Pergamon

0960-0760(94)00094-8

J. Steroid Biochem. Molec. Biol. Vol. 50, No. 5/6, pp. 275–282, 1994

0960-0760/94 $7.00 + 0.00

Human 17β-Hydroxysteroid Dehydrogenase: Overproduction Using a Baculovirus Expression System and Characterization

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Estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD) plays a pivotal role in the synthesis of estrogens. We overproduced human placental estrogenic 17β-HSD using a baculovirus expression system for the study of the enzyme mechanism. A cDNA encoding the entire open reading frame of human 17β-HSD was inserted into the genome of *Autographa californica* nuclear polyhedrosis virus and expressed in *Spodoptera frugiperda* (Sf9) insect cells. Metabolic labeling and Western blot analysis using polyclonal antibodies raised against native human 17β-HSD indicated that a molecule with an apparent mass of 35 kDa was maximally expressed 60 h after infection. At that time interval, intracellular 17β-HSD activity reached 0.26 U/mg of protein in crude homogenate, about 70 times the level measured in human placenta. Purification of recombinant 17β-HSD was achieved by a single affinity fast liquid protein chromatography step yielding 24 mg of purified 17β-HSD protein per liter of suspension culture, with a specific activity of about 8 μmol/min/mg of protein for conversion of estradiol into estrone, at pH 9.2. In addition, the recombinant protein purified from infected Sf9 cells was assembled as a dimer with molecular mass and specific activity identical to those of the enzyme purified directly from placenta. The present data show that the baculovirus expression system can provide active 17β-HSD that is functionally identical to its natural counterpart and easy to purify in quantities suitable for its physico-chemical studies.

J. Steroid Biochem. Molec. Biol., Vol. 50, No. 5/6, pp. 275–282, 1994

INTRODUCTION

In addition to their roles in the development, growth, and function of all tissues involved in reproduction and fertility in men and women, sex steroids promote growth of hormone-sensitive cancers, especially prostate [1] and breast [2] cancers.

By catalyzing their formation and degradation, 17β-HSD regulates the three most important sex steroids (estradiol, testosterone, and androst-5-ene-3β, 17β-diol). Indeed, 17β-HSD catalyzes the interconversion of estrone and estradiol [3, 4], the interconversion of 4-androstenedione and testosterone [5], and the interconversion of dehydroepiandrosterone and androst-5-ene-3β, 17β-diol [2]. This enzyme, because of its ability to alter androgen- and estrogen-dependent processes, represents an important target for cancer therapy.

We chose the estrogenic form of 17β-HSD for our study, which is found to be abundant in the soluble subcellular fraction of the human placenta and has been cloned in our laboratory [6, 7]. This form of 17β-HSD is responsible for the formation of active estrogens, i.e. 17β-estradiol and androste-5-ene-3β, 17β-diol [8–10] which stimulate the proliferation of mammary tumor cells.

To design effective and specific inhibitors of human 17β-HSD, we initiated physico-chemical studies to characterize the structure of its active site or the binding sites of its substrates. Such studies require large quantities of enzyme protein purified in a functionally active state. Although purification of human placental 17β-HSD has improved in recent decades [6, 11–14], Lin *et al.* [15] succeeded in purifying this labile enzyme with high specific activity using FPLC.

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Received 20 Jan. 1994; accepted 9 May 1994.
Abbreviations: 17β-HSD, 17β-hydroxysteroid dehydrogenase; DDT, dithiothreitol; EDTA, (ethylenedinitrilo) tetraacetic acid; FPLC, fast protein liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis. *Enzymes*: 17β-Hydroxysteroid dehydrogenase (EC 1.1.1.62).

However, the entire process requires 3 to 4 days, yielding about 5 mg of pure enzyme protein from 600 g tissue of human placenta. To produce the quantities of enzyme required for our studies and to accelerate the purification, we overproduced 17β-HSD in a baculovirus expression system. This system has been used successfully for the overproduction of other mammalian proteins (for review see Luckow [16]).

This report describes the construction of a recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) containing the 17β-HSD cDNA under the control of the polyhedrin promoter, its use for infection of Sf9 insect cells, as well as the rapid purification and characterization of the recombinant 17β-HSD.

MATERIALS AND METHODS

Materials

Spodoptera frugiperda, purified AcNPV DNA, and transfer vector pVL1393 were purchased from Invitrogen Corporation; Grace's insect cell culture medium, methionine-free Grace's medium, yeastolate, and lactalbumin hydrolysate were from Gibco-BRL; L-[^{35}S]methionine was obtained from New England Nuclear; and sodium cholate, NAD^+, PMSF, and protease inhibitors were from Sigma. Mono Q (HR 5/5), Superose-12 (HR 10/30) columns, blue–Sepharose CL-6B, and protein standards for gel filtration were from Pharmacia-LKB; protein standards for SDS–PAGE were obtained from Bio-Rad. 17β-Estradiol, estrone, β-mercaptoethanol and DTT were from Aldrich.

