGGGAAGAGACCTTTGTGCCTTAAGG exon E1
1681 AGTGGCAACTGGTCAACCCTTGTGGGTAGAGCCTAGGTTGCTTTGGCCGGCTTCCCT
1741 AGGGGACAGACTGTTGGGTGGTATGGGGATTGTTAGGATGGAGCCAGGCCACATGGAT
1801 GATGATGATTCTCCCCACAGGTTGCAACCTCTGACATGGGTGGCTATGCTACTTGGCAT
1861 GACTTCACTGAGGCTGTCAATTGCTGCCTTGGCCCCACCAATAGGCCCTGTCCATACCATG exon E2
1921 TAAGGT

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emerged as a single peak from a Sephacryl S-300HR gel filtration column with an apparent molecular mass of 316 kDa (data not shown). This indicates that the recombinant IDH protein most likely exists as an octamer, 2(2 α , β , γ) with a calculated molecular mass of the octamer of 301 kDa.

Role of IDH β Isoforms in IDH Activity—In order to determine any difference in the two IDH β isoforms' ability to support IDH activity, we constructed the appropriate expression constructs (IDH $\alpha\beta_1\gamma$ and IDH $\alpha\beta_2\gamma$) (Fig. 1) and produced the respective proteins in *E. coli* EB106 (DE3). Substitution of IDH β_1 with IDH β_2 in the co-expression system lowered the optimal pH from 8.0 to 7.6 for IDH activity without altering

their maximum activities (Fig. 7A). This difference in optimum pH was analogous to what was observed in mouse kidney and brain (β_1 prevalent) versus heart (β_2 prevalent) mitochondria (Fig. 7B). These results indicate that IDH β isoforms derived from the tissue-specific alternative splicing can modulate the optimal pH values for IDH activity. The K_m values for IDH $\alpha\beta_2\gamma$ and IDH $\alpha\beta_1\gamma$ proteins in *E. coli* lysate were found to be 3.11 ± 0.09 and 1.28 ± 0.02 mM, respectively. V_{max} values of IDH $\alpha\beta_2\gamma$ and IDH $\alpha\beta_1\gamma$ proteins were calculated to 263.3 ± 5.5 and 96.4 ± 2.9 milliunits/mg protein, respectively. Thus IDH $\alpha\beta_2\gamma$ protein exhibited 2.4- and 2.7-fold higher K_m and V_{max} values than IDH $\alpha\beta_1\gamma$ protein. The significant changes in the K_m and

