Regulation of the Steroid-inducible 3α-Hydroxysteroid Dehydrogenase/Carbonyl Reductase Gene in *Comamonas testosteroni*  

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The *Comamonas testosteroni* 3α-hydroxysteroid dehydrogenase/carbonyl reductase gene (*hsdA*) codes for an adaptive enzyme in the degradation of steroid compounds. However, no information was available on the molecular regulation of steroid-inducible genes nor on the mechanism of steroid signaling in procaryotes. We, therefore, investigated the cis- and trans-acting elements of *hsdA* expression to infer the mechanism of its molecular regulation by steroids. The gene was localized on a 5.257-kilobase EcoRI fragment of *C. testosteroni* chromosomal DNA. The promoter was characterized, and the transcriptional start site was identified. Two palindromic operator domains were found upstream of *hsdA*. A new gene coding for a trans-acting negative regulator (repressor A, RepA) of *hsdA* expression was characterized. The specific interaction between RepA, testosterone, and the operator domain is demonstrated. From our results we conclude that *hsdA* is under negative transcriptional control by an adjacent gene product (RepA). Accordingly, induction of *hsdA* by steroids in fact is a derepression, where steroidal inducers bind to the repressor, thereby preventing its binding to the *hsdA* operator.

*Comamonas* (formerly *Pseudomonas*) *testosteroni* is a Gram-negative bacterium found in soil, mud, and water, but it has also been isolated from the gastrointestinal tract in humans (1, 2). Interestingly, *C. testosteroni* can use a variety of steroid substrates as a sole carbon source through the reaction of a set of steroid-inducible enzymes (3, 4). Although the metabolic intermediates of the steroid substrates have been identified, and the basic catalytic pathway is known, only limited information is available on the molecular structure and function of the participating enzymes (5–11). Moreover, nothing was known about the mechanism of induction of steroid-metabolizing enzymes or about the basis of their transcriptional regulation.

The occurrence of several hydroxysteroid dehydrogenases (HSDs)³ in microbial organisms raises the question of the physiological function of these enzymes in procaryotes. Because steroids simultaneously serve both as signal molecules and as a carbon source, they play a particularly important role in certain procaryotes. The same applies to procaryotic HSDs. On the one hand, procaryotic HSDs may play a regulatory role in the steroid-inducible gene expression. On the other hand, it is established that they participate in steroid degradation.

Enzymatic C3-dehydrogenation of ring A, which occurs at the initial stage of steroid catabolism in *C. testosteroni*, is mediated by 3α-HSD, the production of which has been shown to be inducible by testosterone, progesterone, and lanosterol (12). Since the pioneering work of Marcus and Talalay (13), it is well known that 3α-HSD is one of the first enzymes of the steroid catabolic pathway and that, therefore, it plays a central role in steroid metabolism. 3α-HSD, which was first identified by its activity in converting dihydrocortisone to 3α-tetrahydrocortisone (14), has been found in mammalian cells (15) and in procaryotes such as *Clostridium perfringens* (16), *Eubacterium lentum* (17), *Pseudomonas putida* (18), and *C. testosteroni* (19, 20). Despite similar substrate specificities, eucaryotic and procaryotic 3α-HSDs belong to two different protein superfamilies. Whereas eucaryotic 3α-HSD (EC 1.1.1.213) belongs to the aldo-keto reductase superfamily (21), the procaryotic 3α-HSD (EC 1.1.1.50) is a member of the short chain dehydrogenase/ reductase superfamily (22–24).

In previous investigations, we isolated and characterized the 3α-HSD enzyme from *C. testosteroni* and determined the primary structure by the cloning and sequencing of its gene (10, 19, 20). 3α-HSD has been shown to mediate the oxidoreduction at position 3 of the steroid nucleus of a great variety of C₁₉ to C₂₇ steroids (10). Surprisingly, this enzyme is also capable of catalyzing the carbonyl reduction of nonsteroidal xenobiotic aldehydes and ketones and has therefore been named 3α-HSD/CR (10). Further studies revealed that the substrate pluriactivity of 3α-HSD/CR, as well as its inducibility, not only increase the resistance of *C. testosteroni* to the steroid antibiotic fusidic acid but also enhance the metabolic capacity of insecticide degradation in this organism (26). However, no information is available on the transcriptional regulation of 3α-HSD/CR itself or on the molecular regulation of the complete steroid degradation pathway.