Cell culture and virus

The TNM-FH medium was prepared from Grace's medium by the addition of 3.3 g/l yeastolate and 3.3 g/l lactalbumin hydrolysate. The Sf9 cells were grown as monolayers or in suspension at 28°C in TNM-FH medium [17] supplemented with 10% heat-inactivated fetal bovine serum (BFS, Hyclone). Gentamycin sulfate and amphotericin were routinely added to medium at final concentrations of 50 and 2.5 μg/ml, respectively, in the suspension culture. Cells were infected with virus at a multiplicity of infection (MOI) of 0.1 to 1 pfu to produce virus stocks or at a MOI $\geqslant$10 for maximal protein expression.

Construction of recombinant transfer vector

Transfer vector pVL/17β-HSD (Fig. 1) was constructed as follows: a 1.2-kbp cDNA encoding the human placental 17β-HSD was obtained by digestion of $hpE_2DH16.6$ [6, 7] with *Nco* I (at the initiation codon of the coding region of 17β-HSD) and *Eco*R I (269 nucleotides downstream from the stop codon), filled in with DNA polymerase Klenow fragment, and subcloned into the transfer vector pVL1393 previously opened by *Sma* I and treated with bacterial alkaline phosphatase [18]. Hybrid plasmids were then introduced into competent *Escherichia coli* DH5α cells [19] by transformation [20]. Plasmid DNAs from a few ampicillin-resistant (Ap^r) colonies were purified by a small-scale procedure [21] and recombinant plasmids having insert in the correct orientation, with respect to the polyhedrin promoter in the transfer vector, were identified by restriction endonuclease mapping with *Bam*HI, *Hind*III, and *Pst* I (Fig. 1). The construction of one (pVL/17β-HSD) was finally confirmed by dideoxynucleotide sequencing.

Production and isolation of recombinant baculovirus

AcNPV genomic DNA (1 μg) and pVL/17β-HSD (2 μg), purified by two cycles of CsCl density gradient centrifugation, were used to cotransfect monolayers of Sf9 cells by the calcium phosphate precipitation method [17]. Recombinant baculoviruses were cloned by at least three rounds of plaque purification using 0.01% neutral red dye (Sigma) to facilitate visual screening of recombinant plaques [22].

Metabolic labeling of proteins in infected Sf9 cells

The Sf9 cells were infected with recombinant or wild-type AcNPV virus or were mock-infected. At either 48 or 72 h postinfection, culture medium was removed and replaced by methionine-free medium for 1 h. Cells were then incubated at 28°C for 4 h in methionine-free medium with 0.2 mCi/ml of L-[^{35}S]methionine. Labeled cells were pelleted by centrifugation (10 min at 1000 *g*) and directly resuspended in SDS sample buffer [125 mM Tris, 20% (v/v) glycerol, 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, and 0.001% (w/v) bromophenol blue, pH 6.8]. Total proteins were then simultaneously electrophoresed on two SDS–PAGE using the Mini-PROTEAN II (Bio-Rad). One was stained with Coomassie brilliant blue; the other was dried and autoradiographed for 12 to 18 h.

Time course of recombinant 17β-HSD production

The Sf9 cells were infected with recombinant virus at a MOI >10. At various intervals of infection (24, 36, 48, 60, 72 and 96 h), about 4×10^6 cells were harvested, pelleted by centrifugation (10 min at 1000 *g*), resuspended in 2 ml of 17β/Sf9 buffer [0.25 mM Tris–HCl pH 7.5, 20% (v/v) glycerol, 0.1 mM EDTA, 0.2 mM DTT, 20 μM NAD^+ and 0.2% (w/v) sodium cholate] with a mixture of protease inhibitors (0.5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml antipain, 5 μg/ml aprotinin, 5 mM benzamidine, and 1 mM PMSF) [23] and sonicated on ice (four 15-s bursts with 30-s intervals) with an output at 4, using a sonicator from Sonics & Materials Inc. Unbroken cells and nuclei were pelleted by centrifugation (10 min at 10,000 *g*) and supernatants were assayed for 17β-HSD activity.

17β-HSD assay

Measurement of 17β-HSD activity was performed as described previously [15]. Briefly, aliquots of protein extracts were incubated at room temperature for 3 min in a reaction mixture containing 50 mM $NaHCO_3$–Na_2CO_3, pH 9.2, 25 μM estradiol, and 0.5 mM NAD^+. The reaction was followed by spectrophotometric measurement of the reduction of NAD^+ to NADH indicated by the absorption increase at 340 nm. A reaction mixture containing no estradiol was used as blank. One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of estrone per min under the above-indicated conditions, at 23 ± 1°C.

$$\text{Estradiol} + NAD^+ \overset{17\beta\text{-HSD}}{\rightleftharpoons} \text{Estrone} + NADH + H^+$$

SDS–PAGE and Western blot analysis

Levels of recombinant 17β-HSD produced at various time intervals were determined by Western blot analysis. Total protein extracts were electrophoresed on 12.5% SDS–PAGE and electroblotted onto nitrocellulose membranes (100 V for 1 h). Blots were then probed with polyclonal antibodies raised against purified human placental 17β-HSD. Binding of the primary antibody was detected with ^{125}I-labeled goat antirabbit immunoglobulin G. In SDS–PAGE the 17β-HSD samples contained 10% glycerol and 0.5 to 1% SDS. The gels were stained with Coomassie blue or with High Blue Staining from Pharmacia for greater sensitivity [15].

