TRANSCRIPTIONAL REGULATION OF THE MOUSE ADENYLYL CYCLASE TYPE 4 (Adcy4) IN Y1 ADRENOCORTICAL TUMOR CELLS

By

Xianliang Rui

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Pharmacology and Toxicology
University of Toronto

© Copyright by Xianliang Rui 2010
Transcriptional Regulation of the Mouse Adenylyl Cyclase Type 4 (Adcy4) in Y1 Adrenocortical Tumor Cells

Xianliang Rui
Degree of Doctor of Philosophy, 2010
Department of Pharmacology and Toxicology
University of Toronto

ABSTRACT

Adenylyl cyclase (Adcy) is an important early effector of adrenocorticotropic hormone (ACTH) on the adrenal cortex; however, this enzyme consists of ten isozymes in mammalian cells and the factors governing the expression of different Adcy isozymes have not been well defined. The aim of this study is to investigate the regulation of mouse Adcy4, one of ten isozymes, in Y1 adrenocortical tumor cells and in mutant subclones derived from the Y1 cells. Adcy4 is expressed at a high level in brain but at lower levels in many other tissues including the Y1 cells. Moreover, this isozyme is specifically deficient in Y1 mutants with impaired steroidogenic factor 1 (SF1) activity. These observations support a hypothesis that Adcy4 expression is influenced by both ubiquitously expressed and tissue-specific transcription factors. My sequencing results indicate that mouse Adcy4 is highly homologous to the human and rat counterparts; its gene is located less than 1 kb downstream of Ripk3 and contains 26 exons. Primer extension and in silico analyses suggest that Adcy4 contains a TATA-less promoter and initiates transcription from multiple sites. Luciferase reporter gene assays indicate that Adcy4 promoter activity is mainly stimulated by the proximal GC-rich region but is inhibited by the first intron. This 124 bp GC-rich region is well conserved among several mammalian species and exhibits strong promoter activity in Y1 cells, which is functionally compromised in the Adcy4-deficient mutant. Within this region, three Sp1/Sp3- and one SF1-binding sites have been identified which bind the corresponding proteins Sp1 and Sp3 or SF1 in electrophoretic mobility shift assays (EMSAs). Site-directed mutagenesis reveals that the 5'-most Sp1/Sp3 site enhances Adcy4 promoter activity,
whereas the middle Sp1/Sp3 and SF1 sites each repress this activity. In Y1 mutant cells, mutating the SF1 site restores *Adcy4* promoter activity and knocking down SF1 with shRNA increases *Adcy4* expression. All these data demonstrate that *Adcy4* expression is under the control of the ubiquitous factors Sp1 and Sp3 and the tissue-specific factor SF1 and establish that SF1 is a repressor for *Adcy4* promoter activity. This study is the first to demonstrate a repressor function for SF1 in certain promoter contexts.
I would like to thank my supervisor, Dr. Bernard Schimmer, for providing me precious opportunity to do my Ph. D. research in his lab. Dr. Schimmer has inculcated me with valuable instruction on scientific writing skills and rigorous logical thinking that will certainly benefit me for the rest of my life. His exact research style and perseverance in pursuing perfection have set up a high standard for me to be a good scientist and prompted me to strive for nothing less than high-quality research. Without his excellent supervision, enthusiastic support, as well as his confidence in my ability as an independent researcher, this thesis would not have been possible.

I would also like to thank my thesis advisory committee, Dr. David Riddick and Denis Grant, for their suggestions, guidance and generous help throughout my Ph. D. research. Their expertise, patience and support greatly enhanced both my research and course study.

I am exceedingly grateful for many past and current members in Dr. Schimmer’s Lab for their friendship, scientific discussion, and technical help. More specifically, I would like to express my sincere appreciation to Jenny Tsao, whose excellent technical assistance and generous help have made my whole graduate research much easier. I also owe my gratitude to Martha Cordova for her constant technical advises and frequent help with many experiments. This work has been generously supported by the funding from NSERC, Department of Pharmacology and Toxicology, and University of Toronto.

Finally, I am deeply indebted to my parents and my brothers for their love, encouragement and unaltering support over these years. I would like to thank my lovely daughter, Ting, and my little angel, Rosie, who always give me joys and inspiration. Most
importantly, I would like to thank my wife, Chunhui, for her unconditional love and support, which give me the strength to finish this work.
TABLE OF CONTENTS

TITLE PAGE .....................................................................................i
ABSTRACT .....................................................................................ii
ACKNOWLEDGMENTS .....................................................................iv
TABLE OF CONTENTS .....................................................................vi
LIST OF TABLES ..............................................................................ix
LIST OF FIGURES ...........................................................................x
LIST OF ABBREVIATIONS ................................................................xii
LIST OF PUBLICATIONS .................................................................xv

SECTION I. INTRODUCTION .............................................................1-56
  1. Adenylyl Cyclase .................................................................1
     1.1 Isozymes and Structure .................................................1
     1.2 Regulatory Properties ..................................................6
     1.3 Tissue Distribution and Physiological Function ...............11
     1.4 Gene Structure and Transcriptional Regulation ...............15
  2. Adrenal Steroidogenesis .........................................................17
     2.1 Steroidogenesis in the Adrenal Cortex .........................19
     2.2 Regulation of Adrenal Steroid Biosynthesis .................24
  3. Essential Role of cAMP Signaling in Adrenal Steroidogenesis .28
  4. Essential Role of SF1 in the Regulation of Steroidogenesis ....33
     4.1 SF1 and LRH1 .............................................................36
     4.2 SF1 Expression Profiles and its in vivo Function .............39
     4.3 SF1 Target Genes .........................................................41
     4.4 SF1 Structure and its Functional Regulation .................43
  5. The Roles of Transcription Factors Sp1 and Sp3 in Steroidogenesis .48
  6. Rationale, Hypothesis, and Objectives ...................................52

SECTION II. MATERIALS AND METHODS ..................................57-85
  1. Synthetic Oligonucleotides ................................................57
  2. Plasmids ...........................................................................61
     2.1 Plasmids .......................................................................61
     2.2 Reporter Gene Constructs .............................................61
        2.2.1 Reporter Plasmids with Progressive Truncation of the
              5'-Flanking Region of Adcy4 ......................................62
        2.2.2 p-631/-290 Adcy4 Reporter Plasmids ......................64
        2.2.3 p-404_{Adcy}Luc Reporter Plasmid with Mutations in the
              –404/-320 Region ......................................................64
        2.2.4 p-404_{Adcy}Luc Reporter Plasmid with the Internal
              –135/–19 Region of Adcy4 Deleted ..............................65
        2.2.5 p-404_{Adcy}Luc Reporter Plasmid with the First Intron of
              Adcy4 Deleted ........................................................64
        2.2.6 pTA-Luc-Based Heterologous Reporter Plasmids ..........70
        2.2.7 Reporter Constructs with Sp1A, Sp1B, and/or SF1
              Mutations in the Conserved Region .........................72
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>Purification of Plasmid DNA</td>
</tr>
<tr>
<td>3.</td>
<td>Cell Culture and Transfection</td>
</tr>
<tr>
<td>4.</td>
<td>Preparation of Chromosomal DNA</td>
</tr>
<tr>
<td>5.</td>
<td>Isolation of RNA</td>
</tr>
<tr>
<td>6.</td>
<td>RT-PCR and Real-Time PCR</td>
</tr>
<tr>
<td>7.</td>
<td>DNA Sequencing</td>
</tr>
<tr>
<td>8.</td>
<td>Southern Blot Analysis</td>
</tr>
<tr>
<td>9.</td>
<td>Western Blot Analysis</td>
</tr>
<tr>
<td>10.</td>
<td>Primer Extension</td>
</tr>
<tr>
<td>11.</td>
<td>DNA-protein Interaction</td>
</tr>
<tr>
<td>11.1</td>
<td>Preparation of Nuclear Protein Extracts</td>
</tr>
<tr>
<td>11.2</td>
<td>DNase I Footprinting</td>
</tr>
<tr>
<td>11.3</td>
<td>Electrophoretic Mobility Shift (EMSA) Assays</td>
</tr>
<tr>
<td>11.4</td>
<td>Chromatin Immunoprecipitation (ChIP) Assays</td>
</tr>
<tr>
<td>12.</td>
<td>Luciferase Reporter Gene Assays</td>
</tr>
<tr>
<td>13.</td>
<td>shRNA-Mediated Gene Knockdown</td>
</tr>
<tr>
<td>14.</td>
<td>Statistical Analyses</td>
</tr>
</tbody>
</table>

SECTION III. RESULTS AND DISCUSSION

1. Determination of Mouse Adcy4 Gene Structure and its Expression in Selected Tissues and Cell Lines |
   1.1 Sequences of Mouse Adcy4 cDNA and its Deduced Protein |
   1.2 Intron-exon Organization and Restriction Map of Adcy4 |
   1.3 Conservation of Sequence in the 5′-Flanking Regions of Adcy4 from Different Mammalian Species |
   1.4 Global Integrity of the Adcy4 Locus in Y1 and Mutant Adrenal Cells |
   1.5 Adcy4 Expression in Selected Tissues and Cell Lines |
   1.6 Transcription Start Site(s) of Adcy4 |
   1.7 Discussion |

2. Identification of Regulatory Regions in the 5′-Flanking DNA of Mouse Adcy4 |
   2.1 Consequences of 5′ Truncations and Internal Deletions on the Promoter Activity of Adcy4 5′-Flanking DNA |
   2.2 Nuclear Protein Binding Sites in the Adcy4 Promoter Regulatory Regions |
   2.3 The Contribution of the –404/-336 Region to Adcy4 Promoter Activity |
   2.4 The Contribution of the Conserved Region from –135 to –19 to Adcy4 Promoter Activity |
   2.5 Discussion |

3. Contributions of Sp1, Sp3, and SF1 to Adcy4 Promoter Activity |
   3.1 Sp1, Sp3, and SF1 Binding Sites in the Proximal Region of the Adcy4 Promoter |
   3.2 Contributions of the Sp1, Sp3, and SF1 Binding Sites to
Adcy4 Promoter Activity

3.3 Inhibitory Effects of SF1 on Adcy4 Promoter Activity

3.4 Effects of SF1 Knockdown on the Expression of Endogenous Adcy4 in Adcy4 Deficient Mutant Cells

3.5 Discussion

SECTION IV: GENERAL DISCUSSION AND FUTURE DIRECTIONS

1. General Discussion

2. Future Directions

2.1 Characterize the Regulatory Mechanisms of Sp1 and SF1 Binding Sites and Intron I Element on Adcy4 Promoter Activity

2.1.1 Define the Mechanisms Behind the Repressive Effects of the Sp1B and SF1 Sites on Adcy4 Promoter Activity

2.1.2 Define the Mechanisms Behind the Inhibitory Effect of the first Intron on Adcy4 Promoter Activity

2.1.3 Investigate the Recruitment of Coactivators or Corepressors by the Sp1A, Sp1B and SF1 Sites

2.1.4 Evaluate the Contributions of the Sp1C Site and its Neighboring Unidentified Motif to Adcy4 Promoter Activity

2.2 Explore the Inhibitory Effect of SF1 on Global Gene Expression in Y1 Adrenal Cells

2.2.1 Define the Signature Genes Repressed by SF1

2.2.2 Explore Potential SF1 Binding Sites in the Promoter Regions of SF1-Repressed Target Genes

2.2.3 Validate the Direct Repressor Function of SF1 on the Promoters of SF1-Repressive Genes

2.3 Investigate the Regulatory Role of Gβγ Subunits in the Gene Expression of Mc2r and Adcy4

SECTION V: REFERENCES
LIST OF TABLES

Table I-1. Regulatory properties and tissue distribution of mammalian adenyl cyclases ................................................................. 7
Table I-2. Chromosomal localization of the Adcys ....................................................... 16
Table II-1. Primers for Adcy4 cDNA ................................................................. 57
Table II-2. Primers for Adcy4 gene ................................................................. 58
Table II-3. Oligonucleotides for EMSA ............................................................... 59
Table II-4. Primers targeting vectors and other genes ........................................... 60
Table III-1. RIKEN clones and their tissues of origin ......................................... 102
**LIST OF FIGURES**

| Fig. I-1. | Proposed structure of membrane-bound adenylyl cyclases | 3 |
| Fig. I-2. | Biosynthetic pathways of steroid hormones in the adrenal cortex | 20 |
| Fig. I-3. | Signaling pathways involved in the regulation of steroiogenic gene transcription in adrenocortical cells | 27 |
| Fig. I-4. | Structural organization of nuclear receptors and SF1 | 35 |
| Fig. I-5. | Structural motifs in Sp/KLF factors | 49 |
| Fig. II-1. | Progressive truncation of the 5′-flanking region of Adcy4 | 63 |
| Fig. II-2. | Generation of mutations in the -404/-320 region of Adcy4 | 66 |
| Fig. II-3. | Internal deletion of the -135/-19 region of Adcy4 | 68 |
| Fig. II-4. | Deletion of the first intron of Adcy4 | 69 |
| Fig. II-5. | Construction of pTA-Luc-based luciferase reporter plasmids | 71 |
| Fig. II-6. | Mutation of Sp1A, Sp1B, and/or SF1 sites in the -142/-19 region of Adcy4 | 73 |
| Fig. III-1. | Homology search of the mouse expression tag (est) database with rat Adcy4 cDNA sequence | 87 |
| Fig. III-2. | Mouse Adcy4 cDNA and deduced protein sequences | 89-90 |
| Fig. III-3. | Predicted mouse, rat, and human Adcy4 secondary structures | 91 |
| Fig. III-4. | Mouse Adcy4 gene structure and restriction sites | 93 |
| Fig. III-5. | Conserved 5′-flanking region of Adcy4 among different species | 94 |
| Fig. III-6. | Alignment of the conserved 5′-flanking sequences of Adcy4 among different species | 96 |
| Fig. III-7. | Analysis of the integrity of the Adcy4 gene by Southern-blot | 97 |
| Fig. III-8. | Detection of Adcy4 transcripts in different tissues and cell lines by RT-PCR | 99 |
| Fig. III-9. | Determination of the transcription start sites of Adcy4 by primer extension | 101 |
| Fig. III-10. | Transcription start sites of mouse Adcy4 predicted by in silico analyses | 103 |
| Fig. III-11. | Effects of 5′ deletions on Adcy4 promoter activity in Y1 and mutant 10r9 cells | 112 |
| Fig. III-12. | Effects of 5′ deletions on Adcy4 promoter activity in Y1 and mutant 10r6 cells | 114 |
| Fig. III-13. | Effects of the conserved proximal region and intron I on Adcy4 promoter activity | 116 |
| Fig. III-14. | DNase I footprinting analysis of the Adcy4 5′-flanking region | 117 |
| Fig. III-15. | Assessment of enhancer-like activity in the FP2 region (-631/-290) in reporter gene assays | 119 |
| Fig. III-16. | Effects of the nuclear protein protected -382/-321 region on the Adcy4 promoter activity | 120 |
| Fig. III-17. | Promoter/enhancer-like activity of the highly conserved region in Y1 and mutant 10r6 cells | 122 |
| Fig. III-18. | Identification of nuclear protein interactions in the proximal promoter region of Adcy4 | 129 |
Fig. III-19. Identification of Sp1 and SF1 binding sites in the Oligo2 and Oligo3 ……..131
Fig. III-20. Characterization of Sp1, Sp3 and SF1 binding in the sequences of oligonucleotides 2 and 3 .................................................................132
Fig. III-21. Identification of a Sp1 binding site in oligonucleotide 4 and a SF1 binding site in oligonucleotide 7 .........................................................134
Fig. III-22. The binding of nuclear extracts from Y1 and mutant 10r6 cells to SF1 sites in oligonucleotides 3 and 7 .......................................................136
Fig. III-23. Assessment of SF1 binding in vivo by chromatin immunoprecipitation (ChIP) assay .................................................................137
Fig. III-24. Contributions of the Sp1 and SF1 binding sites to Adcy4 promoter activity .................................................................139
Fig. III-25. Effect of transient SF1 expression on Adcy4 promoter reporter activity in Y1 and 3T3 cells .........................................................141
Fig. III-26. Effect of SF1 knockdown on Adcy4 mRNA levels in mutant 10r6 cells .................................................................143
Fig. IV-1. Schematic model of Adcy4 promoter regulation in Y1 and its forskolin- resistant mutant cells ........................................151
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>ABCG5/G8</td>
<td>ATP-binding cassette G5/G8</td>
</tr>
<tr>
<td>ABST</td>
<td>Apical sodium-dependant bile acid transporter</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Adcy</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>Adx</td>
<td>Adrenodoxin</td>
</tr>
<tr>
<td>AF-1/2</td>
<td>Activation function 1/2</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotension II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASP</td>
<td>Adrenal specific protein</td>
</tr>
<tr>
<td>AT1R</td>
<td>Type I angiotension II receptor</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAF</td>
<td>Brahma-related gene (BRG)/Brahma (BRM)-associated factor</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BRE</td>
<td>TFIIB recognition element</td>
</tr>
<tr>
<td>BRG1</td>
<td>Brahma-related gene 1</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;/CaM</td>
<td>Calcium/calmodulin</td>
</tr>
<tr>
<td>CAGE</td>
<td>Cap analysis of gene expression</td>
</tr>
<tr>
<td>CamK</td>
<td>Calmodulin dependent kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CBP/P300</td>
<td>CREB binding protein/E1A binding protein p300</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol ester transfer protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitaiton</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>COUP-TF1</td>
<td>Chicken ovalbumin upstream promoter transcription factor 1</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element-binding protein</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP-response element modulator</td>
</tr>
<tr>
<td>CRS</td>
<td>cAMP-responsive sequence</td>
</tr>
<tr>
<td>CRSP</td>
<td>Cofactors required for Sp1 coactivation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450 enzyme</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>Cholesterol 7α-hydroxylase</td>
</tr>
<tr>
<td>CYP8B1</td>
<td>Sterol 12α-hydroxylase</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>Cytochrome P450 cholesterol side-chain cleavage</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>11β-Hydroxylase</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>Aldosterone synthase</td>
</tr>
<tr>
<td>CYP17</td>
<td>17α-Hydroxylase</td>
</tr>
<tr>
<td>CYP19</td>
<td>Aromatase</td>
</tr>
<tr>
<td>CYP21</td>
<td>21-Hydroxylase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAX-1</td>
<td>Dosage-sensitive sex reversal/adrenal hypoplasia congenita critical region on the X chromosome</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCE</td>
<td>Downstream core element</td>
</tr>
<tr>
<td>DGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DPE</td>
<td>Downstream core promoter element</td>
</tr>
<tr>
<td>DRIP</td>
<td>Vitamin D interacting protein</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Early growth response protein 1</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>est</td>
<td>Expression tag</td>
</tr>
<tr>
<td>FATE1</td>
<td>Fetal and adult testis expressed 1</td>
</tr>
<tr>
<td>FSHβ</td>
<td>Follicle-stimulating hormone β</td>
</tr>
<tr>
<td>Fsk</td>
<td>Foskolin</td>
</tr>
<tr>
<td>Ftz-F1</td>
<td>Fushi tarazu factor-1</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GATA</td>
<td>GATA element binding protein</td>
</tr>
<tr>
<td>GCN5</td>
<td>General control nonderespressed 5</td>
</tr>
<tr>
<td>GCNF(Nr6a1)</td>
<td>Germ cell nuclear factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled seven transmembrane receptor</td>
</tr>
<tr>
<td>GRIP1</td>
<td>Glucocorticoid receptor interacting protein 1</td>
</tr>
<tr>
<td>α-GSU</td>
<td>α-Subunit of glucoprotein hormone</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>12-HETE</td>
<td>12-Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone response element</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>3β-HSD2</td>
<td>3β-Hydroxysteroid dehydrogenase 2</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>INR</td>
<td>Initiator</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>Jak2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LHβ</td>
<td>Luteinizing hormone β</td>
</tr>
<tr>
<td>12-LO</td>
<td>12-lipoxygenase</td>
</tr>
<tr>
<td>L-LTP</td>
<td>Late-LTP</td>
</tr>
<tr>
<td>LRH1</td>
<td>Liver receptor homolog 1</td>
</tr>
<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP1</td>
<td>MluI-box Binding Protein 1</td>
</tr>
</tbody>
</table>
MC2R: Melanocortin 2 receptor
Med: Mediator complexe
MIS: Müllerian-inhibiting substance
MRP3: Basolateral multidrug resistance protein 3
MTE: Mutation ten element
NCBI: The National Center for Biotechnology Information
NCoR: Nuclear receptor co-repressor
NE: Nuclear extract
NF-κB: Nuclear factor kappa-B
NGF-IB: Nerve growth factor IB
NuRD: Nucleosome remodeling and histone deacetylation complex
NURR1: Nuclear receptor related 1
Oct-1: Mitochondrial intermediate peptidase
Olf-1: Olfactory neuron-specific transcription factor
PA: Phosphatidic acid
PC2: Positive cofactor 2
PCR: Polymerase chain reaction
PE: Phosphatidylethanolamine
PET tag: Paired-end tag
PIC: Preinitiation complex
PIP2: Phosphatidylinositol 4,5-bisphosphate
PIP3: Phosphatidylinositol 3,4,5-triphosphate
PKA: Protein kinase A
PKC: Protein kinase C
PKD: Protein kinase D
PLC: Phospholipase C
PLD: Phospholipase D
POR: Cytochrome P450-oxidoreductase
PSF: PTB-associated splicing factor
Ptx1: Pituitary homeobox 1
5'-RACE: Rapid amplification of 5' complementary DNA ends
Ripk3: Receptor-interacting Ser-Thr protein kinase 3
RNA Pol II: RNA polymerase II
SF1(Nr5a1): Steroidogenic factor 1
SHP: Short heterodimer partner
shRNA: Small hairpin interfering RNA
SMCC: SRB/MED containing cofactor complex
SMRT: Silencing mediator of retinoic acid and thyroid hormone receptor
Sox9: SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)
Sp: Specificity protein
KLF: Kruppel-like factor family
SPH: Sphingosine
SR-BI: Scavenger receptor BI (SR-BI)
Src: Src tyrosine kinase
SRC-1: Steroid receptor coactivator 1
SRY: Sex-determining Region Y
StAR: Steroidogenic acute regulatory protein
STAT: Signal transducer and activator of transcription protein
SUMO: Small ubiquitin modifier
SWI/SNF: SWItch/Sucrose NonFermentable
TAF: TBP-associated factor
TBP: TATA box binding protein
TFIID: Transcription factor IID
Tkt: Transketolase
TRAP: Thyroid hormone receptor associated protein
TreP-132: Transcriptional regulating protein of 132 kDa
VDR: Vitamin D receptor
VMH: Ventromedial hypothalamic nucleus
WT-1: Wilms’ tumor 1

LIST OF PUBLICATIONS


SECTION I. INTRODUCTION

1. Adenylyl Cyclase

Adenylyl cyclases (Adcys) are the enzymes that synthesize the universal second messenger cAMP from ATP. They function as central relay stations which receive and integrate the signals from diverse hormones, neurotransmitters, and drugs (Barzu et al. 1994). The enzymatic activities of the adenylyl cyclases are either stimulated or inhibited by these signals and their product cAMP in turn activates target proteins such as protein kinases, ion channels, and transcription factors. In mammalian cells at least four known types of cAMP effector proteins have been identified: protein kinase A (PKA), exchange proteins activated by cAMP (EPACs), cyclic nucleotide gated ion channels (CNGs and HCNs) (Kopperud et al. 2003), and the recently reported phosphodiesterase type 10 (Gross-Langenhoff et al. 2006). The final effect of these cAMP actions is to control a variety of fundamental physiological functions ranging from cell growth and differentiation, gene transcription, to apoptosis and memory (Antoni 2000; Sunahara et al. 2002).

1.1 Isozymes and Structure

In mammals, adenylyl cyclases have multiple isozymes. The first brain-derived isozyme was cloned by the group of Gilman in 1989 (Krupinski et al. 1989). To date, a total of nine isozymes of the membrane-bound enzymes have been cloned, sequenced and more or less characterized, each with a molecular weight of about 120 kDa (Sunahara et al. 2002). In addition, a soluble isozyme of roughly half the size, which is not responsive to G-proteins or other regulators of the membrane-bound enzymes, was discovered (Buck et al. 1999).
These enzymes, based on the order of their publications, are numbered from Adcy1 to Adcy10. Because different Adcy isoforms demonstrate significant diversity in their regulation, the various family members can be broadly divided into groups according to the similarities in their sequences and regulatory properties. Group 1 contains Adcy1, 3, and 8 and is stimulated by calcium and calmodulin (Choi et al. 1992; Krupinski et al. 1992). Group 2 consists of Adcy2, 4, and 7, which are activated by Gβγ subunits of the heterotrimeric G proteins when the active α subunit of the stimulatory GTP binding protein Gs is also present (Gao et al. 1991; Tang et al. 1991a; Yoshimura et al. 1996). Group 3, consisting of Adcy5 and 6, are the predominant Adcys in the heart. These two Adcy isoforms are inhibited by low concentration of calcium, by the Gαi subunit and by PKA (Ishikawa et al. 1992; Katsushika et al. 1992). Group 4 has only one isoform, Adcy9, and is insensitive to either calcium or Gβγ subunits, but is regulated by calcineurin (Paterson et al. 1995). The last group is comprised of the soluble isozyme Adcy10, which is not regulated by many of the known activators of Adcys (fluoride, Gα subunit, forskolin). This enzyme is activated by bicarbonate and calcium, and is highly expressed only in spermatozoa (Chen et al. 2000).

