Characterization of cDNAs for Human Estradiol 17β-Dehydrogenase and Assignment of the Gene to Chromosome 17: Evidence of two mRNA Species with Distinct 5′-Termini in Human Placenta

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Human placental estradiol 17β-dehydrogenase (E_2DH) cDNA clones were isolated from a λgt11 expression library by screening with 33 mer synthetic oligonucleotides derived from the amino acid sequence of the catalytic site of E_2DH and with polyclonal antibodies raised against the enzyme purified from human placenta. Using ^{32}P-labeled fragments from the coding and 5′-untranslated regions, two mRNA species have been identified in poly(A)$^+$ RNA from human placenta, a major species migrating at 1.3 kilobases (kb) while a minor one is found at 2.2 kb. Primer extension analysis identifies the major mRNA as starting 9–10 nucleotides upstream from the in-frame ATG initiating codon while the longer mRNA has at least 814 noncoding nucleotides at its 5′-terminus. Sequence analysis of the longest cDNA clone (2092 base pairs) shows that this clone possesses identical coding and noncoding sequences in the regions of overlap with the shorter cDNA clones. The ^{32}P-labeled 5′-noncoding fragment hybridized only to the 2.2 kb band, thus providing additional evidence for the existence of two distinct mRNA species which differ only in their 5′-noncoding regions. Using hpE_2DH36 cDNA as a probe for *in situ* hybridization, the human E_2DH gene was localized to the q11-q12 region of chromosome 17. The cloned cDNAs encode E_2DH, a 327-amino acid protein having a calculated molecular weight of 34,853. Since E_2DH is the enzyme required for the formation of 17β-estradiol, the availability of the cDNA encoding the enzyme should permit a detailed investigation of the factors regulating the expression and activity of this crucial enzyme in both normal and malignant tissues, especially breast cancer.
(Molecular Endocrinology 3: 1301–1309, 1989)

0888-8809/89/1301-1309$02.00/0
Molecular Endocrinology

INTRODUCTION

Estrogens play a predominant role in the development, growth, and function of all tissues involved in reproduction and fertility in women. In addition, the action of estrogens in promoting the growth of breast cancer (1–3) and a series of other malignant and nonmalignant hormone-sensitive diseases (4, 5) is well recognized. The most important estrogen, 17β-estradiol, is synthesized from its weak precursor estrone through the action of estradiol 17β-dehydrogenase (E_2DH) (EC 1.1.1.62). Despite its recognized key role and its purification to homogeneity two decades ago (6–9), the only information available about the structure of E_2DH was limited to its amino acid composition (10), a 5-amino acid sequence at the NH_2 terminus (10), a heptadecapeptide possibly involved in coenzyme binding (11) and a 20-amino acid sequence at the catalytic site (12). We report the molecular cloning and sequencing of cDNAs encoding human E_2DH. The availability of this cDNA offers the opportunity of studying in detail the factors controlling the expression of this crucial enzyme, not only in gonadal but also in several peripheral estrogen target tissues. It should also permit to elucidate the molecular alterations responsible for E_2DH deficiency in the human and could well lead to the development of inhibitors of estradiol biosynthesis.

RESULTS

Approximately 5×10^5 independent recombinants obtained from a human placental λgt11 cDNA library were screened by hybridization with two ^{32}P-labeled 33 mer oligonucleotides derived (13) from the amino acid sequence of the catalytic site of E_2DH (12). The first screening yielded five positive clones which were isolated and identified as hpE_2DH6,17,31,36, and 53, respectively. Polyclonal antibodies raised against E_2DH purified to homogeneity from human placenta by affinity chromatography (Fig. 1) showed cross-reactivity with fusion proteins produced by hpE_2DH6 and hpE_2DH31 (Fig. 2B). A good correlation was observed between the size of the fusion proteins and the length of the corresponding inserts (Fig. 2B). Clone hpE_2DH6 contains an insert of 654 base pairs (bp) in length while clone hpE_2DH31 has an insert of 988 bp.

A second screening of the human placenta λgt11 cDNA library was performed using hpE_2DH36 as the probe in order to obtain clones containing longer transcripts. Among 65 clones isolated, three clones which contained 2, 128, and 814 nucleotides 5′ to the ATG initiating codon were characterized in detail by a combination of restriction endonuclease mapping and DNA sequence analysis according to the strategy described in Fig. 3. Clones possessing identical sequences in their overlapping regions are also indicated in Fig. 3.