Large-scale production and purification of 17β-HSD from insect cells

The Sf9 cells in suspension culture (2×10^6 cells/ml) were pelleted by centrifugation and infected with recombinant virus at a MOI ⩾10 for 1 h at room temperature. Cells were then diluted to a density of 1×10^6 cells/ml with fresh medium and incubated at 28°C. At 60 h postinfection, cells were harvested, washed with serum-free medium and resuspended in 1/10 vol of 17β/Sf9 buffer (see above). The cells were then fractionalized and frozen at −70°C. Before each purification of 17β-HSD, one or several such cell fractions were thawed, diluted three to four times, and sonicated as mentioned above. Unbroken cells and nuclei were then removed by a 10,000 *g*/10 min centrifugation. Unless otherwise mentioned, a buffer solution containing 40 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.2 mM DTT, and 20% glycerol was used for protein purification as the low-ionic-strength buffer, referred to herein as buffer A (see Results).

Superose-12 gel filtration of recombinant 17β-HSD

The Superose-12 (HR 10/30) column was equilibrated with buffer A in the presence of 0.15 M NaCl for both 17β-HSD and protein standards. The sample volume was between 0.1 and 0.2 ml. A flow rate of 0.4 ml/min and a fraction size of 0.2 ml were used.

RESULTS

Construction and isolation of recombinant baculovirus encoding human 17β-HSD

The recombinant transfer vector pVL/17β-HSD (Fig. 1), which contains the entire 17β-HSD cDNA coding sequence downstream from the baculovirus polyhedrin promoter of pVL1393, was constructed as described in Materials and Methods. Because the length of the 5′ noncoding sequence of the foreign gene to be inserted influences the level of expression [24], the insert was digested at the *Nco*I site (C CATG G) overlapping the initiation codon of the 17β-HSD coding sequence, leaving only one extra nucleotide upstream from the ATG start codon. Then the Sf9 cells were cotransfected with the transfer vector pVL/17β-HSD and wild-type baculovirus DNA. Four independent putative recombinant viruses that express 17β-HSD instead of the baculovirus polyhedrin protein were initially identified by plaque morphology screening under a dissection microscope and then isolated by three or four rounds of plaque purification. One of them, named VL/17β-HSD, was chosen for further studies and expressed experiments.

Expression of recombinant 17β-HSD

Detection of recombinant 17β-HSD was first demonstrated by *in vivo* metabolic labeling. The Sf9 cells were infected with wild-type AcNPV or with recombinant virus VL/17β-HSD or were mock-infected. At both 48 and 72 h postinfection, cells were labeled for 4 h with L-[^{35}S]methionine and homogenized in SDS sample buffer. Total cell extracts were resolved by SDS–PAGE as described in Materials and Methods. Coomassie blue gel staining revealed a protein with an apparent molecular mass of about 35 kDa (Fig. 2, left, lanes VL/17β-HSD) that was not present in mock-infected cells (Fig. 2, left, lanes Mock) or in wild-type virus-infected cells (Fig. 2, left, lanes AcNPV) and that corresponds to the apparent molecular mass of human 17β-HSD [6, 7]. Although the level of expression of this protein is much lower than that of polyhedrin (strong band at 32 kDa in Fig. 2, left, lanes AcNPV), it is a major constituent of total cellular protein. Autoradiography of the gel showed that the recombinant 35-kDa protein was the most abundant newly expressed protein in VL/17β-HSD-infected cells at both 48 and 72 h (Fig. 2, right).

The time course of recombinant 17β-HSD production was monitored by analyzing 17β-HSD enzymatic activity in infected cells (Fig. 3). Intracellular 17β-HSD activity became detectable by 36 h postinfection and reached a maximum by 60 h. Thereafter, enzymatic activity decreased rapidly to return to basal