Deduced from the amino acid composition, all membrane-bound Adcy isoforms share the characteristic secondary structure depicted in Fig. I-1 (Hanoune et al. 1997; Patel et al. 2001). They all contain a short and variable N-terminal region, followed by two hydrophobic transmembrane spans (M1 and M2) and two long cytoplasmic domains (C1 and C2), each of about 40 kDa. The transmembrane regions each contain predicted six membrane-spanning helices that usually consist of short sequences (20-25 aa) rich in hydrophobic residues and are not homologous among various isoforms from different
Fig. I-1. Proposed structure of membrane-bound adenylyl cyclases
families. Except for their potential role in membrane localization, the function of M1 and M2 is largely unknown despite the twelve-transmembrane domain topology reminiscent of the ABC family of transporters. The C1 and C2 cytoplasmic domains can be subdivided into a and b subdomains. The C1a and C2a domains are well conserved among the mammalian enzymes with 50-70% identity in amino acids. They are also homologous to each other and contain all of the catalytic apparatus. The C1b and C2b domains are less conserved. The C1b region is relatively larger (~15 kDa), variable, and contains several regulatory sites, while the C2b region is extremely short, only present in Adcy1, 2, 3, and 8 isozymes, and lacks identified functions (Hurley 1999). Studies revealed that expressing half of the adenylyl cyclase molecule separately (i.e. M1C1 or M2C2) did not give any adenylyl cyclase activity (Katsushika et al. 1993; Tang 1995). However, when the two halves of the adenylyl cyclase molecule (i.e. M1C1 and M2C2) were co-expressed (Katsushika et al. 1993; Tang 1995), or the C1a and C2a regions were expressed as a fusion protein with a linker (Tang et al. 1995; Scholich et al. 1997), the adenylyl cyclase activity was reconstituted, and could be regulated by GTP bound GoS subunit and forskolin. Moreover, the individually expressed C1a and C2a, when mixed together after purification, also form an active enzyme (Whisnant et al. 1996; Yan et al. 1996). These observations demonstrate that the two cytoplasmic domains are essential for the enzyme activity and that they have to interact with each other to form a catalytically active core.

In the last ten years, the determination of the crystal structure of the catalytic core, together with site-directed mutagenesis analysis, have provided a new context for understanding the mechanism of function and the actions of many regulators of Adcys (Kamenetsky et al. 2006). The first structure of Adcy catalytic domain to be solved was an
enzymatically inactive mammalian C2 homodimer (Zhang et al. 1997), followed by the crystal structure of a heterodimer formed by the C1a region of Adcy5 and the C2 region of Adcy2, a breakthrough in understanding the catalytic process (Tesmer et al. 1997; Tesmer et al. 1999). Thereafter, structures for several mycobacterial Adcys and a cyanobacterial soluble Adcy homolog also were characterized (Steegborn et al. 2005; Kamenetsky et al. 2006). These studies revealed that even though the topologies of the membrane bound and soluble Adcy isozymes are quite different, the catalytic core structure as well as most residues involved in substrate binding and catalysis are well conserved among them, suggesting that they share a common catalytic mechanism. The two cytoplasmic domains appear to interact head-to-tail, forming a pseudosymmetrical structure in the active conformation. The catalytic site is located at one end of the dimer interface and formed by residues from both cytoplasmic domains. The other end is degenerate, and in membrane bound Adcys, this degenerate center is part of the binding site for forskolin, which allosterically activates most of the membrane bound mammalian Adcys (Tesmer et al. 1997; Tesmer et al. 1999). At the catalytic core, six amino acids (Asp396 and Asp440 from C1a and Lys938, Asp1018, Arg1029 and Asn1025 from C2a) serve the principal functions in catalysis. Two highly conserved aspartate residues (Asp396 and Asp440) and the phosphate tails of the bound substrate ATP accommodate the two metal cofactor ions (Mg\(^{2+}\) or Mn\(^{2+}\)), which enable a nucleophilic attack of the 3’–OH group of the ribose on the \(\alpha\) phosphoryl group of ATP. These two aspartates are contributed by the C1 cytoplasmic domain (Tesmer et al. 1997; Tesmer et al. 1999). The four other residues are donated by the C2 domain. Lys938 and Asp1018 bind to the adenine moiety and are replaced in guanylyl cyclases by Glu and Cys, respectively. Substitutions of the corresponding amino acids in guanylyl
cyclases convert the substrate specificity to ATP (Tucker et al. 1998). These data suggest that these two residues are crucial for substrate selection (Linder 2005). The last two amino acids, Arg1029 and Asn1025, are believed to stabilize the transition state. Similarly, the forskolin binding site is also formed by the participation of about eight amino acids derived from both the C1a and C2a domains (Tesmer et al. 1997). By binding to the residues in both domains, forskolin stabilizes the interactions between the two heterodimer domains and hence augments the activity of the enzyme.

1.2 Regulatory Properties

Despite their structural similarities, the mammalian Adcy enzymes are differentially modulated by multiple primary and secondary regulators (Antoni 2000; Hanoune et al. 2001; Sunahara et al. 2002). The regulatory properties of the different Adcys are listed in Table 1-1. The membrane bound Adcy isozymes are primarily regulated by G proteins via ligand bound GPCR; however, they also receive signals from other sources, including Ca^{2+} loaded calmodulin, protein kinases (PKA, PKC and calmodulin kinase), phosphatases (calcineurin), and other smaller molecules such as forskolin, calcium and P site inhibitors. Thus these isozymes are able to cross-talk and integrate signals from different regulatory pathways. In contrast, soluble Adcy cannot be activated by G proteins, forskolin, and many of the above regulators; it is specifically regulated by bicarbonate and possibly calcium.

Heterotrimeric G proteins (Gαs, Gαi and Gβγ) are the major regulators of mammalian Adcys. Gαs-GTP directly activates all nine membrane bound Adcy isozymes by a factor of 3 to 20 and this activation is synergistic, not competitive, with respect to forskolin (Gilman 1987; Linder 2006). GTP bound Gαs displays a tenfold greater affinity for
<table>
<thead>
<tr>
<th>Group</th>
<th>Isozymes</th>
<th>Tissue distribution</th>
<th>Stimulators</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Adcy1</td>
<td>Brain (neuron), adrenal gland (medulla)</td>
<td>$\text{Go}_s$, Fsk, Ca$^{2+}$/CaM, PKC (weak)</td>
<td>$\text{Go}_i$, Go$_z$, Gi$\gamma$, CaM kinase IV, P-site analogs</td>
</tr>
<tr>
<td></td>
<td>Adcy3</td>
<td>Brain, olfactory epithelium, male germ cells, pancreas, brown adipose tissue, uterus</td>
<td>$\text{Go}_s$, Fsk, Ca$^{2+}$/CaM, PKC (weak)</td>
<td>$\text{Go}_i$, CaM kinase II, P-site analogs, Gi$\gamma$?</td>
</tr>
<tr>
<td></td>
<td>Adcy8</td>
<td>Brain, Lung, testis, adrenal, uterus, heart</td>
<td>$\text{Go}_s$, Fsk, Ca$^{2+}$/CaM</td>
<td>$\text{Go}_i$, P-site analogs, Gi$\gamma$?</td>
</tr>
<tr>
<td>Group 2</td>
<td>Adcy2</td>
<td>Brain, skeletal muscle, lung, heart</td>
<td>$\text{Go}_s$, Fsk, G$\beta\gamma$, PKC</td>
<td>P-site analogs</td>
</tr>
<tr>
<td></td>
<td>Adcy4</td>
<td>Brain, heart, kidney, liver, lung, brown adipose tissue, uterus</td>
<td>$\text{Go}_s$, Fsk, G$\beta\gamma$</td>
<td>P-site analogs, PKC</td>
</tr>
<tr>
<td></td>
<td>Adcy7</td>
<td>Brain, platelets, widespread</td>
<td>$\text{Go}_s$, Fsk, G$\beta\gamma$, PKC</td>
<td>P-site analogs</td>
</tr>
<tr>
<td>Group 3</td>
<td>Adcy5</td>
<td>Heart, brain, kidney, liver, lung, uterus, adrenal</td>
<td>$\text{Go}_s$, Fsk, PKC-$\alpha$ and -$\zeta$</td>
<td>$\text{Go}_i$, Go$_z$, Ca$^{2+}$, PKA, P-site analogs, G$\beta\gamma$</td>
</tr>
<tr>
<td></td>
<td>Adcy6</td>
<td>Widespread</td>
<td>$\text{Go}_s$, Fsk</td>
<td>$\text{Go}_i$, Go$_z$, Ca$^{2+}$, PKA, PKC, P-site analogs</td>
</tr>
<tr>
<td>Group 4</td>
<td>Adcy9</td>
<td>Brain, skeletal muscle, widespread</td>
<td>$\text{Go}_s$</td>
<td>Calcineurin, P-site analogs</td>
</tr>
<tr>
<td>Group 5</td>
<td>sAdcy</td>
<td>Testis, other tissues</td>
<td>Bicarbonate, Ca$^{2+}$</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Adapted from Hanoune et al. (2001), Patel et al. (2001) and Sunahara et al. (2002)

\(^b\) In the tissues underlined, the corresponding isozymes are expressed at high level.
activating Adcs compared to the Gαs-GDP form (Sunahara et al. 1997). Crystal structure reveals that the main contact site for Adcy and Gαs occurs between a crevice formed by part of C2 and the N-terminal portion of C1a in Adcy and the short α2-helix of Gαs, which is part of a switch II structure that undergoes a conformational change upon GTP binding (Sprang 1997; Tesmer et al. 1997). In contrast, members of Gαi family act as secondary regulators and selectively inhibit only Adcy1, Adcy5, Adcy6, and possibly Adcy8 isozymes (Taussig et al. 1993; Taussig et al. 1994). They apparently do not affect basal activities of these enzymes, but rather the activities stimulated by physiological regulators. The brain enriched Gαo, however, can inhibit Ca^{2+}/calmodulin activated Adcy1 and Adcy8. Interestingly, the mode of these inhibitions is not through direct competition with Gαs; it appears that Gαi docks onto a site, symmetrical to the Gαs binding site, which is located on the opposite site of the C1a/C2 dimer (Dessauer et al. 1998). Similar to Gαo, the Gβγ subunits of heterotrimeric G proteins are a secondary or conditional regulator of Adcy activity, but it shows a complex pattern of modulation. Released mainly through the activation of inhibitory G proteins Gαi, the Gβγ subunits stimulate the activity of Adcy2, Adcy4 and Adcy7 when Gαs is coactivated (Gao et al. 1991; Tang et al. 1991a). This regulation enables Gβγ to establish a synergistic relationship with Gαs, whereby the presence of Gβγ might significantly increase the ability of Gαs to activate these Adcy isozymes. Such synergistic “cross talk,” which showed enhanced activation of Adcy2 and Adcy4 by simultaneous activation of Gαs- and Gαi-coupled receptors has been reported in transfected cell lines (Chen et al. 1995). At the same time, Gβγ can also function as a powerful inhibitory regulator of Adcy1 and Adcy8 (Sternweis et al. 1984). This regulation is particularly relevant for the physiology of brain, where Gβγ can markedly inhibit the effects
of forskolin, $\text{G}\alpha_s$, and $\text{Ca}^{2+}$/calmodulin on Adcy activity. In cotransfection experiments $\text{G}\beta\gamma$ was shown to inhibit the activity of Adcy5 and Adcy6, but a direct interaction between these G protein subunits and the Adcys has not been demonstrated (Bayewitch et al. 1998).

$\text{Ca}^{2+}$ is the second-most characterized regulator of mammalian Adcy activity. $\text{Ca}^{2+}$/calmodulin can work as a primary stimulator that strongly activates Adcy1 and Adcy8 (Tang et al. 1991b; Cali et al. 1994). Although the structural basis for $\text{Ca}^{2+}$/calmodulin-induced changes is unclear, the binding sites of $\text{Ca}^{2+}$/calmodulin have been mapped to different sites: at C1b in Adcy1 and at both the N-terminal region and the very C-terminal part in Adcy8 (Gu et al. 1999). $\text{Ca}^{2+}$/calmodulin also acts as a weak secondary activator of Adcy3, especially when the enzyme is preactivated by $\text{G}\alpha_s$ or forskolin (Choi et al. 1992). It is of note that not all intracellular $\text{Ca}^{2+}$ signals enhance the activity of these isozymes. $\text{Ca}^{2+}$ released from IP3-regulated stores is not effective for this regulation, whereas $\text{Ca}^{2+}$ from capacitative entry or from voltage-gated ion channels is very efficient for the activation of these isozymes. In addition to these stimulatory effects, $\text{Ca}^{2+}$/calmodulin can also inhibit Adcy1 and Adcy3 indirectly via phosphorylation by CaM kinases (Wayman et al. 1995; Wayman et al. 1996). This negative regulation is most likely a feedback mechanism that controls the $\text{Ca}^{2+}$-mediated stimuli. Interestingly, mammalian Adcys are all inhibited by high, millimolar concentration of free $\text{Ca}^{2+}$. This low affinity inhibition is probably caused by competition of $\text{Ca}^{2+}$ with the metal cofactor in vitro, and is unlikely to have any physiological meaning. But a high affinity and possibly physiological inhibition of Adcy5 and Adcy6 by free $\text{Ca}^{2+}$ has been reported (Cooper et al. 1998). These two isozymes are predominant in the heart, and inhibition of their activity by the rise in cytosolic $\text{Ca}^{2+}$ has been linked to function within the cardiac cycle.
Posttranslational modification of selective Adcy isozymes by protein kinases (i.e. PKA and PKC) and Ca\(^{2+}\)/calcineurin forms another mode of regulation for the activity of these enzymes. PKA, which is directly activated by the second messenger cAMP, can negatively regulate Adcy5 and Adcy6 activity by phosphorylation of these molecules, perhaps serving as a feedback inhibition mechanism (Iwami et al. 1995b). Treatment of cells with phorbol ester can stimulate the activities of Adcy1, Adcy2, Adcy3, and Adcy5, but inhibit those of Adcy4 and Adcy6, suggesting that PKC can regulate adenylyl cyclase in an isozyme-specific manner (Yoshimura et al. 1993; Zimmermann et al. 1996). It has been reported that this regulation for Adcy2, Adcy5, Adcy6, and possibly Adcy4 is through direct phosphorylation by PKC. Another potential posttranslational regulation of Adcy is through the Ser/Thr protein phosphatase calcineurin. Adcy9 has been reported to be inhibited by Ca\(^{2+}\) through calcineurin in some systems mainly expressing this isozyme; however, whether or not calcineurin directly dephosphorylates Adcy9 is still unclear (Antoni et al. 1995).

Adcy activity can also be modulated by small molecules such as forskolin or P-site inhibitors. Forskolin is a diterpene compound isolated from coleous roots plants that potently activates all isozymes of mammalian membrane bound Adcy with the exception of Adcy9 (Seamon et al. 1986). For most of these forskolin sensitive isozymes, activation by forskolin is synergistic with G\(\alpha_s\)-mediated coactivation, whereas for Adcy1, Adcy3, and Adcy8, activation by forskolin and G\(\alpha_s\) is only additive (Sunahara et al. 1996). As mentioned before, forskolin binds to the degenerated core site opposite of the active site in the cleft. It activates the enzyme by inducing binding of the two cytoplasmic domains using a combination of hydrophobic and hydrogen bonding interactions. Interestingly, the residues
of the forskolin binding pocket are absolutely conserved in Adcy1-8 and differ only subtly in Adcy9. Mutating just two amino acids of Adcy9 back to the corresponding conserved residues, i.e. an Ala→Ser and a Tyr→Leu, restored forskolin sensitivity to the level seen with other Adcys (Yan et al. 1998). In contrast to the stimulatory effect of forskolin, P-site inhibitors are a class of adenosine analogs that potently inhibit the activity of mammalian membrane bound Adcys (Dessauer et al. 1999). Studies of crystal structures revealed that P-site inhibitors bind to the active site along with the reaction product pyrophosphate (Tesmer et al. 2000). The inhibition capacity of P-site inhibitors is thus dramatically influenced by the activity state of the cyclase and is significantly potentiated by the presence of pyrophosphate. By a dead-end, uncompetitive manner, P-site inhibitors lock the enzyme in a closed inactive conformation and inhibit the activity of all membrane bound isozymes.

Regulation of soluble Adcy diverges significantly from its membrane bound relatives in that it is unresponsive to hormones, G proteins, and forskolin. Soluble Adcy is uniquely regulated by bicarbonate and calcium (Chen et al. 2000). It is widely expressed at low levels in many tissues, but is very highly expressed in sperm cells, consistent with its important physiological role in sperm maturation and activation (Garbers et al. 1980). In addition, the concentration level of bicarbonate that enables the activation of recombinant soluble Adcy is well within the range found in epididymal fluid (Chen et al. 2000). However, the precise binding site of bicarbonate in the soluble Adcy is still unclear.

1.3 Tissue Distribution and Physiological Function

It is evident that the various Adcy isozymes are not equally expressed in all tissues, and that their distribution profiles are distinctive for each isozyme. Because specific high-
affinity antibodies are not readily available, it is still difficult to precisely determine the expression pattern for each of the ten mammalian isozymes. Thus, tissue distribution has generally been determined by mRNA expression studies as shown in Table 1-1 (Defer et al. 2000). In general, all membrane bound Adcy isozymes can be found in brain cells, although some, especially the Ca\(^{2+}\)/calmodulin activated Adcy1, Adcy3, and Adcy8, are more specific to this organ (Hanoune et al. 1997). Adcy3 is also preferentially enriched in the olfactory neurons (Xia et al. 1992), whereas Adcy5 and Adcy6 are the predominant isozymes in heart (Ishikawa et al. 1992; Katsushika et al. 1992). Adcy9 has been found to be abundant in the zona fasciculata of the rat adrenal cortex and some specific areas of brain (Hanoune et al. 1997); the soluble Adcy is highly expressed in spermatozoa (Jaiswal et al. 2001). The broad distribution of Adcy isozymes suggests that any given cell contains multiple isozymes.

Largely due to the multiplicity of the isotype expression and complicated effects of regulators in the intracellular milieu, it is extremely difficult to assess the physiological roles of the individual isozymes in intact cells, tissues or organisms. The majority of functional data are mainly derived from the studies of genetically altered animals: i.e. gene knockout mice which are designed to disrupt isozyme-specific genes and transgenic mice which are modified to overexpress a particular isozyme (Patel et al. 2001). Disruption of Adcy1 in mice results in a significant loss of calcium-sensitive Adcy activity in the cerebellum, cortex, and hippocampus, and this is accompanied by a dampening of the long-term potentiation (LTP) in hippocampus and a blockade of cerebellar LTP (Wu et al. 1995; Storm et al. 1998). These findings suggest that Adcy1 is critical for the regulation of some synaptic plasticity and LTP. However, formation of the late-LTP (L-LTP) and long-term memory (LTM) has been shown to require the function of both Adcy1 and Adcy8 (Storm et al.
Gene knockout mice lacking either Adcy1 or Adcy8 exhibit normal L-LTP and LTM; but double knockout mice in which both Adcy1 and Adcy8 genes have been disrupted lose L-LTP or LTM. In addition, injection of forskolin into the hippocampus of these mice, which activates all the other isozymes of Adcy except Adcy9, restores normal LTM (Storm et al. 1998). These data clearly show the obligatory roles of these calcium stimulated Adcys in L-LTP and LTM.

Studies on Adcy3 knockout mice demonstrate that this enzyme plays a critical role in olfaction and olfaction related responses (Wong et al. 2000). Mice with Adcy3 deficiency lost electro-olfactogram responses to a number of cAMP and IP3 stimulatory agents, and failed olfaction-based avoidance tests when compared with their wild type controls. In addition, other studies on these Adcy3-null mice suggest that Adcy3 is also an important mediator for prostaglandin E2-induced growth inhibition of arterial smooth muscle (Wong et al. 2001).

Besides the knockout approach, several investigators have employed the transgenic techniques to study the physiological roles of specific isozymes such as Adcy5, Adcy6, and Adcy7 (Roth et al. 1999; Tepe et al. 1999; Yoshimura et al. 2000). It has been reported that during heart failure, the level of Adcy6 decreases while that of Adcy5 remains constant. Over-expression of Adcy6 in hearts of mice increases sensitivity to the activation by epinephrine and enhances cardiac function (Roth et al. 1999). In the Gaq over-expressing mice, cardiac overexpression of Adcy6 leads to an improvement of function impaired by the cardiac hypertrophy in these mice and restores the cAMP generating capacity in response to catecholamines (Roth et al. 1999). In contrast, overexpression of Adcy5 only leads to increased Adcy activity, without further improvement in sensitivity to epinephrine and an
impaired cardiomyopathy (Tepe et al. 1999). These results demonstrate an important difference of function between the two prominent isozymes of the heart. Using the same technique to over-express Adcy7 in the central nervous system reveals that this enzyme enhances acute responsiveness and tolerance to morphine (Yoshimura et al. 2000), suggesting its involvement in drug addiction.

In recent years novel techniques such as small interfering RNA-mediated gene silencing, real-time PCR, and gene expression profiling have been employed to determine the function of specific Adcy isozymes in different tissues and/or cell lines (Tovey et al. 2008; Gottle et al. 2009; Strazzabosco et al. 2009). The advantages of these techniques lie in their ability to accurately quantitate or manipulate the expression levels of each isozyme, and thus providing more definitive information about its function. For example, using siRNA-mediated inhibition of Adcy5 levels within the nucleus accumbens, Kim et al demonstrated that this isozyme was sufficient to produce an anxiolytic response, suggesting an essential role of Adcy5 for maintaining normal levels of anxiety in this brain structure (Kim et al. 2008). Similar approaches have been used to study the function of several other isozymes and have brought in a wealth of new functional information for Adcy isozymes 6, 8, 9 and soluble Adcy (Tovey et al. 2008; Geng et al. 2009; Strazzabosco et al. 2009). Parallel to these advances, isozyme-selective stimulatory and/or inhibitory compounds for Adcy enzymes have been developed to pharmacologically modulate the activity of these enzymes in organ- or tissue-specific manner (Pavan et al. 2009; Pierre et al. 2009). These studies have lead to a promising strategy to target adenylyl cyclase isozymes for the treatment of various clinical disorders, such as congestive heart failure, neuropathic pains, and asthma (Pierre et al. 2009).
1.4. Gene Structure and Transcriptional Regulation

The intron-exon organization of various mammalian Adcy isozymes was mainly determined during or after the completion of the human genome project. Before that, few Adcy genomic structures were reported in the literature; however, various cDNAs were successfully cloned and shown to share a considerable degree of homology (Patel et al. 2001). In the meantime, different approaches were used to determine the chromosomal localization of these genes (Hanoune et al. 1997; Patel et al. 2001). The results, as presented in Table 1-2, demonstrate that the various Adcy genes are not clustered in a single chromosome, but are distributed independently throughout the genome. Now, the sequence and detailed genomic structure of each Adcy isozyme for several mammalian species are readily available in public databases and will facilitate future studies as to how the different isozymes are regulated in a given cell or an organ.

As mentioned before, due to the distinctive tissue distribution pattern and specific regulatory properties of Adcy family members, changes in their expression profile would have a fundamental influence on cellular responses and organ functions. Despite this, very little information is available. It has been reported that the protein and mRNA levels of Adcy1 undergo a circadian oscillation in the pineal gland and Adcy1 may play a pivotal role in regulating nocturnal melatonin synthesis (Tzavara et al. 1996). Similarly, circadian variation of Adcy2 mRNA levels occurs in the rat suprachiasmatic nuclei (Cagampang et al. 1998). In the rat brown adipose tissue, activation of the β adrenergic receptor specifically increases the accumulation of Adcy3 mRNA (Granneman 1995). However, the underlying mechanisms for all of these regulations have not been well clarified. Ludwig et al. analyzed the proximal promoter regions of human Adcy genes and found that most of these genes
Table I-2. Chromosomal localization of the Adcys

<table>
<thead>
<tr>
<th>Adcy isozyme</th>
<th>Human chromosome</th>
<th>Mouse chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adcy1</td>
<td>7p12</td>
<td>11A2</td>
</tr>
<tr>
<td>Adcy2</td>
<td>5p15</td>
<td>13C1</td>
</tr>
<tr>
<td>Adcy3</td>
<td>2p22-24</td>
<td>12A-B</td>
</tr>
<tr>
<td>Adcy4</td>
<td>14q11.2</td>
<td>14D3</td>
</tr>
<tr>
<td>Adcy5</td>
<td>3q13.2-q21</td>
<td>16B5</td>
</tr>
<tr>
<td>Adcy6</td>
<td>12q12-13</td>
<td>15F</td>
</tr>
<tr>
<td>Adcy7</td>
<td>16q12-13</td>
<td>8C3-D</td>
</tr>
<tr>
<td>Adcy8</td>
<td>8q24</td>
<td>15</td>
</tr>
<tr>
<td>Adcy9</td>
<td>16p13.3</td>
<td>16B1</td>
</tr>
<tr>
<td>sAdcy</td>
<td>1q24</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Hanoune et al. (1997) and Patel et al. (2001)
lack a canonical TATA box (Ludwig et al. 2002). For Adcy1-5 and Adcy9 the promoter sequences are GC-rich and contain, among other transcription binding sites, a putative GC box, suggesting a potential role of Sp1 factors in the regulation of basal transcription. Apart from the work reported in this thesis, this hypothesis has not yet been tested experimentally. Besides this, the factors that determine the optimal expression of different members of the Adcy family and contribute to their tissue-selective distribution are also largely unknown. Studies of Adcy3 regulation reveal that less than 1 kb of 5’ flanking DNA is sufficient for expression of this enzyme (Abdel-Halim et al. 1998). The olfactory expression of Adcy3 is regulated by a transcription factor (Olf-1) that participates in the coordinate expression of a set of neuronal cell-specific olfactory genes (Wang et al. 1993). In contrast, in the case of Adcy8, 10 kb of 5’-flanking DNA is needed to obtain tissue specific expression in transgenic mice; but the cAMP-stimulated expression of Adcy8 in specific regions of mouse brain and in rat neuronal cells is probably regulated via a CRE element located in the very proximal promoter region (Muglia et al. 1999). Interestingly, like Adcy3, Adcy1 expression in neurons and pinealocytes is also regulated by a short promoter less than 1 kb in size (Chan et al. 2001). Within this promoter, a 15 bp compound regulatory element comprised of a nuclear-receptor-like binding motif and an E-box is critical for the neuro- and pineal-directed expression of this enzyme.