The present paper provides the first data on the molecular mechanism of steroid signaling in procaryotes. It reports on the regulation of the steroid-inducible gene (*hsdA*) of 3α-HSD/CR, one of the first enzymes in steroid degradation in *C. testosteroni*. 3α-HSD/CR, 3α-HSD/carbonyl reductase; kb, kilobase(s); CAT, chloramphenicol acetyltransferase; pm, mutant promoter; HPLC, high pressure liquid chromatography; RepA, repressor A; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s); Co, control.
teroni. The results suggest that hsdA in C. testosteroni is under negative transcriptional control by a repressor protein, the gene of which has an opposite orientation to that of hsdA and which overlaps the hsdA sequence.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Host strains Escherichia coli HB101 (Promega) and C. testosteroni ATCC 11996 (Deutsche Sammlung für Mikroorganismen) were used for gene cloning and expression. Subcloning of fragments was carried out in plasmids pUC18 and pUC19 (Life Technologies, Inc.), pBluescript II (Amerham Pharmacia Biotech) containing the cat gene, and pBBR1MCS-2 (27). Special importance was the broad host range cloning vector pBBR1MCS-2, which contains the kanamycin resistance gene and which was found in our investigations to be able to replicate not only in E. coli but also in C. testosteroni ATCC 11996. pK18, which contains the kanamycin resistance gene, was a gift obtained from CIBA-Geigy AG, Department of Biotechnology, Basel. The plasmid copy numbers determined were as follows: 80 copies per cell of pUC18 and pUC19 in E. coli, 10 copies per cell of pKK232-8 in E. coli, 6 copies per cell of pBBR1MCS-2 in both E. coli and C. testosteroni, 80 copies per cell of pK18 in E. coli. Copy numbers remained unchanged, notwithstanding the size of the inserted fragments.

General Media and Growth Conditions—Bacterial cells were grown in standard I nutrient broth medium (Merek) or standard LB medium at 37 °C (E. coli) or at 30 °C (C. testosteroni).

Restriction Enzymes and Other Reagents—Restriction enzymes, ligase, shrimp alkaline phosphatase, and S1 nuclease were obtained from Roche Molecular Biochemicals, Biolabs, MBI Fermentas, and Amerham Pharmacia Biotech, and used according to the manufacturers’ instructions. Ampicillin and kanamycin antibiotics were from AGS Biochemicals kit. Primer extension was performed by hybridizing 20,000 cpm-labeled pRse for 20 min at 65 °C with 5 μg of total RNA of each preparation, followed by 10 min at room temperature. Annealed primer was extended by reverse transcriptase (Promega) for 1 h at 41 °C. The resulting mixture was separated on a sequencing gel. As a reference, DNA sequence reactions of pX12 and pAX1 were performed with the same primer (pRse).

DNA Manipulations—Recombinant DNA work was carried out following standard techniques according to Sambrook et al. (28).

Subcloning of the C. testosteroni hsdA Regulatory Region—Subcloning of the C. testosteroni hsdA regulatory region and the preparation of respective plasmids are shown in Fig. 1. The 5.257-kb EcoRI fragment, which has been isolated from C. testosteroni chromosomal DNA in previous investigations (25), was cloned into pUC18 to get p6. p6 was digested with Hinfl and XmnI, and the resulting 2.979- and 2.937-kb fragments were ligated again into pUC18 to give pH2 and pX12, respectively. AvrII (restriction site on the p6 C. testosteroni insert upstream of hsdA) and XbaI (restriction site in pUC18) were used for double digestion of the p6 fragment and ligated bases. The AvrII/XbaI fragment from p6 was then cloned into pBBR1MCS-2, and a corresponding 2.741-kb SphI-ClaI fragment was cloned into pBBR1MCS-2, to generate pBBK2. The same cloning strategies as described above were performed to subclone respective inserts from p6 into pBBR1MCS-2, to obtain pBB21B, pBBH1, pB8X1, and pBBX7. The AvrII/XbaI-ClaI fragment was also cloned into pK18 to get pKAX1. The 2.708-kb EcoRI-ClaI fragment from p6 was cloned into pBBR1MCS-2, and a corresponding 2.741-kb SphI-ClaI fragment was cloned into pBBR1MCS-2, to yield pBBK2. The same cloning strategies as described above were performed to generate pBBK1, pB8X5, and pBBKX1, which represent descendants from pKK232-8 containing the Hinfl-ClaI 0.430 kb, XmnI-ClaI 0.385 kb, and AvrII/XbaI-ClaI 0.185 kb fragments, respectively, from p6 upstream of the cat gene. For getting deleted pBBR1MCS-2/CAT reporter constructs, the cat gene fragment from pKK232-8 was first subcloned with BamH1 and AvrII into pBBR1MCS-2 to yield pBBK1 (data not shown). A 2.735-kb XbaI-ClaI fragment from p6 was then ligated to pBBK1 upstream of cat to receive pBBK4. Similarly, constructs of the Hinfl-ClaI, XmnI-ClaI, and AvrII/XbaI-ClaI fragments plus the cat gene yielded pBBK13, pBBK7, and pBBKX1.

