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Steroidogenic activity of a peptide specified by the reversed sequence of corticotropin mRNA

(complementary peptide/antisense peptide/molecular recognition theory/peptide mimetic/molecular code)

BENJAMIN L. CLARKE AND J. EDWIN BLALOCK*

Department of Physiology and Biophysics, University of Alabama at Birmingham, UAB Station BHSB 896, Birmingham, AL 35294

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ABSTRACT **The molecular recognition theory predicts that a reversed (3′→5′) reading of an mRNA should yield a peptide that is structurally and functionally similar to that specified in the 5′→3′ direction. We tested this idea by synthesizing a corticotropin (ACTH) analogue using a reverse reading of bovine mRNA for ACTH-(1–24). This peptide, designated ACTH-3′→5′, had a similar hydropathic profile to native ACTH-5′→3′ but had only 30% sequence homology and eight different charge substitutions. ACTH-3′→5′ specifically bound to the surface of mouse Y-1 adrenal cells and to polyclonal anti-ACTH antibody. Additionally, ACTH-3′→5′ stimulated cAMP synthesis and steroidogenesis in adrenal cells. These findings show that ACTH-3′→5′ mimics the corticotropic properties of native ACTH, thereby further validating the molecular recognition theory.**

The molecular recognition theory proposes that complementary nucleotide sequences encode peptides (termed complementary peptides) that interact as a result of a genetically determined inversion of their respective hydropathic profiles (1, 2). This idea was initially validated by showing that corticotropin (ACTH) would bind with specificity and high affinity to a synthetic peptide (HTCA-5′→3′) specified in the 5′-to-3′ direction by RNA complementary to that of ACTH. Furthermore, antibody to peptide HTCA bound the ACTH receptor, indicating a structural similarity between the complementary peptide and the receptor binding site (1). Subsequently, the generality of these findings have been shown in a number of different ligand–receptor systems (for review, see ref. 3). An unexpected revelation from this theory is that, in terms of binding, the particular amino acid sequences of two complementary peptides are probably less important than their respective hydropathic profiles. This finding was graphically demonstrated by showing that when amino acids are assigned in the nonconventional 3′-to-5′ direction for HTCA RNA, a peptide (termed HTCA-3′→5′ or generically antisense peptide) results that is hydropathically identical to HTCA-5′→3′ and yet shares only 25% amino acid homology (4). Interestingly, HTCA-5′→3′ and HTCA-3′→5′ bound ACTH with equal affinity. We and others have shown that such antisense peptides are antigenically related (5, 6). The hydropathic similarity of antisense peptides derives from the observation that the middle base of a codon signifies the hydropathic character of the amino acid. Thus, reading a single mRNA in opposite directions will result in hydropathically similar peptides with different amino acid sequences (4). The distinguishing characteristic will be the respective orientation of their amino and carboxyl termini, but this can be overcome by switching the termini of one peptide relative to the other. Because structural similarity was observed for the receptor-like antisense peptides (i.e., HTCA), we tested whether a similar situation existed for the hormonal counterpart (i.e., ACTH). Here we report that this approach yields a peptide (ACTH-3′→5′) with little primary structural homology, yet with considerable hydropathic and functional similarity to ACTH. Specifically, the peptide elevated glucocorticoid levels in adrenal cells and was recognized by antibody to ACTH as well as by the ACTH receptor. These findings suggest another way to design peptide mimetics and describe an intrinsic quality of genetic information.

MATERIALS AND METHODS

Peptide Synthesis. The ACTH-3′→5′ peptide was synthesized by Multiple Peptide Synthesis (San Diego, CA) using solid-phase 9-fluorenylmethoxycarbonyl (f-Moc) chemistry. Purity was assessed as >98% by HPLC with a C_{18} reverse-phase column and 10–60% acetonitrile linear gradient in water/0.1% trifluoracetic acid. A subsequent mass spectral analysis showed that all protective groups had been removed. Sequence analysis of ACTH-3′→5′ was done by the University of Alabama Cancer Center Protein Analysis Core Facility, Birmingham, and confirmed that the synthesis was correct. The ACTH analogue [Phe^2, Nle^4]ACTH (where Nle is norleucyl) was synthesized and radiolabeled with ^{125}I as described (7). Cortrosyn was obtained from Organon.