2. Adrenal Steroidogenesis

Steroid hormones are lipid products synthesized from cholesterol by steroidogenic enzymes that consist of several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases. Mammalian steroid hormones can be
divided into five groups: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestins (Miller 1988; Sanderson 2006). They are separately synthesized from numerous organs such as the adrenal gland, gonads, brain, placenta, and adipose tissue. The adrenal gland, testis, and ovary are the three major endocrine organs specialized for de novo steroid production (Sanderson 2006; Ghayee et al. 2007).

Steroid hormones are essential for many physiological functions ranging from regulation of normal sexual development to modulation of immune responses. Both androgens and estrogens produced mainly from testis and ovary are very important for sexual differentiation and development, secondary sex properties, and sexual behavior (Jensen et al. 1972; Carani et al. 1997; Nelson et al. 2001; Simpson 2003). The major function of progestins (progesterone), predominantly secreted by corpus luteum, is to prepare the endometrium for implantation and potentiate estrogen feedback effects on hypothalamus and anterior pituitary (Jensen et al. 1972). The mineralocorticoids and glucocorticoids largely function to regulate fluid and salt balance, carbohydrate metabolism, inflammation reactions and stress responses, and they are produced by the adrenal cortex (Vinson 2003; Ghayee et al. 2007). Interestingly, all these hormones are lipid soluble and act via very similar molecular mechanisms. They get into the cytoplasm of target cells by diffusion and bind to corresponding steroid hormone receptors, which are ligand-activated proteins regulating transcription of selected genes. The activated receptors are then dissociated from their chaperone proteins such as heat shock proteins and are translocated into the nucleus, where they bind as homo- or heterodimers to specific DNA sequences identified as hormone response elements (HREs), leading to altered rates of transcription of the associated genes (Walters et al. 2004; Hammes et al. 2007).
2.1 Steroidogenesis in the Adrenal Cortex

Unlike the gonads, the adrenal gland is essential for survival. Adrenal insufficiency, known as Addison disease, can rapidly cause death within 1-2 weeks in the absence of steroid hormone replacement therapy (Lovas et al. 2008). The adrenal gland is responsible for production of three characteristic steroids, all of which are synthesized in the adrenal cortex that is histologically and functionally divided into three concentric zones (Neville et al. 1985; Vinson 2003; Sanderson 2006). The outer zone, the zona glomerulosa, makes the 21-carbon mineralocorticoid aldosterone, which is essential for life as it tightly regulates salt and water balance in extracellular fluids. The intermediate zone, the zona fasciculata, makes the glucocorticoid cortisol (corticosterone in rodents), which is important in metabolic homeostasis and the stress responses of the organism and also is important in the regulation of vascular response to catecholamines and modulators of the immune system. Finally, the inner zone, the zona reticularis, produces 19-carbon androgen precursors such as dehydroepiandrosterone (DHEA) and androstenedione. Interestingly, this zone-specific synthesis of adrenal steroids is regulated by different external stimuli and more importantly by the expression of distinct steroidogenic enzymes within these adrenal zones.

Steroid hormone biosynthesis generally begins with cholesterol (Fig. I-2) and the first reaction takes place at the inner mitochondrial membrane (Miller 1988; Sewer et al. 2003; Payne et al. 2004). It is believed that the rate-limiting step in this process is the delivery of free cholesterol from outside of mitochondria to the first reaction site (Lin et al. 1995; Sewer et al. 2007). In most cells cholesterol can be synthesized de novo from acetate or taken from circulating lipoprotein particles such as high density lipoprotein (HDL) and
Fig. I-2. Biosynthetic pathways of steroid hormones in the adrenal cortex  
(Adapted from Payne and Hales, 2004)
low density lipoprotein (LDL) (Miller 2007). Esterified cholesterol molecules packaged in HDL and LDL are transferred into cytoplasm via the HDL- and LDL-receptors in humans (Connelly et al. 2004), but in rodent these processes are mediated by scavenger receptor BI (SR-BI) and LDL receptor, respectively (Azhar et al. 2002). Extra cholesterol is stored in lipid droplets in the form of cholesterol ester.

Once the cells receive signals for steroidogenesis, cholesterol ester is digested by cholesterol esterase, known as hormone sensitive lipase (HSL), releasing the free cholesterol as a steroid substrate (Kraemer et al. 2002). The accumulated free cholesterol is then imported to the inner mitochondrial membrane by a macromolecular protein complex formed at the outer mitochondrial membrane, which includes several members such as steroidogenic acute regulatory protein (StAR), mitochondrial translocator protein (TSPO), TSPO-associated protein RAP7, and the regulatory subunit of PKA (Liu et al. 2006). Among these proteins, StAR is a cycloheximide-sensitive, mitochondrial protein that is acutely regulated by pituitary hormone ACTH, a strong regulator for adrenal steroidogenesis, and plays a critical role in this first and rate-limiting step of steroid hormone production by facilitating the import of free cholesterol (Clark et al. 1995; Stocco et al. 1996; Stocco 2001). ACTH-stimulated acute steroid hormone production is always accompanied by a rapid increase in StAR mRNA levels which precedes the increase in other steroidogenic enzymes in the adrenal cortex cells. Patients with mutations of the StAR gene that lead to the expression of a short, inactive StAR protein results in congenital lipoid adrenal hyperplasia (Lin et al. 1995). Moreover, targeted disruption of the StAR gene in mice severely impairs their ability to produce steroid hormones, with consequent male pseudohermaphroditism, and ultimately leads to death within 1 week after birth (Caron et al.
suggesting an essential role of StAR in the early process of steroid hormone biosynthesis.

Once transferred to the inner mitochondrial membrane, free cholesterol is subjected to a set of sequential enzymatic reactions and transformed into the mineralocorticoids, glucocorticoids or adrenal androgens depending on the cell types in the specific zones of the adrenal cortex (Sewer et al. 2007). In the human adrenal cortex, five distinctive steroid cytochrome P450 hydroxylases and one 3β-hydroxysteroid dehydrogenase (3β-HSD) participate in the steroidogenic pathways (Payne et al. 2004; Sanderson 2006; Sewer et al. 2007). All the P450 enzymes are membrane-bound, being located either in the inner membrane of the mitochondria or in the endoplasmic reticulum. The type I P450 enzymes, including CYP11A1 (P450sc), CYP11B1 (P450c11β), and CYP11B2 (P450cAldo), reside in the mitochondria and use adrenodoxin as their electron transfer system. The type II enzymes, consisting of CYP17 (P450c17) and CYP21 (P450c21), are located in the smooth endoplasmic reticulum. They use cytochrome P450-oxidoreductase (POR) for electron transfer (Ghayee et al. 2007). However, the 3β-HSD enzyme is found in both mitochondria and smooth endoplasmic reticulum (Sanderson 2006). In addition, each adrenal zone contains a distinctive expression profile of these P450 enzymes, which is the major determinant of zone-specific production of different steroid hormones.

The first reaction of all steroid hormone biosynthesis starts with the conversion of cholesterol to pregnenolone by CYP11A1 (Parker et al. 1995; Payne et al. 2004). As mentioned before, CYP11A1 is located in the inner membrane of mitochondria and is expressed in all steroidogenic tissues but is undetectable in nonsteroidogenic tissues. Once it is formed, pregnenolone is converted to progesterone by 3β-HSD, an enzyme that consists
of two isozymes, which are regulated in a tissue- and species-specific pattern (Simard et al. 2005). The 3β-HSD that is predominantly expressed in steroidogenic tissues is the type 2 isozyme in human and type I isozyme in mice (Bain et al., 1993). Both pregnenolone and progesterone can serve as the precursors for the synthesis of other steroids in the adrenal cortex.

The zona glomerulosa of the adrenal cortex does not express CYP17 that is required for the biosynthesis of glucocorticoids (cortisol) and adrenal androgens (DHEA and androstenedione) (Voutilainen et al. 1986; Reincke et al. 1998); however, zona glomerulosa cells uniquely express aldosterone synthase (CYP11B2), an enzyme that converts corticosterone to aldosterone, the most potent mineralocorticoid (Ogishima et al. 1992). Therefore, the main function of zona glomerulosa is to synthesize the principal mineralocorticoid, aldosterone, in response to the signals of specific stimuli such as angiotensin II and potassium.

In contrast, the human zona fasciculata and zona reticularis do not express aldosterone synthase (CYP11B2), and thus these tissues cannot produce aldosterone. Instead, both these zones express CYP17 (Voutilainen et al. 1986; Reincke et al. 1998; Rainey 1999) and CYP11B1, which converts the precursor 11-deoxycortisol to cortisol in both zones, particularly in the zona fasciculata (Ogishima et al. 1992; Erdmann et al. 1995). The function of these two zones is therefore to produce cortisol, the major glucocorticoid. However, CYP17 is not expressed in the zona fasciculata and zona reticularis of mouse or rat adrenal glands. The adrenals of these animals produce corticosterone instead of cortisol (Perkins et al. 1988; Brock et al. 1999). In addition to its ability to hydroxylate pregnenolone and progesterone, P450 CYP17 also contains the 17,20-lyase activity, which is low in the
adult adrenal cortex and is exclusive to the zona reticularis in humans. This zone-dependent difference in activity is not determined by the CYP17 enzyme itself but is determined by the differential contribution of an accessory protein cytochrome b\(_5\) in these zones (Auchus et al. 1998; Brock et al. 1999). In humans, cytochrome b\(_5\) can act as an allosteric effector of the CYP17-oxidoreductase complex and increase the Vmax of the lyase activity when 17\(\alpha\)-hydroxypregnenolone is utilized as the substrate instead of 17\(\alpha\)-hydroxyprogesterone (Auchus et al. 1998). Thus, the major androgen precursor produced in human adrenal reticularis is DHEA (Auchus et al. 1998; Brock et al. 1999).

**2.2 Regulation of Adrenal Steroid Biosynthesis**

Adrenocortical steroidogenesis is under tight control by numerous signaling regulators such as trophic hormones, extracellular ions, and a variety of neuropeptides. Among them, the most important regulators include angiotensin II (Ang II), extracellular potassium, and the pituitary hormone, ACTH (Stocco et al. 2005; Sewer et al. 2007). The production of aldosterone in the adrenal zona glomerulosa is mainly regulated by Ang II and by small increases in the concentration of extracellular potassium (Bassett et al. 2004c; Foster 2004; Spat et al. 2004). In contrast, the production of adrenal glucocorticoids and androgens from the zona fasciculata and reticularis is regulated predominantly by ACTH (Gallo-Payet et al. 2003; Sewer et al. 2003; Sewer et al. 2007). ACTH also contributes to the acute increase of aldosterone production both in vivo and in isolated cells, while chronically it decreases plasma aldosterone levels in humans and lowers adrenal expression of aldosterone synthase (CYP11B2) in animals (Bassett et al. 2004c). The regulation of steroid hormone (e.g. aldosterone and cortisol) production in the adrenal cortex by these peptide
hormones can be divided into acute and chronic steroidogenic responses (Simpson et al. 1983; Cherradi et al. 1998). Acute steroidogenesis is clearly detectable within minutes after Ang II and ACTH stimulation, and is often seen in the body in response to stress. Unlike secretion of peptide hormones from cells that usually store a large amount of hormone within secretary vesicles ready for rapid release, steroid-producing cells store steroids in minimal quantities. Thus, the rapid secretion of steroid hormones seen in response to hormone stimulation reflects a rapid synthesis of new steroids, and is initiated by the mobilization and delivery of the substrate, cholesterol, from the outer to the inner mitochondrial membrane where it is metabolized to pregnenolone by the cytochrome P450scc (CYP11A1) (Stocco et al. 1996; Miller 2007). This cholesterol mobilization process is a rate-limiting step in the hormone-stimulated acute response and has an absolute requirement for de novo synthesis of the StAR protein.

The exact mechanism of StAR-mediated cholesterol transport is still unclear. StAR is a mitochondrial protein encoded by the nuclear genome and synthesized in the cytosol as a 37 kDa precursor (Pon et al. 1986; Clark et al. 1994). During the transport of cholesterol to the internal mitochondrial membrane, it has been found that the 37 kDa StAR precursor is phosphorylated and proteolytically processed into a 30 kDa protein (Mathieu et al. 2002; Lehoux et al. 2003). Models for this StAR-mediated process have been reviewed recently and support the involvement of both protein-phospholipid and protein-protein interactions (Lehoux et al. 2003; Miller 2007). In addition, consistent with its role in the acute regulation of steroidogenesis, StAR is predominantly expressed in the adrenal cortex and gonads, which have the acute steroidogenic responses, but not expressed in the placenta, which does not have an acute response (Sugawara et al. 1995; Pollack et al. 1997). The regulation of
StAR activation in adrenal gland and gonads is quite different. In the gonads and inner zones of the adrenal cortex StAR is mainly activated by the hormone-mediated cAMP signaling pathway (Manna et al. 2003), whereas StAR in the zona glomerulosa is activated by Ang II and potassium via the Ca\(^{2+}\)/calmodulin kinase cascade (Bassett et al. 2004; Sewer et al. 2007).

The chronic steroidogenic response takes hours, and involves the increased transcription of the genes that encode steroidogenic enzymes and proteins employed in electron transfer (Fig. I-3). Thus, the chronic production of aldosterone is regulated by Ang II and extracellular potassium at the level of expression of aldosterone synthase (CYP11B2), an enzyme exclusively expressed in the zona glomerulosa of the adrenal cortex (Denner et al. 1996; Kakiki et al. 1997; Bassett et al. 2004); and the chronic secretion of cortisol in the zona fasciculata and zona reticularis is regulated by the pituitary hormone ACTH, leading to the increased expression of CYP11B1 and other steroid hydroxylases, 3β-HSD2, and the mitochondrial electron transport protein adrenodoxin (Adx) (Sewer et al. 2003). In addition, under in vivo conditions, Ang II and ACTH also work as growth factors for their targeted adrenocortical cells, stimulating cell proliferation (Lotfi et al. 1997; Gallo-Payet et al. 2003; Hunyady et al. 2006). Together, these chronic effects of Ang II and ACTH increase the overall capacity of the adrenal cortex to produce corresponding steroids.

Both Ang II and potassium increase CYP11B2 expression via a calmodulin- and calmodulin kinase-dependent pathway. Ang II binds to the type I Ang II receptor (AT1R), a typical G-protein coupled seven transmembrane domain receptor (GPCR), and mediates the activation of phospholipase C through G\(_{q/11}\), resulting in the increased production of inositol 1,4,5-trisphosphate (IP\(_3\)) (Hajnoczky et al. 1992; Hunyady et al. 2006). IP\(_3\) stimulates the
Multiple signaling systems coordinately regulate gene expression of steroidogenic enzymes. ACTH stimulates cAMP/PKA pathway and increases the production of cortisol and adrenal androgens via induction of the expression of many steroidogenic enzymes; Ang II increases intracellular levels of Ca\(^{2+}\) which activate calmodulin kinase (CaMK), leading to the increased expression of CYP11B2 and increased production of aldosterone. Ang II may activate alternate pathways (e.g. 12-LO and the Src kinase family) that can also increase CYP11B2 expression.

Fig. I-3. Signaling pathways involved in the regulation of steroidogenic gene transcription in adrenocortical cells (Adapted from Sewer et al. 2003)
rapid release of Ca\textsuperscript{2+} from intracellular stores and causes an increase in cytosolic Ca\textsuperscript{2+}, which in turn activates calmodulin and calmodulin dependent kinases I and IV (CamKI and CamKIV). Calmodulin and the CamKs appear to be the key factors that activate specific transcription factors such NURR1/NGFIB and ATF-1/CREB and that regulate CYP11B2 transcription in the zona glomerulosa (Pezzi et al. 1997; Condon et al. 2002). In contrast, small increases in potassium concentration depolarize the cell membrane and directly activate Ca\textsuperscript{2+} entry through voltage-dependent T- and L-type Ca\textsuperscript{2+} channels without release of Ca\textsuperscript{2+} from intracellular stores (Foster et al. 1982; Rossier et al. 1996; Yagci et al. 1996). Like Ang II, this Ca\textsuperscript{2+} signaling leads to the activation of calmodulin and CamKs and increases the transcription of CYP11B2 (Pezzi et al. 1997; Condon et al. 2002). In addition to Gq/phospholipase C signaling, Ang II elicits many other cellular responses. Stimulation of these pathways leads to a range of downstream effects, including activation of phospholipase D (PLD), 12-lipoxygenase, src tyrosine kinases, the Jak2/STAT pathway, and MAP kinases (Bassett et al. 2004; Hunyady et al. 2006). These diverse pathways also influence the capacity for the acute and chronic production of aldosterone by the adrenal gland.

3. Essential Role of cAMP Signaling in Adrenal Steroidogenesis

Although adrenocortical steroidogenesis is modulated by trophic hormones or extracellular potassium via numerous signaling mechanisms, the cAMP and perhaps the calcium pathways are widely regarded as the most important intracellular messenger cascades involved in ACTH action, both interacting closely through positive feedback loops to enhance steroid secretion (Gallo-Payet et al. 2003; Enyeart 2005). For example, both the
acute and chronic effects of ACTH on steroid production in the adrenal zona fasciculata and reticularis mainly utilize cAMP and calcium as the second messengers (Miller 1988; Sewer et al. 2001). In the zona glomerulosa, these signaling pathways are also responsible for the ACTH-mediated acute expression of StAR and aldosterone secretion (Aguilera 1993). However, calcium is probably not a first second messenger per se; rather, recent studies suggest that ACTH-induced increase in [Ca$^{2+}$]; appear to occur secondary to cAMP production through cAMP-induced modulation of specific ionic conductances and membrane potential (Enyeart et al. 1993; Mlinar et al. 1993; Enyeart et al. 2002). Like Ang II, ACTH is a ligand for a GPCR. When bound to its cognate melanocortin 2 receptor (MC2R), ACTH activates adenylyl cyclases, leading to an increase in intracellular cAMP. Along one pathway, cAMP may mediate inhibition of specific background K$^{+}$ channels that set the membrane potential, leading to membrane depolarization and Ca$^{2+}$ entry through T-type or L-type Ca$^{2+}$ channels (Mlinar et al. 1993; Guyot et al. 2000). More importantly, cAMP activates the cAMP-dependent protein kinase A (PKA) which in turn phosphorylates multiple downstream targets (Gallo-Payet et al. 2003). For example, PKA may phosphorylate the cAMP-response element-binding (CREB) protein or the cAMP-response element modulator (CREM), promote their association with CREB-binding protein (CBP), and quickly induce transcription and expression of StAR, a protein that is critical for the acute steroidogenic response (Sugawara et al. 2006). PKA is also proposed to target many other cellular factors that act cooperatively to increase the transcription of all steroidogenic genes and/or the activity of steroidogenic enzymes (Sewer et al. 2003; Sewer et al. 2007). This transcriptional pressure on the steroidogenic enzymes is essential for maintaining
optimal steroidogenic capacity of the adrenal glands, and thus is the main mechanism of the chronic steroidogenic response induced by the trophic hormone ACTH (Waterman 1994).

ACTH-mediated cAMP production and accumulation in the adrenal cortex is modulated by several Adcy isozymes (Gallo-Payet et al. 2003). These isozymes are usually regarded as important immediate effectors of ACTH in its action on the regulation of steroidogenesis. Three groups of Adcys have been found in the adrenal cortex based on *in situ* hybridization and immunohistochemistry (Shen et al. 1997; Burnay et al. 1998; Cote et al. 2001): the Ca\(^{2+}\)- inhibited Adcy5 and Adcy6 in both human and rat adrenal glands; the Ca\(^{2+}\)- stimulated Adcy1 and Adcy3 in human adrenal gland, and Adcy3 alone in rat and bovine adrenal glands; and finally the G\(\beta\gamma\)-regulated Adcy2 and Adcy4 in rat and human adrenals. The contribution of each Adcy isozyme to cAMP production and adrenocortical function is largely unknown. It has been reported that ACTH treatment increases the accumulation of Adcy5 and Adcy9 mRNA in rat adrenal cortex, implying that they are involved in ACTH regulation of this tissue (Shen et al. 1997). Other studies suggested that cAMP production induced by ACTH is regulated by multiple signaling molecules, including different G proteins (G\(\alpha\_s\) and G\(\alpha\_i\), and G\(\beta\gamma\)) and intracellular Ca\(^{2+}\) concentration acting on the different Adcy isozymes (Cote et al. 2001). Based on various biochemical studies, it has been proposed that the rapid and sustained increase in cAMP levels in adrenocortical cells is obtained via first activation of the Ca\(^{2+}\)- inhibited Adcy5 and Adcy6, then by the Ca\(^{2+}\)- stimulated Adcy3, and finally by the G\(\beta\gamma\) - sensitive Adcy2 and Adcy4 (Gallo-Payet et al. 2003). Whether these hypotheses are in agreement with the observations in vivo still needs further investigation. The increased cAMP and perhaps calcium signals are then linked to multiple downstream pathways that mediate the action of ACTH in the adrenal glands.
The developmental stage at which the cAMP-dependent pathway is established as a key regulator of adrenal steroidogenic enzyme expression and steroid hormone production is not well addressed. Numerous studies suggest that transcriptional regulation of all the steroidogenic genes in the adrenal cortex requires the activities of factors working at multiple levels, including those that influence developmental, tissue-specific, and cAMP-dependent regulation (Sewer et al. 2003; Sewer et al. 2007). During the embryonic stage, it is clear that both the developmental and tissue-specific regulation of steroidogenic genes are largely dependent on the expression of steroidogenic factor 1 (SF1), an orphan nuclear receptor that binds as a monomer to variations of an AGGTCA motif through a zinc finger DNA binding domain (Morohashi et al. 1992; Parker et al. 1997). This orphan nuclear receptor is also essential for organogenesis of steroidogenic tissues since disruption of its encoding gene in mice leads to adrenal and gonadal ageneses (Luo et al. 1994). However, in the adult adrenal cortex, transcriptional control of steroidogenic genes by the ACTH-activated cAMP/PKA signaling pathway appears to be more predominant and mediated by effects on the activity and/or concentration of many different transcription factors, including SF1.

The mechanisms regarding how the ACTH/cAMP signaling coordinately regulate all the steroidogenic genes in the adrenal tissue have been extensively studied for decades and seem more complicated than might have been expected. Given that the cAMP/PKA system rapidly induces the transcription of many genes through a well-defined CRE/CREB pathway (Roesler et al. 1988), it was expected that a common CRE element would exist in the promoter regions of all steroidogenic genes that were regulated by cAMP. However, many studies have shown that the mechanism of ACTH/cAMP-dependent transcription of most of
the steroidogenic genes is quite distinct from the classical cAMP/CRE/CREB regulatory system. First, the ACTH/cAMP-mediated increase in steroidogenic mRNA expression takes four to six hours and does not reach the plateau until 12 hours (John et al. 1986; Waterman et al. 1989). In contrast, the CRE/CREB-induced expression of immediate early genes, such as c-Fos and Jun-B, is much faster, taking place within minutes and is maximal between thirty minutes and two hours (Roesler et al. 1988; Viard et al. 1992). Second, the ACTH/cAMP steroidogenic pathway and the CRE/CREB pathway have a significant difference in response to the protein synthesis inhibitor, cycloheximide (CHX). The CRE/CREB responsive genes can be superinduced by cAMP signals when CHX is also applied, whereas, the ACTH/cAMP-mediated transcription of steroidogenic genes is significantly inhibited by CHX (Waterman 1994). The delayed, CHX-sensitive induction of steroidogenic genes by ACTH/cAMP stimulation suggests that new protein synthesis may be required before the increased transcription of steroidogenic genes. However, a newly synthesized protein required for the induced transcription has not been identified. Third and most strikingly, unlike the CRE/CREB-regulated genes, ACTH-stimulated steroidogenic genes usually do not have a consensus CRE element. Instead, each gene seems to utilize a unique cAMP-responsive sequence (CRS) found in its promoter, which is linked to the binding of a distinct but diverse array of transcription factors (Waterman 1994). Various trans-acting factors such as nuclear receptor family members (Sewer et al. 2003; Kurihara et al. 2005), adrenal specific protein (ASP) (Kagawa et al. 1992), Sp family members (Ahlgren et al. 1999; Cheng et al. 2000; Lin et al. 2001), homeodomain proteins (Bischof et al. 1998), CREBP and GATA factors (Bassett et al. 2000; Sher et al. 2007) have all been demonstrated to be required for the increased transcription of different steroidogenic genes. Among the list
of these transcription factors, which is ever increasing, the nuclear orphan receptor SF1 and the Sp family member Sp1 and Sp3 appear to be the most commonly used factors in the basal- and/or cAMP-dependent transcription of steroidogenic enzymes (Sewer et al. 2007). As mentioned before, SF1 is involved in the expression of almost all these enzymes; however, SF1 itself in most cases is not the only key mediator of cAMP-dependent transcription. Instead, this cAMP-mediated regulation is usually dependent on the cooperative action between SF1 and a set of other distinctive factors (Sewer et al. 2007). Recent studies further suggest that ACTH/cAMP can dynamically modulate the binding of many nuclear protein complexes in the promoter regions of steroidogenic genes, which contain multiple transcription factors dependent on each gene (Winnay et al. 2006; Dammer et al. 2007). Thus, the formation and binding of these protein complexes are probably the target sites of the ACTH/cAMP signaling pathway that confers cAMP-dependent transcription.