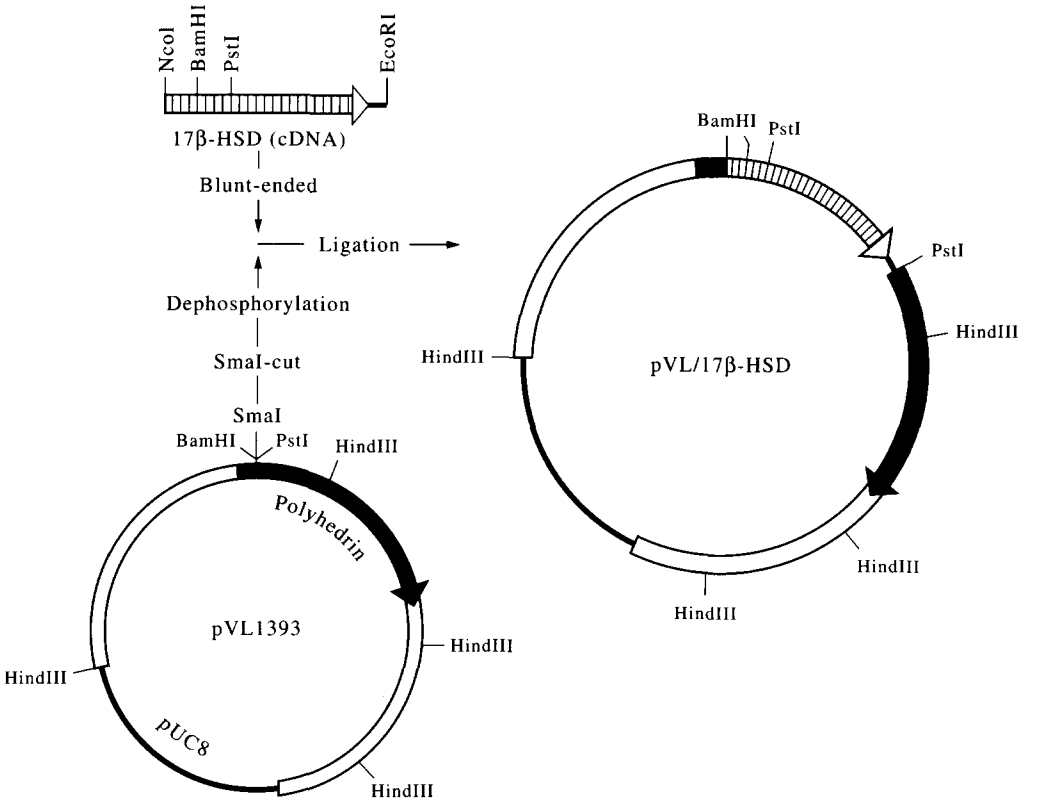

Fig. 1. Construction of recombinant transfer vector pVL/17β-HSD. Transfer vector pVL/17β-HSD was constructed as follows: the cDNA encoding human placental 17β-HSD was obtained by digestion of hpE$_2$DH16.6 with *Nco*I and *Eco*RI (see text), filled in with DNA polymerase Klenow fragment, and subcloned into the transfer vector pVL1393 previously opened by *Sma*I and treated with bacterial alkaline phosphatase. Plasmid DNAs from a few ampicillin-resistant (Ap^r) colonies were purified by a small-scale procedure. Recombinant plasmids having an insert in the correct orientation, with respect to the polyhedrin promoter in the transfer vector, were identified by restriction endonuclease mapping with *Bam*HI, *Hind*III and *Pst*I. The construction of one (pVL/17β-HSD) was confirmed by dideoxynucleotide sequencing.