Cell Culture. The adrenal glands from decapitated male Sprague–Dawley rats (150–200 g) were rapidly excised and stored in sterile RPMI 1640 medium (GIBCO)/1% bovine serum albumin on ice. The adipose tissue was removed before squeezing the medulla and fasciculata-reticularis layers away from the capsule. The inner adrenal tissue was suspended in 1 ml of RPMI 1640 medium/1% bovine serum albumin per two glands and minced by opposing slices with two razor blades; collagenase type IV (Sigma) and DNase I (Sigma) were then added to a final concentration of 1 mg/ml and 0.1 mg/ml, respectively. The digestion mix was incubated for 15 min at 37°C with mild agitation, then spun to a pellet, resuspended in fresh RPMI 1640 medium/1% bovine serum albumin, and finally poured through a sterile (50-mesh) sieve. Viable cells were isolated by centrifugation on Ficoll/Hypaque and cultured for 3–5 days in RPMI 1640 medium/10% fetal calf serum before use in bioassays.

Binding on mouse Y-1 adrenal cells was assayed in 24-well plates (Costar, Mark II). Cells were grown in RPMI 1640 medium/10% fetal calf serum to a density of ≈500,000 cells per well before measuring ligand binding. Binding of ^{125}I-labeled ACTH to the surface of Y-1 mouse adrenal cells was measured essentially as described for antibody (7) but instead in the presence of ACTH-5′→3′ or ACTH-3′→5′. The cells were mixed with various concentrations of nonlabeled peptide plus 140 pM radiolabeled probe and incubated for 30 min at room temperature, washed three times with RPMI 1640

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Abbreviations: ACTH, corticotropin; GHRH, growth hormone-releasing hormone.

*To whom reprint requests should be addressed.

medium/10% fetal calf serum (GIBCO), and then assayed for cell-associated radioactivity.

General Information. All chemicals were of reagent grade unless otherwise specified. ACTH binding to rabbit anti-ACTH antibody (Incstar, Stillwater, MN) was detected by using anti-rabbit antibody-coated microspheres (Kirkegaard and Perry Laboratories, Gaithersburg, MD), cAMP was detected by radioimmunoassay (Rainin Assay System, DuPont, Billerica, MA), and corticosterone was detected by radioimmunoassay (Radioassay Systems Labs, ICN). Centrifugations were performed in either a Sorvall RT6000B refrigerated table-top unit or in a Sorvall Microspin model 24S.

RESULTS

Fig. 1*A* illustrates the concept used to design an ACTH mimetic; the bovine ACTH mRNA sequence and the resulting peptides when the mRNA is read in the 5'-to-3' and 3'-to-5' direction (ACTH-5'→3' and ACTH-3'→5', respectively) are shown. Clearly the amino acid sequences differ—only 8 of 24 residues are identical between the two peptides. Interestingly, these similarities arise only from palindromic codons in the mRNA. In contrast, the hydropathic profiles (Fig. 1*B*) for the two peptides are extremely similar and, when analyzed by pairing hydropathic scores of corresponding amino acids in the sequences, give a correlation coefficient of 0.8022 with $P < 0.01$. This finding argues strongly for similar hydropathic properties of both peptides and quite possibly for similar secondary structure. Indeed, an Edmundson wheel (9) for ACTH-3'→5' is almost identical to ACTH-5'→3' (10), implying that both peptides have the potential to form amphipathic α-helical structures (11).

To experimentally test for similar biochemical activity, we first determined whether ACTH-3'→5' would compete for binding sites with ACTH-5'→3'. Fig. 2 shows that ACTH-3'→5' (as designed in Fig. 1) could competitively block specific ^{125}I-labeled-ACTH-5'→3' binding to the surface of Y-1 mouse adrenal cells in a radioreceptor assay. The 50% inhibitory dose for ACTH-5'→3' was compared with reported findings on rat adrenal cells (12), and the IC_{50} dose for ACTH-3'→5' was ≈2.5 logarithmic units less potent. Such inhibition was observed in four separate radioreceptor assays. The slopes of the binding-inhibition curves of these four radioreceptor assays were not significantly different for ACTH-5'→3' or ACTH-3'→5' (−28.28 ± 5.3 and −35.25 ± 7.5, respectively). A second binding analysis was performed by using another known ACTH binding protein, an antibody to ACTH-5'→3'. Fig. 3 shows that the anti-ACTH IgG binds both ACTH-5'→3' and ACT-3'→5' in a radioimmunoassay (RIA). In this assay, the ACTH-3'→5' performed as a more potent inhibitor because the competition curves only differed by 1.5 logarithmic units or a factor of 32.