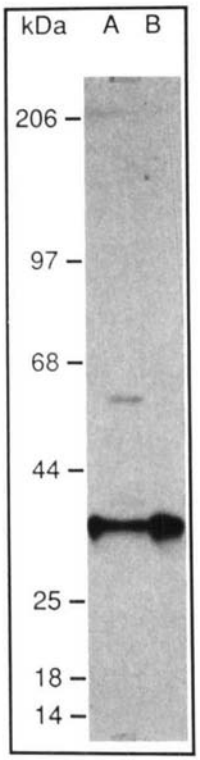

Fig. 1. Immunoblot Analysis Using Antiserum Raised in Rabbit against Purified Human Placental E_2DH

Human placental homogenate (lane A) and purified E_2DH (lane B) were separated by NaDodSO_4/PAGE, electroblotted onto nitrocellulose, treated with rabbit anti-E_2DH serum diluted 1/1000 and exposed to ^{125}I-labeled goat anti-rabbit IgG. The autoradiograph was obtained after exposing the filters to Kodak X-OMAT films for 14 h at −80 C with intensifying screens.

The first in-frame ATG codon (designated as position 1 in Fig. 4) is located 815 nucleotides from the 5′-end of the longest cDNA (hpE_2DH216). The predicted amino acid sequence of human E_2DH is illustrated in Fig. 4. Translation from the first in-frame AUG codon predicts a protein of 327 amino acids. Translation from the second in-frame AUG codon, on the other hand, would predict a protein of 209 amino acids. The second in-frame AUG codon lacks a purine at the critical −3 position of the eukaryotic consensus translation initiation sequence (14). This, in addition to the unique N-terminal amino acid sequence, indicates that the second in-frame AUG codon does not function as an initiation codon.

This protein contains the 17 and 20 amino acid sequences of the suggested coenzyme binding (11) and catalytic (12) sites, respectively. It also contains the sequence of the 23-amino acid N-terminus determined directly by Edman degradation of the purified enzyme (Fig. 4). A single amino acid difference between the N-terminal sequence (Ala-Glu-Thr-Val-Val) reported by Burns *et al.* (10) and the predicted N-terminal amino acid sequence (Ala-Arg-Thr-Val-Val) is observed. An Arg at position 2 from the initiation codon (ATG) is predicted from the cDNAs instead of Glu as previously reported by protein sequencing (10). These data show that human E_2DH is 327 amino acids long.

The ATG initiation codon is preceeded at −41 by a TATA box-like motif (ATATCAA) (15–17) and a GC-rich (93%) region at nucleotides −51 to −80 containing the GC box consensus sequence (GGGGCGGGGC). Analysis of the promoter region of viral and cellular genes has identified the consensus sequence $^{G}_{T}$GGGGCGG$^{GGC}_{AAT}$ or its inverted repeat termed the GC box. This consensus sequence has been shown to interact with transcription factor Sp1 and is commonly found in the promoter sequences of housekeeping genes (18, 19). The GC box present at nucleotides −61 to −52 could well act as an Sp1-binding site for transcription of the shorter mRNA transcript. There is a putative inverse CAAT box (GATTG) at −101 (20).

As evidenced by sequencing of the corresponding 5′-flanking region of the human E_2DH gene, the 5′-noncoding region of the cDNA sequence shown in Fig. 4 (nucleotides −814 to −1) is identical to the genomic nucleotide sequence (Luu-The, V., and F. Labrie, unpublished data). This, to our knowledge, could represent one of the rare examples where one promoter region of a gene is potentially transcribed into a long mRNA transcript of the same gene (21, 22). Usually, mRNAs having different 5′-noncoding regions originate from alternative tissue splicing (23).

The sequence CACCATGG containing the initiating codon ATG corresponds to the sequence found as being the optimal sequence for initiation by eukaryotic ribosomes (14). This strong initiation site allows for efficient translation of the predominant placental mRNA containing only nine to ten 5′-noncoding nucleotides. In the 5′-region, there is no potential in-frame ATG