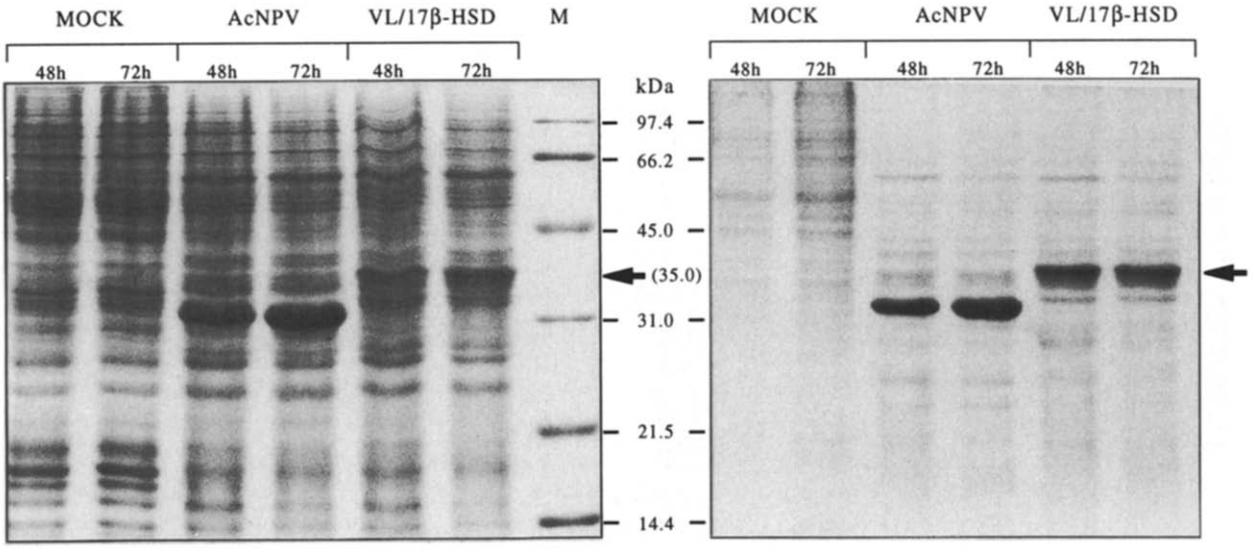

Fig. 2. Evaluation of the level of 17β-HSD expression by recombinant baculovirus. The left photograph shows SDS–PAGE of extracts from infected Sf9 insect cells 48 or 72 h postinfection. Coomassie blue gel staining revealed a polypeptide with an estimated molecular mass of 35 kDa (lanes VL/17β-HSD), which was not present in mock-infected cells or in wild-type virus-infected cells (lanes AcNPV). The right photograph shows the same time course using labeling with [^{35}S]methionine.

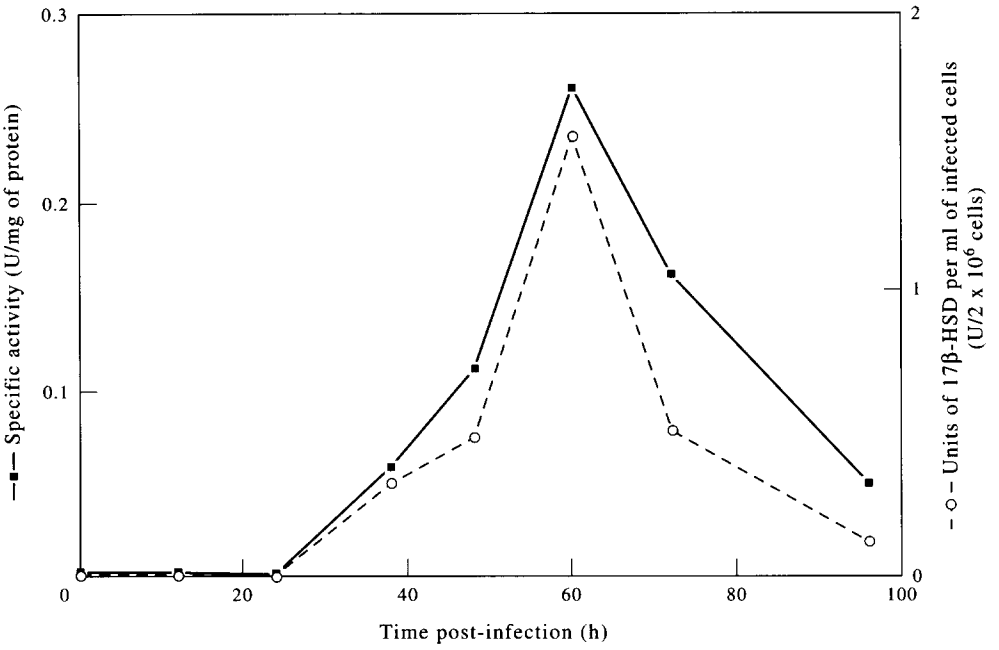

Fig. 3. Time course of 17β-HSD production in infected insect cells. Intracellular 17β-HSD activity appeared by 36 h postinfection and reached a maximal value by 60 h postinfection, when its specific activity reached a level approx. 70-fold higher than in human placenta, the richest source of 17β-HSD.

levels 110 h postinfection. At 60 h postinfection, 17β-HSD specific activity reached a maximal value of 0.26 U/mg protein in the cell extract; this value corresponds to an enrichment of about 70-fold compared with the concentration of the enzyme in the human placenta (0.0039 U/mg protein), the richest natural source of human 17β-HSD. No activity was detectable in wild-type virus-infected cells.

The nature of the recombinant 17β-HSD produced at various intervals was also examined by Western blot analysis with specific antibody raised against human 17β-HSD [6, 7]. In agreement with the measurements of 17β-HSD activity, the presence of recombinant protein was detected as a single band at 36 h postinfection, and maximal levels were observed at 60 h (Fig. 4). Thereafter, the band of immunoreactivity progressively disappeared. Long term exposure (20 h) of the immunoblot revealed that recombinant 17β-HSD was already expressed 24 h postinfection, whereas no signal was detectable in the controls (results not shown).

Purification of recombinant 17β-HSD

17β-HSD homogenization was achieved using a single blue–Sepharose chromatography on FPLC.