4. Essential Role of SF1 in the Regulation of Steroidogenesis

The orphan nuclear receptor SF1 belongs to the nuclear receptor superfamily, one of the largest classes of transcription factors in metazoans. Nuclear receptors are phylogenetically conserved in different species, with 21 genes in the complete genome of the fly Drosophila melanogaster (Adams et al. 2000), 48 genes in humans, 49 genes in the mouse (Robinson-Rechavi et al. 2001), and, unexpectedly, more than 270 genes in the Caenorhabditis elegans (Sluder et al. 1999). The diversity of nuclear receptors can be grouped into seven subfamilies (NR0-NR6) based on their phylogenetic conservation (Robinson-Rechavi et al. 2003). However, most of these receptors usually share a common
The activity of most nuclear receptor superfamily members is often modulated by the binding of small hydrophobic ligands such as steroid and thyroid hormones, retinoids, fatty acids, leukotrienes and prostaglandins (Escriva et al. 2000). However, a number of nuclear receptors, identified through sequence similarity to known receptors, have no identified natural ligands and thus are referred to as orphan nuclear receptors (Gustafsson 1999; Kliewer et al. 1999). The functional regulation of these orphan receptors is not quite clear and is often influenced by a number of factors such as posttranslational modifications and
Fig. I-4. Structural organization of nuclear receptors and SF1
(Adapted from Sadovsky and Dorn, 2000)

Nuclear receptors share a common structural feature consisting of the N-terminal region (A/B domain), DNA binding domain (DBD), hinge region (D domain), and ligand binding domain (LBD). Some receptors may also contain an F domain at the C-terminus. Most nuclear receptors have two activation function (AF) domains. SF1 lacks the N-terminal A/B domain and a functional AF-1 domain, and its transcriptional activation function thus resides within the C-terminal region of AF-2.
binding of potential physiological ligands. Upon activation nuclear receptors may undergo a conformational change, resulting in the dissociation of co-repressor complexes and recruitment of co-activator complexes, which in turn facilitate the transcription of target genes embedded in chromatin structures (Robinson-Rechavi et al. 2003). By regulating the expression of each specific subset of genes, nuclear receptors control a diversity of functional processes ranging from homeostasis, reproduction, to development and metabolism.

4.1 SF1 and LRH1

SF1 and liver receptor homolog-1 (LRH1) are homologues of the fushi tarazu factor-1 (Ftz-F1) of Drosophila and are categorized into the NR5A, or Ftz-F1 subfamily (Fayard et al. 2004). The NR5A subfamily now contains at least four identified members (NR5A1-NR5A4) (Fayard et al. 2004), in which Drosophila Ftz-F1 (NR5A3) is the first identified member and works as a key transcriptional regulator of the Drosophila homeobox gene fushi tarazu (ftz), an essential gene for segmentation during development (Kuroiwa et al. 1984; Wakimoto et al. 1984). Genes homologous to the Drosophila Ftz-F1 was subsequently isolated from numerous species in different phyla. In Zebrafish, for example, four Ftz-F1 homologues (ff1a, ff1b, ff1c and ff1d) have been identified (von Hofsten et al. 2005) and these homologues, as revealed by phylogenetic analysis, may have been derived from a single ancestral gene through a round of genome amplification and several rounds of duplication, followed by loss of chromosomal segments (Kuo et al. 2005); however, in the mammalian genome, only two homologues, SF1(NR5A1) and LRH1(NR5A2), have been
characterized, each with several isoforms derived from differential splicing or alternative promoter usage (Ikeda et al. 1993; Ninomiya et al. 1995; Zhang et al. 2001).

SF1 and LRH1 share very similar properties in many aspects (Fayard et al. 2004). They have an overall 60% amino acid similarity and have a virtually identical DNA binding domain. They both bind to identical DNA consensus sequences as monomers. SF1 and LRH1 also have a much conserved 3D structure in their LBDs and are able to bind similar phospholipids as potential ligands (Wang et al. 2005). These shared features imply that they have the potential to target overlapping genes and regulate similar metabolism pathways. However, SF1 and LRH1 have a quite different tissue expression pattern, suggesting they are involved in different physiological functions.

LRH1 is expressed predominantly in tissues of endodermal origin, such as the liver, the exocrine pancreas, and the basal compartment of the gastrointestinal tract, justifying its functional classification as an enterohpetic nuclear receptor (Fayard et al. 2004). During embryonic development, LRH1 maintains stem cell pluripotency and contributes to the specification of the enterohpetic axis (Gu et al. 2005). In adult mammals, LRH1 has been shown to regulate a set of target genes encoding proteins important for high density lipoprotein metabolism and reverse cholesterol transport, including cholesterol ester transfer protein (CETP) (Luo et al. 2001), apolipoprotein AI (Delerive et al. 2004), and scavenger receptor BI (Schoonjans et al. 2005). In coordination with two other nuclear receptors, farnesoid X receptor (FXR) and short heterodimer partner (SHP), LRH1 also has been shown to drive the expression of both cholesterol 7α-hydroxylase (CYP7A1) (Song et al. 2008), which catalyzes the first and rate-limiting step in bile acid synthesis and sterol 12α-hydroxylase (CYP8B1) (del Castillo-Olivares et al. 2000), which determines the synthesis of
cholic acid. In addition, LRH1 transactivates the expression of a set of genes essential for
the biliary secretion of sterols and ileal transport of bile acids, including the ATP-binding
cassette G5 and G8 proteins (ABCG5/8) (Freeman et al. 2004), the apical sodium-dependant
bile acid transporter (ABST) (Chen et al. 2003), and the basolateral multidrug resistance
protein 3 (MRP3) (Inokuchi et al. 2001). Therefore, LRH1 is an essential factor for the
development of enterohepatic organs and a master transcriptional regulator in the control of
cholesterol and bile acid homeostasis.

In contrast, SF1 is expressed predominantly in the seroidogenic tissues and in
hypothalamo-pituitary-adrenal axis (Parker et al. 1997). Initially identified as an essential,
tissue-specific regulator for the genes encoding steroid hydroxylases, SF1 subsequently was
found to have a much broader role in sex differentiation and endocrine specification.
Targeted disruption of SF1 in mice results in failed development of the adrenal gland and
gonads, male-to-female sex reversal, absence of the differentiated ventromedial
hypothalamic nucleus (VMH), and impaired expression of gonadotropins in the anterior
pituitary gland (Morohashi et al. 1992; Parker et al. 1997). In adults, SF1 modulates the
biosynthesis of mineralocorticoids, glucocorticoids and sex steroids by regulating the
expression of many of the steroidogenic enzymes (Hammer et al. 1999a; Parker et al. 2002).
Thus SF1 has been established as a pivotal, global regulator of endocrine differentiation and
function at multiple levels, particularly with respect to reproduction.

In recent years, LRH1 was also found to express in some of the steroidogenic tissue
such as in mouse and human ovaries (Sirianni et al. 2002; Hinshelwood et al. 2003; Peng et
al. 2003), human and rat testis (Sirianni et al. 2002; Pezzi et al. 2004), and, at lower levels,
in the human placenta and adrenals (Wang et al. 2001), raising the possibility that both
LRH1 and SF1 receptors could be involved in the regulation of steroidogenesis in these tissues. Indeed, it has been shown that these two receptors can target identical genes and seem to have similar actions on rat granulosa cell steroidogenesis, where they are both expressed (Saxena et al. 2007). Further studies, however, suggested that even in these tissues subtle differences exist in the cell-specific distribution and potential functions of SF1 and LRH1. In the human and rat testis, LRH1 is more abundant than SF1 in Leydig cells and spermatocytes but is less abundant than SF1 in Sertoli cells (Sirianni et al. 2002; Pezzi et al. 2004). In rat and human ovaries, LRH1 is more abundant and highly restricted to estrogen-producing granulosa cells and to corpus luteal cells, where it may play as essential role in the regulation of corpus luteum steroidogenesis (Peng et al. 2003; Saxena et al. 2007). In contrast, SF1 is expressed most highly in steroids-producing theca cells and interstitium (Takayama et al. 1995; Hinshelwood et al. 2003). Thus, LRH1 may exert some regulatory effect on steroidogenesis in a tissue-specific and even cell-specific manner, but SF1 indisputably works as a master regulator in all steroidogenic tissues.

4.2 SF1 Expression Profiles and its in vivo Function

To better understand the function of SF1 in vivo, the tissue and cell distribution of SF1 in the fetus and adult has been extensively studied. Consistent with its proposed regulatory role in steroidogenesis, SF1 expression has been found to be restricted in the primary steroidogenic organs and cells, including the adrenal cortex, testicular Leydig cells, and ovarian theca and granulosa cells (Ikeda et al. 1993; Sasano et al. 1995; Takayama et al. 1995). However, SF1 transcripts have also been found in the testicular Sertoli cells, pituitary gonadotrope, hypothalamic ventromedial nucleus (VMH), spleen, and hippocampus.
(Morohashi et al. 1984; Ingraham et al. 1994; Morohashi et al. 1994; Asa et al. 1996; Roselli et al. 1997; Wehrenberg et al. 2001), suggesting additional roles for SF1 in vivo which may be beyond the coordinated regulation of steroidogenesis.

In mice, SF1 transcripts were detected as early as E9 in the urogenital ridge, a region that ultimately contributes cells to the adrenal cortex, gonads and kidneys (Hatano et al. 1994; Ikeda et al. 1994). During embryonic development, SF1 was expressed in the adrenal primordium when it first appears as a distinct structure at ≈E10-10.5, about one day before the onset of steroid hydroxylase expression, and remained at a constant high level exclusively within the adrenal cortex throughout gestation and postnatal life. In parallel, SF1 transcripts were seen in both male and female gonads during the indifferent stage (E9-E11) (Hatano et al. 1994; Ikeda et al. 1994). Then, coincident with formation of the testicular cords at E12.5, SF1 expression persisted in the testes but diminished in ovaries until postnatal follicle formation (Shen et al. 1994; Hatano et al. 1996). Interestingly, SF1 was also expressed in the embryonic diencephalon – the precursor to the endocrine hypothalamus and in the developing anterior pituitary gland (Ikeda et al. 1994; Ikeda et al. 1995; Shinoda et al. 1995). Taken together, this unexpected wide pattern of expression implies that SF1 may play an essential role in the regulation of the entire hypothalamus-pituitary-steroidogenic organ axis at various levels.

As expected, SF1 knockout mice have female internal and external genitalia, irrespective of genetic sex, and adrenocortical insufficiency, evidenced by volume depletion, low corticosteroid levels, and elevated ACTH concentration in the blood (Luo et al. 1994; Luo et al. 1995; Sadovsky et al. 1995). Without the administration of exogenous corticosteroids, the SF1 null mice usually die within one week after birth. These data
indicate that SF1 is essential for the organogenesis of the primary steroidogenic tissues and plays an important role within specific hypothalamic nuclei. Finally, SF1 knockout mice exhibit impaired expression of a number of gonadotropin genes of the pituitary gland that regulate reproduction, including luteinizing hormone β (LHβ), follicle-stimulating hormone β (FSHβ), and the α-subunit of glycoprotein hormone (α-GSU) (Ingraham et al. 1994; Shinoda et al. 1995). These peptides also work as the key trophic hormones for the controlled production of sex steroids in the gonads. Thus SF1 has been established as a master regulator of endocrine development and function.

4.3 SF1 Target Genes

Identification of the target genes that are regulated by SF1 is an important step toward the better understanding of SF1 function in vivo. Originally identified as a tissue-specific transcriptional regulator of the cytochrome P450 steroid hydroxylases within adrenocortical cells, SF1 has been found to be involved in transcriptional regulation of a variety of genes in the tissues and cells where it is expressed (Parker et al. 2002). In the adrenal cortex and gonads, SF1 controls the expression of almost all the major enzymes involved in steroidogenesis, including P450scc (CYP11A1), 11β-hydroxylase (CYP11B1), aldosterone synthase (CYP11B2), 17α-hydroxylase (CYP17), 21-hydroxylase (CYP21), 3β-HSD2, and aromatase (CYP19) (Sewer et al. 2007). SF1 also regulates the transcription of the ACTH receptor and StAR protein in the adrenal cells (Beuschlein et al. 2001; Sewer et al. 2007). In addition to influencing the basal level expression of steroidogenic genes, SF1, as mentioned above, is proposed to mediate ACTH-stimulated up-regulation of these genes, most probably via the cAMP-protein kinase A pathway (Sewer et al. 2007). The optimal
expression of all these steroidogenic genes is believed to be essential for the regulated steroid production in their corresponding tissue – the adrenal cortex or gonads.

However, these studies are mainly based on promoter -reporter gene assays in transfection experiments, and thus have the possibility to overemphasize the significance of SF1 (Parker et al. 2002). To overcome this limitation, Li et al. (2004b) further studied the SF1 effect on gene regulation in cultured human adrenal H295R cells by constitutively expressing a dominant negative form of SF1. They found that inhibition of the endogenous SF-1 activity significantly reduced basal and inducible transcription of CYP17, CYP21B and CYP11B1, but exhibited little effects on StAR and CYP11A1 expression, suggesting that these steroidogenic genes are subject to the regulation by SF1 but may have differential sensitivity to its activity in cultured cell lines (Li et al. 2004b). Despite these concrete findings, the central role of SF1 in regulating steroidogenic gene expression in vivo is still controversial due to the early postnatal death of SF1 double knockout mice, which precludes efforts to examine the roles of SF1 in differentiated animals. In humans, partial loss of function caused by point mutations in SF1 revealed XY sex reversal and severe adrenal insufficiency, demonstrating a dose-dependent manner of SF1 function (Achermann et al. 1999; Biason-Lauber et al. 2000). Similarly in mice, deletion of one SF1 allele results in profound defects in adrenal development leading to adrenal insufficiency (Bland et al. 2000). However, the steroidogenic capacity per cell in SF1 heterozygous (+/-) adrenals was elevated, concomitant with the unexpected up-regulation of some SF1 target genes (Bland et al. 2004). This study strongly suggests that alternative, compensatory mechanisms exist to increase expression of many SF-1 target genes in the SF1 heteroinsufficiency adrenal tissue (Bland et al. 2004).
SF1 also targets genes in non-steroidogenic tissues and cells. During embryonic sex differentiation, SF1 promotes the expression of Müllerian-inhibiting substance (MIS) in the Sertoli cells of the testis, a prototypic male-specific gene responsible for regression of the female Müllerian duct (which gives rise to the upper 1/3 of the vagina, uterus, and fallopian tubes) (Shen et al. 1994; De Santa Barbara et al. 1998). Mutations in SF1 or the response element in the MIS promoter abrogate this activation in cell culture assays and in transgenic mice (Shen et al. 1994; Giuili et al. 1997). In addition, SF1 regulates the expression of the Sex-determining Region Y (SRY) and the SRY-related Sox9, two mammalian testis-determining genes which are sufficient to activate a cascade of events leading to male sexual development (Parker et al. 2002). In the gonadotrope of the pituitary gland, SF1 determines the expression of at least four genes, including LHβ, FSHβ, α-GSU, and the receptor for gonadotropin-releasing hormone (Barnhart et al. 1994; Halvorson et al. 1996; Halvorson et al. 1998; Ngan et al. 1999), which are involved in the regulation of steroidogenesis in the adrenal and gonads.

4.4 SF1 Structure and its Functional Regulation

The overall modular structure of SF1 is quite similar to most other nuclear receptors, except that it lacks an amino-terminal A/B domain harboring activation function 1 (AF-1), which is responsible for ligand independent transactivation (Fig. I-4). In the N-terminal region of SF1, there is a highly conserved DNA binding domain (DBD), composed of two classic Cys2-Cys2 zinc fingers (Parker et al. 1997). However, unlike many other nuclear receptors that bind their cognate DNA response elements as either homodimers or heterodimers, SF1 belongs to a subgroup of receptors, including SF1, LRH1, ERR, NGFI-B,
and ROR, that bind to DNA as monomers, requiring only one DNA half-site to bind with high affinity and recognizing variations of the DNA consensus motif, PyCAAGGTCA. Adjacent to the second zinc finger motif of the DBD is an additional 30-amino acid extension, designated A box, that helps to recognize the 5’ variations in the PyCAAGGTCA motif and increase binding fidelity (Ueda et al. 1992; Wilson et al. 1992). In the C-terminal region of SF1, a large ligand binding domain (LBD) can be readily identified even though it remains controversial whether SF1 transcriptional activity is regulated by small physiological ligands (Hammer et al. 1999a). Particularly, like other nuclear receptors, SF1 contains an activation function domain (AF-2) located at the C-terminus of its LBD (Sadovsky et al. 2000). The precise conformation of the AF-2 domain in SF1 and other nuclear receptors usually determines the transcriptional status of a receptor. Between the DBD and LBD of SF1 lies a large hinge region, whose functional role has not yet been well defined.

The mechanisms by which SF1 transactivates target genes have become an active research area. In the absence of a clear-cut ligand, SF1 is classified as an orphan nuclear receptor and is proposed to activate gene expression in a ligand-independent manner (Mangelsdorf et al. 1995). Early structural studies suggest that SF1 can adopt an active conformation without ligand binding (Desclozeaux et al. 2002). Cell-based reporter assays revealed that SF1 appears to be constitutively active because it stimulates transcription in the absence of any exogenous ligand. However, recent x-ray crystallographic studies carried out using bacterially expressed SF1 have shown that phospholipids are present in the ligand binding pocket (Krylova et al. 2005; Li et al. 2005; Wang et al. 2005). After extensive studies in this area, it is now more widely accepted that the ability of SF1 to transactivate
target genes is regulated by several mechanisms, including posttranslational modifications, cofactor associations, and perhaps ligand binding.

Posttranslational modifications such as phosphorylation, acetylation, and conjugation by small ubiquitin modifier (SUMO) can modulate SF1 transcriptional activity (Parker et al. 2002). It has been reported that phosphorylation at serine 203 by mitogen-activated protein kinase (MAPK) is important for the transcriptional activity of SF1 in some cell types (Hammer et al. 1999a). Activating the MAP kinase pathway increased SF1 transcriptional activity, while mutation of Ser203 diminished SF1 activity, resulting in a significant 50-80% drop in transcriptional activity when assayed on the MIS promoter and the mouse steroidogenic enzyme P450 21-hydroxylase promoter. A recent study suggested that this Ser203 phosphorylation was largely dependent on phospholipid binding and was directly mediated by the cyclin-dependent kinase 7, a component of the basal transcription factor TFIIF, thus linking the ligand association and phosphorylation of SF1 to the basic transcriptional machinery (Lewis et al. 2008). Other reports demonstrated that SF1 can be acetylated in vivo by the cAMP/P300 pathway or by SF1-recruited GCN5 (general control nonderepressed 5) (Jacob et al. 2001; Chen et al. 2005), leading to the increase of its activity in cell-based assays. In contrast, sumoylation of SF1 represses its activity (Chen et al. 2004; Komatsu et al. 2004), though the importance of this regulation remains elusive.

Interacting with different co-regulatory proteins is another important way to regulate SF1 activity. Indeed, a number of transcription factors have been shown to interact directly with SF1 (Parker et al. 2002), including CREB-Binding Protein (CBP), pituitary homeobox 1 (Ptx1), Sp1, GATA-4, Wilms’ tumor 1 (WT-1), early growth response protein 1 (Egr-1), Sry-related gene 9 (SOX9), and the orphan nuclear receptors dosage-sensitive sex
reversal/adrenal hypoplasia critical region on chromosome X gene 1 (DAX-1) and small heterodimer partner (SHP). For example, SF1, Sp1 and the CBP are found to cooperatively mediate cAMP-dependent transcription of CYP11A1 in steroidogenic cells (Guo et al. 2007). Similarly, Egr-1 is required for LHβ synthesis in gonadotropes. The binding of both SF1 and Egr-1 to their respective elements within the LHβ promoter is essential for LHβ gene expression (Halvorson et al. 1996; Dorn et al. 1999). In the same promoter, strong synergism between Ptx1 and SF1 is also observed (Tremblay et al. 1998). WT-1 is a protein critical for normal development of the kidneys and gonads. WT-1 is found to interact directly with SF1 to synergistically activate the MIS promoter in Sertoli cells (Nachtigal et al. 1998). However, DAX-1, a gene on the human X chromosome that encodes another nuclear receptor involved in sex determination, physically interacts with SF1, and actually inhibits SF1-mediated transcription of many of the SF1-responsive promoters (Ito et al. 1997; Crawford et al. 1998).

Besides interactions with the above tissue-specific and nonspecific transcription factors, SF1 is also able to recruit co-activator and co-repressor complexes which are usually regarded as critical regulators of nuclear receptor transcriptional activity (Parker et al. 2002). Recruitment of co-activators, such as SRC-1, CBP/P300, GRIP1, and MBP1, to the promoter-bound complexes potentiates the transactivation activity of SF1 (Crawford et al. 1997; Monte et al. 1998; Hammer et al. 1999a; Kabe et al. 1999). In contrast, interactions with co-repressor complexes, e.g. SMART or N-CoR, diminish the transcriptional activity of SF1 (Crawford et al. 1998; Hammer et al. 1999a). It has been proposed that SF1, through differential interactions with a specific set of transcription factors and cofactors, determines the distinctive expression of its target genes in various tissues (Parker et al. 2002).
Modulation of SF1 transcriptional activity by small molecule ligands is receiving increasingly more attention. Lala and colleagues first reported that hydroxycholesterol derivatives, known suppressors of cholesterol biosynthesis, increase SF1-dependent transcriptional activity in CV-1 cells by 2-10-fold (Lala et al. 1997). Others, however, failed to duplicate these results in other cells, suggesting that these effects of oxysterols are unique to certain nonsteroidogenic cells and may not be physiologically relevant (Christenson et al. 1998; Mellon et al. 1998). Recently, structural studies of bacterially expressed SF1 by several groups (Krylova et al. 2005; Li et al. 2005; Wang et al. 2005) indicated that SF1 preferentially binds phosphatidyl inositols PIP2 (phosphatidylinositol 4,5-bisphosphate) and PIP3 (phosphatidylinositol 3,4,5-trisphosphate) (Krylova et al. 2005), as well as phosphatidic acid (PA) and phosphatidylethanolamine (PE), which have fatty acyl chains of between 12 and 18 carbons (Li et al. 2005). These phospholipids can be readily exchanged and modulate the interaction of SF1 with co-activators. Mutations that reduce the SF1 pocket size or disrupt its phospholipid interaction sites abolished SF1/coactivator association and reduced SF1 transcriptional activity (Li et al. 2005). In searching for the endogenous ligands, Sewer’s group demonstrated that SF1 binds to sphingosine (SPH) and phosphatidic acid (PA) in the human H295R adrenal cells (Urs et al. 2006; Li et al. 2007). SPH is bound to the receptor under basal condition and acts as an endogenous antagonist. Activation of ACTH/cAMP signals rapidly decreases SPH binding but at the same time increases nuclear diacylglycerol kinase (DGK) activity and PA production. PA works as an agonist of SF1 and stimulates SF1-dependent transcription of CYP17 and several other steroidogenic genes (Li et al. 2007). These results suggest that the constitutive activity of SF1 may be subject to regulation by endogenous lipid ligands.
5. The Roles of Transcription Factors Sp1 and Sp3 in Steroidogenesis

Sp1 and Sp3 belong to the large Sp/KLF (specificity protein/Kruppel-like factor) family of transcription factors that consist of at least eight Sp proteins and 15 KLF factors. These Sp/KLF members are united by a particular combination of three conserved Cys2-His2 zinc fingers that form the sequence-specific DNA binding domain (Philipsen et al. 1999; Bouwman et al. 2002; Suske et al. 2005). Each of the three zinc fingers has been shown to recognize three bases in one strand and a single base in the complementary strand, thus, making the family members bind to the same GC-(GGGCGGGG) and GT-(GGTGTGGGG) boxes with different affinities (Pavletich et al. 1991; Narayan et al. 1997). Based on their modular structures (Fig. I-5), Sp/KLF members can be divided into several subgroups (Philipsen et al. 1999): Sp1 – Sp4 form a subgroup which contains several distinctive features/regions such as an activation domain (AD), the C-terminal zinc finger DNA binding region, and an inhibitory domain (ID). Sp5 – Sp8 are structurally similar and appear to be truncated forms of Sp1 – Sp4 in which portions of the N-terminal region have been deleted. The 15 KLF factors exhibit considerable structural variability though they all contain the three zinc finger motifs. Within the Sp/KLF transcription factors, Sp1 and Sp3 are ubiquitously expressed in mammalian cells (Marin et al. 1997; Bouwman et al. 2000), but Sp4, Sp7 and factors like EKLF (erythroid Kruppel-like factor) show tissue-restricted expression patterns (Nuez et al. 1995; Gollner et al. 2001; Nakashima et al. 2002). Acting
Fig. I-5. Structural motifs in Sp/KLF factors (Adapted from Li et al. 2004)

The Sp/KLF factors all contain the characteristic three zinc finger domains (*black-filled boxes*) at their C-terminal region but other regions are variable. The buttonhead boxes (*Btd*) are conserved only in Sp factors (Sp1-Sp8) and are absent in KLF factors. *AD* and *ID* represent activation and inhibitory domains, respectively. *A*, *B*, *C*, and *D* modules of Sp1 (Courey et al. 1988) are marked with black bars. Two short forms of Sp3 (M1-, M2-Sp3) are indicated.
through the GC/GT-box in the promoter region, Sp/KLF factors regulate expression of many different genes, including housekeeping, tissue-specific, viral, and inducible genes.