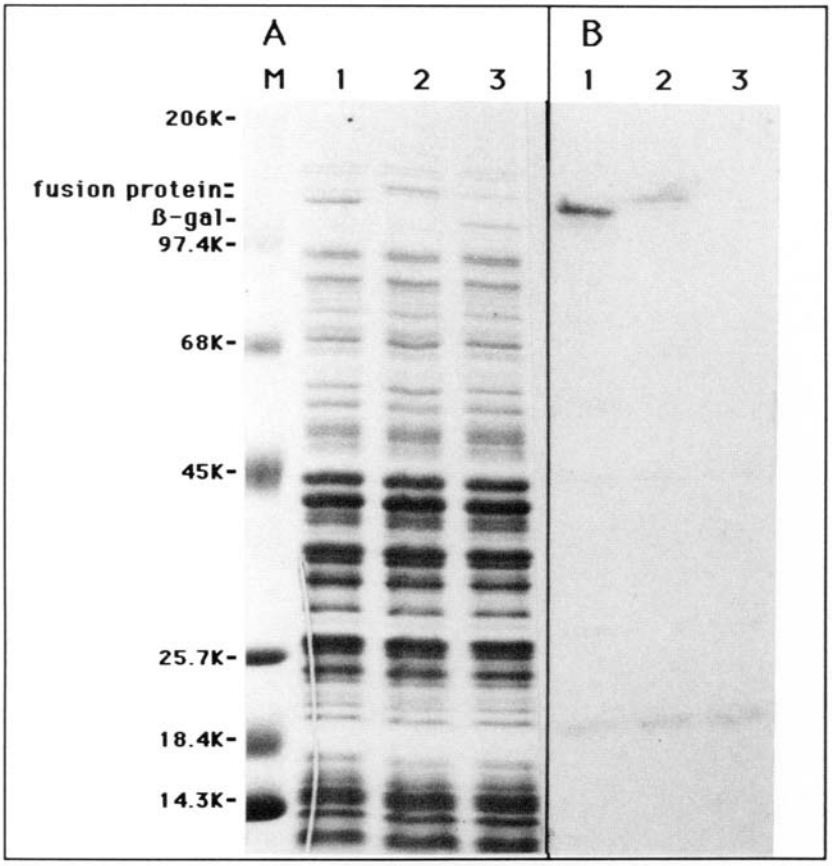

Fig. 2. Identification of hpE_2DH6 and hpE_2DH31 Clones by Immunoblot of β-Galactosidase Fusion Proteins

Lysogens and β-galactosidase fusion proteins were prepared as described in *Materials and Methods*. A, Patterns of Coomassie blue-stained lysate proteins of phage lysogens separated by $NaDodSO_4$/PAGE. B, Autoradiograms of the same preparations after electroblotting onto nitrocellulose, treatment with rabbit anti-E_2DH serum, and exposure to ^{125}I-labeled goat anti-rabbit IgG. Protein markers (lane M), clone hpE_2DH6 (lanes A1 and B1), clone hpE_2DH31 (lanes A2 and B2), nonrecombinant clone (lanes A3 and B3).

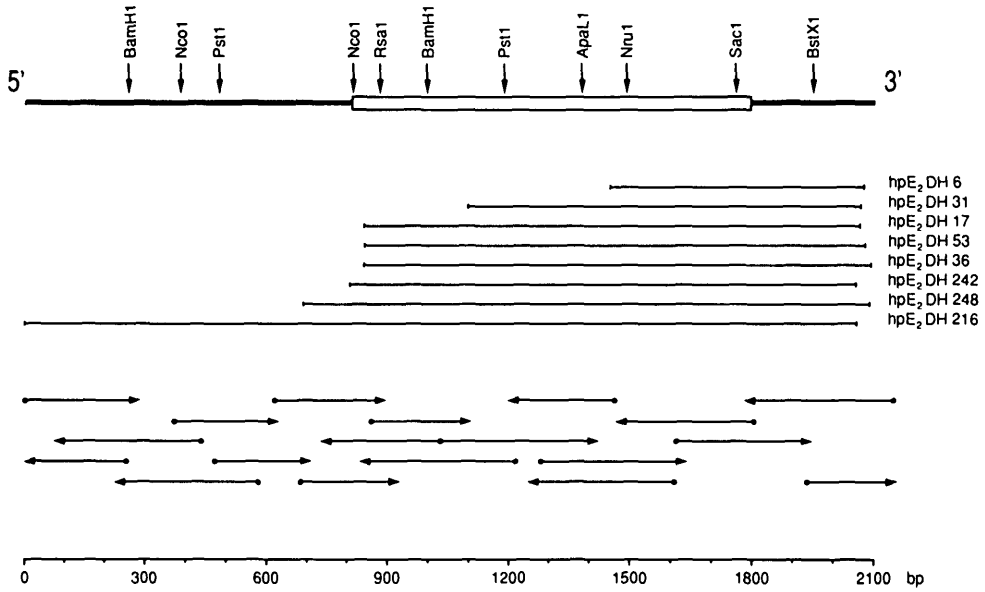

Fig. 3. Restriction Map of Human Placenta cDNA Clones Encoding E_2DH and Sequence Analysis Strategy

A diagrammatic representation of the longest full-length cDNA is shown at the *top*. The protein coding region is represented by the *open box* and the 5′- and 3′-noncoding regions by the *solid line*. The clones numbered from 1 to 100 (6, 17, 31, 36, 53) and above 200 (216, 242, 248) were obtained from the first and second screenings, respectively. The *arrows* beneath the schematic cDNA indicate the direction and extent of sequencing using synthetic oligonucleotide primers. A scale in base pairs is given below the restriction map.