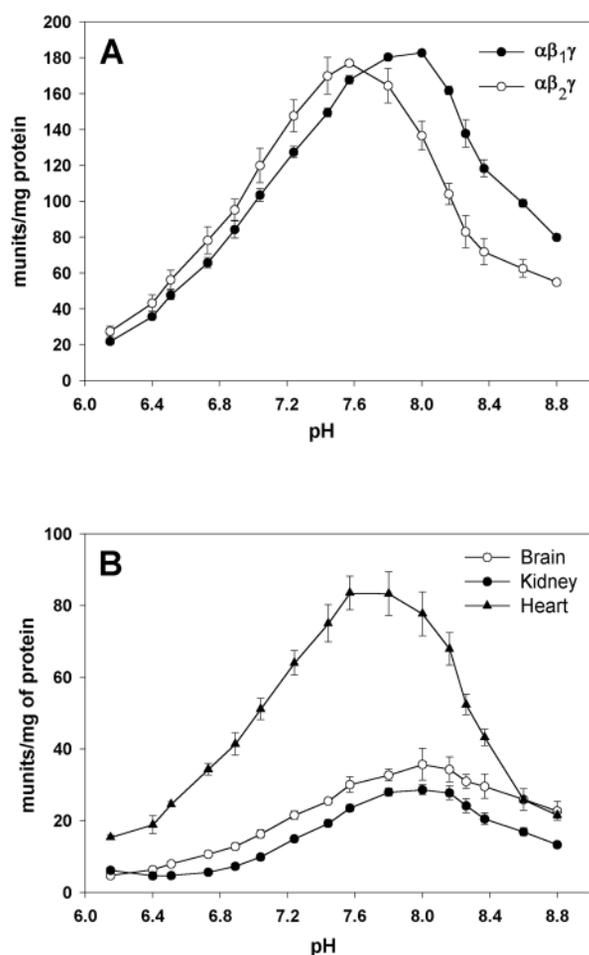


FIG. 7. Optimal pH values for IDH activity for the recombinant H-IDH proteins and in mouse tissues. A, optimal pH profiles for IDH activity of the recombinant H-IDH proteins produced in *E. coli* using the plasmid pIDH $\alpha\beta_1\gamma$ and pIDH $\alpha\beta_2\gamma$. B, optimal pH profiles for the mitochondrial IDH activity in mouse tissues. IDH activity was measured at 25 °C as NADH production at 340 nm in the reaction mixture containing 50 mM MOPS buffer containing 5 mM three-D_s-ISOCITRATE, 35.5 mM triethanolamine, 2 mM NAD⁺, 1 mM ADP, 2 mM MgCl₂ and 1 μ g/ml rotenone, as described by Rutter and Denton (25).

V_{\max} values caused by the substitution of IDH β isoforms further support the role of IDH β in the regulation of IDH activity in a tissue-specific manner.

pH-Dependent Expression of H-IDH β_2 in Cultured Cells—To determine whether changes in pH levels can cause a shift in the alternative splicing patterns of the IDH β mRNA transcript, the plasmid pIDH β GFP, in which GFP was allowed to be expressed only under the same manner of alternative splicing for IDH β_2 mRNA was constructed (Fig. 8) and then stably transfected into HT 1080 cells. At about 70% confluence, HT1080 cells were subjected to growth for another 16 h under fresh culture media at different pH values (pH 6.2, 6.6, 7.0, 7.4, and 7.8). As shown in Fig. 9, A and B, the levels of GFP expression from pIDH β GFP in HT1080 cells decreased as the pH of the culture media increased. Relative intensity assessed by GFP fluorescence at pH 6.2 was 1.7-fold higher than that at pH 7.8 (Fig. 9B). These results indicate that pH levels can regulate the levels of IDH β isoforms by favoring one splicing pattern over another. A similar pH-dependent splicing was observed in human ATP synthase γ subunit (28).

DISCUSSION

To investigate the functional role of each of the three IDH α , IDH β , and IDH γ subunits for IDH activity, we isolated and

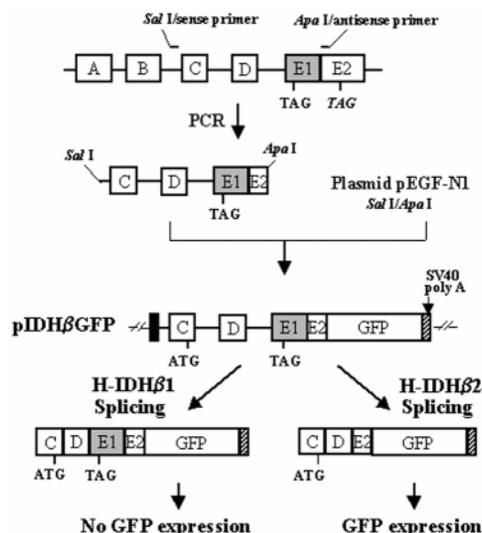


FIG. 8. Schematic explanation of GFP expression by the alternative splicing. Using the partial human genomic DNA for H-IDH β (Fig. 6) as a template, DNA fragment containing exon C, D, E1 and E2 were amplified by the PCR primers. The amplified DNA was ligated to the *SalI/ApaI* cleaved plasmid pEGFPN-1 to generate plasmid pIDH β GFP. For the translation of GFP gene, ATG in the exon C was used as a translation initiation codon. In the HT1080 fibrosarcoma cells permanently harboring the pIDH β GFP gene, the expression of GFP should be only allowed when E1 was excluded as an intron by the same manner found in the alternative splicing of H-IDH β_2 transcript.

characterized nearly full-length cDNA clones for human IDH β and IDH γ subunits in this study. Using the co-expression system that we developed (Fig. 1), we have successfully produced IDH proteins of various combinations together with the IDH α subunit (17). Based on the relative activities of IDH proteins of various subunit combinations, our data established that all three IDH subunits are required for the maximal IDH activity and that IDH α is the catalytic subunit, while IDH β or γ subunit has an essential supporting role in constituting IDH activity. This conclusion is also in agreement with our previous data (17) of structural similarity between H-IDH α and yeast IDH2 subunit, the catalytic subunit in yeast IDH (11). Furthermore, the catalytically active recombinant H-IDH $\alpha\beta_1\gamma$ protein (316 kDa) most likely exists as an octamer of 2($2\alpha, \beta, \gamma$) (301 kDa) (32, 33), although we cannot rule out other possible octameric structures, such as 2($\alpha, 2\beta, \gamma$) or 2($\alpha, \beta, 2\gamma$). However, our data clearly dispute the possibility of a heteropentamer of 2 $\alpha, 2\beta, \gamma$ (25) or the mixture of other oligomeric structures (34).