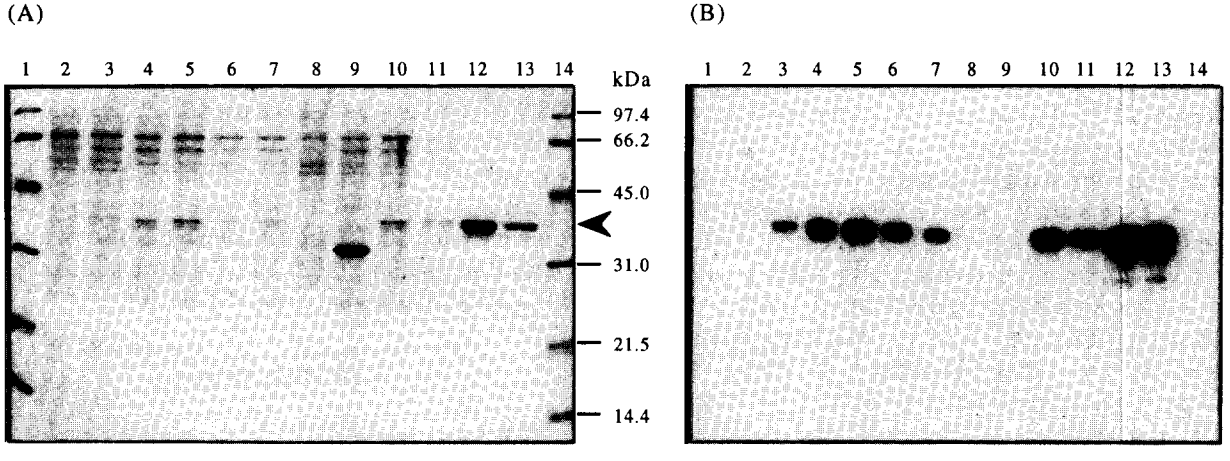

Fig. 4. Immunoblot analysis of 17β-HSD synthesized in Sf9 cells infected by recombinant baculovirus VL/17β-HSD. Cells were infected with VL/17β-HSD, and extracts were prepared 24 (lane 2), 36 (3), 48 (4), 60 (5 and 10), 72 (6), and 96 (7) h postinfection. Extracts from Mock (8) and wild-type virus (9)-infected cells were used as negative controls. Purified 17β-HSD from human placenta (12), supernatant of the 140,000 *g* ultracentrifugation (11), and purified enzyme from infected insect cells (13) were used as positive controls. Equivalent proportions of each fraction were separated on two 12.5% SDS–PAGE gels. In A the proteins were stained with Coomassie blue; in B, electroblotted onto nitrocellulose membrane. The presence of 17β-HSD was detected by using specific antibody raised against human enzyme.

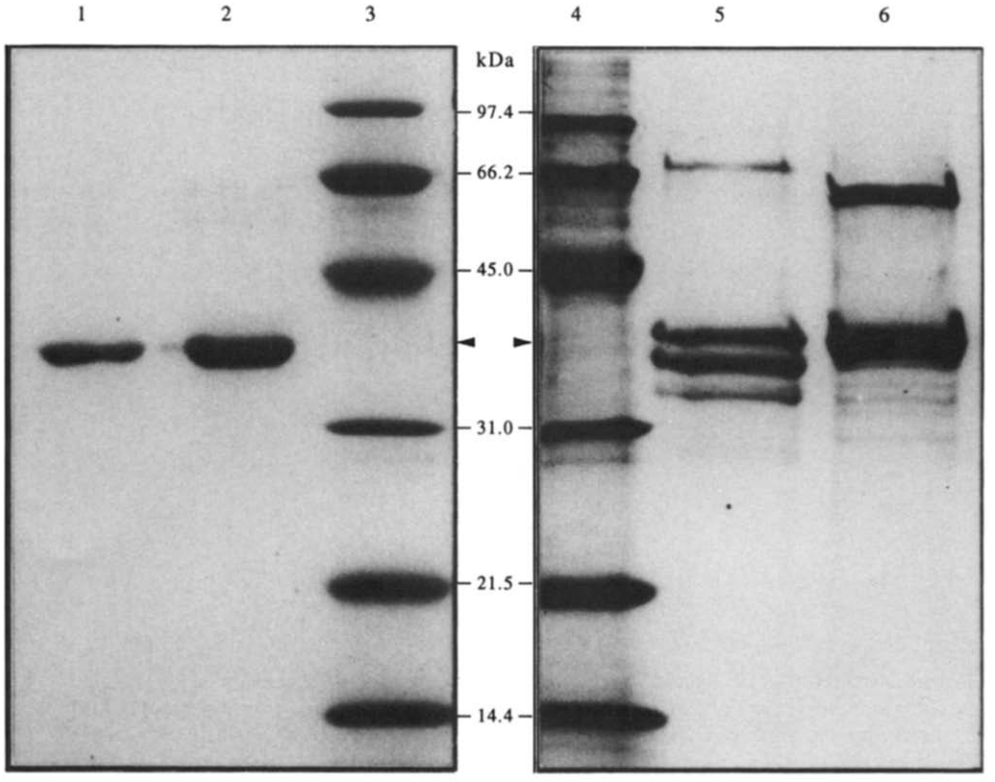

Fig. 5. SDS–PAGE of 17β-HSD. (1, 2) 17β-HSD overproduced in a baculovirus expression system and purified by blue–Sepharose CL-6B chromatography with different sample loading for these two lanes; (3, 4) protein standards; (5) 17β-HSD expressed in HeLa cells and partially purified; (6) 17β-HSD overproduced in a baculovirus expression system and partially purified on a Mono Q HR 5/5 column. The arrows in the middle indicates the apparent molecular mass of 17β-HSD: 34.5 kDa. The gels were stained by Coomassie blue.