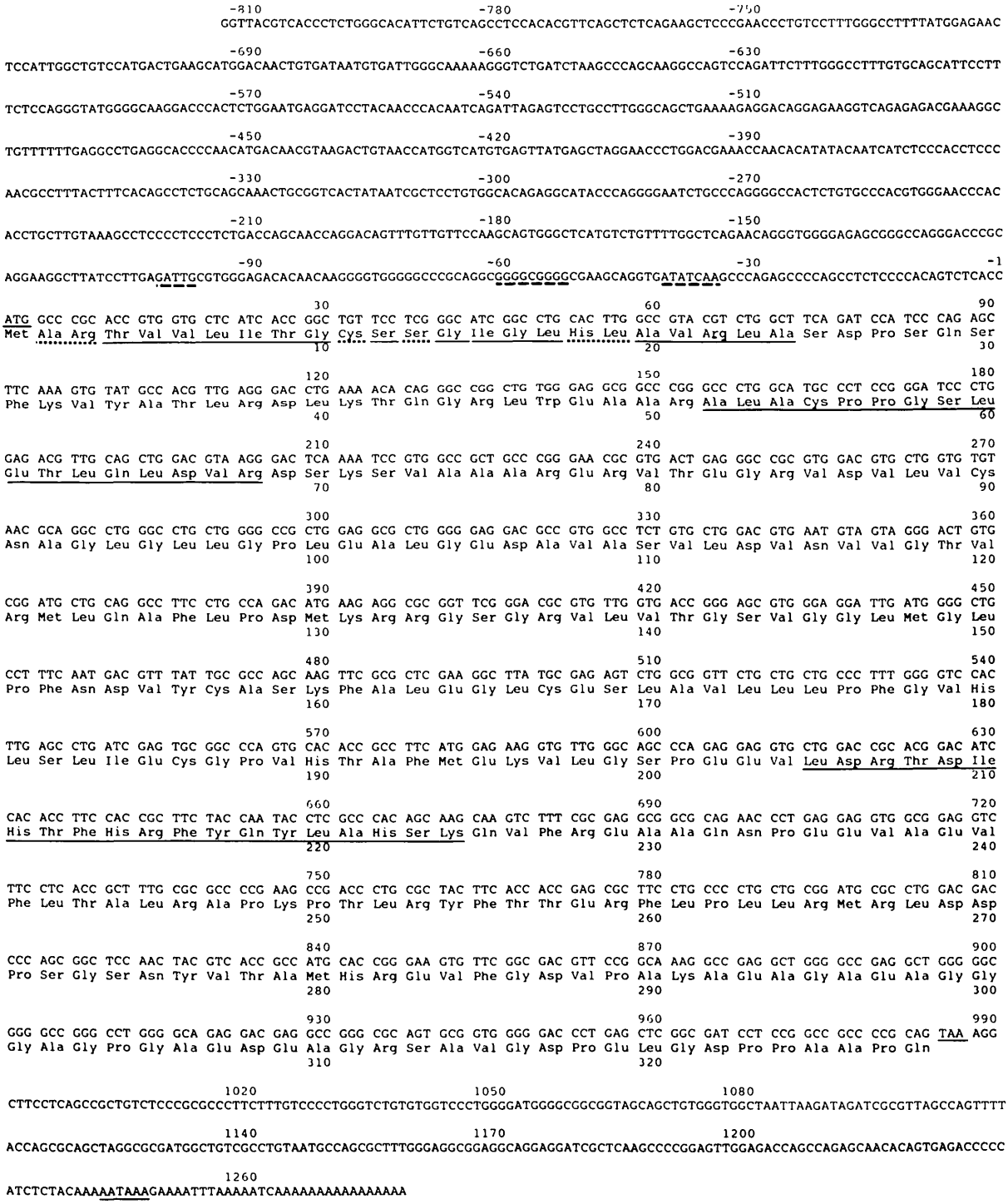

Fig. 4. Nucleotide Sequence of the cDNA Encoding Human E_2DH and the Predicted Amino Acid Sequence

The single open reading frame beginning at the ATG codon (nucleotide 815 of the cDNA) is shown below the nucleotide sequence which is numbered in the 5′- to 3′-direction. Nucleotides are numbered above the sequence while amino acids are numbered below the sequence. Nucleotides 5′ of the ATG codon are given negative numbers. The amino acid sequences of the coenzyme binding site (11), of the catalytic site (12), as well as the NH_2-terminus determined from the purified enzyme are underlined. The nucleotides corresponding to the translation stop codon TAA, the putative polyadenylation signal AATAAA, the TATA and inverse CAAT boxes as well as the GC box are also underlined.

initiating codon. There are, however, 10 ATG codons at positions −729, −706, −695, −679, −590, −567, −452, −430, −424, and −415 which could well modulate the efficiency of translation of the long mRNA (14). Only 5–10% of eukaryotic mRNAs possess ATG triplets upstream of the true ATG starting codon (14). The presence of upstream ATG codons could inhibit initiation at the proper downstream starting codon (14, 24).