For many cell types and promoters, Sp1 and Sp3 are the major GC/GT box-binding factors. They may act as positive or negative regulators of gene transcription and their activity at any specific promoter is governed by several variables (Philipsen et al. 1999; Suske 1999). First, the Sp1 and Sp3 abundance and their relative ratio (Krikun et al. 2000; Ling et al. 2001; Pang et al. 2004) may play important role in gene regulation. In mammalian cells, Sp1 typically acts as an activator, while Sp3 serves as a repressor or an activator (Philipsen et al. 1999; Li et al. 2004a). This dual activity of Sp3 is probably related to the expression of different Sp3 isozymes (Kennett et al. 1997). In addition to the full-length protein that is structurally similar to Sp1, Sp3 has two short isozymes. These short isozymes are derived from internal translational initiations and are expressed in all mammalian cells and tissues along with the long isozyme. The short Sp3 isozymes lack the transactivation A domain, but do have the B domain and the inhibitory domain believed to be responsible for their repression function (Kennett et al. 1997; Kennett et al. 2002).

Second, like many other transcription factors, Sp1- and Sp3-mediated regulatory activity is dependent on their ability to recruit and interact with specific nuclear factors and protein complexes. Sp1 can form stacked multimers at the DNA-binding site (Mastrangelo et al. 1991) and communicate directly with the basal transcription machinery by interacting with several of its cofactors, such as the TATA binding protein (TBP) (Emili et al. 1994), dTAF110/hTAF130, hTAF55, and hTAF250 (Chiang et al. 1995; Tanese et al. 1996). Sp1 and Sp3 proteins have also been shown to functionally interact with many sequence-specific transcription factors. Examples include ubiquitous factors like Oct-1, NF-κB, and E2F-1.
(Shao et al. 1995; Karlseder et al. 1996; Pazin et al. 1996); tissue-specific regulators like MEF-1 and GATA (Merika et al. 1995; Grayson et al. 1998); and even nuclear receptors like ER, VDR, and SF1 (Safe et al. 2004). Interactions with these transcription factors can cooperatively activate or repress expression of various target genes. Finally, posttranslational modification of Sp proteins (Jackson et al. 1988), recruitment of chromatin remodeling complexes by Sp1 and Sp3 (Naar et al. 1998), and methylation of GC sequence of the binding sites (Brandeis et al. 1994) all have been suggested to be involved in Sp1- and Sp3-mediated transcriptional regulation, adding additional layers of complexity to the function of these transcription factors.

By regulating the expression of a large number of genes that have GC-rich promoters, Sp1 and Sp3 transcription factors may take part in virtually all facets of cellular function (Kaczynski et al. 2003). In steroidogenic tissues, Sp1 and Sp3 have been reported to regulate the expression of at least three P450 steroid hydroxylases, CYP11A1, CYP17, and CYP21. Cooperating with SF-1 and CBP, Sp1 can mediate the cAMP-dependent transcription of CYP11A1 (Liu et al. 1999). In the CYP17 promoter, Sp1, Sp3 and nuclear factor-1C (NF-1C) are essential for optimal basal transcription of this enzyme (Lin et al. 2001). Studies on the cAMP-dependent transcription of CYP21 show that the binding of adrenal specific protein (ASP) and Sp1 to the promoter and their synergistic actions are required for maximal gene induction (Zanger et al. 1992). However, the overall function of the Sp1 and Sp3 proteins on the expression of steroidogenic genes is not well known.
6. Rationale, Hypothesis, and Objectives

Although the Adcy isozymes and their product cAMP, are essential for the action of ACTH on the adrenal cortex, the mechanisms determining the tissue-specific expression pattern of these isozymes and the contribution of each isozyme to adrenocortical function and steroidogenesis are still largely elusive. Dr. Schimmer’s laboratory has investigated the roles of these Adcy isozymes in adrenocortical function using the mouse adrenal Y1 tumor cell line as a model system. This cell line was isolated from a culture-adapted mouse adrenocortical tumor (Yasumura et al. 1966; Rainey et al. 2004), which originally had the ability to produce corticosterone as one of the major steroid products and responded to ACTH with increased rates of steroidogenesis (Cohen et al. 1957). Subsequent passages of the tumor in isogenic mice, however, resulted in loss of its metastatic property and the capacity to produce corticosterone (Bloch et al. 1960). Y1 cells in culture produce 20α-dihydroxyprogesterone and 11β, 20α- dihydroxyprogesterone instead of corticosterone produced in normal the mouse adrenal glands (Pierson 1967; Kowal et al. 1968). Prolonged incubation of these cells leads to the synthesis of 11keto, 20α- dihydroxyprogesterone, a product generated by 11β-hydroxysteroid dehydrogenase from the accumulated 11β, 20α- dihydroxyprogesterone. This abnormal steroid profile may result from a deficiency in the CYP21 coupled with an increase in 20α-hydroxysteroid dehydrogenase activity (Pierson 1967; Parker et al. 1985); transfection of Y1 cells with genomic DNA encoding CYP21 restores activity and the capacity for corticosterone production in the cell line (Parker et al. 1985; Szyf et al. 1990).

Despite the minor deviation from normal adrenal cells in steroid production, Y1 cells express most of other genes essential for steroidogenesis and exhibit ACTH-regulated
steroid synthesis that is similar to the regulation by seen in cells cultured from normal adrenal glands (Schimmer 1979). Prolonged stimulation with ACTH or with other agents that raise intracellular levels of cAMP leads to the induction of a number of genes that are supportive for steroid biosynthesis. Examples include genes encoding the MC2R, CYP11A1, CYP11B1, StAR, adrenodoxin and HDL receptor SR-BI (Rainey et al. 2004). Moreover, the Y1 cell line is readily amenable to genetic manipulation, making it possible to change the phenotype of the cell line through mutations or gene transfer (Parker et al. 1985). This cell line has, therefore, become one of the most useful cellular models to study the regulation of steroidogenesis by ACTH.

To facilitate the genetic studies of the ACTH/cAMP signaling pathways involved in the adrenocortical function in Y1 cells, Dr. Schimmer’s lab isolated and characterized four ACTH receptor-deficient mutant clones (known as Y6, OS3, 10r6 and 10r9). Clones Y6 and OS3 were spontaneous mutants and were isolated from a transplanted tumor (Yasumura 1968) and from the Y1 cell line itself (Schimmer 1969), respectively; whereas, clones 10r6 and 10r9 were isolated from Y1 cells in the presence of the diterpene forskolin, a direct adenylyl cyclase activator (Schimmer et al. 1984). Treatment of Y1 cells with forskolin stimulated Adcy activity, increased the intracellular level of cAMP that in turn inhibited cell growth and caused the cells to round up and detach from the culture dishes. These specific effects of forskolin permitted the isolation and identification of mutants that are stably resistant to the diterpene (Schimmer et al. 1984). Remarkably, all the four mutants shared very similar phenotypes despite their apparently independent origins (Rainey et al. 2004). They all exhibited Adcy activities that were resistant to the activation by ACTH and forskolin, but fully responsive to sodium fluoride (Schimmer et al. 1984; Schimmer et al.
1987; Qiu et al. 1996). They fail to express the genes encoding ACTH receptor and Adcy4 (Qiu et al. 1996; Al-Hakim et al. 2004). They are deficient in $G\alpha_s$ and $G\alpha_i$ at the protein level but not the mRNA level (Qiu et al. 1996). Most interestingly, all these mutants appear to have similar underlying mutation(s) that impaired the activities of $G\beta\gamma$ subunits (Mitchell et al. 1992; Qiu et al. 1998) and the transactivation function of SF1 (Frigeri et al. 2000).

The underlying mutation(s) of these mutant cells have not been identified yet, but their ACTH-resistance appears to result from the defective SF1 function that prevents the expression of the gene encoding the ACTH receptor (Frigeri et al. 2000). The mutation is not in SF1 per se, and does not alter the ability of SF1 to bind its DNA recognition sites; rather it impairs the ability of SF1 to interact functionally with co-activators such as GRIP1 (Frigeri et al. 2000). The defect in SF1 function also affects the expression of many other SF1-target genes such as those encoding CYP11A1, CYP11B1 and StAR (Frigeri et al. 2000); however, the loss of $G\alpha_s$ and $G\alpha_i$ in the mutants is only linked to the loss of ACTH receptors, which is supported by the findings that $G\alpha_s$ and $G\alpha_i$ are restored to normal levels upon transfection of the mutants with cDNA expressing the ACTH receptor or the $\beta_2$-adrenergic receptor (Qiu et al. 1996). Thus, the G-protein coupled receptors may exert stabilizing effects on their associated $G\alpha$ subunits in the plasma membrane.

On the other hand, forskolin-resistance seen in the mutants is largely due to the loss of Adcy4. It has been shown that forskolin-induced growth-inhibitory and morphological effects in normal Y1 cells are mediated by cAMP (Schimmer et al. 1984). In all the four mutants, these cellular effects of forskolin are severely impaired, most probably due to the failure in forskolin-induced cAMP accumulation rather than the defect in cAMP action (Schimmer et al. 1984). Analyses of the Adcy isozyme composition revealed that the four
mutants were all specifically deficient in Adcy4; the levels of other Adcy isozymes (Adcy1, Adcy3 and Adcy5/6) were comparable to the levels in parent Y1 cells (Al-Hakim et al. 2004). Transfection of mutant cells with a rat Adcy4 expressing vector increased Adcy4 expression, restored the sensitivity of Adcys to the forskolin stimulation and rescued the defects in the forskolin-induced changes in cell morphology and growth (Al-Hakim et al. 2004). These results demonstrate the important role of Adcy4 in the forskolin-resistant phenotype of the mutant clones.

The concurrent loss of ACTH receptor and Adcy4 expression appear to be independent consequences of the same underlying mutation, which can be partially suppressed by expression of the Gβγ subunits (Rui et al. 2004). Mutant cells transfected with a plasmid encoding ACTH receptor can restore the ACTH-responsive Adcy activity, but remain Adcy4 deficient and forskolin-resistant (Qiu et al. 1996). Similarly, mutant cells transfected with an Adcy4 expression plasmid only restore their responsiveness to forskolin; the ACTH receptor still remains deficient (Al-Hakim et al. 2004). Interestingly, the expression of both MC2R and Adcy4 can be restored by transfection of mutant cells with vectors encoding Gβγ subunits (Qiu et al. 1998; Rui et al. 2004). This unexpected regulation in the mutant cells makes the model system especially useful for the further study of Adcy4 gene regulation.

Because Adcy4 is widely expressed, my first hypothesis is that the mechanisms controlling its expression within different tissues are largely similar and may involve some ubiquitous transcription factors. Based on the observation that Adcy4 is expressed in Y1 mouse adrenocortical tumor cells but is not expressed in mutant Y1 derivatives with defective SF1 function (Frigeri et al. 2000; Al-Hakim et al. 2004), my second hypothesis is
that SF1 influences Adcy4 expression in Y1 cells and the loss of SF1 function leads to the loss of Adcy4 expression in the mutant cells.

This study is designed to investigate the activities of the promoter/regulatory region of *Adcy4* in Y1 adrenal cells and identify the regulatory elements and the transcription factors that govern or contribute to the expression of this gene. Meanwhile, the contributions of the regulatory elements and transcription factors to gene expression in Adcy4 deficient mutants have also been evaluated to determine the basis of the indirect loss of Adcy4 expression. My original objectives were to:

1. Determine the intron exon organization of *Adcy4*
2. Identify the transcription start sites of *Adcy4* in Y1 adrenocortical tumor cells and other mouse tissues
3. Determine the contribution of 5’ flanking DNA to *Adcy4* expression in Y1 cells
4. Identify the transcription factors that affect the expression of *Adcy4* in Y1 cells
5. Determine the regulatory elements and transcription factors that are responsible for the loss of *Adcy4* expression in Adcy4 deficient mutant cells
SECTION II. MATERIALS AND METHODS

1. Synthetic Oligonucleotides

Table II-1. Primers for Adcy4 cDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS157</td>
<td>TGCTGAGAGAACTTCTGGGAGC</td>
<td>47-67</td>
</tr>
<tr>
<td>BS159</td>
<td>CGTTCTCTGTGGAGTCTTGG</td>
<td>1231-1251</td>
</tr>
<tr>
<td>BS160</td>
<td>CCTGGAAGAACTTCTCTTCACC</td>
<td>1733-1712</td>
</tr>
<tr>
<td>BS161</td>
<td>CGTTCTCTGTGGAGTCTTGG</td>
<td>296-315</td>
</tr>
<tr>
<td>BS163</td>
<td>GTGTGAGTAGTAGTAGTAGTAG</td>
<td>401-420</td>
</tr>
<tr>
<td>BS164</td>
<td>TATCCTTCCTTGCTGCTGCTG</td>
<td>802-821</td>
</tr>
<tr>
<td>BS165</td>
<td>TCTATCTCTGAGTCTTGAG</td>
<td>1552-1571</td>
</tr>
<tr>
<td>BS166</td>
<td>AAGATTTTGACTTGTGGG</td>
<td>2036-2054</td>
</tr>
<tr>
<td>BS167</td>
<td>GCCCTTTATCAAAGCCCTTCCG</td>
<td>2421-2440</td>
</tr>
<tr>
<td>BS168</td>
<td>TGCTGACTTTGGATGACTG</td>
<td>2803-2822</td>
</tr>
<tr>
<td>BS169</td>
<td>GCAAGATCCTGAAGAGGCTG</td>
<td>3189-3209</td>
</tr>
<tr>
<td>BS170</td>
<td>TAGGATGCTGAGGGAGGATCC</td>
<td>3312-3293</td>
</tr>
<tr>
<td>BS171</td>
<td>TCCAGAGGATGATTTAAG</td>
<td>2911-2892</td>
</tr>
<tr>
<td>BS172</td>
<td>TATCCTTCCTTGCTGCTG</td>
<td>2569-2551</td>
</tr>
<tr>
<td>BS173</td>
<td>CAGATGACACATTTGGAAGCC</td>
<td>2207-2188</td>
</tr>
<tr>
<td>BS174</td>
<td>CTGCTTCCATATGGGTTG</td>
<td>1319-1300</td>
</tr>
<tr>
<td>BS175</td>
<td>CCAGATGATGGGCTGAGCC</td>
<td>956-937</td>
</tr>
<tr>
<td>BS176</td>
<td>TTGCTGCCACTTGCTGAG</td>
<td>641-622</td>
</tr>
<tr>
<td>BS177</td>
<td>ATCAGCGTGGGTACTGTTG</td>
<td>202-182</td>
</tr>
<tr>
<td>BS179</td>
<td>TAGGCCAGCGAGCAGAAGTTC</td>
<td>426-407</td>
</tr>
<tr>
<td>BS180</td>
<td>AAGAGATCTTCGCTGGGG</td>
<td>159-140</td>
</tr>
<tr>
<td>BS577</td>
<td>TGAGGAGAAGAACGAGAGAG</td>
<td>1465-1484</td>
</tr>
<tr>
<td>BS578</td>
<td>TGTCAGGGGGCTCTAGGCC</td>
<td>1607-1588</td>
</tr>
</tbody>
</table>

*, Refers to positions targeted on Adcy4 cDNA (accession no. AF442771)
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS209</td>
<td>CGCTTTGGGTCAAGCTCC</td>
<td>+92/+75</td>
</tr>
<tr>
<td>BS237</td>
<td>cggaatTCTCTGAGGTTGCAATCCAGGG</td>
<td>-958/-939</td>
</tr>
<tr>
<td>BS238</td>
<td>cgggatccTGTGTGGTGCTAGAAGCC</td>
<td>-572/-592</td>
</tr>
<tr>
<td>BS239</td>
<td>cggaattcGGTGAAGGAAGTGCTCAGG</td>
<td>-631/-611</td>
</tr>
<tr>
<td>BS240</td>
<td>cgggatccAGGAAAGCCTGGGAGAAAGC</td>
<td>-290/-309</td>
</tr>
<tr>
<td>BS241</td>
<td>cggaattcCTCCCAGGCCRTCTCTCTGG</td>
<td>-304/-287</td>
</tr>
<tr>
<td>BS242</td>
<td>cgggatccCAAAGCTAGGCTCCAGGAG</td>
<td>+60/+41</td>
</tr>
<tr>
<td>BS459</td>
<td>GCCAGGTCAAGGATAGTGGATCTCCTACCTCTTGCTC</td>
<td>-356/-395</td>
</tr>
<tr>
<td>BS460</td>
<td>GCCGGGGGTCACGTGACATCGCCATTTCCCTTC</td>
<td>-444/-383</td>
</tr>
<tr>
<td>BS461</td>
<td>GGGGTCCTCCTGATGTTGAGGATCCGGGGTGGGCG</td>
<td>-321/-358</td>
</tr>
<tr>
<td>BS462</td>
<td>CGGGAGGTGGGGAGITGAGTTGGATCCTCCTCTTTGGTCCC</td>
<td>-309/-446</td>
</tr>
<tr>
<td>BS464</td>
<td>GAGAAACACAGAGGTTAGGGATATGCTATCTCTGACCTCGC</td>
<td>-208/-225</td>
</tr>
<tr>
<td>BS465</td>
<td>CAGACAGAATGTGGGATAGTGGATCTCCTACCTCTCTTGGCG</td>
<td>-395/-356</td>
</tr>
<tr>
<td>BS466</td>
<td>GAACCGGAAATGTGGGATAGTGGATCTCCTACCTCTCTGACCTCGC</td>
<td>-383/-444</td>
</tr>
<tr>
<td>BS467</td>
<td>CGCCCAACCCGGGATAGTGGATCTCCTACCTCTCTCAGGACACC</td>
<td>-358/-321</td>
</tr>
<tr>
<td>BS468</td>
<td>CCGGCCCAGCTGGGGAGITGAGTTGGATCCTCCTCTCTCTGCC</td>
<td>-446/-309</td>
</tr>
<tr>
<td>BS771</td>
<td>gggtaccGAATGCAATCTGCAGCATGG</td>
<td>-142/-124</td>
</tr>
<tr>
<td>BS772</td>
<td>cggctcgagCTAGTGGCTCAGGGCTGC</td>
<td>-2/-20</td>
</tr>
<tr>
<td>BS773</td>
<td>CACCTGCTCATCGACATCG</td>
<td>-199/-178</td>
</tr>
<tr>
<td>BS774</td>
<td>CCTTTGCTAGTGGCCTACG</td>
<td>+4/-15</td>
</tr>
<tr>
<td>BS775</td>
<td>GAGGCAAGCTATGATAACACC</td>
<td>+2548/+2568</td>
</tr>
<tr>
<td>BS776</td>
<td>TCCATATTCTTCCTGTGACGATC</td>
<td>+2766/+2744</td>
</tr>
<tr>
<td>BS781</td>
<td>CAGGGTCTCAAGACGAGAGATGACTGTGCTGGCTGTATCG</td>
<td>-106/-72</td>
</tr>
<tr>
<td>BS782</td>
<td>CGATAACGCCAGCTACTCTCTCTCTGGAGACCTG</td>
<td>-72/-106</td>
</tr>
<tr>
<td>BS783</td>
<td>GTATCGGGGGGAAAGTGCTAGACGCGCTTTGCGCCCAAG</td>
<td>-77/-41</td>
</tr>
<tr>
<td>BS784</td>
<td>CTTGGCGCAAGGCGTCTAGCAGCTCCCCTCCCGCTATAC</td>
<td>-41/-77</td>
</tr>
<tr>
<td>BS785</td>
<td>GGAAGTGGGAGGGCTCTCTGCGAAAGAGGGAAG</td>
<td>-68/-34</td>
</tr>
<tr>
<td>BS786</td>
<td>CCTCTTCTTGGCGCAAGAGGCCGCCCTCTCCACCTCTCCC</td>
<td>-34/-68</td>
</tr>
<tr>
<td>BS787</td>
<td>CGGGGAAGTGCTAGACGCGCTTTGCGCCCAAGAGGGAAG</td>
<td>-71/-34</td>
</tr>
<tr>
<td>BS788</td>
<td>CTTCCCTCTTGGCGCAAGAGGCCCTCTAGCACCTCCCGCA</td>
<td>-34/-71</td>
</tr>
</tbody>
</table>

The underlined sequences or nucleotides are mutations introduced to replace the original sequences or nucleotides; small letters represent 5’-overhang for added restriction sites. *, Refers to relative position to the major transcription start site of Adcy4 in Y1 cells.
Table II-3. Oligonucleotides for EMSA

<table>
<thead>
<tr>
<th>Oligo Probe</th>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo1</td>
<td>BS561</td>
<td>gatccATCTGAGCATGGGAAGCAGCCAGGGa</td>
<td>-135/-121</td>
</tr>
<tr>
<td></td>
<td>BS562</td>
<td>gatctCCCTGGCTTCCATGCTCAGATg</td>
<td>-121/-135</td>
</tr>
<tr>
<td></td>
<td>BS563</td>
<td>gatccGAACGGGAAGGGCTGGCTGATCa</td>
<td>-97/-73</td>
</tr>
<tr>
<td></td>
<td>BS564</td>
<td>gatctGATAACAGCCCAGCCCTCCGTTTg</td>
<td>-73/-97</td>
</tr>
<tr>
<td></td>
<td>BS565</td>
<td>gatccGGGGAAGTGGGAGGGGGCTTGGGa</td>
<td>-70/-46</td>
</tr>
<tr>
<td>Oligo2</td>
<td>BS566</td>
<td>gatctCGCCAAGGGCCCCTCCACCTCCCg</td>
<td>-46/-70</td>
</tr>
<tr>
<td></td>
<td>BS567</td>
<td>gatccGGGGAAGTGGGAGGGGGCTTGGGa</td>
<td>-45/-19</td>
</tr>
<tr>
<td></td>
<td>BS568</td>
<td>gatctCCGGGCGACTGGCACTTCCCTTGGg</td>
<td>-19/-45</td>
</tr>
<tr>
<td></td>
<td>BS569</td>
<td>gatccCAACGGGAGTCAGGCTCAGAAGCCGa</td>
<td>-116/-91</td>
</tr>
<tr>
<td>Oligo3</td>
<td>BS570</td>
<td>gatctCCCCTCTGAGCTGACTCCCTCCCTGg</td>
<td>-91/-116</td>
</tr>
<tr>
<td></td>
<td>BS571</td>
<td>gatccGGGGCTGTATCCTCCAGAAGCCGa</td>
<td>-82/-58</td>
</tr>
<tr>
<td></td>
<td>BS572</td>
<td>gatctCTCCCCTTCCCGATACCGCCCGg</td>
<td>-58/-82</td>
</tr>
<tr>
<td></td>
<td>BS573</td>
<td>gatccGGGGCTGTATCCTCCAGAAGCCGa</td>
<td>-57/-42</td>
</tr>
<tr>
<td>Oligo5</td>
<td>BS574</td>
<td>gatctACTTCCCTCTTGGCCAGGCCCCg</td>
<td>-42/-57</td>
</tr>
<tr>
<td></td>
<td>BS575</td>
<td>gatccGAACGGGAGTCAGGCTCAGAAGCCGa</td>
<td>-97/-73</td>
</tr>
<tr>
<td>Oligo2 Sp1m</td>
<td>BS578</td>
<td>gatctGATAACAGCCCAGCTACCCTCCCTGTCg</td>
<td>-73/-97</td>
</tr>
<tr>
<td>Oligo3 Sp1m</td>
<td>BS579</td>
<td>gatccGGGGCTGTATCCTCCAGAAGCCGa</td>
<td>-70/-46</td>
</tr>
<tr>
<td></td>
<td>BS580</td>
<td>gatctCGCCAAGGGCCCCTCCACCTCCCg</td>
<td>-46/-70</td>
</tr>
<tr>
<td>Oligo3 SF1m</td>
<td>BS581</td>
<td>gatctCGCCAAGGGCCCCTCCACCTCCCg</td>
<td>-70/-46</td>
</tr>
<tr>
<td></td>
<td>BS582</td>
<td>gatctCGCCAAGGGCCCCTCCACCTCCCg</td>
<td>-46/-70</td>
</tr>
<tr>
<td>Oligo4 Sp1m</td>
<td>BS583</td>
<td>gatccGGGCGACTGGCACTTCCCTTGGg</td>
<td>-45/-19</td>
</tr>
<tr>
<td></td>
<td>BS584</td>
<td>gatccGGGCGACTGGCACTTCCCTTGGg</td>
<td>-19/-45</td>
</tr>
<tr>
<td>Sp1</td>
<td>BS576</td>
<td>ATTCGATCGGGGCGGGCCGAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BS577</td>
<td>GCTCGGGGGGGGGGATCGAAT</td>
<td></td>
</tr>
<tr>
<td>SF1</td>
<td>BS122</td>
<td>CTTTACTCAAGGGCTTTAGGATAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BS123</td>
<td>TTTATCTAAACCTTTGAGTAAAG</td>
<td></td>
</tr>
<tr>
<td>SF1m</td>
<td>BS110</td>
<td>CTTTACTCAAGGGCTTTAGGATAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BS111</td>
<td>ATTCCTAACAGGGCTTTAGGATAAA</td>
<td></td>
</tr>
</tbody>
</table>