The preceding studies showed that ACTH-5'→3' and ACTH- 3'→5' were antigenically related and also probably bound to the same receptor. To show that the peptides are functionally related requires that they cause similar biological activities. ACTH stimulates cAMP production in adrenal cells as a committing step toward corticosterone release (13). Stimulation of corticosterone synthesis by ACTH-3'→5' would be strong evidence that a receptor-mediated event is involved. Accordingly, we found that 1 nM ACTH-3'→5'

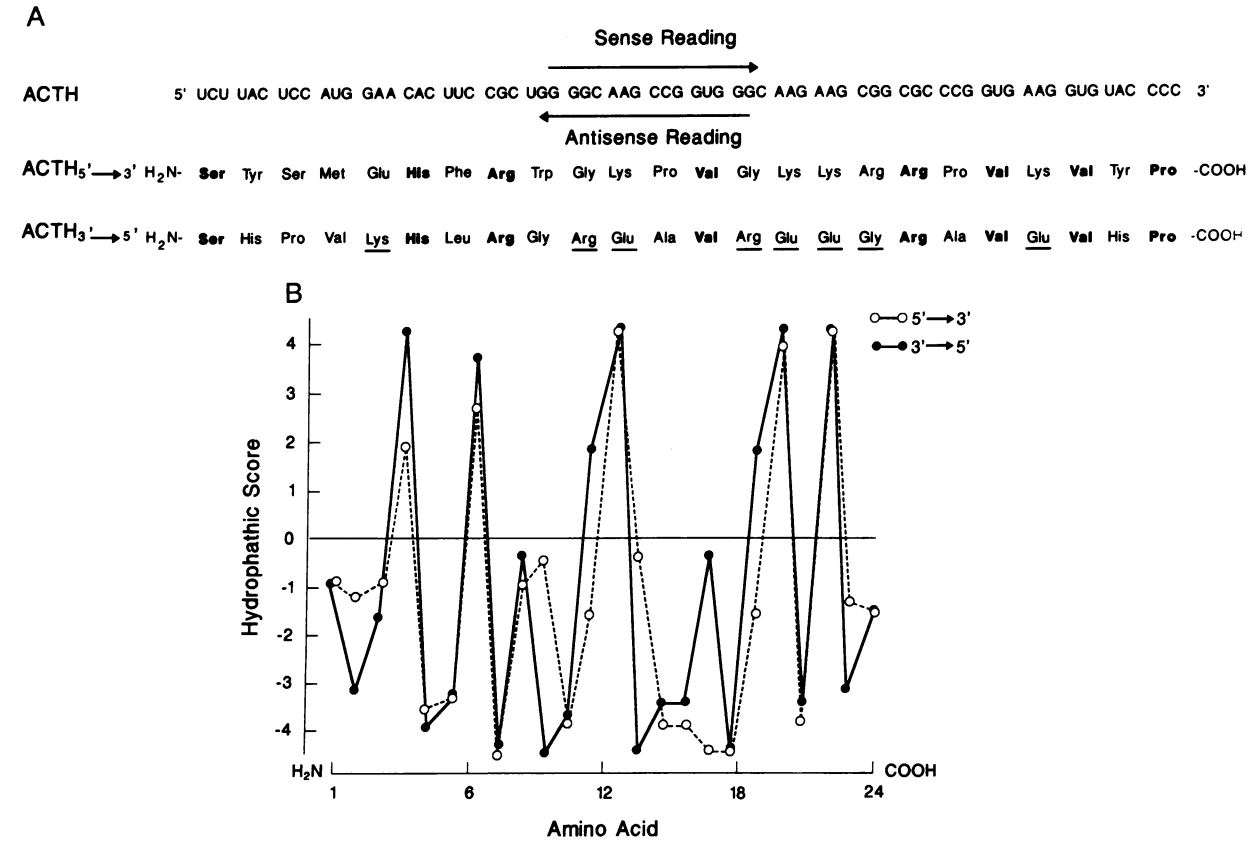

FIG. 1. Design and synthesis of hydropathically similar ACTH peptides derived from mRNA read in the 5'→3' and 3'→5' directions. Syntheses of two different ACTH analogues were patterned after the same mRNA by reading the codon sequence in the opposite direction. (*A*) Codon sequence for bovine ACTH-(1–24) mRNA and the corresponding amino acids decoded from reading in the 5'→3' direction or the 3'→5' direction. Amino acids labeled in boldface represent residues not changed by reversed reading; underlined amino acids represent changes in charged residues. (*B*) Hydropathic profile of ACTH-3'→5' superimposed over the hydropathic profile of ACTH-5'→3'. The hydropathic scoring is based on the Kyte and Doolittle scale (8).