Subcloning of the hsdA Repressor Gene—Subcloning of the C. testosteroni hsdA repressor gene and the preparation of respective plasmids are also shown in Fig. 1. A 2.451-kb KpnI-NdeI fragment of p6 was cloned into either pUC18 or pUC19 to yield pKpN6 or pKpN7, respectively. Plasmids pKAN10 and pKAN12 (containing a 1.030-kb insert) were obtained by excising a 1.421-kb KpnI-AvrII fragment from pKpN6 or pKpN7, respectively.

Transformation of Bacteria—All constructs were verified by restriction digestion of the isolated plasmids. Plasmids were purified with the QiagenTip-100 kit (Qiagen, Hilden, Germany). Ligation was transferred into E. coli or C. testosteroni. Competent cells were prepared by the CaCl2 or electroporation methods. Double plasmid transformations were performed by exploiting the kanamycin resistance gene of pK18 and pBBR1MCS-2 and the ampicillin resistance gene of pUC18, pUC19, and pKK232-8. In these cases both antibiotics were added to the culture medium. Plasmid isolation and agarose gel electrophoresis were performed to prove successful double transformations.

Construction of Mutant Promoters (pM) by Polymerase Chain Reactions—The mutant promoters (pMx, where x indicates the plasmid number) were generated by overlap extension polymerase chain reaction mutagenesis (29). The following set of primers was used (see Table I): pL1 and pR2 for hsdA promoter mutations, pL1 and pR4 for wild type hsdA promoter-cat constructs, pM1 and pMR for the mutated promoter-cat constructs, and pM0 and pR2 for promoterless hsdA constructs. Mutant plasmids were then transferred into E. coli HB101, and 3s-HSD/CR activity was assayed by HPLC analysis as described previously (30) for determining the mutant promoter activity with the CAT reporter assay, mutant promoter-cat constructs were transformed into C. testosteroni, and the CAT activities were assayed as described below. All mutant promoters generated in this study were confirmed by DNA sequencing (MWG Biotec, Ebersberg, Germany).

Primer Extension—Total RNA was prepared from E. coli HB101, previously transformed with plasmids p6, pX12, or pAX1, after growth for 6, 9, 12, or 16 h. The 15-mer oligonucleotide primer pRse (complementary to a sequence within hsdA and later used as the sequencing primer; see Table I) was end-labeled with 32P-ATP and subsequently digested with XbaI to yield a single-stranded 32P-labeled 185-mer. The oligonucleotide (50,000 cpm) was mixed in hybridization buffer with 10 μg of total RNA from E. coli HB101, previously transformed with plasmids p6, pX12, or pAX1, after growth for 6, 9, 12 or 16 h. The mixture was denatured at 85 °C for 10 min and then incubated at 37 °C for 6 h. The annealed oligonucleotide/mRNA hybrid was treated with 200 units of S1 nuclease (Roche Molecular Biochemicals) at 37 °C for 1 h, and the resulting products were separated on a sequencing gel. As a reference, DNA sequence reactions of pX12 and pAX1 were performed with the sequencing primer pRse.

Point and Frameshift Mutations of repA—Point mutations of both possible ATG start codons (resulting in pRm1 and pRm3) and shift mutations of the internal base within the repA sequence (resulting in pRm2 and pRm4) were generated by two-step polymerase chain reaction mutagenesis (29) according to standard procedures. The resulting plasmids were used in cotransformation experiments together with pAX1, and 3s-HSD/CR protein production was measured by ELISA.

Specific Binding of Testosterone to RepA—E. coli HB101 cells, carrying pKpN6, were grown for 18 h, and total protein was isolated. Ten μg of protein (10 μl) was mixed with 0.1 μl of [3H]testosterone at room temperature for 20 min. Nonlabeled testosterone was used in increasing concentrations as a specific binding competitor. The samples were washed on nitrocellulose filters with 0.2 × TEN (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0) and 0.5% Tween 20 buffer and mixed with a scintillator, and the cpm were determined.

Preparation of 5-Nucleotide and 10-Nucleotide Deleted Operator Constructs—Two-step polymerase chain reaction mutagenesis was used to prepare 5-nucleotide and 10-nucleotide deleted mutants (29). All primers and oligonucleotides were prepared by MWG Biotec. The polymerase chain reaction fragments were cloned into p6 for the preparation of pL10n (deletion at 9.935 kb) and pL5n (TGGGC deleted on location 2.569 kb). All clones prepared were sequenced by MWG Biotec.