The amino acid composition of the protein deduced from the nucleotide sequence (Table 1) is in good

Table 1. Amino Acid Composition of E_2DH

	Estimated Residues/mol Subunit	
	Determined by Amino Acid Analysis[a]	Deduced from cDNA
Lys	10	9
His	7	7
Arg	22	23
Cys	6	6
Asp	{21}	17
Asn		5
Thr	16	16
Ser	19	19
Glu	{28}	23
Gln		8
Pro	19	20
Gly	31	32
Ala	36	38
Val	32	32
Met	4	6
Ile	4	4
Leu	41	42
Tyr	6	6
Phe	13	13
Trp	1	1
Unknown	3	
Total residues	319	327

[a] Data from Ref. 10.

agreement with the amino acid composition previously reported (10). The calculated mol wt of E_2DH of 34,853 is also in agreement with the mol wt of 33,500 ± 1,000 estimated by $NaDodSO_4$/polyacrylamide gel electrophoresis (PAGE) (25). Ultracentrifugation studies reveal that the enzyme in solution is a dimer of identical subunits of about 68,000 daltons (10, 25). Clones hpE_2DH36 and hpE_2DH248 contain a 3′-untranslated region of 264 nucleotides. A consensus polyadenylation signal AATAAA (26) is located 15 nucleotides upstream of the poly(A) tail.

Using a probe (probe 2) exclusive to the 5′-end of the longest cDNA, it can be seen in Fig. 5A that a mRNA species is detected at 2.2 kb which specifically hybridizes to the unique 5′-probe (Fig. 5A). However, when the probe corresponding to the coding region is used (probe 1), mRNAs of both 1.3 and 2.2 kilobases (kb) are detected. The present data confirm the presence of two mRNA transcripts differing in size by approximately 1000 bp. While the short mRNA is predominant in placenta, the long form of the mRNA is abundant in other tissues including the breast cancer cell line ZR-75-1, prostate and adrenals (Labrie, C., unpublished data). Moreover, the present data indicate that the difference is due to variable 5′-ends of the transcripts. The mRNA population was further analyzed by primer extension using poly(A)$^+$ RNA prepared from human placenta. Using a 17 bp oligonucleotide primer corresponding to positions 92 to 75 of the cDNA, the predominant products obtained by extension with reverse transcriptase are approximately 103 bp long, thus indicating the presence of 9 to 10 nucleotides upstream of the starting ATG codon in this mRNA species.

In order to localize the chromosomal position of the human E_2DH gene in normal cells, we used the [^{3}H] hpE_2DH36 clone. In the 168 metaphase cells examined by *in situ* hybridization, 256 silver grains were associated with chromosomes and 24 of them (9.3%) were located on chromosome 17 (Fig. 6). The distribution of grains on this chromosome was not random since 50% were located on the q11–q12 region of the long arm with a maximal concentration in the 17q12 band. The present data thus assign the E_2DH gene to chromosome 17.

Since E_2DH is well known to be associated with membranes, a hydropathy plot was generated according to the algorithm of Kyte and Doolittle (27). Such analysis (Fig. 7) predicts that the N-terminal region of E_2DH is hydrophobic while the C-terminal region is hydrophilic. The hydrophobic region (residues 90 to 200) adjacent to the catalytic site (residues 203 to 223) might be involved in steroid binding.

DISCUSSION

Based on the predicted amino acid sequence of the cDNA clone which contains the 20 amino acid sequence of the catalytic site of E_2DH (12), an heptadecapeptide possibly involved as coenzyme binding site (11), and a 23 N-terminal amino acid fragment determined by Edman degradation of the purified enzyme, we can conclude that the present cDNA clones encode E_2DH. As additional support, polyclonal antibodies raised against the purified enzyme react with the β-galactosidase fusion proteins produced by cDNA clones (Fig. 2). Moreover, the mol wt (34.853) and the amino acid composition (Table 1) obtained from the deduced amino acid sequence are in a good agreement with the mol wt (33,500 ± 1,000) and amino acid composition previously reported for the purified enzyme (25).

There is no evidence for the presence of carbohydrates in the E_2DH molecule (10). Accordingly, no consensus sequences corresponding to potential N-glycosylation sites (Asn-X-Ser or Thr) are evident within the E_2DH open reading frame. After completion of this work, the sequence of a 1325 bp E_2DH cDNA having a short 5′-noncoding segment has been reported (28).

In addition to its role in the placenta, special attention has been given to E_2DH in the endometrium (4) and breast (5). It is in fact thought that the beneficial effect of treatment with progestins in endometrial cancer is due to the stimulation of E_2DH activity which increases the formation of inactive estrone from the highly potent 17β-estradiol (4). E_2DH is also likely to play a major role in breast development and functions as well as in the growth of breast cancer (3, 4).