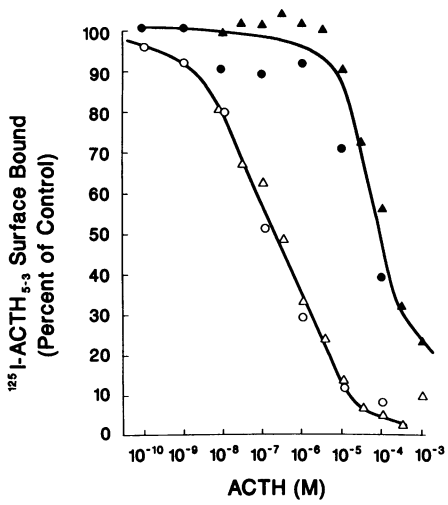

FIG. 2. ACTH-3'→5' blocks ACTH-5'→3' binding to ACTH receptors. The binding of ^{125}I-labeled ACTH to the surface of Y-1 mouse adrenal cells was measured essentially as described for antibody (7) but rather with ACTH-5'→3' (○) or ACTH-3'→5' (●). Cells were mixed with various concentrations of nonlabeled peptide plus 140 pM radiolabeled probe and incubated for 30 min at room temperature, washed three times with RPMI 1640 medium/10% fetal calf serum (GIBCO), and then assayed for cell-associated radioactivity. Circles and triangles are replicate experiments.

stimulated Y-1 mouse adrenal cells to produce 280 pmol of cAMP per 10^6 cells per 10 min compared with cells in the presence of 1 nM ACTH-5'→3' that produced 350 pmol of cAMP per 10^6 cells per 10 min. A corresponding production of corticosterone was also measured; the ACTH-5'→3' titer was ≈30 times greater than the ACTH-3'→5' titer (Fig. 4). The induction of steroidogenesis was specific because it occurred with ACTH-5'→3' or ACTH-3'→5', whereas four other peptides had a negligible effect (Table 1).

To test the generality of this observation, the same approach was used to synthesize an antisense peptide for growth hormone-releasing hormone (GHRH) residues 1–23 (GHRH-3'→5', 17% homology with GHRH-5'→3'). GHRH-3'→5', but not ACTH-releasing hormone, luteinizing hormone-releasing hormone, or vasoactive intestinal peptide

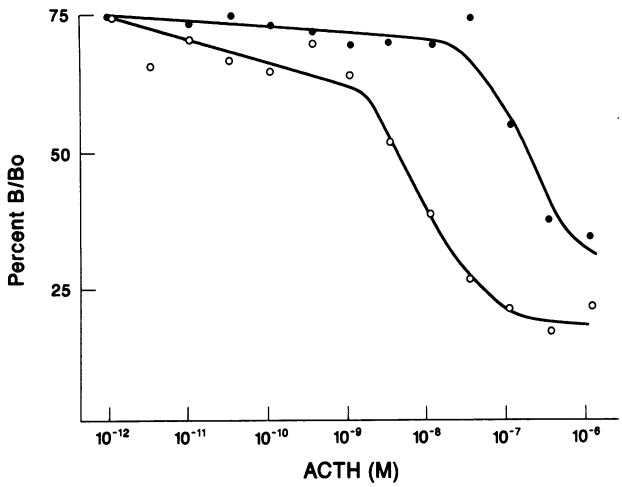

FIG. 3. Both ACTH-3'→5' and ACTH-5'→3' are recognized by anti-ACTH polyclonal antibody. An RIA procedure was used to measure binding of rabbit anti-ACTH antisera to ^{125}I-labeled ACTH in the presence of various concentrations of ACTH-5'→3' (○) or ACTH-3'→5' (●). Immunocomplexes were pelleted by centrifugation with anti-rabbit IgG microspheres and assayed for precipitated radioactivity. B/Bo, bound/free.

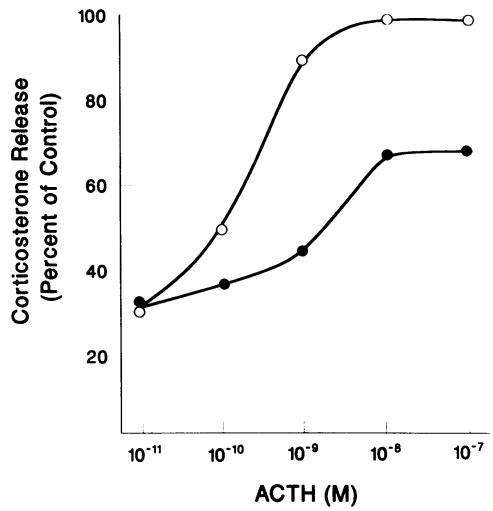

FIG. 4. Isolated rat adrenal cells are stimulated to release corticosterone by ACTH-3'→5'. Rat adrenal glomerulosa/reticularis cells (≈1 × 10^5 cells per sample) were treated with various concentrations of ACTH-5'→3' (○) and ACTH-3'→5' (●) for 24 hr. The culture medium was then sampled and assayed for corticosterone by an RIA procedure. Each point represents the mean of triplicate samplings normalized to a maximal response of 450 ng of corticosterone per 10^5 cells per 24 hr at 10^{-7} M ACTH.