Mobility Shift Assays—Three 34-bp double-stranded DNA fragments (Op1, Op2, and Op3) for gel shift experiments with RepA were prepared by the annealing of six oligonucleotides synthesized by MWG Biotec. Oligonucleotides (100 pmol) were incubated pairwise in TEN buffer at 95 °C for 10 min and allowed to cool down slowly to 25 °C in 1.5 h. Finally, 4 pmol of DNA was labeled with digoxigenin-11-dUTP. Electrophoretic mobility was performed according to the instructions from Roche Molecular Biochemicals. 8% polyacrylamide was used in gel shift assays. Op1 contained the 10-bp palindromic motif TCAAGGGC at 0.935 kb, Op2 contained the 10-bp palindromic motif TGGGCTTTGA at 2.568 kb, and Op3 represented a region outside the operator motifs and served as a control.

ELISA of 3s-HSD/CR—For the quantification of 3s-HSD/CR pr-
Fusion expression, an ELISA was established, and respective antibodies were generated. Rabbit antibodies directed against 3α-HSD/CR from C. testosteroni were prepared according to standard methods. ELISA plates were coated with protein extracts containing 3α-HSD/CR in coating buffer. After washing, antibodies against 3α-HSD/CR were added in 1:10,000 dilution. The further procedure corresponded to that of the CAT ELISA kit from Roche Molecular Biochemicals.

**Assay of 3α-HSD/CR Activity and CAT Reporter Gene Expression—**

3α-HSD/CR enzyme activity was assessed by HPLC as described previously (10). CAT expression from pBBKAX1 was measured by the ELISA kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Because nontransformed E. coli and C. testosteroni cells exhibited some background CAT activity, fresh competent cells were transformed and then cultivated for 16 h until the cells showed some increase in CAT activity. The results of enzymatic activities (HPLC) and CAT reporter gene expression were determined by both ELISA and HPLC. For CAT reporter gene expression, the entire CAT ELISA kit from Roche Molecular Biochemicals was used, and a single band representing the 5′-end was observed. For the primer extension reaction (Fig. 3A) the sequencing primer pRse (Table 1) complementary to a region within the hsdA coding region (positions +93 to +78), was used, and a single band representing the 5′-end was determined. For the S1 nuclease protection assay (Fig. 3A), a 32P-labeled antisense DNA probe that extended from the AcrII site (at 2.532 kb) to the ClaI site (at 2.708 kb) on the entire EcoRI C. testosteroni fragment of plasmid p6 was used together with total RNA as described above. The 5′-end was thus localized 28 bp upstream of the ATG codon. The 5′-end (marked by an arrow in Fig. 3B) is preceded by a 26 bp spacer. The 32P-labeled probe, which was used for the primer extension reaction (Fig. 3A), was generated by priming the S1 nuclease digestion with the 3′-end primer pRse (Table 1) and the 5′-end primer pRse (Table 1).

**Point Mutations for Determination of the Promoter Sequence**—Site-specific mutations in the –35 (TAGCCCT) and –10 (TGTGAT) motifs were introduced by polymerase chain reaction and are shown in Table II. Mutant promoter fragments were inserted into pAX1 descendants and transferred into E. coli HB101 to determine 3α-HSD/CR activity, or pMx-cat constructs (in plasmid pBBKAX1) were transferred into C. testosteroni to test for CAT expression. As compared with respective control experiments, all point mutations except one resulted in a decrease of 3α-HSD/CR activity or CAT reporter expression (Table II). For example, a T to G transition of the first T in the –10 motif resulted in a residual 3α-HSD/CR activity of only 1.8%, and a T to G transition of the first T in the –35 motif gave a residual CAT expression of only 13%. Interestingly, the C to A transition in the –35 motif yielded an enhanced activity of up to 135% (3α-HSD/CR) and 123% (CAT).

As controls, two mutations located 2 and 3 bp upstream of the –35 sequence were introduced to give plasmids pα-2 and pα-3, which were transferred into E. coli and C. testosteroni. No changes in the promoter activity could be observed, as revealed by both 3α-HSD/CR and CAT determinations (Table II). In addition, we prepared mutant plasmids (pM0 and pBBK1) in which the entire promoter sequence (downstream of the AcrII restriction site) was deleted. Cells transformed with these plasmids showed no 3α-HSD/CR activity or very low CAT activities, respectively (Table II).
These experiments identified the promoter region of hsdA and demonstrated that the 2.35 and 2.10 regions are critical for full activity. These sequences are separated by 17 bp, the optimal distance (17 ± 2 bp) for promoters recognized by the σ70 RNA polymerase holoenzyme.