The availability of a full-length cDNA encoding human E_2DH opens the possibility of studying in detail the mechanisms which control the expression of this enzyme in a large series of tissues already known to

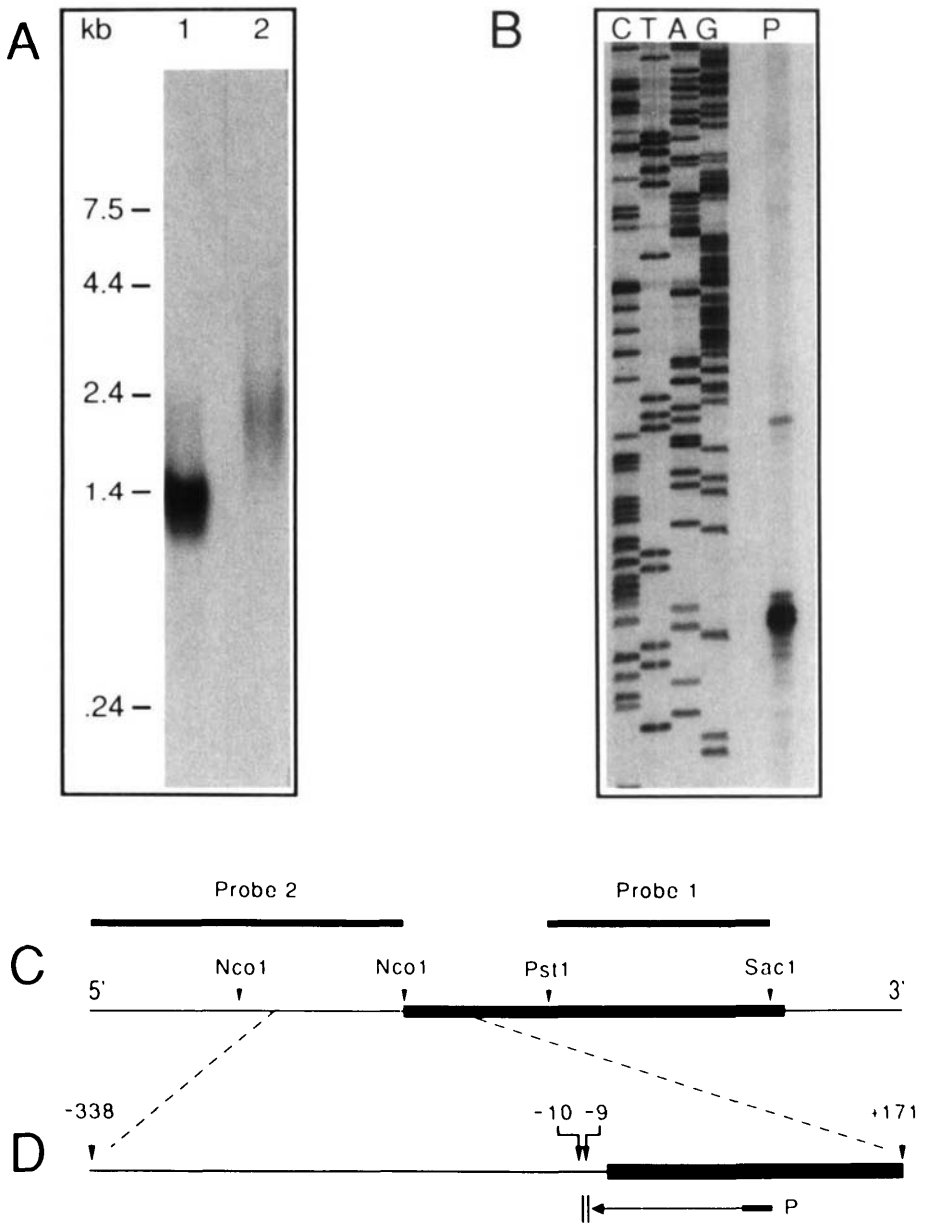

Fig. 5. RNA Blot and Primer Extension Analysis of Human Placental E_2DH mRNA

A, As described under *Materials and Methods*, 20 μg poly(A)$^+$ RNA purified from human placenta were hybridized under stringent conditions with ^{32}P-labeled cDNA probes corresponding to (lane 1) the coding sequence of hpE_2DH36 (probe 1 in C), or (lane 2), the 5′-noncoding sequence of hpE_2DH216 (probe 2 in C). Elements of the BRL 0.24–9.5 kb RNA ladder were used as molecular size markers. B, Poly(A)$^+$ RNA (3 μg) from human placenta was used for extension by AMV reverse transcriptase (42) using the 17 bp [^{32}P] 5′-end-labeled oligonucleotide corresponding to nucleotides 75 to 92 of the hpE_2DH216 cDNA. Lane P, Major extension products; lanes C, T, A, G, sequence of clone hpE_2DH216 obtained by the dideoxy chain termination sequencing method using the same 17 bp primer. C, Schematic representation of the E_2DH cDNA. The coding sequence is represented by a thick line while the thin lines indicate the untranslated regions. Hybridization probes 1 and 2 are depicted above the cDNA map (see A). D, Detailed scheme of the primer extension analysis. The primer P is indicated by the *solid line*, and the extended products are indicated by the *arrow*. *Vertical lines* indicate the termination points at nucleotides −9 and −10.