Briefly, ultracentrifugation for 30 min at 140,000 *g* of a 60 ml 10,000 *g* supernatant yielded a clear supernatant. The latter was diluted about 3 times and loaded onto the affinity column (100 × 16 mm i.d.). The column was first washed with 80 mM NaCl, before stepwise elution with $NADP^+$. 17β-HSD eluted at about 35 μM $NADP^+$ and resulted in a homogenous preparation (Fig. 5).

Further characterization of recombinant 17β-HSD

Superose-12 gel filtration. A Mono Q fraction of 17β-HSD was used for the gel filtration which was obtained in our earlier experiments as follows: the 10,000 *g* supernatant from 1.25×10^6 Sf9 cells was diluted in 7.5 ml of buffer A and loaded on a Mono Q HR 5/5 column. The column was then washed by buffer A containing 0.5 mM PMSF, and a 26-ml gradient of 0 to 0.5 M NaCl in the same buffer was applied. Active 17β-HSD eluted at about 0.25 M NaCl, thus yielding a fraction of up to 70% homogeneity as evaluated by SDS–PAGE and gel scanning (Fig. 5).

Both the cell extract and the Mono Q fraction of the overproduced 17β-HSD in Sf9 cells were chromatographed. Enzymatic activities eluted at the same elution volume, very close to the BSA standard (67 kDa) (Fig. 6). The K_{av}, that is, the fraction of the stationary gel volume available for diffusion of a given solute species [25], was plotted against the logarithm of molecular mass of each protein standard, thus establishing the standard curve (data not shown). The K_{av} for 17β-HSD is 0.297 ± 0.020 and its molecular mass thus estimated at 68 kDa, in coincidence with the 17β-HSD molecular mass evaluation on pore gradient native gels [15]. Since the apparent molecular mass determined by SDS–PAGE is 35 kDa, such results indicate dimerization of the enzyme. Moreover, since the overproduced enzyme is expressed from a single cDNA encoding a 34.5-kDa protein [6, 7], these data further support its homodimeric structure.

Recombinant 17β-HSD specific activity. The homogeneous 17β-HSD from Sf9 cells catalyzes the oxidation of about 8.0 μmol estradiol/min/mg of enzyme protein (the maximum velocity), a value similar to that obtained for native placental 17β-HSD.

DISCUSSION

The present data clearly demonstrate that the overproduced 17β-HSD is identical to its natural counterpart isolated from placenta in terms of subunit structure, molecular weight and catalytic activity.

It is well known that sex steroids can stimulate cell proliferation in many hormone-sensitive cancers, especially breast [2], endometrial [26], and prostate [1] cancers. Many studies have been initiated to control the