The underlined nucleotides are mutations introduced to disrupt predicted Sp1 or SF1 binding site; small letters are added sequences to generate cohesive sites for labeling or cloning. *, Refers to relative position to the major transcription start site of Adcy4 in Y1 cells. Oligo probe Sp1 contains the Sp1 consensus sequence (Schafer et al. 2003); Oligo probe SF1 contains the SF1 binding site from Mc2r (Frigeri et al. 2000).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene/vector</th>
<th>Accession no.</th>
<th>Sequence (5' to 3')</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS35</td>
<td>pBluescript II SK L08787</td>
<td>L08787</td>
<td>ATTAACCCTCACTAAAG</td>
<td>790-774</td>
</tr>
<tr>
<td>BS43</td>
<td>pBluescript II SK L08787</td>
<td>L08787</td>
<td>ATACGACTCACTATAGGGCG</td>
<td>627-647</td>
</tr>
<tr>
<td>BS86</td>
<td>pGL3-Basic U47295</td>
<td>U47295</td>
<td>CTAGCAAAATAGGCTGTC</td>
<td>5198-5217</td>
</tr>
<tr>
<td>BS112</td>
<td>pBluescript II SK L08787</td>
<td>L08787</td>
<td>GTAAAACGACGGCCAGT</td>
<td>600-616</td>
</tr>
<tr>
<td>BS113</td>
<td>pBluescript II SK L08787</td>
<td>L08787</td>
<td>AACAGCTATGACCAGT</td>
<td>823-807</td>
</tr>
<tr>
<td>BS425</td>
<td>NR5a1 (SF1) NM_139051</td>
<td>NM_139051</td>
<td>TGGACTATTCGACTCACG</td>
<td>33-53</td>
</tr>
<tr>
<td>BS426</td>
<td>NR5a1 (SF1) NM_139051</td>
<td>NM_139051</td>
<td>GACTGTGCCTTGAAGAAC</td>
<td>154-135</td>
</tr>
<tr>
<td>BS246</td>
<td>NR6a1 (GCNF) NM_010264</td>
<td>NM_010264</td>
<td>TGATAGCTTCTCCTGTCATG</td>
<td>639-619</td>
</tr>
<tr>
<td>BS247</td>
<td>NR6a1 (GCNF) NM_010264</td>
<td>NM_010264</td>
<td>ATCGAGCTGAAACAAGCAA</td>
<td>406-425</td>
</tr>
<tr>
<td>BS398</td>
<td>Tkt NM_009388</td>
<td>NM_009388</td>
<td>TTGCTAACATCCGAATGCC</td>
<td>1019-1038</td>
</tr>
<tr>
<td>BS399</td>
<td>Tkt NM_009388</td>
<td>NM_009388</td>
<td>CGAGAAGGTGGAATTCTTG</td>
<td>1170-1150</td>
</tr>
<tr>
<td>BS575</td>
<td>Gapdh NM_008084</td>
<td>NM_008084</td>
<td>TTCAACGGGCACAGTCAGG</td>
<td>218-236</td>
</tr>
<tr>
<td>BS576</td>
<td>Gapdh NM_008084</td>
<td>NM_008084</td>
<td>TTCAACGGGCACAGTCAGG</td>
<td>342-321</td>
</tr>
</tbody>
</table>
2. Plasmids

2.1 Plasmids

Plasmids BF782774 and BE291206 are two I.M.A.G.E Consortium mouse est clones obtained from ATCC, which contain sequences highly homologous to the published rat Adcy4 cDNA. Plasmids pGL3-Basic, pGL3-Promoter, and pGL3-Control are luciferase reporter vectors, each with a firefly luciferase gene driven by no promoter, SV40 core promoter, and SV40 enhancer and core promoter, respectively (Promega, Madison, WI). Plasmid pTA-Luc is another luciferase reporter vector with a firefly luciferase gene governed by a TA minimal promoter (Clontech Laboratories, Mountain View, CA). Luciferase reporter pPro36-Luc contains a luciferase gene under control of the core element of the prolactin gene (Ikeda et al. 1993). Plasmid p0196L22R (accession no. AZ420139) is a mouse genomic clone containing approximately 10 kb of 5’-flanking DNA of the mouse Adcy4 gene and was kindly provided by Dr. R. B. Weiss, University of Utah Genome Center, University of Utah. General cloning vectors pBluescript II SK(+) and pBluescript II KS(+) were obtained from Stratagene (La Jolla, CA). Three hsRNA expression plasmids, pPRNAT-SF1-R1, -R2, and -R3, targeting nucleotides 151/171 (SF1 hsRNA #1), 565/585 (SF1 hsRNA #2) and 1375/1395 (SF1 hsRNA #3) respectively in the mouse SF1 transcript (accession no. AF511594), and one hsRNA expression plasmid, pRNAT-GCNF-G2, targeting nucleotides 716/736 in GCNF (accession no. NM_010264) were prepared previously in our lab.

2.2 Reporter Gene Constructs
2.2.1 Reporter Plasmids with Progressive Truncation of the 5’-Flanking Region of Adcy4.

The luciferase reporter construct p-5364_{AdcyLuc} was generated by subcloning two fragments, a 4782 bp XhoI-XbaI fragment and a 942 bp XbaI-NcoI fragment, both released from p0196L22R, into the XhoI-NcoI sites of pGL3-Basic (Fig. II-1). The resultant plasmid contained 5364 bp of Adcy4 5’-flanking DNA plus exon I, intron I, and part of exon II until the translation initiation ATG codon (+354) in front of the coding region of the firefly luciferase gene. The p-5364_{AdcyLuc} was used as starting material for making the progressively truncated constructs. Reporter plasmids p-4536_{AdcyLuc}, p-1459_{AdcyLuc}, p-723_{AdcyLuc}, and p-336_{AdcyLuc} were generated by digesting p-5364_{AdcyLuc} with SmaI (-4536), NheI (-1459), SacI (-723), or KpnI (-336) and religating the plasmid. For construction of the plasmids p-970_{AdcyLuc}, p-585_{AdcyLuc}, p-404_{AdcyLuc}, p-180_{AdcyLuc}, and p-18_{AdcyLuc}, the reporter plasmid p-1459_{AdcyLuc} was first digested with HincII, XbaI, MscI, DrdI, or BglII respectively, blunt-ended when necessary, and then digested with NcoI to release the fragments containing different lengths of Adcy4 5’-flanking DNA plus sequence up to the translation initiation ATG codon. These fragments were gel purified and ligated directionally into the SmaI-NcoI sites of the pGL3-Basic vector. Plasmid p-3562_{AdcyLuc} was made by digesting p-5364_{AdcyLuc} with XhoI and BglIII, and then blunt-ending and religating the vector. For the plasmid p-2227_{AdcyLuc}, a 2584 bp NcoI fragment was retrieved from p-5364_{AdcyLuc} and inserted into NcoI site of pGL3-Basic. The correct orientation of the insert was determined by restriction digestion with HindIII. To construct p-1903_{AdcyLuc}, a SacI fragment of 1185 bp was released from p-2227_{AdcyLuc} and inserted into the SacI site of p-
Fig. II-1. Progressive truncation of the 5'-flanking region of *Adcy4*

Two parental luciferase constructs, p-5364\textsubscript{Adcy}Luc and p-1459\textsubscript{Adcy}Luc, with available restriction sites within the 5' flanking region of *Adcy4* for making progressive truncation of this region are presented. Promoter-less luciferase vector pGL3-Basic and its cloning sites also are shown. Arrows indicate translation start sites.
723_{Adcy}Luc. The correct orientation of the insert was determined by digestion with NheI and NcoI.

2.2.2 p-631/-290 Adcy4 Reporter Plasmids

The region flanking Adcy4 from –631 to –290 was placed upstream of the pGL3-Promoter vector (see Section 2.1) by T/A cloning (Zhou et al. 2000). Briefly, the -631/-290 region was amplified from p-1459_{Adcy}Luc by PCR with sense (BS239) and antisense (BS240) primers (Table II-2). The pGL3-Promoter vector was linearized with SmaI and a single dT was added to each end by incubation with Taq DNA polymerase (New England Biolabs Inc. Mississauga, ON) at 72 C for 20 min in the presence of dTTP. The gel purified PCR product was ligated with the “T” ended reporter vector and the resultant recombinants were sequenced to identify inserts in forward and reverse orientation. The plasmids were named p-631/-290SVLuc and p-290/-631SVLuc respectively to reflect the different orientations of the insert and the presence of the minimal SV40 promoter in this vector. The –631/-290 Adcy4 flanking region also was placed in front of pPro36-Luc, containing the prolactin core promoter and luciferase reporter gene. The –631/-290 sequence was retrieved from p-631/-290SVLuc by digestion with XhoI and BamHI and cloned into the same restriction sites of pPro36Luc. The resultant plasmid was designated p-631/-290ProLuc.

2.2.3 p-404_{Adcy}Luc Reporter Plasmid with Mutations in the –404/-320 Region

Four Mutants of the reporter plasmid p-404_{Adcy}Luc, each with 12-bp mutations in the –404–320 region (M1-M4) were made by PCR using mutated oligonucleotide primers as described previously (Ho et al. 1989). Briefly, a SacI-HindIII fragment (–404/+233)
containing the target region, exon I and part of intron I from p-404_{Adcy}Luc was subcloned into the SacI-HindIII sites of pBluescript II (SK+), resulting in the intermediate plasmid p-404/+233Bluescript II (SK+) (Fig. II-2). This intermediate plasmid was used as a template for all four mutations. Four sets of complementary primers, namely BS465/BS459, BS466/BS460, BS467/BS461, and BS468/BS462, with each set containing a 12-bp mutation block (Table II-2), were designed to make these four mutations. First, each set of primers was used in PCR in combination with the flanking primers BS113 (Table II-4) and BS242 (Table II-2) to amplify two fragments which overlapped at the end containing the mutated sequence (Fig. II-2). These two fragments were then denatured, annealed, and extended using four cycles of PCR to generate a longer fused template; a nested PCR was performed with primers BS35 (Table II-4) and BS464 (Table II-2) to amplify the fragment which carried the 12 bp mutated sequences (Fig. II-2). Next, the amplified fragment was digested with SacI and XcmI and cloned back into the same sites of the intermediate plasmid p-404/+233Bluescript II (SK+) ; the mutated sequence between SacI and XcmI was confirmed by dideoxynucleotide sequencing (Sanger et al. 1977). Finally, the intermediate plasmids harboring each mutation were digested with SacI and HindIII to release 638 bp fragments (Fig. II-2). These fragments were cloned back into p-404_{Adcy}Luc at the corresponding sites, replacing the original sequence.

2.2.4 p-404_{Adcy}Luc Reporter Plasmid with the Internal –135/-19 Region of Adcy4 Deleted

Reporter plasmid p-404_{Adcy}Luc with the -135/-19 region deleted was constructed using the available BsmI and BgII restriction sites flanking the conserved region as shown in
The intermediate plasmid p-404/+233Bluescript II (SK+) carrying a 638 bp fragment derived from Adcy4, which contains a -404 bp of 5' flanking region, the first exon (E1), and part of Intron I, was used as template for generation of mutations in the -404/-320 region. Complementary primers, each with a 12 bp mutation block (double x marked arrows), and regular flanking primers (arrows) for the PCR-based mutation process are presented. The cross-hatched box denotes the highly conserved region of Adcy4. Details on the mutation and subcloning steps are described in the text.
Fig. II-3. The intermediate plasmid p-404/+233Bluescript II (SK+) was linearized separately with BsmI and BglII, blunt ended and then each was digested with HindIII. The larger fragment purified from the BsmI/HindIII digestion which contained the vector DNA and the –404/–136 sequence of Adcy4 was ligated to the smaller fragment purified from the BglII/HindIII digestion which contained the –18/+233 region of Adcy4 (Fig. II-3). The resultant plasmid was digested with SacI and HindIII to release a fragment which contained a deletion in –135/-19 region. This fragment was cloned into p-404AdcyLuc to replace the original sequence. The incorporation of the deletion into p-404AdcyLuc was confirmed by restriction digestion with SacI and HindIII enzymes.

2.2.5 p-404AdcyLuc Reporter Plasmid with the First Intron of Adcy4 Deleted

Reporter plasmid p-404AdcyLuc with the first intron deleted was generated by fusion of two separate regions – one from –404 to +92 (i.e., to the end of first exon of Adcy4 genomic DNA) and the other from the non-coding region of Adcy4 cDNA. As shown in Fig. II-4, these two regions were amplified from the intermediate plasmid p-404/+233Bluescript II (SK+) and from the Adcy4 cDNA clone BF782774 with primer sets BS35/BS209 (Table II-4 and 2-2), or BS157/BS176 (Table II-1) respectively. The amplified fragments had a 41 bp overlap at the end of the first exon. These overlapping fragments were denatured, annealed, and extended using four cycles of PCR to generate a longer template with the entire intron I deleted. A nested PCR using primers BS241 (Table II-2) and BS179 (Table II-1) was performed to amplify the intron I deleted fragment. This fragment was digested with XcmI and HindIII and subcloned back into the same sites of p-404/+233Bluescript II (SK+) to replace the intron I containing fragment. The XcmI-HindIII region was sequenced.
Fig. II-3. Internal deletion of the -135/-19 region of Adcy4

The intermediate plasmid p-404/+233Bluescript II (SK+) as described in Fig. II-2 and appropriate restriction sites available in the highly conserved region used to delete the -135/-19 region are presented. Details are provided in the text.
Fig. II-4. Deletion of the first intron of Adcy4

A genomic region in p-404/+233Bluescript II SK(+) as described in Fig. II-1 and a full-length Adcy4 cDNA in pSport6-Adcy4 were used to generate a fused fragment with specific deletion in the first intron of Adcy4 via a PCR-based method. The primer pairs and appropriate restriction sites used for this procedure are indicated. Details are provided in the text.
to confirm the deletion mutation. The SacI-NcoI fragment with intron I deleted was then subcloned into the same restriction sites of p-404AdcyLuc to replace the original Adcy4 promoter element.

2.2.6 pTA-Luc -Based Heterologous Reporter Plasmids

To place the –142/-2 flanking region of Adcy4 in front of pTA-Luc reporter vector, which contains a minimal TATA box and a heterologous luciferase gene, this region was first amplified from p-404AdcyLuc with primers BS771 and BS772 (Table II-2; Fig. II-5) and cloned into the EcoRV site of p-Bluescript II (KS+) by T/A cloning (Section 2.2.2). DNA sequencing was performed to make sure that mutations were not introduced into the amplified fragment. Because primers BS771 and BS772 had KpnI or Xhol restriction site at their respective 5’ ends, this fragment was then released by digestion with these two enzymes and subcloned into the corresponding sites of pTA-Luc, resulting in the reporter plasmid p-142/-2TALuc (Fig. II-5).

To replace the minimal TATA box element of p-142/-2TALuc or pTA-Luc with Adcy4-derived element, the Adcy4 regions from –142 to +92 and from –18 to +92 were amplified from plasmids p-404AdcyLuc and p–18AdcyLuc with primer set BS771/BS209 and BS86/BS209, respectively (Fig. II-5). The amplified fragments were subcloned into EcoRV site of pBluescript II (KS+) by T/A cloning. Plasmids containing inserts in the correct orientation were identified by restriction digestion, and the sequences of the inserts were confirmed by dideoxynucleotide DNA sequencing. These two fragments with correct sequences were then subcloned into the KpnI and HindIII digested vector pTA-Luc, replacing the TATA box with Adcy4-derived sequences, but leaving the translation
Fig. II-5. Construction of pTA-Luc-based luciferase reporter plasmids

The Adcy4 promoter-luciferase constructs used for generating different promoter elements via PCR amplification or restriction digestion are presented as well as pTA-Luc reporter vector. Primers used for specific promoter-luciferase templates, relevant restriction sites in each plasmid, and the relative positions for the ends of different promoter elements generated also are indicated. TA, TATA box of a minimal promoter element; E1, exon I of Adcy4. Bent Arrows represent translation start sites and cross-hatched boxes denote the highly conserved regions. Details of plasmid construction are provided in the text.
initiation signals of the vector intact (Fig. II-5). The resultant plasmids were designated as p-142/+92AdcyLuc and p-18/+92AdcyLuc.

To re-introduce the first intron into the heterologous reporter constructs, the Adcy4-derived region from −142 to +354, which contains the highly conserved region, the first exon and first intron, and the non-coding region of exon II, were retrieved from p-180AdcyLuc by excising a BsmI (blunt-ended)–NcoI fragment and subcloning this fragment into the SmaI and NcoI digested vector pTA-Luc (Fig. II-5), resulting in reporter plasmid p-142/+354AdcyLuc. As a control, the Adcy4-derived region from -18 to +354 was released from p-18AdcyLuc by double digestion with KpnI and NcoI and subcloned into the same restriction sites of pTA-Luc (Fig. II-5), resulting in p-18/+354AdcyLuc.

2.2.7 Reporter Constructs with Sp1A, Sp1B, and/or SF1 Mutations in the Conserved Region

To mutate the Sp1A, Sp1B, and/or SF1 binding sites in the conserved region, the intermediate recombinant plasmid p-142/+92Bluescript II (KS+) (Section 2.2.6) was used as template. As shown in Fig. II-6, three sets of primer pairs containing point mutations for Sp1A (BS781/BS782), Sp1B (BS783/BS784), and SF1 (BS785/BS786) only (Table II-2) were used to make single mutations by the two step PCR method described in Section 2.2.3. After the sequences for all the single mutations were confirmed, the Sp1A mutation plasmid was then used as template to make double or triple mutations with primer sets containing point mutations for Sp1B, SF1, or Sp1B and SF1 (BS787/BS788; Table II-2) by the same PCR method. The sequences of all the double and triple mutations were confirmed and the fragments were released by digestion with KpnI and HindIII and inserted into the
Fig. II-6. Mutation of Sp1A, Sp1B, and/or SF1 sites in the -142/-19 region of *Adcy4*

The binding sequences of the Sp1A, Sp1B and SF1 sites (boxed) and the introduced mutations to disrupt these sites are shown at the top. Mutations were introduced using the PCR-based method as described in Fig. II-2 with p-142/+92Bluescript II (KS+) as template, which contains a 235 bp fragment derived from *Adcy4* (shown at the bottom). The complementary primers, each with single or double binding site mutations (x-marked arrows), and regular flanking primers (arrows) as well as restriction cloning sites also are indicated. Detailed procedures are provided in the text.
corresponding sites of pTA-Luc (Fig. II-5), removing the minimal TATA box, but leaving the translation initiation signals of the vector intact.

### 2.3 Purification of Plasmid DNA

Plasmids used for routine molecular cloning manipulations or DNA sequencing were isolated from individual bacterial clones using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON) following the manufacturer’s instructions. Plasmids used for cell transfection were purified in large quantity by alkaline lysis followed by ultracentrifugation through a cesium chloride-ethidium bromide gradient (Ausubel et al. 2001). Briefly, overnight cultures (1L) of bacteria containing each plasmid were harvested by centrifugation and the cell pellets were resuspended in 30 ml of Solution I (50 mM glucose, 25 mM Tris.Cl, pH 8.0, 10 mM EDTA, 25mg/ml lysozyme). The bacterial cells were then lysed by adding 60 ml of freshly prepared Solution II (0.2 N NaOH, 1% sodium dodecyl sulfate) at room temperature for 10 min followed by adding 45 ml of Solution III (3 M potassium acetate, pH4.8) for neutralization. The bulk of chromosomal DNA and cell debris were removed by centrifugation and the plasmid DNA was precipitated with isopropanol. The DNA pellet was dissolved in 16 ml of TE buffer, and 17.8 g of CsCl and 1.6 ml of ethidium bromide (10 mg/ml) were added to make the solution with final density of 1.57 g/ml. The solution was divided into two Beckman quick-seal ultracentrifuge tubes and centrifuged at 55,000 rpm at 20 C for 16 h followed by 40,000 rpm for 1 h in a Beckman Ti 70.1 fixed-angle rotor. The plasmid band was collected with a 20-G needle and made up to 20 ml with CsCl/TE solution (density=1.57 g/ml) and 0.8 ml of ethidium bromide (10 mg/ml). The solution was recentrifuged and the plasmid DNA was collected again as described above.
After removal of the ethidium bromide with salt-saturated isopropanol, plasmid DNA was repeatedly precipitated with ethanol and dissolved in TE buffer. For repeat assays, large-scale preparations of plasmid DNA also were prepared with Qiagen Plasmid Maxi Kits (Qiagen Mississauga, ON) according to the manufacturer’s instructions.

3. Cell Culture and Transfection

Wild type Y1 cells and the forskolin-resistant mutant OS3 are stable subclones isolated from the mouse adrenocortical tumor cell line first described by Yasumuara et al. (Yasumura 1968; Schimmer 1979). Mutant 10r6 and 10r9 cells were obtained by selective growth of Y1 cells in the presence of 10 μM forskolin. Cells were routinely cultured at 37°C under humidified atmosphere with 5% CO₂ in F10 medium supplemented with 15% heat-inactivated horse serum and 2.5% heat-inactivated fetal bovine serum (Invitrogen Canada, Burlington, ON). All complete media contained penicillin G sodium (0.12 mg/ml) and streptomycin sulfate (0.27 mg/ml). For transient transfections, cells (2X10⁵) were plated in 60 mm tissue culture dishes containing 4 ml of alpha minimal essential medium supplemented with sera and antibiotics as described above. After growing for 3 days at 37°C under 5% CO₂, cells were replaced with the same fresh medium and transfected with supercoiled plasmid DNA using a high-efficiency calcium phosphate precipitation method (Ausubel et al. 2001). Briefly, plasmid DNA (0.41 pmoles) was mixed with 200 μl of 0.25 M CaCl₂ and added drop-wise into the same volume of 2XBES (50 mM N,N-bis[2-hydroxyethyl]-2-aminoethane-sulonic acid, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.95). The calcium phosphate-DNA complex was allowed to form at room temperature for 20 min. Cells were incubated with DNA precipitates for 18-24 h at 36.5°C under humidified
atmosphere of 3% CO₂ to facilitate DNA uptake. Then, the transfected cells were washed twice and incubated at 36.5 C with 5% CO₂ for additional 48 h before analysis.

4. Preparation of Chromosomal DNA

High-molecular-weight genomic DNA was prepared from cultured cells a described elsewhere (Ausubel et al. 2001). Cell monolayers were trypsinized and transferred to conical tubes for centrifugation. Cell pellets were thoroughly resuspended in 1ml digestion buffer/10^8 cells (100 mM NaCl, 10 mM Tris.Cl, pH8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, and 0.1 mg/ml proteinase K) and incubated with shaking at 50 C for 12-18 h. Samples were extracted with an equal volume of phenol/chloroform. Genomic DNA was precipitated with ethanol, dissolved in TE and quantitated by UV spectrophotometry.

5. Isolation of RNA

Total RNA from cultured cells (100 mm dishes) or large pieces of mouse liver was extracted with guanidinium thiocyanate and purified by density-gradient centrifugation through a 5.7 M CsCl cushion. Briefly, cells or tissues were lysed or homogenized in a 4 M guanidine solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5%(w/V) N-lauroylsarcosine, and 0.1 M 2-mercaptoethanol). The viscosity of the solution was reduced by drawing the lysate through a 21-G needle 10 times to shear genomic DNA. The RNA was pelleted at 15 C by centrifugation at 35,000 rpm for 20 h through a CsCl cushion (5.7 M Cesium chloride in 50 mM sodium citrate, pH 5.2) and dissolved in DEPC-treated water. Alternatively, total RNA from cells cultured in 60 mm dishes or from smaller pieces of liver or adrenal tissues was prepared using the RNeasy Mini Kit (Qiagen Mississauga, ON)
following the manufacturer’s specifications. Poly(A)-enriched RNA from Y1 cells or mouse liver was isolated by two rounds of affinity chromatography over oligo(dT) cellulose (New England Biolabs Ltd., Pickering, ON).

6. RT-PCR and Real-Time PCR

Total RNA (5 µg) was reverse transcribed into cDNA using SuperScript II RNase H reverse transcriptase (200U; Invitrogen Canada, Burlington, ON) and an oligo-dT18 (100 pmol) primer for 1 h at 42 C in a 20 µl reaction, which contained 20 mM Tris.Cl, pH 8.4, 50 mM KCl, 3 mM MgCl2, 10 mM DTT, 400 µM each dATP, dGTP, dCTP and dTTP). The first strand cDNAs (2 µl) were amplified using Platinum Taq Polymerase (Invitrogen Canada, Burlington, ON) for 35 to 40 cycles under following conditions: 94 C 1 min, appropriate annealing temperature 1 min, and 72 C 1 min. In the subset of selective gene knockdown experiments in the mutant 10r6 cells, the expression levels of SF1, GCNF, and Adcy4 transcripts were quantitatively analyzed by real-time PCR using Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen Canada, Burlington, ON) and a thermal cycler (ABI 7300 Real Time PCR System). Standard curves were generated from cDNA fragments of these genes and were used to quantify the transcript levels. Experiments were repeated at least four times in triplicate. As an internal control, levels of TKT transcripts were used to normalize each individual sample.