Identification of a Gene Coding for a Negative Regulator of hsdA Expression—As is the case with many negatively regulated enzymes, the coding region of a putative repressor protein was suspected to lie adjacent to hsdA. To test this hypothesis, the upstream and downstream regions of hsdA were searched for potential repressor genes in separate experiments. First, the 2.708-kb EcoRI-ClaI upstream fragment of hsdA was subcloned into pBBR1MCS-2 to generate pBB7 (Fig. 1). Levels of 3α-HSD/CR expression of p6, pH2, pH2H31, pX12, pBBX1, pAX1, and pBBAX7 were compared after double plasmid transformation with either pBBR1MCS-2 (control) or pBB7 in medium containing ampicillin and kanamycin. From the fact that pBB7 did not affect 3α-HSD/CR expression, it was obvious that no repressor gene is located on the 2.708-kb upstream fragment of hsdA (data not shown).

To search for the coding region of the repressor protein downstream of hsdA, we fused the EcoRI-ClaI fragment from p6 (lacking hsdA) to the cat gene in pKK232–8 (Fig. 1). pKK232–8–controlled and the resulting plasmid descendants pBBK12, pBBKH1, pBBX8, and pBBAX1 were each double-transformed into E. coli with the second plasmids pBBR1MC-
S-2 (control), pBB7, pBBAX7, and pBB21B in medium containing ampicillin and kanamycin. As has already been found with \(3^\alpha\)-HSD/CR expression, the EcoRI-ClaI upstream fragment of \(hsdA\) (pBB7) failed to inhibit CAT expression after double plasmid transformation with pBKHX1, pBKX8, and pBKAX1. In contrast, strong inhibition of CAT expression was obtained when either the downstream fragment of \(hsdA\) (pBBAX7) or the complete 5.257-kb EcoRI fragment (pBB21B) were double-transformed with the cat plasmids (data not shown). These results indicate that the coding region of the repressor protein must be located downstream of the AvrII site (at 2.532 kb) on the EcoRI fragment.

To define the exact coding region of the repressor protein, several plasmids containing deleted constructs of the EcoRI fragment downstream of the ClaI site were prepared. Cotransformation experiments with pKpN6 and pAX1 suggest that the repressor gene is present between the KpnI (at 1.111 kb) and NdeI (at 3.562 kb) restriction sites on the EcoRI fragment (Fig. 4). The presence of an open reading frame (orf4) between 2.065 kb and 3.328 kb gave rise to the possibility that orf4 is the coding gene for the repressor protein, which we named RepA. This was confirmed by cotransformation experiments with pKpN7, which contains the lacZ promoter upstream of orf4 (Fig. 1) and which did not affect \(hsdA\) expression (Fig. 4). To specify the active region of RepA, plasmids pKAN10 and pKAN12 were generated; both plasmids code for a C-terminal truncated form of RepA but differ in their lacZ orientation (Fig. 1). The resulting \(hsdA\) expression data (Fig. 4) proved that RepA is encoded by orf4, which has an opposite orientation to \(hsdA\) and which, interestingly enough, overlaps with the \(hsdA\) sequence. Obviously, the N-terminal half of RepA contains the repressor active region.

RepA Acts as Repressor of \(hsdA\)—Further verification for RepA acting as negative regulator of \(hsdA\) was achieved by point and frameshift mutations generated within the RepA gene (repA) (Fig. 5). Two possible translation start sites are found within the repA sequence at 2.929 and 3.328 kb. Point mutations of both ATGs to GTGs (pRm1 and pRm3) decreased the inhibitory effect of RepA on \(hsdA\) expression compared with respective controls (pKpN6) with full RepA activity and low \(hsdA\) expression (Fig. 5). The generation of a frameshift mutation by insertion of an additional base after the putative start codons of repA (pRm2 and pRm4) led, in both cases, to a complete loss of RepA activity and full \(hsdA\) expression. These results indicate the translation start point of repA to be located at 3.328 kb. Moreover, the fact that a frameshift mutation within repA provides full \(hsdA\) expression proves that RepA acts as a repressor protein and excludes the possibility of an antisense RNA effect.

The repA gene is 1.263 kb long, and the deduced amino acid sequence comprises 420 amino acid residues with a calculated molecular mass of 45.4 kDa. A homology search of the predicted primary structure of RepA against protein data bases failed to detect significant identities to any other known protein.
Regulation of 3α-HSD/CR Gene Expression in C. testosteroni

Cell extracts of *E. coli* HB101 (transformed with p6-derived mutant promoter fragments) and of *C. testosteroni* (harboring mutant promoter-cat constructs derived from pBBKAX1) were prepared and tested for 3α-HSD activity or CAT expression as described under “Experimental Procedures.” Single point mutations are underlined and indicated in italics. 3α-HSD activities (nmol × min⁻¹ × mg⁻¹) of protein are given as percentages relative to control values obtained with plasmids pAX1 and pBBKAX1. The data correspond to averages ± S.D. of at least four independent determinations.