Based on the significant difference in its C-terminal sequence from monkey IDH β (29) and pig IDH (16), careful analyses of the nucleotide and deduced protein sequences of H-IDH β_1 cDNA revealed the presence of an intron-like cassette located at near the 3'-end of the cDNA sequence (Fig. 2). Surprisingly, the C-terminal 26 amino acids deduced from the alternatively spliced IDH β_2 variant (Fig. 3) were highly homologous to those of monkey IDH β (29) and the partial tryptic peptide of pig IDH (16). The actual presence of the two IDH β cDNA isoforms was verified using DNA amplification by PCR and subsequent sequencing of the amplified DNA fragments near the 317 bp in question (Figs. 2 and 3). This was further supported by Northern analyses for human and mouse tissues (Fig. 5).

It is well established that multiple mRNA transcripts and their corresponding protein isoforms can be produced by a variety of different mechanisms (18). For example, a different set of mRNA transcripts can be generated by using alternative promoters (35) or from spatially separated exons in the genome (30, 35) by either mutually exclusive splicing as in the cases of

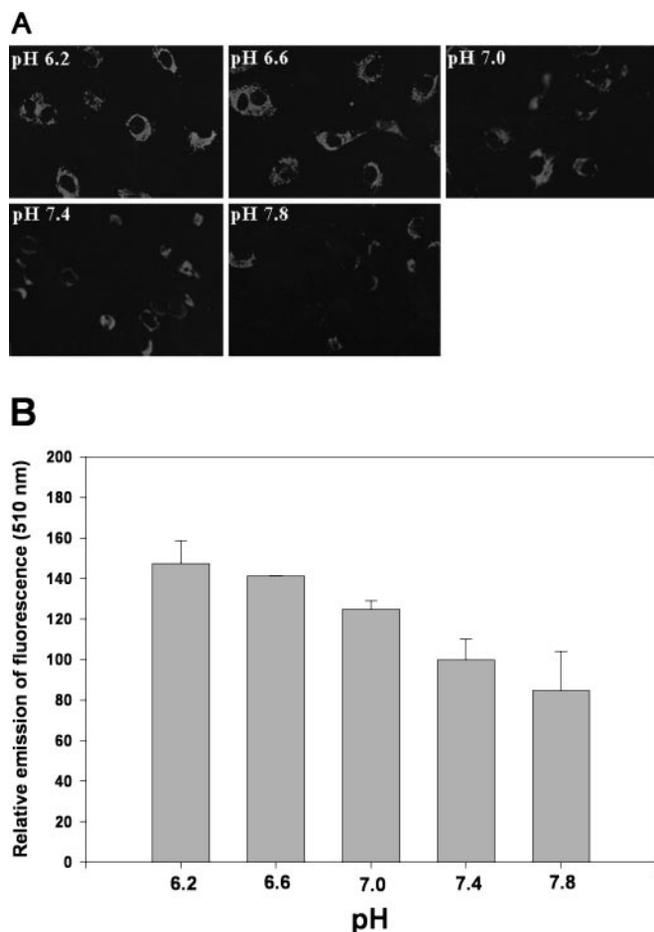


FIG. 9. Effect of pH on the expression of IDH β isoform by alternative splicing. Human HT1080 fibrosarcoma cells with permanently transfected pIDH β GFP gene were grown in a culture media at pH 7.4 pre-adjusted by sodium bicarbonate (15 mM). Upon 70% confluence, cells were grown in new media at various pH values pre-adjusted by different concentrations of sodium bicarbonate, as described by Endo *et al.* (28). (A) The photomicroscopy of GFP fluorescence in the cells (x 200) were taken by an Inverted microscope equipped with a GFP filter. (B) The relative intensities of GFP fluorescence from the cells cultured at various pH values were compared with GFP intensity from the cells grown at pH 7.4. The intensity of GFP fluorescence for each cell lysate (0.1 mg protein/ml) was determined using a fluorescence spectrometer with excitation and emission at 488 and 510 nm, respectively.