7. DNA Sequencing

DNA sequencing was performed with the dideoxy-chain-termination method (Sanger et al. 1977), using a modified T7 DNA polymerase, Sequenase™, following the
directions in the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemicals, USA). All the reaction buffers and sequencing reagents were also prepared according to this kit. Briefly, one pmol of template plasmid (2-4 µg) was denatured with 0.2 M NaOH, 0.2 mM EDTA, precipitated with ethanol, and annealed with 1 pmol of sequencing primer. The labeling reaction was carried out at room temperature, in the presence of 5 µCi of [α-35S]dATP (1250 Ci/mmol, PerkinElmer Canada, Woodbrige, ON), and then divided into four different stop reactions each with a termination buffer containing one of the four individual ddNTPs. Samples were resolved on 6% denaturing poly-acrylamide sequencing gels and exposed to Kodak X-OMAT AR films (Mandel Scientific Company Inc., Guelph, Ont.). Alternatively, the full-length Adcy4 cDNA and most double or triple mutations in the conserved region of the Adcy4 promoter were sent out for sequencing by the services from the Hospital for Sick Children or ACGT Corp.(Toronto, Ont.), using their Licor or ABI sequencer facilities.

8. Southern Blot Analysis

Genomic DNA (25 µg) from parent or mutant cells was digested with 125U of XhoI, KpnI, or HindIII for 2h and electrophoresed on 0.5% agarose gel at 30V for 16-18h. DNA fragments on the gel were denatured for 45 min in several volumes of 1.5 M NaCl, 0.5 M NaOH, and neutralized for 30 min with 1 M Tris.Cl, pH 7.4, 1.5 M NaCl before blotting onto Nytran SuperCharge nylon membranes (Schleicher and Schuell Bioscience Inc., Keene, NH). The radiolabeled full-length 3.4kb Adcy4 cDNA was used as the hybridization probe. This Adcy4 cDNA fragment was isolated from IMAGE Consortium clone BF782774 by digestion with XbaI/SalI restriction enzymes and labeled by nick-translation with [α-
Hybridization was carried out at 42°C overnight. The blot was washed in 2XSSC, 0.1% SDS at room temperature for 1 h and then in 0.1XSSC, 0.1% SDS at 60°C for 1.5 h.

9. Western Blot Analysis

Monolayers of cell culture were lysed by a radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (9.1 mM Na$_2$HPO$_4$, 1.7 mM NaH$_2$PO$_4$, 150 mM NaCl, pH 7.4, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 5 mM EDTA, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride and 5 mM benzamidine). Equal amounts of protein samples were heated at 90°C for 10 min in SDS sample loading buffer, separated on 8% SDS-polyacrylamide gels, and electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% fat-free milk in 20 mM Tris.Cl, pH 7.6, 140 mM NaCl, 0.05% Tween-20. After three washes, membranes were incubated overnight at 4°C with anti-SF1 antiserum (kindly provided by Dr. K. Morohashi, National Institute for Basic Biology, Japan) diluted in blocking buffer (1:200). The blots were rinsed three times in blocking buffer and then incubated with 1:10,000 diluted horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h. Signals were detected with Renaissance Enhanced Luminor Reagent (NEN Life Science Products, Boston, MA) following the manufacturer’s directions.

10. Primer Extension

Primer extension used for the determination of the transcription start site(s) of Adcy4 was carried out using an end-labeled 20-mer oligonucleotide primer (BS180, Table II-1) complementary to the Adcy4 transcript 47 bp downstream of the initiator ATG. The primer
(12.8 pmol) was labeled with $\left[\gamma^{32}\right]P$ATP (50 µCi; 3000 Ci/mmol) using T4 polynucleotide kinase (20 U; New England Biolabs Ltd.) and purified through a stacked column of DE53/AG50W-X8 ion exchange resin. About 0.5 pmol of labeled primer was hybridized at 55 C for 1.5 h with 50 µg of total RNA or 10 µg of poly(A)-enriched RNA from adrenal Y1 cells or mouse liver and then ethanol precipitated. Primer extension was performed for 1 h at 42 C in the presence of dNTPs, first-strand buffer and 40 units of SuperScript II reverse transcriptase (Invitrogen Canada, Inc., Burlington, ON). Excess RNA was digested with RNase A and the reaction was terminated by extraction with phenol/chloroform. Extended products were separated by electrophoresis on a 6% acrylamide/7 M urea gel together with a sequencing reaction as a marker to estimate their lengths. The sequencing reaction was carried out using the same primer with the mouse Adcy4 cDNA clone BF782774 as template, which includes 112 bp of 5’ untranslated sequence.

11. DNA-protein Interaction

11.1 Preparation of Nuclear Protein Extracts

Nuclear protein extracts were prepared according to the method of Dignam et al (1983) with minor modifications. Cultured cells were collected in PBS, pelleted and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9 at 4 C, 1.5 mM MgCl$_2$, 10 mM KCl, 2 µM pepstatin, 0.6 µM leupeptin, 25 mU/ml aprotinin, 0.5 mM PMSF, 0.5 mM DTT). After 10 min incubation on ice, cells were homogenized using a Dounce homogenizer. Cell nuclei were collected by centrifugation and resuspended in one volume of low salt buffer (20 mM HEPES, pH7.9 at 4 C, 25% glycerol, 1.5 mM MgCl$_2$, 20 mM KCl, 0.2 mM EDTA,
2 μM pepstatin, 0.6 μM leupeptin, 25 mU/ml aprotinin, 0.5 mM PMSF, 0.5 mM DTT). One half volume of high salt buffer (the same as low salt buffer, except with 800 mM KCl instead of 20 mM KCl) was then added dropwise with gentle mixing and nuclear proteins were extracted during a period of 30 min. Debris was removed by centrifugation and the nuclear extracts were stored at –80°C. For the repeat of some gel mobility shift assays, nuclear extracts were also prepared with a modified protocol as previously described (Frigeri et al. 2000). Briefly, cell membrane were lysed by incubation with a NP-40 buffer (10 mM Tris.Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 2 μM pepstatin, 0.6 μM leupeptin, 25 mU/ml aprotinin, 0.5 mM PMSF, 0.5 mM DTT) instead of using a Dounce homogenizer. Nuclear proteins were extracted with a sodium chloride buffer (20 mM HEPES, pH 7.9 at 4°C, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 μM pepstatin, 0.6 μM leupeptin, 25 mU/ml aprotinin, 0.5 mM PMSF, 0.5 mM DTT) for 30 min at 4°C.

11.2 DNase I Footprinting

Three DNase I footprinting probes, FP1 (-958/-572), FP2 (-631/-290) and FP3 (-304/+60), were prepared by PCR amplification of Adcy4 genomic DNA in the presence of high fidelity AccuPrime Pfx DNA polymerase (Invitrogen Canada, Burlington, ON) using primer sets (Table II-2) BS237/BS238, BS239/BS240 and BS241/BS242 respectively. The PCR fragments were digested with BamHI, a restriction site added to all the reverse primers mentioned above, end-labeled with [α-32P]dCTP and the Klenow fragment of DNA polymerase I, and purified through a Sephadex G-50 Nick Purification Column (Pharmacia Biotech Biotech. Inc., Princeton, NJ). DNase I footprinting analyses were performed using 5 fmol of labeled probe, 8-16 μg of nuclear extracts, and 0.33 U or 0.17 U DNase I
(Boehringer Mannheim GmbH, Mannheim, Germany) at 22 C for 5 min in a buffer containing 10 mM Tris.Cl, pH 8.0, 5 mM MgCl₂, 1mM CaCl₂, 50 mM KCl, 2 mM DTT, 50 µg/ml BSA, 2 µg/ml salmon sperm DNA, and 0.05 µg/ml poly[dl-dC]. In control reactions, labeled probe DNA was treated with DNase I in the absence of nuclear extract. To identify the protected nucleotides, sequencing reactions were performed in parallel using the same reverse primers as were used in PCR for each probe. Footprinting reactions and sequencing marker were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography.

11.3 Electrophoretic Mobility Shift (EMSA) Assays

Electrophoretic mobility shift assays (EMSA) were performed as described by Frigeri et al (2000) with minor modifications. Oligonucleotide probes were prepared by annealing complementary oligonucleotides in 10 mM Tris.Cl pH8.0, 1 mM EDTA and 50 mM NaCl at 95 C for 5 min in a heat block and then slowly cooling the reaction to room temperature. The probes were labeled by filling the 3’-BamHI/BglII ends using the Klenow fragment of DNA polymerase I in the presence of [α-³²P]dCTP (3000 Ci/mmol). In binding reactions, 40 fmol (20,000 cpm) of labeled probe was incubated for 20 min at 25 C with 5 µg nuclear extract in a total of 20 µl containing 2 µg poly[dl-dC], 225 µg/ml BSA, 7.5%(v/v) glycerol, 20 mM HEPES pH7.9, 50 mM KCl, 50 mM MgCl₂, 1 mM EDTA, and 1 mM DTT. For competition experiments, a 25-, 50-, 100-, or 150-fold molar excess of unlabeled double-strand oligonucleotide was added to the reaction before the addition of the radiolabeled probe. For supershift assays, 1 µg of rabbit antibodies against Sp1, Sp3 (Upstate Cell Signaling Solution, Lake Placid, NY), and/or 1µl of SF antiserum were added
after the addition of the probe. Reactions were electrophoresed on 4% nondenaturing polyacrylamide gels and visualized by exposure to X-ray film with the aid of an intensifying screen.

**11.4 Chromatin Immunoprecipitation (ChIP) Assays**

Antibody precipitated DNA samples used for ChIP assays were kindly provided by Dr. G. Hammer (Winnay et al. 2006). Briefly, Y1 adrenal cells stably transfected with a plasmid encoding SF-1 with tandem hemagglutinin epitope tags were grown to saturation density, serum deprived for 48 h and then treated with 2.5 µM α-amanitin (Sigma Chemical Co., St. Louis, MO) for 2 h followed by a 30 min wash-out to remove the α-amanitin. Cells were rinsed and chromatin was cross-linked by incubating the cells with formaldehyde at a final concentration of 1% at 37 C for 10 min. Cells were rinsed, nuclei were isolated and lysed in 150 µl of a buffer containing 1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1 x protease cocktail (Sigma Chemical Co.) per 2 x 10^6 cells. Samples were sonicated so as to shear chromosomal DNA to an average length of 500 bp, cleared by centrifugation and then immuno-cleared with 15 µl of pre-immune serum, 2 µg of sheared salmon sperm DNA (Invitrogen) and 80 µl protein A-Sepharose, added as a 50% slurry. Immunoprecipitation was carried out overnight at 4 C and immune complexes were recovered by absorption to protein A-Sepharose beads for 1 h at 4 C. The beads then were washed extensively and immune complexes were extracted using 1% sodium dodecyl sulfate, 0.1 M NaHCO₃. Extracts were heated overnight at 65 C to reverse cross-linking and DNA fragments were isolated by extraction with phenol-chloroform and then precipitated. DNA products were assayed for *Adcy4* fragments in 25 µl PCR reactions containing 0.5 mM
MgCl₂ and 25 pmol each of primer pair BS773/BS774 spanning –199 to +4 or BS775/BS776 spanning a 205 bp region within intron 4 (Table II-2). PCR reactions were initiated by incubating samples at 94 C for 4 min and then were continued for 40 cycles—each cycle consisted of incubations at 94 C for 45 sec, 58 C for 30 sec and 72 C for 30 sec. Samples were then incubated at 72 C for 10 min and cooled to 4 C.

12. Luciferase Reporter Gene Assays

Transfected cells in 60-mm cell culture dishes were harvested in 500 µl lysis buffer containing 50 mM Tris.2-[N-morpholino]ethanesulfonic acid, pH 7.8, 1% Triton X-100, 4 mM EGTA and 1 mM dithiothreitol. Cells were disrupted by vortexing and clarified by centrifugation at 4 C for 12 min. Luciferase activity in cell extracts was assayed with a Berthold Lumat LB Luminometer in a 250 µl cocktail containing 46 mM Tris.2-[N-morpholino]ethanesulfonic acid, pH 7.8, 9.2 mM magnesium acetate, 3 mM ATP, 600 mM KPO4, and 60 µM luciferin. Experiments in triplicates were repeated at least three times and results were normalized to the activity of a control plasmid with a luciferase reporter gene driven by the SV40 core promoter and enhancer (pGL3-Control, see Section 2.1).

13. hsRNA-Mediated Gene Knockdown

Selective knockdown of SF1 expression was performed using plasmid-based RNA interference techniques. Three hsRNA expression constructs targeting three different regions of SF1 transcript (see Section 2.1) were used to transfect mutant 10r6 cells and the stable transformants were selected by growing cells in G418 medium. A GCNF hsRNA expression vector, targeting the 716/736 region in GCNF, was used as a negative control in the
experiment. Total RNA from individual cell clones was extracted using RNeasy Mini Kit (Qiagen Mississauga, ON). The hsRNA-mediated selective gene knockdown was confirmed by real-time PCR analysis.

14. Statistical Analyses

Significant differences of mean values between and within multiple groups were determined by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test when only one independent variable was involved, or by two-way ANOVA followed by the Bonferroni test when two independent variables were involved, using GraphPad Prism 4.0 software (GraphPad Software, Inc. La Jolla, CA). A p value <0.05 was considered statistically significant.
SECTION III. RESULTS AND DISCUSSION

1. Determination of Mouse Adcy4 Gene Structure and its Expression in Selected Tissues and Cell Lines

1.1 Sequences of Mouse Adcy4 cDNA and its Deduced Protein

Prior to undertaking a detailed transcriptional analysis of the mouse Adcy4 promoter, we first characterized the mouse Adcy4 cDNA using an in silico approach. A homology search of the mouse expressed tag (est) database with the published rat Adcy4 sequence (Gao et al. 1991) using the NCBI web-based BLAST program (Altschul et al. 1990; Madden et al. 1996) identified two I.M.A.G.E Consortium mouse cDNA clones BF782774 and BE291206 (Fig. III-1). These two est clones were derived from mouse kidney and mammary ductal carcinoma cDNA libraries respectively, and the 5’ ends of their partial sequences were shown to be about 90% homologous to rat Adcy4 cDNA, suggesting they are mouse Adcy4 cDNA clones. Using the same rat Adcy4 sequence to search the high-throughput genome screening (HTGS) database for potential mouse Adcy4 genomic sequence, we identified a candidate genomic clone RP23-2C24 which was derived from mouse chromosome 14 (accession no. AC098877). Alignment of rat Adcy4 cDNA and mouse genomic sequence revealed multiple potential exons for mouse Adcy4 (data not shown). Based on the sequences of these potential exons, twenty primers (BS157, BS159-161, BS163-177 and BS179; see Table II-1) spanning the entire predicted mouse Adcy4 cDNA were synthesized and used to sequence the BF782774 and BE291206 mouse est clones from both strands. Sequencing analysis suggested that both clones were derived from Adcy4
Fig III-1. Homology search of the mouse expression tag (est) database with rat Adcy4 cDNA sequence

Published full-length sequence of the rat Adcy4 cDNA (rAdcy4) (accession no. M80633) was used to search for homologous clones from the mouse est database using NCBI BLAST software. Many I.M.A.G.E Consortium mouse cDNA clones were identified based on the homology of their 5’ partial sequences to rat Adcy4 cDNA. Among them, two clones (arrows) were selected as candidate mouse Adcy4 cDNAs for further sequencing analysis.
mRNA; clone BF782774 corresponded to a full-length sequence, extending from the 5’ untranslated region to the 3’ poly(A) tail (Fig. III-2; accession no. AF442771) whereas clone BE291205 contained a partial sequence, missing 1129 bp from the 5’ end of the full-length cDNA (Fig. III-2).

Adcy4 cDNA and protein were highly conserved among mouse, rat and human species. For example, at the nucleic acid level, mouse Adcy4 was 93% identical to rat and 85% identical to human Adcy4; at the protein level, this similarity was even higher (96% and 92% respectively). The deduced protein sequence of mouse Adcy4 shared the same length as the human counterpart (1077 aa), but was 13 aa longer than the rat Adcy4 (Fig. III-3). In addition, Adcy4 proteins from rat, mouse and human all have very similar predicted topologies as do other membrane-bound Adcy4 isoymes, which contain two transmembrane domains, each with six helical clusters and two cytoplasmic domains necessary for the formation of the catalytic core (Fig. III-3). In contrast, Adcy4 is less similar to other Adcy isoymes from the same species. By comparison, mouse Adcy4 was 62% identical to mouse Adcy2 (accession no. NM_153534) and 61% identical to mouse Adcy7 (accession no. NM_001037724). These two isoymes belong to the same group as Adcy4 which are all regulated by Gβγ proteins and are more closely related to each other in phylogenetic analysis. When compared with isoymes out of the group, Adcy4 is even less conserved; for instance, the identities of Adcy4 with the Ca^{2+}-stimulated Adcy1 (accession no NM_009622) and the Ca^{2+}-inhibitory Adcy5 (accession no. NM_001012765) were about 51% and 50%, respectively. These results were consistent with the reported data (Hanoune et al. 1997).
Fig. III-2. Mouse Adcy4 cDNA and deduced protein sequences

The sequence of mouse Adcy4 cDNA was determined by dideoxy sequencing of both strands of the insert in the mouse est clone BF782774. The predicted coding region and the deduced amino acid sequence of mouse Adcy4 were determined by GeneWorks® Release 2.5.1 (Oxford Molecular Group Inc. Campbell, CA). This sequence has been deposited in the NCBI database (accession no. AF442771).
Fig. III-3. Predicted mouse, rat, and human Adcy4 secondary structures

Deduced amino acid sequences of Adcy4 from rat (rAdcy4, accession no. M80633), mouse (mAdcy4, accession no. AF442771) and human (hAdcy4, accession no. AF497516) were analyzed by the online SMART program (http://smart.embl-heidelberg.de/) for their potential secondary structures. TM1 and TM2 represent two predicted transmembrane domains, each with six transmembrane spans (vertical black bars). Following each transmembrane domain is a cytoplasmic domain (labeled as Cycc); the two cytoplasmic domains are predicted to form the catalytic center.
1.2 Intron-exon Organization and Restriction Map of Adcy4

To determine the restriction map of the mouse Adcy4 gene, mouse genomic sequence extracted from the clone RP23-2C24 was analyzed with the online software, Webcutter 2.0 (http://www.firstmarket.com/cuter/cut2.html), and the restriction sites for XhoI, HindIII and KpnI are indicated in Fig. III-4. The intron-exon organization of the mouse Adcy4 gene was constructed by aligning the same genomic sequence with the mouse Adcy4 cDNA as described above. As shown in Fig. III-4, mouse Adcy4 contains 26 exons spanning a total of 15014 bp of DNA; the putative first exon is situated 978 bp downstream of another gene encoding a receptor-interacting Ser-Thr protein kinase (Ripk3, accession no. NM_019955), suggesting that the promoter/regulatory region of Adcy4 is contained within this 978 bp of flanking DNA.

1.3 Conservation of Sequence in the 5’-Flanking Regions of Adcy4 from Different Mammalian Species

Highly conserved promoter regions are usually considered to play important roles in gene expression or regulation (Das et al. 2007). To determine if there is any such region in the 5’-flanking DNA of Adcy4, the intergenic sequence between Ripk3 and Adcy4 from rat, human, chimpanzee, rhesus monkey and dog were aligned with the mouse counterpart. Results shown in Fig. III-5 indicated that the 5’-flanking region of mouse Adcy4 was significantly homologous to the counterpart of rat Adcy4 but was much less homologous to those of Adcy4 genes from other species. However, a stretch of 144 bp immediately upstream of Adcy4 was highly conserved among all these species (≥76% identity, Fig. III-5). These observations suggest that the highly homologous 144 bp region may contain
Fig. III-4. Mouse *Adcy4* gene structure and restriction sites
(Adapted from Al-Hakim et al. 2004)

Restriction sites for *XhoI* (X), *KpnI* (K), and *HindIII* (H) within the 32 kb region containing mouse *Ripk3* and *Adcy4* genes were analyzed *in silico* from genomic sequence (accession no. AC098877) (A). The intron-exon organization of *Adcy4* gene was determined by aligning the cDNA sequence of mouse *Adcy4* (accession no. AF442771) with the genomic sequence (B). Vertical bars denote exons and lines denote introns. The arrow and asterisk (B) represent translation start and stop sites, respectively, within the *Adcy4* transcript.
Fig. III-5. Conserved 5’-flanking region of \textit{Adcy4} among different species

The 5’-flanking sequence between \textit{Ripk3} and \textit{Adcy4} from mouse (accession no. NT_039606) was compared with corresponding sequences flanking the \textit{Adcy4} from rat (accession no. NT_039606), human (accession no. NT_026437), chimpanzee (accession no. NW_001224596), rhesus monkey (accession no. NW_0011211199), and dog (accession no. NW_8876327). The sequences were analyzed using the online program rVISTA (http://rvista.dcode.org/) with windows of 100 bp; the regions of homology are displayed as shaded boxes (cutoff ≥ 76%).
regulatory elements that are involved in the regulation of *Adcy4* gene expression. The sequence alignments of this region are shown in Fig. III-6.

### 1.4 Global Integrity of the Adcy4 Locus in Y1 and Mutant Adrenal Cells

To eliminate the possibility that Adcy4 deficiency in the forskolin-resistant mutant cells resulted from gross gene rearrangement in the *Adcy4* locus, the global integrity of this gene in both parent Y1 and mutant adrenal cells was assessed by Southern-blot analysis (Fig. III-7). High molecular weight genomic DNA from Y1 cells and two forskolin-resistant mutants (clones 10r9 and OS3) was digested with *HindIII, XhoI* or *KpnI* restriction endonucleases and analyzed by Southern-blot hybridization using the full-length mouse Adcy4 cDNA as probe. The profiles of digested fragments generated from the *Adcy4* genes of the mutant cells were exactly the same as those from Y1 cells, with their molecular weights corresponding to the predicted sizes for the *Adcy4* gene (Fig. III-7). Furthermore, the hybridization intensities of the digested DNA fragments from Y1 and mutant cells also were similar. These results indicate that the loss of Adcy4 expression in the mutant cells was not due to gross gene deletion or alteration in the *Adcy4* locus.

### 1.5 Adcy4 Expression in Selected Tissues and Cell Lines

*Adcy4* is widely expressed in many organs and tissues with highest abundance seen in the brain, heart, kidney, liver, lung, and brown adipose tissue (Gao et al. 1991). The relative levels of *Adcy4* in Y1 and mutant cells were assessed using semi-quantitative RT-PCR. As shown in Fig. III-8A, the Adcy4 transcript level in Y1 adrenal cells was relatively lower than the
Fig. III-6. Alignment of the conserved 5’-flanking sequences of *Adcy4* among different species (From Rui et al. 2008)

The conserved proximal 144 bp of DNA sequences flanking the *Adcy4* from all the six species (derived from Fig. III-5) were aligned using a Clustal W alignment tool provided with Mac Vector™ 7.2 software (MacVector, Inc., Cary, NC). The shaded letters represent the conserved sequences. Part of the sequence from exon I of the rhesus monkey *Adcy4* (underlined) is included to complete the comparison.
High molecular weight genomic DNA samples (25 μg) from parent Y1 and mutant 10r9 and OS3 cells were digested with XhoI, KpnI or HindIII, separated by electrophoresis on agarose gels, and transferred onto supercharged nylon membranes. The membranes were analyzed by Southern blot hybridization with $^{32}$P labeled mouse Adcy4 cDNA probe as described in “Materials and Methods.” The uncut genomic DNAs from these cell lines were also included as controls.
levels in the mouse adrenal gland or liver where Adcy4 is highly expressed. The level of Adcy4 transcripts in mouse 3T3 fibroblasts also is lower than the levels found in the adrenal gland and liver; in contrast, in the mutant 10r9 and 10r6 cells, only trace amounts of Adcy4 were detected. To test the integrity of Adcy4 mRNA in these tissues and cell lines, three partially overlapping fragments that covered the entire coding region of Adcy4 were amplified by RT-PCR. The electrophoresis of the amplified fragments is shown in Fig. III-8B. Each of the three PCR fragments amplified from different tissues and cell lines was similar in size and with expected length according to Adcy4 sequence. The levels of these Adcy4 transcripts were consistently different among these tissues and cell lines as mentioned above. These results suggested that the Adcy4 transcripts within these tissues and cell lines were the same, though small splice variants beyond the resolution of this method may exist.

1.6 Transcription Start Site(s) of Adcy4

To determine the transcription start site(s) of Adcy4, 5’ RACE experiments were repeatedly performed to clone and sequence the 5’ end fragments of Adcy4 in both Y1 cells and mouse liver RNAs. Sequencing these 5’ RACE products showed no alternative splicing between the exon I and exon II in both Y1 and liver RNAs but the cloned 5’ end fragments were all 46 bp shorter than the Adcy4 cDNA clone BF782774 within the putative first exon, suggesting that this site may not reflect the true transcription start site.