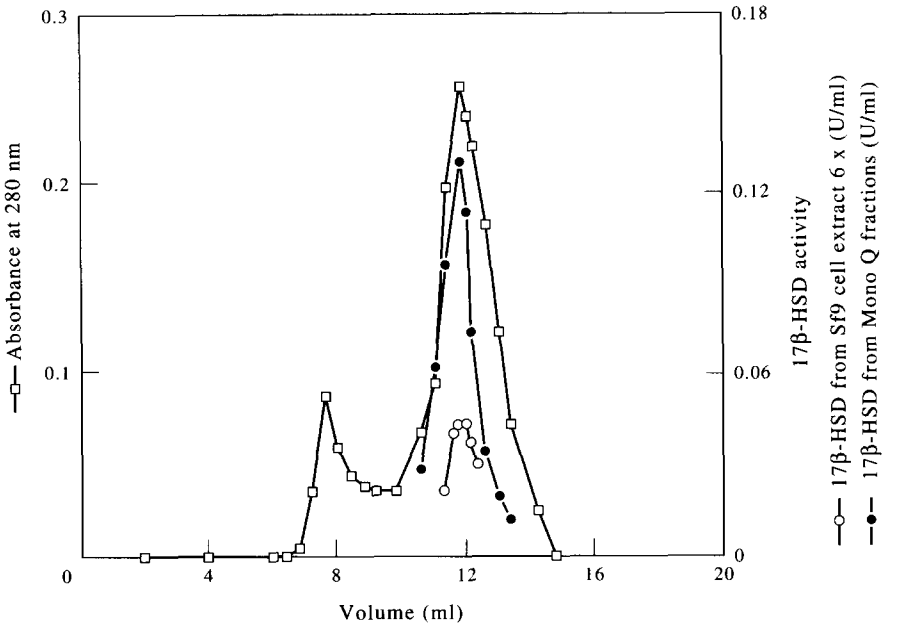

Fig. 6. Elution profile of Superose-12 gel filtration of overproduced 17β-HSD. 0.1–0.2 ml of enzyme or standard proteins were loaded on a HR 10/30 column equilibrated with buffer A in the presence of 0.15 M NaCl at 0.4 ml/min, □ protein standards (667 kDa and 67 kDa), ○ and ● represent activities from cell extract and Mono Q fractions of infected Sf9 cells, respectively.

intracellular concentration of sex steroids via the inhibition or inactivation of enzymes responsible for their conversion. By catalyzing the synthesis as well as degradation of active steroid hormones (estradiol, testosterone, and androst-5-ene-3β,17β-diol), human 17β-HSD plays a crucial role in regulating the intracellular concentration of sex steroids and thus represents an important target in cancer therapy. Several groups have attempted to synthesize inhibitors of 17β-HSD [27–29], but the lack of knoweldge about the structures involved in the binding of its substrates prevented a rational design of selective inhibitors or inactivators.

Physico-chemical studies initiated in our laboratory open the way for the design of appropriate inhibitors. Because such studies require large quantities of the purified enzyme, we used the baculovirus expression system to overproduce 17β-HSD. As mentioned above, this system has been successfully used to express many eukaryotic proteins [16, 24]. Most of these expressed proteins were biologically and immunologically active. Moreover, the overproduction and purification require only a few days. We then characterized the recombinant enzyme to determine if the molecule had retained the properties characteristic of 17β-HSD purified from human placenta.

The apparent molecular mass of 35-kDa is in perfect agreement with that calculated from the amino acid sequence (34 853) deduced from the primary structure of cDNA coding for human 17β-HSD [6, 7]. Polyclonal antibodies raised against 17β-HSD purified from human placenta reacted specifically with this protein as determined by electrophoretic blot. We can thus conclude that the protein, expressed exclusively in insect cells infected by our recombinant baculovirus, is genuine human 17β-HSD.

In the expression of recombinant 17β-HSD, the decrease in activity from 60 h could reflect the intracellular protein degradation that occurs later in the infection period that may be due to endogenous proteases or viral-encoded protease activity. However, as noted for β-galactosidase by Licari and Bailey [30], the absence of degradation products at 72 and 96 h by Western blot analysis suggests that 17β-HSD (or proteolysis products) has been released to the extracellular medium because of leakage or cell lysis. At 60 h postinfection, recombinant 17β-HSD accounted for 3 to 5% of the total soluble Sf9 proteins. The level of expression achieved in this study far exceeds that observed in any human tissue or that obtained by transient expression in mammalian HeLa cells [31] and it allowed us to purify the overproduced 17β-HSD to homogeneity with only one affinity chromatography; overall yield was more than 70%. Thus, more than 24 mg of homogeneous recombinant enzyme protein can be obtained from 1 l (2×10^9 Sf9 cells) of suspension culture. The whole purification takes about 6 h, including the cell disruption and the ultracentrifugation steps. This very fast purification procedure probably eliminated protein microheterogeneity caused by *in vitro* modifications, such as oxidation–reduction effects or partial proteolysis [32, 33] and can explain the higher specific activity measured for homogeneous preparations of recombinant 17β-HSD (about 8.0 U/mg of protein) compared with those reported previously in the literature using conventional procedure, for the purified human placental enzyme (see [15] for review). The reductive activity (at optimal

pH 6.5) is also identical for the recombinant protein and the native one from placenta (about 7 U/mg). In contrast to the enzyme purified from human placenta found in the soluble fraction, 17β-HSD expressed in insect cells seemed associated with an insoluble fraction, which was verified by analyzing the 10,000 *g* supernatant and pellet with anti17β-HSD antibodies (data not shown). The addition of sodium cholate to 0.2% permits solubilization of the recombinant protein and keeps it in the soluble fraction after the ultracentrifugation step. The molecular mass of 68-kDa for the native recombinant 17β-HSD estimated by gel filtration demonstrates its correct assembly into a dimeric complex [15]. The similar specific activity also confirm the identity of the recombinant 17β-HSD to the naturally occurring human enzyme molecule. The availability of this expression system makes possible site-directed mutagenesis experiments to characterize the catalytic mechanisms and the ligand binding sites of 17β-HSD as well as detailed structure–function studies.

Acknowledgements—This work was supported in part by the Medical Research Council of Canada (MRC) in the form of a Group Grant in Molecular Endocrinology, the Fonds de la Recherche en Santé du Québec (FRSQ) and Endorecherche. We thank Dr Van Luu-The for helpful discussions.

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