display enzymatic activity. Furthermore, this should permit to obtain additional information about the steroid dehydrogenase(s) responsible for the synthesis of androgens in mammalian tissues. Such data should have major implications for a better understanding of the development, growth, and function of the gonadal as well as accessory sex organs. Moreover, such information should be crucial for a better knowledge of the reproductive processes and for the design of specific inhibitors of sex steroid formation which could play a major role in the treatment of sex steroid-dependent cancers, especially breast and endometrial cancer.

MATERIALS AND METHODS

Screening of the Human Placental cDNA Library

A mixture of two oligodeoxyribonucleotide probes of 33 mer (5′-CAG-GTA-CTG-GTA-GAA-ACG-GTG- GAA-[AG]GT-GTG-

GAT-3′) predicted (13) from the amino acid sequence of the catalytic site of E_2DH (12) were synthesized with a Biosearch DNA synthesizer. The purified oligonucleotides were 5′-end-labeled and used as probes to screen the human placental cDNA library (Clontech Laboratories, Inc., Palo Alto, CA). Hybridization was performed at 45 C in 6× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), 1× Denhardt's (1× Denhart's = 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 0.02% BSA), 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate. Filters were washed twice in 3× SSC, 0.05% sodium pyrophosphate at 55 C for 30 min.

Lysogen Construction and Immunoblot Analysis

Lysogens of positive λgt11 recombinant clones were generated in the *Escherichia coli* strain Y 1089 and the β-galactosidase fusion proteins were prepared as described by Huynh *et al.* (29). Cell lysate proteins from each lysogen were separated on 5–15% $NaDodSO_4$/PAGE (30) and electroblotted onto nitrocellulose. The protein blots were then washed and treated with rabbit anti-E_2DH serum and ^{125}I-labeled goat anti-rabbit immunoglobulin G (IgG) as described (31).

DNA Sequencing

Complementary DNA clones were sequenced by the dideoxy chain termination method (32) using modified T7 DNA polymerase (33) (Sequenase kit, United States Biochemical Corp., Cleveland, OH). Fragments were subcloned in the Bluescript SK vector (Stratagene, San Diego, CA) and synthetic oligonucleotides were used as sequencing primers as indicated in Fig. 3.

RNA Blot Analysis

Total RNA was isolated from human placenta by homogenizing tissue in guanidinium isothiocyanate followed by centrifugation through a cushion of 5.7 M CsCl (34–38). Poly(A)$^+$ RNA was purified by two successive cycles of chromatography through an oligo(dT)-cellulose column (39). RNA blot analysis was

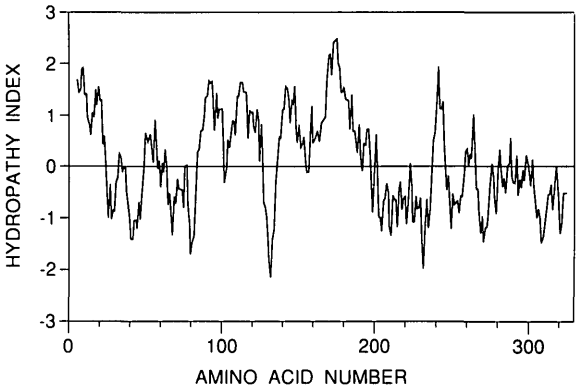

Fig. 7. Hydropathy Profile of E_2DH Calculated according to Kyte and Doolittle (27)