competed with GHRH-5'→3' for binding to both the GHRH receptor and antibody to GHRH. In contrast with ACTH-3'→5', GHRH-3'→5' was an antagonist that inhibited GHRH-5'→3'-induced growth hormone mRNA synthesis and growth hormone release from pituitary cells (D. A. Weigent, B.L.C., and J.E.B., unpublished work). An interesting future question will be to determine why one antisense peptide is an agonist while another antisense peptide is an antagonist.

DISCUSSION

Our objective in this report was to test the functional significance of a fundamental property of the genetic code as it relates to the molecular recognition theory (1–4, 11). A subtle designation for a peptide's secondary structure is intrinsic to the second base of each of its codons. Codons with uridine in the middle-base position virtually always code for hydrophobic amino acids; in contrast, codons with adenine in the middle-base position virtually always code for hydrophilic amino acids. A weaker correlation exists for codons with

Table 1. Steriodogenic effects of various peptides

Peptide	Length, no. of amino acids	Corticosterone production, ng per 10^5 cells per 24 hr ± SD
ACTH-3'→5'	24	256 ± 8
ACTH-5'→3'	24	412 ± 20
GHRH	20	75 ± 28
RWEAPRNAL	9	107 ± 13
LHRH	10	71 ± 26
HTCA-5'→3'	24	146 ± 41
None	NA	100 ± 11

Corticosterone production in the presence of 10 nM of peptide was measured as described in Fig. 4. Sequences for ACTH-3'→5' and ACTH-5'→3' are in Fig. 1, GHRH is reported in ref. 14, luteinizing hormone-releasing hormone (LHRH) is [Ac-D-*p*-Cl-Phe^{1,2},D-Trp^3, D-Arg^6,D-Ala^{10}]LHRH described in ref. 15, peptide HTCA is described in ref. 1, and RWEAPRNAL is a control sequence in single-letter code. NA, not applicable.

cytosine at the middle position, which codes for a collection of weakly hydrophilic amino acids, and guanosine, which codes for weak and strong hydrophilic amino acids. Assuming that the hydropathic profile is a fundamental property driving formation of secondary structure (16), the putative conformation can be approximated by reading the second base of each codon from the mRNA for any peptide. In this study, we have taken advantage of the symmetrical properties found in a linear array of four coding elements that are grouped into sets of three. Reading a set of three bases in opposite directions translates different amino acids except for the case of palindromic triplets. However, reading directions do not greatly alter the hydropathic profile because the sequence of middle bases is not affected by this reversal; only the polarity with respect to amino and carboxyl termini is reversed in this new peptide. Because the first two bases determine identity of the amino acid with few exceptions (4), reversed reading will change the amino acid sequence; but because the hydropathic profile remains virtually unchanged, the secondary structure should also remain similar. In contrast to hydropathicity, there were eight different changes of charged amino acids but with only a half-charge change in the net dipole. This difference in charge profile may explain why ACTH-3′→5′ was not totally equivalent to ACTH-5′→3′ in terms of agonist potency. Other experimental evidence suggests that charge and pH may play an important role for peptide interactions with membranes (17), and this effect of charge substitution has been seen for ACTH-5′→3′ (18).

The observation that a radically different peptide derived from reversed mRNA sequence, only 30% homologous and with eight different charge substitutions compared with native ACTH, has 1/30th the specific activity and can elicit ≈70% of the maximal response to ACTH strongly suggests that the genetic code may contain more information than previously thought. Perhaps this imprint of information in the noncoding direction is an evolutionary remnant that preceded the polarity of genetic information. Regardless of its origins, however, it presents a practical and simple way to design peptide mimetics. Furthermore, the findings imply that the linear pattern of amino acid hydropathy rather than the amino acid sequence *per se* may define secondary and tertiary structure, thereby dictating function. Minor changes in primary sequence may then serve only to increase or decrease affinity.

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