| Original Sequences | Mutated sequences | CAT activity | Promoter activity |
|--------------------|-------------------|--------------|-------------------|
| TAGCCT             | pM101             | 0.21 ± 0.03  | 3α-HSD           |
| TGGAT              | pM102             | 0.24 ± 0.20  | 0.5%             |
| TGGAT              | pM103             | 6.22 ± 0.74  | 5.8%             |
| TTTGAT             | pM104             | 3.48 ± 0.16  | 55%              |
| TTTGAT             | pM105             | 8.15 ± 0.30  | 9.6%             |
| TTTGAT             | pM106             | 1.03 ± 0.35  | 22%              |
| TTTGAC             | pM351             | 2.35 ± 0.51  | 40%              |
| ATGTAGCCT          | pM352             | 1.51 ± 0.41  | 10%              |
| ATGTCCGCT          | pM353             | 1.22 ± 0.22  | 12%              |
| ATGTAGCCT          | pM354             | 0.47 ± 0.20  | 4.2%             |
| ATGTAGACT          | pM355             | 0.09 ± 0.05  | 0.52 ± 0.21      |
| ATGTAGCAT          | pM356             | 0.8%         | 3.3%             |
| ATGTAGCCG          | pM-2               | 15.5 ± 2.04  | 20.3 ± 4.33      |
| ATGTAGCCG          | pM-3               | 0.36 ± 0.09  | 13%              |
| ATGTAGCCG          | pM-0               | 3.1%         | 5.2%             |
| ATGTAGCT           | pBBK1              | 9.86 ± 1.42  | 16.6 ± 1.73      |
| ATGTAGCT           | pUC18              | 4.1%         | 18%              |
| ATGTAGCT           | pBBR1MSC-2         | 9.82 ± 0.55  | 17.8 ± 1.07      |

**FIG. 4. Repression of hsdA by RepA.** 3α-HSD/CR (μg/mg of protein) was assayed by ELISA in *E. coli* after pAX1 cotransformations with pK18, pKpN6, pKpN7, pKAN10, and pKAN12. Cotransformation of p6 and pK18 served as a control. Four independent plates were transformed, and each extract was assayed in duplicate. Repression of hsdA by RepA (gene product of orf 4 in Fig. 1) is observed if the lacZ promoter is at the Ndel site (pKpN8 and pKAN10) but not at the KpnI (pKpN6) or AvrII (pKAN12) sites. Obviously, the repressor active region is located on the N-terminal half of RepA, due to hsdA repression in cells carrying pKAN10.

**Binding of Testosterone to RepA**—The binding of testosterone to RepA was assayed by mixing protein extracts from *E. coli* HB101 cells, carrying pKpN6 (repA) or pK18 (control), with [3H]testosterone (Fig. 6). Nonlabeled testosterone served as a specific competitor and decreased [3H]testosterone binding to RepA in a concentration-dependent manner, finally leading to control values (pK18) at an excess of 100-fold nonlabeled steroid (Fig. 6).

*Two 10-bp Palindromic Sequences as Cis-acting Elements for hsdA Regulation—*Two palindromic 10-bp motifs were localized at 0.935 kb (TCAAAGCCCA = Op1) and at 2.568 kb (TGGGCTTTGA = Op2) upstream of hsdA and were tested as cis-acting operator elements for hsdA regulation (Figs. 7 and 8). The amount of 3α-HSD/CR produced in *E. coli* cells transformed with plasmids carrying deletions in these sequences is shown in Fig. 7. Deletion of Op1 (pL10n) resulted in an increase in hsdA expression compared with p6. Upon addition of the steroid testosterone (Fig. 7, + ster.), hsdA expression increased 8-fold in strains carrying either p6 or pL10n, compared with “noninduced” conditions, *i.e.* without steroid. Because Op2 overlaps the −10 binding site of the σ70 RNA polymerase by 5 bp, only half of Op2 could be deleted (pL5n) without affecting the hsdA promoter structure. Here, no alterations in hsdA expression could be observed, even in the presence of testosterone. However, the effect of testosterone “induction” of hsdA is seen in *E. coli* strains cotransformed with pAX1 and pKpN6 (the latter coding for RepA) as well as in wild-type *C. testosteroni* cells (Fig. 7). In these cases, testosterone led to a reversal of hsdA repression. Together, these results point to a specific
Interaction between RepA, testosterone, and the operator sequences.