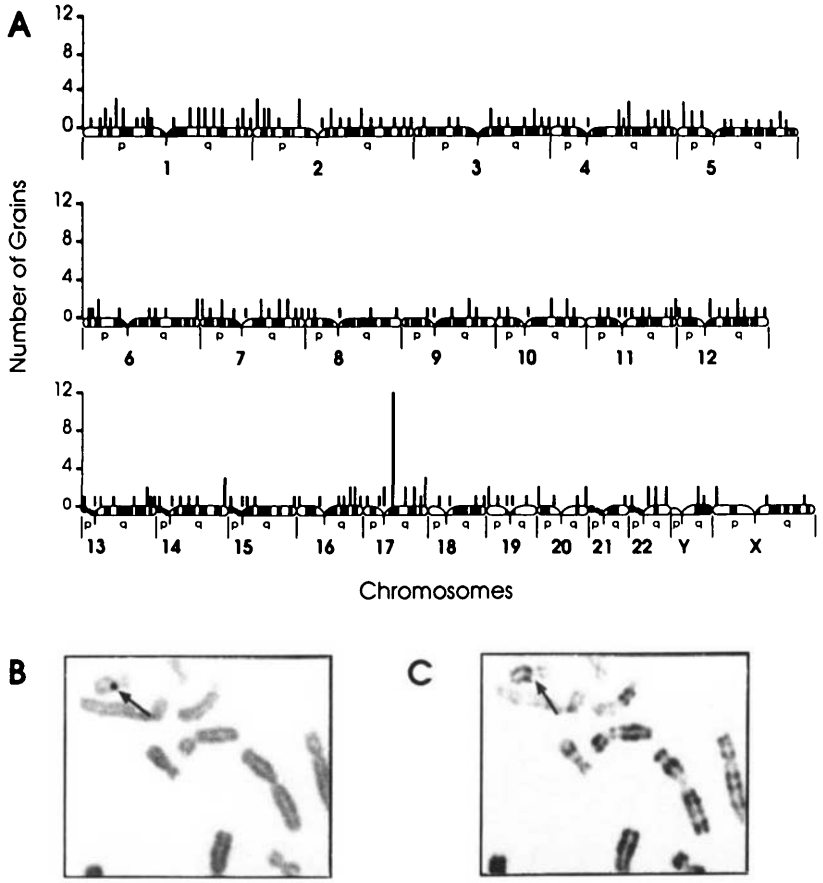

Fig. 6. Assignment of the E_2DH Gene to Chromosome 17 by *in Situ* Hybridization

A, *In situ* distribution of silver grains among the human chromosomes. The major hybridization is associated with the long arm of chromosome 17. B, Partial metaphases showing Giemsa-stained chromosomes with silver grains (indicated by *arrows*) illustrating the specific site of hybridization of the hpE_2DH36 probe on chromosome 17. C, Same metaphases after subsequent R-banding by fluorochrome photolysis Giemsa method shows that the labeled region is located on q11-q12 subbands.

performed as previously described (37, 38). Briefly, 20-μg samples of poly(A)$^+$ RNA isolated from human placenta were electrophoresed on a 1.2% agarose/2.2 M formaldehyde gel and immobilized on a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL). Hybridization with either the 588 bp *Pst*I-*Sac*I fragment corresponding to a section of the coding sequence of hpE$_2$DH36 cDNA (probe 1) or the 823 bp 5′-noncoding *Eco*RI-*Nco*I-*Nco*I fragment of cDNA hpE$_2$DH216 (probe 2) was performed at 42 C for 16 h. The fragments were labeled with [α-^{32}P]dCTP to a specific activity of 1×10^9 dpm/μg using the random primer method (40). The probes were added to hybridization buffer (37) at a concentration of 2×10^6 cpm/ml. Three micrograms of BRL (Gaithersburg, MD) 0.24-9.5 kb RNA ladder were blotted alongside the placental poly(A)$^+$ RNA and hybridized with ^{32}P-labeled lambda DNA. The filters were washed twice for 15 min in 2× SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature followed by two 15 min washes in 0.1× SSC/0.1% SDS at room temperature and two 15-min washes in fresh 0.1× SSC/0.1% SDS at 65 C. The autoradiograph was obtained after exposing the filters to Kodak X-OMAT AR films for 8.5 h at −80 C between two intensifying screens.

Chromosome Localization

In situ hybridization was performed on chromosome preparations obtained from phytohemoglutinin-stimulated human lymphocytes cultured for 72 h as previously described (41). 5-Bromodeoxyuridine was added for the last 7 h of culture (30 μg/ml) to ensure high quality posthybridization chromosomal banding. The hpE$_2$DH36 clone was ^{3}H-labeled by nick-translation to a specific activity of 1.0×10^7 dpm/μg. The labeled probe was hybridized to metaphase spreads at a final concentration of 25 ng/ml hybridization solution as described (41). After coating with nuclear track emulsion (Kodak NTB$_2$), the slides were exposed for 24 days at 4 C. Subsequently, chromosome spreads were stained with buffered Giemsa solution and the metaphases were photographed. R-Banding was then performed by the fluoro-chrome-photolysis-Giemsa method and metaphases were photographed before analysis.

Acknowledgments

The authors are grateful to Dr. Paul Trotta (Schering Plough Corporation, Bloomington, NJ) for determining the N-terminal amino acid sequence, Drs. M. V. Govindan, S. Guérin, and L. Lagacé for helpful discussions, and Ms. Elaine Leclerc for typing the manuscript.

Received April 17, 1989. Revision received May 22, 1989. Accepted May 22, 1989.

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