β -tropomyosin (36) and MEF2D (37) or single exon-excluding splicing for ATP synthase $F_1\gamma$ subunit (28), myocyte-specific enhancer factor 2A (MEF2A) (38), and neural cell adhesion molecule (N-CAM) genes (39). In the case of the two IDH β mRNA transcripts, it is most likely that the two isoforms are produced from a single gene via an alternative splicing by using a common 5'-splicing site (GT dinucleotide at nucleotides 819 and 820 in Fig. 6A) and different 3'-splicing sites (AG dinucleotides) in a tissue-specific manner: AG at nucleotides 1503 and 1504 for H-IDH β_1 and at 1820 and 1821 for H-IDH β_2 . Therefore, the H-IDH β_2 transcript is likely to be produced by the exclusion of a part of one exon (exon E1 in Fig. 6B) like an intron cassette. A similar pattern of the alternative splicing has been reported in several genes and has been previously categorized as an alternative splicing mechanism mediated by the internal intron acceptor site (18).

Recently, the tissue-specific alternative splicing of ATP synthase $F_1\gamma$ mRNA isoforms in the heart and liver has been reported to be dependent on the extracellular pH and other physiological factors such as a transactivating factor produced via the protein kinase C-mediated pathway in HT1080 fibrosarcoma cells (28). In acidic pH, the heart-specific ATP syn-

these $F_1\gamma$ transcript is mainly produced through the exclusion of exon 9, whereas the liver-specific transcript containing the exon 9 is predominantly expressed in alkaline pH (28). In addition, the possible roles of MyoD in this pH-dependent alternative splicing in cultured mouse myoblasts have been suggested (40), although the significance of the alternatively spliced $F_1\gamma$ transcript isoforms relative to the regulation of ATPase activity was not clearly demonstrated (41). Our current data indicate that the alternative splicing of H-IDH β transcripts, as measured by the level of GFP expression in HT1080 cells, is also inversely affected by increasing the extracellular pH. The GFP expression at pH 6.2 was about 1.7-fold higher than that expressed at pH 7.8 (Fig. 9, A and B). In contrast to the ambiguous biological implication for the alternatively spliced heart- and liver-specific ATPase $F_1\gamma$ transcript isoforms, the substitution of H-IDH β_1 (liver-, brain-, and kidney-specific) with H-IDH β_2 (heart- and muscle-specific) in the recombinant IDH $\alpha\beta\gamma$ protein lowered the optimal pH value for IDH activity from pH 8.0 to 7.6 (Fig. 7A). This result is consistent with the values observed in mouse tissues: pH 7.6 for the heart and pH 8.0 for the brain and kidney (Fig. 7B). The substitution of H-IDH β_1 with H-IDH β_2 in the recombinant IDH $\alpha\beta\gamma$ protein also increased the K_m and V_{max} values by 2.4- and 2.7-fold, respectively. These data together with the tissue-specific expression of IDH β transcripts (Fig. 5) indicate a potential regulatory role for the IDH β subunit in constituting IDH activity. Because of the differences in amino acid sequences and secondary structures of the C termini (Fig. 3) between H-IDH β_1 and H-IDH β_2 , it can be concluded that the C-terminal region of IDH β , expressed in a tissue-specific manner, probably plays an important role in constituting the total IDH activity.

In conclusion, the data presented provided evidence for at least two different IDH β isoforms existing in a tissue-specific manner via an alternative splicing mechanism using a part of an exon as an intron-like cassette. Functional analyses of the recombinant IDH proteins suggested that the IDH β or - γ subunit performs a supporting role for constituting IDH activity with the catalytic subunit IDH α . Furthermore, the replacement of one β isoform with the other in the recombinant IDH $\alpha\beta\gamma$ protein suggested that IDH β isoforms appear to have a regulatory role in determining optimal pH values, as previously observed for the mitochondrial IDH in various mouse tissues. The pH-dependent regulation of the alternative splicing of IDH β isoforms also indicated a complex regulatory mechanism for the production of IDH β isoforms. To our knowledge, this is the first report of a pH-regulated alternative splicing pattern with the resultant isoforms causing a different pH optimum of the enzyme.

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