**Gel Mobility Shift Experiments**—The specific interaction between RepA, testosterone, and the operator sequences was proved by gel mobility assays (Fig. 8). Three 34-bp double-stranded DNA fragments, Op1 and Op2 (containing the two palindromic 10-bp operator sequences on locations 0.935 kb and 2.568 kb, respectively) as well as Co (a sequence 17 bp upstream of Op2 that served as a control), were used. As shown in Fig. 8B (lanes 1 and 6), both Op1- and Op2-containing DNA fragments gave band shifts upon the presence of RepA, indicating the binding of RepA to the operator sequences. The specificity of this protein-DNA interaction was confirmed by mixing a 100-fold excess of nonlabeled Op1- or Op2-containing fragments to the assays. The band shift of both Op1 and Op2 disappeared with nonlabeled Op1 and Op2 (and *vice versa*) as specific competitors (lanes 2, 3, 7, and 8). Fragment Co, which did not contain the palindromic operator motif, could not compete for specific RepA binding (Fig. 8B, lanes 4 and 9). Finally, protein extracts from *E. coli* carrying pK18 (lacking repA) did not result in band shifts, either with Op1 or with Op2 (Fig. 8B, lanes 5 and 10). The interaction between RepA and Op2 was then investigated in the presence or absence of testosterone (Fig. 8C). The reversal of RepA binding to Op2 by testosterone was clearly demonstrated.

**A Model on the Regulation of hsda Expression**—The factors controlling the expression of hsda in *C. testosteroni* are summarized in Fig. 9. A negative regulator of hsda is encoded by a gene, repA, which has an opposite orientation to hsda and which overlaps the hsda sequence. In the absence of “inducing” steroids, the RepA protein binds to operator sequences (Op1 and Op2) and blocks hsda transcription. In the presence of appropriate steroids, however, these bind to RepA, thereby reducing its ability to bind to the operator region. Hence, induction of hsda expression by steroids in fact is a derepression, where steroid “inducers” prevent the binding of a repressor protein to the operator. Upon dissociation of the repressor from the operator, the polymerase binds to the promoter, and transcription of 3α-HSD/CR mRNA is initiated.

**DISCUSSION**

Microbial metabolism of steroids is of considerable interest academically and because of the potential applications of this process in the pharmaceutical and food industries as well as in human medicine and environmental biotechnology. Because biotransformation of steroids in procaryotes is so important, there is considerable interest in the mechanism of its regula-
**FIG. 8.** Gel mobility shift experiments. The specific interaction between RepA, testosterone, and the two operator sequences, at 0.935 kb (Op1, TCAAAGC-CCA) and at 2.568 kb (Op2, TGGGCTTTG-TGA), was proved by gel mobility assays. A, three 34-bp double-stranded DNA fragments (containing Op1 and Op2, as well as a sequence 17 bp upstream of Op2 that served as a control (Co)) were used. B, both Op1- and Op2-containing DNA fragments gave band shifts upon the presence of RepA (lanes 1 and 6). Upon addition of a 100-fold excess of nonlabeled Op1 or Op2, the band shifts disappeared (lanes 2, 3, 7, 8). Fragment Co could not compete for specific RepA binding (lanes 4 and 9). Extracts from *E. coli* carrying pK18 (lacking repA) did not result in band shifts (lanes 5 and 10). C, the interaction between RepA and Op2 was tested in the presence or absence of testosterone. The reversal of RepA binding to Op2 by testosterone is clearly shown.
hsdA binds to operator sequences (Op1 and Op2) and blocks testosterone. In the absence of inducing steroids, the RepA protein promoter, and transcription of 3RepA, which is then released from the operator region. Upon dissociation. In the presence of appropriate steroids, however, these bind to HSD/CR mRNA is initiated. Hence, induction of 3aHSD/CR transcriptional regulation in procaryotes, either on hsdA or on the complete steroid degradative pathway.

To follow up our previous work on the molecular biology and structure of 3α-HSD/CR (10, 19, 24, 25, 31), we investigated the transcriptional regulation of this important enzyme in C. testosteroni. In this study, we present the first data on the molecular mechanism of steroid induction in procaryotes. Southern blot experiments revealed the 3α-HSD/CR gene (hsdA) to be located on a 5.257-kb EcoRI genomic fragment of C. testosteroni ATCC 11996 (25). Immediately downstream of hsdA, the gene of another steroid-metabolizing enzyme was found, Δ5–3-ksi, which has been cloned and sequenced previously (32).

Analysis of the C. testosteroni hsdA regulatory region led to the identification of the operator and promoter region upstream of hsdA. Interestingly, we could identify a gene (repa) that codes for a negative regulator protein for hsdA expression. The repA gene has an opposite orientation to hsdA and overlaps the hsdA sequence. The repressor protein (RepA) obviously binds to the operator region under noninduced conditions, thus preventing binding of the RNA polymerase to the hsdA promoter. It is suggested that in the presence of appropriate steroid inducers, these bind to RepA, thereby altering its conformation and reducing its affinity to the cis-regulating operator sequences. Upon dissociating from the operator, the polymerase binds to the promoter, and transcription of 3α-HSD/CR mRNA is initiated. Hence, induction of 3α-HSD/CR expression in C. testosteroni by steroids actually appears to be a derepression by preventing the binding of a repressor protein to regulatory DNA regions (Fig. 9), like that of the well known lac operon in E. coli. Corresponding to this idea, Arai et al. (33) suggested that the expression of the genes for phenol degradation in C. testosteroni TA 441 are under negative control by AphS, which belongs to the GntR family of transcriptional regulators (34).

Interestingly, downstream of hsdA is another gene encoding a steroid-metabolizing enzyme, the gene for Δ5–3-ketosteroid isomerase (32, 35). Δ5–3-ketosteroid isomerase (steroid Δ-isomerase, EC 5.3.3.1) catalyzes the isomerization of the double bond of, e.g. 5-androstene-3,17-dione to 4-androstene-3,17-dione by a direct intramolecular and stereospecific transfer of the C-4β proton to the C-6β position. The resulting double bond is then in conjugation with the 3-keto group, a fact that is important prior to ring A aromatization and ring B opening.

From an examination of the proposed catabolic pathway (36), it can be estimated that the complete degradation of the steroid nucleus requires more than 20 enzymatic reactions. With a pathway of this size, it is conceivable that the encoding genes are clustered into operons or belong to a steroid-dependent regulon. In this respect, the close proximity of the two genes, hsdA and Δ5–3-ksi, is intriguing. Interestingly, parts of a bile acid-inducible operon have been identified in Eubacterium sp. strain VPI 12708. In this procaryote, at least six new polypeptides of different sizes have been identified by one- and two-dimensional gel electrophoresis, and their encoding genes have been cloned, sequenced, and localized to overlapping fragments of chromosomal DNA (37). From these and some other observations, the authors concluded that all six open reading frames are transcribed as a polycistronic message from a single bile acid-induced operon. In addition, evidence was provided that the genes of some steroid-metabolizing enzymes, 3-oxo-steroid Δ1-dehydrogenase and 3-oxo-steroid Δ4–5α-dehydrogenase, in Arthrobacter simplex and C. testosteroni (38) are clustered to one operon.

It is meanwhile well established that HSDs act as pre-receptor control devices by promoting either the synthesis or degradation of steroid hormones, thereby regulating their physiological action. Therefore, like other short-chain dehydrogenase/reductase proteins, 3α-HSD/CR from C. testosteroni may also have an important function in the conversion of signaling molecules (steroids) to either the active or inactive state. Moreover, the analysis of short-chain dehydrogenase/reductase protein sequences revealed an unexpected relationship between steroid dehydrogenases and NodG and FixR proteins, signal molecules that mediate nodulation of legume roots by nitrogen-fixing (22, 23, 25). The nodulation process is initiated by the secretion from the plant of a flavonoid, which stimulates the transcription of nodulation (nod) genes in the target rhizobia. The remarkable similarities between the actions of flavonoids in the communication between plants and rhizobia to that of steroid-mediated actions in vertebrates (39), together with the occurrence of steroid-binding proteins and HSDs in bacteria (40, 41), are relevant to the question whether the elements for transcriptional activation or inactivation of genes by steroid-like molecules are also present in procaryotes.

Steroid hormones were originally assumed to be exclusively the products of vertebrate endocrine organs, implying a recent evolutionary origin. However, several steroid hormones have been isolated from plant sources, where they are considered to play a prominent part in plant growth, development, and flowering (42, 43). Interestingly, recent findings provide evidence for the existence in microbial cells of fully developed functional signal transduction pathways that are capable of recognizing and responding to the corresponding mammalian hormones (44). Detailed analysis of steroid-protein interactions in bacteria might therefore provide insight into the evolution of the hormonal systems found in more complex life forms. Recognition may also lead to the discovery of new systems for the microbial transformations of steroids and identification of...
novel ligands, which, through genetic manipulation, can be effectively utilized in health care or biotechnology.

Hence, steroids play a particularly important role in certain procaryotes, because they may simultaneously serve both as signal molecules and as a carbon source. The same applies for procaryotic HSDs, such as 3α-HSD/CR from C. testosteroni. These enzymes, on the one hand, have their role in procaryotic steroid hormone signal transduction, and, on the other hand, participate in steroid degradation.

In conclusion, induction of hsda in C. testosteroni actually appears to be a derepression of gene transcription by specific binding of the steroid inducer molecule to a repressor protein. Under noninduced conditions the repressor protein (encoded by repA) prevents transcription by binding as a trans-acting negative regulator to operator sequences upstream of hsda. The present paper is the first to give insights into the genetics of steroid-dependent gene regulation in procaryotes and provides a firm foundation for further investigations into the genetics of steroid catabolism in bacteria.

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Regulation of the Steroid-inducible 3α-Hydroxysteroid Dehydrogenase/Carbonyl Reductase Gene in \textit{Comamonas testosteroni}

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