Thus, primer extension was performed using a radiolabeled Adcy4 oligonucleotide probe and RNA from either Y1 cells or from mouse liver as described in “Materials and
RT-PCR was performed on 5 μg of total RNA to assess Adcy4 expression in different tissues and cell lines as indicated. Specific PCR products were separated by electrophoresis on agarose gels and visualized by staining with ethidium bromide. In panel (A), Adcy4 cDNA was amplified with the primer set BS159/BS160 that spanned a region from 1231 to 1733 bp within the Adcy4 transcript (accession no. AF442771). In panel (B), three regions of the Adcy4 cDNA as indicated were amplified with primer pairs, BS157/BS174, BS159/BS172, and BS167/BS170, which overlappingly covered the entire protein coding sequence within the Adcy4 transcript. A 100 bp DNA ladder (M) was used to size the RT-PCR products. GAPDH transcripts were amplified in separate reactions using the same RNA preparations to normalize for RNA input among these samples.
Methods.” In either sample of total RNA, only one extended product was generated which mapped the major transcription start site to a position that was 12 bp longer than the site predicted from the Adcy4 cDNA clone described above (accession no. AF442771). This major start site was therefore designated +1 (Fig. III-9). When poly(A)-enriched RNAs from both Y1 and mouse liver were used for primer extension analysis, two additional, minor extended products were generated which mapped 13 bp upstream and 11 bp downstream of the major start site respectively (Fig. III-9). These latter observations suggest that Adcy4 may use multiple transcription start sites in Y1 cells and mouse liver.

To further define the transcription start sites and map the first exon of Adcy4, twelve full-length RIKEN clones (Bono et al. 2002; Carninci et al. 2003; Hayashizaki 2003) derived from different tissues (Table III-1) with their 5’-sequences homologous to mouse Adcy4 cDNA were retrieved from the NCBI est database by performing a BLAST search. Sequences for these clones were aligned with the mouse genomic sequence (accession no. AC098877) and their start sites are shown in Fig. III-10A. This analysis indicated that, in different tissues, Adcy4 was transcribed from different start sites, resulting in first exons with variable 5’-ends. However, these transcription start sites were clustered within the –41/+15 region, overlapping the highly conserved sequence and flanking the major and minor transcription start sites determined by primer extension of Y1 adrenal cell and mouse liver RNA. Sequence inspection revealed that the vicinity of these start sites contained no canonical TATA motif, but a GC-rich region with three Sp1-like motifs centered at –86, -57 and –23 bp (named as Sp1A, Sp1B and Sp1C) and one SF1-like site centered at -51 bp that partially overlapped the Sp1B motif was identified. Interestingly, the sequence from –135 to the end of the first exon (+92) had no ATG (an ATG desert area) (Lee et al. 2005),
Fig. III-9. Determination of the transcription start sites of Adcy4 by primer extension
(From Rui et al. 2008)

Total (T) and poly(A)-enriched (M) RNAs from Y1 cells and mouse liver were analyzed by primer extension with the $^{32}$P-labeled primer BS180. The extended products were separated by electrophoresis on a denaturing sequencing gel. A sequencing reaction performed by sequencing the complementary strand of an Adcy4 cDNA clone (accession no. AF442771) using the same primer served as a size marker. The sequences in capital letters are derived from the complimentary strand of the Adcy4 cDNA; the sequences in small letters are derived from the vector; the positions of the major (arrow) and minor (asterisks) transcription start sites relative to the sequencing ladder are indicated. Results were replicated six times for total RNA and two times for poly(A)-enriched RNA.
Table III-1. RIKEN clones and their tissues of origin

<table>
<thead>
<tr>
<th>Tissues</th>
<th>RIKEN clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole body (E9)</td>
<td>CJ064367</td>
</tr>
<tr>
<td>Whole joint</td>
<td>BY346497, BY346402, BY345677</td>
</tr>
<tr>
<td>Lung</td>
<td>CJ066484, BB605514</td>
</tr>
<tr>
<td>Olfactory</td>
<td>BY003200</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>BY28370, BY290735</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>CJ049940</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>BB585863</td>
</tr>
<tr>
<td>Placenta</td>
<td>BY035898</td>
</tr>
</tbody>
</table>
Fig. III-10. Transcription start sites of mouse Adcy4 predicted by in silico analyses  
(Adapted from Rui et al. 2008)

(A) The 5’ ends of several full-length RIKEN cDNA clones obtained from transcripts of various tissues and identified by their accession nos. were mapped to a region of the Adcy4 genomic clone, accession no. AC098877, as shown. The primer extended products obtained from Y1 cells and mouse liver transcripts also are displayed. The 5’-end of the major transcript identified in Y1 cells and mouse liver is arbitrarily designated +1. Minor transcripts are denoted with asterisks. Three putative Sp1 binding sites (underlined and labeled Sp1A, Sp1B and Sp1C) and a putative SF1 binding site that partially overlaps the Sp1B site (boxed) were identified by searching the TransFac® Professional data base with the PATCH™ algorithm. (B) Eighty sequenced 5’-ends of full-length enriched cDNA clones (CAGE tags) of Adcy4 derived from various tissues were extracted from the FANTOM3 database and mapped to the Adcy4 genomic region along with their CAGE tag frequencies. The major transcription start site (arrow) in Y1 cells and mouse liver also is indicated.
suggesting that the multiple transcription start sites used by different tissues had no effect on the protein coding sequence.

Cap analysis of gene expression (CAGE) is another technique used to determine transcription start sites, and at the same time, to profile promoter usage by counting a large number of sequenced 5’ ends of full-length cDNA (Shiraki et al. 2003; Kodzius et al. 2006). Searching the FANTOM3 database for such sequence tags, termed CAGE tags (Carninci et al. 2006), derived from the mouse Adcy4 gene identified a cluster of 80 CAGE tags from the Adcy4 transcripts expressed in various tissues. As shown in Fig. III-10B, the ends of these tags spread across a 70 bp region (−50/+20), with most located 6 to 11 bp upstream of the primary start site that I identified by primer extension. These results were quite consistent with the analysis of RIKEN clones. Taken together, all of these data suggested that Adcy4 contains a TATA-less promoter with multiple transcription start sites broadly distributed within a highly conserved region and that the Sp1- and SF1-like motifs identified in this region may be important determinants of Adcy4 expression.

1.7 Discussion

To facilitate the study of Adcy4 expression in Y1 adrenocortical cells and its forskolin-resistant mutants, I determined the mouse Adcy4 cDNA sequence and its intron-exon organization. I also identified the transcription start sites of mouse Adcy4 by primer extension and in silico analysis.

My sequencing data and in silico analysis demonstrated that the cDNA fragment contained in the mouse est clone BF782774 corresponds to a full-length mRNA of mouse Adcy4. The whole sequence of this clone (Fig. III-2) is highly homologous to the rat and
human Adcy4s at both the cDNA level and deduced protein level. The predicted topology of the deduced protein has a similar structure to all other membrane-bound Adcy proteins (Fig. III-3). Moreover, the 5’ flanking region of the this gene shares extensive homology with the counterpart of the rat Adcy4 (Fig. III-5). All these data support the conclusion that this cDNA encodes mouse Adcy4. It has been reported that different Adcy isozymes usually share considerable similarities in protein sequence and predicted topology (Hanoune et al. 1997; Patel et al. 2001). Of the ten characterized Adcy enzymes, nine are membrane-bound isozymes that exhibit the same secondary structure, with two transmembrane domains and two cytoplasmic domains. The overall similarity of the amino acid sequences among these membrane-bound Adcy isozymes is quite high, reaching up to 60%. The sequences located in the cytoplasmic domains (C1a and C2a) are most conserved, ranging from 50% to 90% in similarity. In addition, the evolutionary conservation of the same isozyme among different mammalian species is often higher than that of different Adcy isozymes in the same species, with the average similarity more than 90% among some species (Hanoune et al. 1997). These observations are consistent with my data, which showed that mouse Adcy4 is 95% identical to the rat Adcy4 and 85% to the human one at the cDNA level. In contrast, when compared with other Adcy isozymes in mouse, this conservation is much less: Adcy4 is about 61% identical to Adcy2 and Adcy7, the two other Gβγ regulated isozymes, and is only about 50% identical to Adcy1 and Adcy5, which belong to different groups of isozymes. These data give additional support that the cDNA fragment in the est clone BF782774 is indeed derived from mouse Adcy4 transcript.

Although highly conserved, the human, rat and mouse Adcy4 proteins still have subtle differences: the deduced mouse Adcy4 protein has 1077 amino acids, the same as
human Adcy4 protein but is 13 amino acids longer that rat Adcy4 protein, suggesting the existence of potential splicing variants for this gene. Indeed, multiple molecular forms of mRNAs have been reported for some Adcy isozymes, including Adcy4. In the N-terminal domain of Adcy5 two distinct splicing products have been described, resulting in quite different first putative cytoplasmic regions without necessarily changing the biochemical properties of this enzyme (Ishikawa et al. 1992; Premont et al. 1992; Iwami et al. 1995a). In the coding region of Adcy8, at least two splicing variants have been identified with internal in-frame deletions of 90 and 198 bp respectively (Defer et al. 1994; Cali et al. 1996). With RT-PCR analysis, Adcy4 from both rat uterine and cultured myometrial cells also has been found to have two splicing variants, each containing 8-amino acid insertions at two closely located sites within the C2a region (Emala et al. 1998). Using RT-PCR to analyze the mouse Adcy4 transcripts, I failed to identify this or other potential splicing variants in both mouse liver and Y1 adrenal cells (Fig. III-8B). One reason is that this splicing variant may be species-specific in rat; another major reason is that my PCR products are all too big to see the differences caused by such small differential splicing variants. Designing specific primer pairs covering much smaller regions for this site and the whole Adcy4 cDNA is needed to screen for potential splicing variants in this tissue or cell line. In addition, sequencing more cDNA clones of Adcy4 may also help to identify splicing variants that occur at low frequency. Fortunately, my 5’ RACE experiments and full-length RIKEN clone analyses indicated no splicing variants at the 5’ end of Adcy4; thus, splicing variants like those seen with rat Adcy4 are less likely to affect Adcy4 promoter usage and influence my subsequent Adcy4 promoter regulation studies.
Alignment of mouse Adcy4 cDNA with its candidate genomic sequence reveals that mouse \textit{Adcy4} contains 26 exons and is encoded by a 15 kb region (Fig. III-4) within a BAC clone derived from mouse chromosome 14 DNA. This chromosomal location of \textit{Adcy4} is consistent with a previous report, which localized this gene to 14D3 by fluorescence \textit{in situ} hybridization (Edelhoff et al. 1995). Long before the completion of the human and mouse genome projects, the chromosomal localization of the various Adcy isozymes were extensively studied. Each isozyme was found to be coded by a gene localized on a different chromosome. For example, human \textit{ADCY} 1, 2, 3, and 4 are encoded by chromosomes 7, 5, 2, and 14 respectively (Stengel et al. 1992; Villacres et al. 1993; Gaudin et al. 1994; Haber et al. 1994); and human \textit{ADCY} 5, 6, 7, 8, and 9 are encoded by chromosomes 3, 12, 16, 8, and 16 respectively (Stengel et al. 1992; Haber et al. 1994; Hellevuo et al. 1995; Premont et al. 1996). The orthologous genes of human and mouse are usually localized in regions of known homology, so the human and mouse \textit{Adcy4} genes are encoded by chromosome 14, in regions of shared synteny (Gaudin et al. 1994; Edelhoff et al. 1995).

Using 5’-RACE to define the transcription start site of Adcy4 failed to reach the 5’-end of its mRNAs. One possibility is that the longer transcripts are GC-rich and form more stable secondary structures which may influence either the 5’ extension by reverse transcriptase or the tailing efficiency by terminal deoxynucleotidyl transferase, two reactions both critical for the success of 5’ RACE (Frohman 1993; Schaefer 1995). Thus, primer extension experiments were used and the results demonstrate that mouse \textit{Adcy4} has at least one major and two minor transcription start sites in Y1 adrenal cells and B57BL/6 mouse liver (Fig. III-9). The major transcription start site resides at a position that is 12 bp longer than the 5’ end of the Adcy4 cDNA clone BF782774 and at the base “A” of a “CA”
sequence (Fig. III-10A), which is frequently seen in transcription start sites of many other genes (Suzuki et al. 2001). However, primer extension assay has its limitations; it only determines the relative positions of transcription start sites by comparison to a sequencing marker and it does not reveal possible splicing variants that often appear in the 5’ end of various genes (Calzone et al. 1987; Boorstein et al. 1989). Fortunately, neither 5’-RACE experiments nor analysis of full-length RIKEN clones showed splicing variants at the 5’-ends of Adcy4 RNA. Therefore, the transcription start sites determined by the primer extension assays may reflect the true initiation sites of Adcy4 in mouse liver and Y1 adrenal cells.

*In silico* analyses of the 5’-ends of Adcy4 transcripts from a variety of other tissues confirmed that Adcy4 has multiple transcription start sites. In recent years, several novel technologies have been developed to define gene boundaries on a genome-wide scale. Most of them involve new strategies to enrich full-length cDNAs and sequence the 5’ ends derived from capped RNAs, such as the 5’ RIKEN est clones (Hayashizaki 2003), short CAGE tags (Shiraki et al. 2003; Harbers et al. 2005) and 5’-SAGE (serial analysis of gene expression) (Hashimoto et al. 2004). Other strategies involve sequencing the paired 5’-3’ ends derived from same RNA transcripts, such as PET tags (paired-end tags) (Ng et al. 2005). The advantage of these methods is that they are able to identify the transcription start sites at the base pair level and at the same time to measure the dynamic usage of these start sites by specific tissues. Examination of the selected RIKEN clones suggest that mouse Adcy4 is transcribed from multiple start sites. These sites are clustered within a small contiguous region of approximately 56 bp and are utilized in a tissue-selective manner (Fig. III-10). *In silico* analysis of the 5’ ends of Adcy4 transcripts using the mouse CAGE
database also indicate that Adcy4 has multiple transcription start sites clustered within 50 bp of the major site utilized by Y1 cells and mouse liver (Fig. III-10).

Owing to their strong link with core promoters, transcription start sites are usually regarded as position markers for core promoter elements. Recent genome-wide bioinformatics studies reveal that RNA Pol II core promoter structures are highly divergent, consisting of various combinations of core promoter elements (Kadonaga 2002; Smale et al. 2003; Juven-Gershon et al. 2006). These elements include TATA box, INR (initiator), BRE (TFIIB recognition element), DCE (downstream core element), DPE (downstream core promoter element), and MTE (mutation ten element). Based on the distribution of transcription start sites, Pol II promoters can be roughly separated into two classes: single peak promoters, which initiate transcription from a single base pair or at tightly defined positions within a few base pairs and broad peak promoters, which show an array of closely located transcription start sites spreading over around 50-100 bp (Juven-Gershon et al. 2006; Kawaji et al. 2006). Single peak promoters are often associated with TATA boxes and are involved in tissue-specific regulation of gene expression. In contrast, broad peak promoters usually contain CpG-rich islands without TATA motifs and are associated with ubiquitously expressed genes. According to this standard, mouse Adcy4 resembles a CpG-rich broad peak promoter. The proximal portion of the Adcy4 5’-flanking DNA is GC–rich and is highly conserved in several mammalian species (Fig. III-5 and III-6). The multiple transcription start sites of mouse Adcy4 are clustered within the 3’ portion of this conserved region. In the vicinity of these start sites there is no identifiable TATA box, but there is a consensus DPE motif further downstream at +38 bp (Fig. III-10). This spread of transcription start sites
without a TATA box or initiator-like elements is often associated with ubiquitously expressed genes (Smale et al. 2003).

Interestingly, within the GC-rich conserved region we found three Sp1-like binding sites and one SF1-like binding site (Fig. III-10). This observation, together with the evidence that Adcy4 is expressed in many tissues and cell lines including Y1 adrenal cells, prompted us to propose that Adcy4 may be subjected to regulation by ubiquitous factors and that these Sp1-like motifs may be the important determinants of Adcy4 expression in adrenal cells. In addition, we reported previously that Adcy4 and MC2R might share some similar regulations. Mutant Y1 cells isolated on the basis of their resistance to forskolin all have a defective SF1 function and are deficient in a cluster of SF1-regulated genes such as Mc2r, StAR, Cyp11a1 and Cyp11b1 (Frigeri et al. 2000). These cells are also specifically deficient in Adcy4; other Adcy isozymes expressed are unaffected (Al-Hakim et al. 2004). Transient expression of Gβγ subunits in the mutant cells partially restores the levels of both MC2R and Adcy4 (Rui et al. 2004). Therefore, it is possible that Adcy4 may also be subjected to regulation by some tissue-specific factors and that the SF1 motif within the GC-rich region may contribute to Adcy4 gene regulation in Y1 cells.
2. Identification of Regulatory Regions in the 5’-Flanking DNA of Mouse Adcy4

2.1 Consequences of 5’ Truncations and Internal Deletions on the Promoter Activity of Adcy4 5’-Flanking DNA

In order to identify promoter regulatory regions that contribute to Adcy4 expression, fragments containing exon I, intron I, and the non-coding part of exon II (Fig. III-4) plus various lengths of the 5’-flanking region of Adcy4 were put in front of a luciferase reporter gene, and promoter activities were evaluated in both Y1 and Adcy4-deficient mutant cells. In Y1 cells, the reporter gene p-1459\textsubscript{Adcy}Luc, which contained 1459 bp of flanking DNA extending into the last intron of the upstream Ripk3 gene, had about 5.5-times the activity of pGL3-Basic (p<0.001), a promoter-less reporter vector. Further extending this flanking region upstream into intron I of Ripk3 did not enhance the activity of the luciferase reporter, whereas extending to –5364 bp into the 5’-flanking region of the Ripk3 (p-5364\textsubscript{Adcy}Luc), i.e. to the –576 bp flanking the potential major transcription start site of Ripk3 as revealed by CAGE analysis (data not shown), resulted in the total loss of reporter gene activity, possibly due to competition between the Ripk3 and Adcy4 promoters. These data indicate that the p-1459\textsubscript{Adcy}Luc construct contains regulatory elements necessary for the promoter activity of Adcy4 in Y1 cells (Fig. III-11).

Progressive 5’ truncation of p-1459\textsubscript{Adcy}Luc revealed three regions that appeared to contribute to Adcy4 promoter function (Fig. III-12). Truncation to -970 reduced promoter activity in Y1 cells by 25.4% (p<0.05). Further deletion from –970 to –404 did not change the activity of the promoter in parent Y1 cells, but truncation from –404 to –336 and from
Y1 and mutant 10r9 cells were transfected with the indicated 5’ deletion series of luciferase expression vectors (0.41 pmol/dish) created from p-5364_{Adcy}Luc which contained exon I (E1), intron I and part of exon II (E2) up to the initiator ATG and 5364 bp length of DNA flanking the transcription start site (Adcy4 arrow). The intron-exon organization of Ripk3 gene (heavily hatched) and the restriction sites used to progressively truncate the 5’ end of the Adcy4 promoter are shown. Results are expressed as mean percentages of the activity obtained with the pGL3-control vector ± SEM. Depending on the plasmid, data were compiled from 3 to 11 independent experiments each in triplicate. Statistically significant differences were determined by ANOVA followed by the Newman-Keuls Multiple Comparison Test. All of the Adcy4 promoter reporter constructs, except p-5364_{Adcy}Luc, had activities that were significantly different from that of the control vector, pGL3-Basic, in Y1 cells (P<0.05-0.001), but not in 10r9 cells.
–180 to –18 dramatically reduced promoter activities by 78% (p<0.01) and 97.5% (p<0.001) respectively, and yielded, in both truncation products, the remaining activities that were not significantly different from that of the pGL3-Basic (Fig. III-12). Paradoxically, truncation from –336 to –180 increased promoter activity by 20.3%, which was no longer statistically different from that obtained by the p-404\textsubscript{Adcy}Luc (Fig. III-12).

The activities of all these deletion constructs also were evaluated in Adcy4–deficient mutant 10r9 or 10r6 cells (Figs. III-11 and III-12). None of these constructs produced luciferase activity that was significantly higher than the promoterless vector, suggesting that the mutants harbored a defect affecting the activity of the Adcy4 promoter, thereby compromising Adcy4 gene expression. With the exception of the –5364, –336 and -18 promoter constructs, all of the constructs had significantly greater activity in parent Y1 cells than in mutant 10r9 or 10r6 cells (Figs. III-11 and III-12). These results implied that the –5364/-4536, -404/-336 and –180/-18 regions were candidate positions for the cis-elements with defective function in the mutant cells. However, the –5364 construct, as mentioned above, was also devoid of activity in parent Y1 cells and contained a 576 bp 5’ flanking DNA of Ripk3 which may compete away the Adcy4 promoter activity. The –336 construct still showed statistically differential activity in parent and mutant cells when using Student T test so that the failure to see a difference in activity between parent and mutant cells may have resulted from a type II error. Thus, the –180/-18 region which contained the 144 bp of highly conserved sequence appears to be more important for Adcy4 promoter regulation and may be related to the loss of Adcy4 activity in mutant cells.
Fig. III-12. Effects of 5’ deletions on \textit{Adcy4} promoter activity in \textit{Y1} and mutant \textit{10r6} cell (From Rui et al. 2008)

\textit{Y1} and mutant \textit{10r6} cells were transfected with the indicated 5’ deletion series of luciferase expression vectors created from \textit{p-1459\textsubscript{Adcy}Luc} as described in Fig. III-11. The last exon of \textit{Ripk3} is indicated, as is the sequence between –142 and –19 that is conserved among species (boxed). The restriction sites used to progressively truncate the 5’ end of the \textit{Adcy4} promoter also are shown. Results are expressed as mean percentages of the activity obtained with the pGL3-control vector ± SEM. Depending on plasmid and cell line, data were compiled from 4 to 20 independent experiments each in triplicate (\(n = 7\) to 20 in \textit{Y1} cells and 4 to 9 in \textit{10r6} cells). Statistical analysis was performed as described in Fig. III-11. All of the constructs, except \textit{p-336\textsubscript{Adcy}Luc}, \textit{p-18\textsubscript{Adcy}Luc} and the control pGL3-basic, had activities that were significantly higher (\(p<0.001\)) in parent \textit{Y1} cells than in mutant \textit{10r6} cells; * denotes a significant difference between the activities of the indicated plasmids in \textit{Y1} cells, \(P<0.05-0.001\).
To test this hypothesis, I deleted most of the conserved sequence (from –135 to -19) in the context of p-404\textsubscript{AdcyLuc}. This deletion reduced promoter activity to a level that was not different from p-18\textsubscript{AdcyLuc} and abolished differential activity seen in Y1 and mutant cells (Fig. III-13). I also deleted intron I (+93/+321) from the p-404\textsubscript{AdcyLuc} and found that this deletion proportionally increased Adcy4 promoter activity by 3.4- and 3.1-fold in parent Y1 and 10r6 mutant cells, respectively, suggesting an inhibitory effect of the intron I on this promoter (Fig. III-13).

2.2 Nuclear Protein Binding Sites in the Adcy4 Promoter Regulatory Regions

To identify potential regulatory factor binding sites within the Adcy4 promoter region, three partially overlapping probes (FP1, FP2 and FP3) covering the entire intergenic sequence between Ripk3 and Adcy4 and part of exon I of Adcy4 were each labeled with \textsuperscript{32}P at the end of the sense strand and analyzed for their interactions with the nuclear extracts from Y1 cells by DNase I footprinting assays. As seen in Fig. III-14, probe FP1 did not produce any protected site, consistent with the absence of promoter regulatory activity in the –958/-572 region (e.g., Fig. III-12). In contrast, probe FP2 generated at least two large protected sites--one between 416/-400 and one between –382/-321 (Fig. III-14). The latter protected site resides within the -404/-336 region, which appeared to be important for Adcy4 promoter activity in luciferase reporter gene assays (Fig. III-12). When using probe FP3, sites at -95/-45, -30/-2, and +2/+58 were efficiently protected by Y1 nuclear proteins (Fig. III-14). These nuclear protein binding sites were located either in the highly conserved region which was critical for Adcy4 expression or in the first exon which at least has a DPE-like element centered at +38 bp. Thus, the co-occurrence of these protected sites with the
Fig. III-13. Effects of the conserved proximal region and intron I on Adcy4 promoter activity (Adapted from Rui et al. 2008)

The highly conserved proximal region from -135 to -19 or the intron I of Adcy4 was internally deleted from p-404_{Adcy}Luc. The resultant reporter genes and two control plasmids, p-404_{Adcy}Luc and p-18_{Adcy}Luc, were transfected into Y1 and mutant 10r6 cells and assayed for luciferase activity as described in Fig. III-11. Results were compiled from 3-7 independent experiments depending on plasmids and cell line each in triplicate and are expressed as means ± SEM. Bars marked a denote P<0.001 compared to p-404_{Adcy}Luc; bars marked b denote P<0.001 compared to p-18_{Adcy}Luc. Significant differences in activities between Y1 and 10r6 cells were only seen with p-404_{Adcy}Luc and the Δ Intron I construct.
Fig. III-14. DNase I footprinting analysis of the Adcy4 5'-flanking region

Three partially overlapping probes, FP1, FP2 and FP3, which together covered the entire intergenic region between Ripk3 and Adcy4 and the first 60 bp of exon I were 5’-end-labelled on the coding strand. DNase I protection experiments were performed by incubating ~5 fmol of each labeled probe with BSA as a control or with 2, 4, 8 and 16 µg of nuclear proteins extracted from Y1 cells as described under "Materials and Methods." Sequencing reactions on each probe template were run as size marker along with the corresponding DNase I protected samples. The protected regions and their positions relative to the major transcription start site are indicated by vertical bars on the right of the autoradiogram. Experiments were repeated three times for FP1 and six times for FP2 and FP3 with similar results.
critical regulatory regions identified by promoter deletion analysis suggested that at least some of these sites bind to specific nuclear proteins that are essential for *Adcy4* expression in Y1 cells.

### 2.3 The Contribution of the –404/-336 Region to *Adcy4* Promoter Activity

To further evaluate the functional significance of the large protected sites within the probe FP2, the whole fragment of this probe was put in front of a minimal SV40 or a minimal prolactin promoter and their activities were assessed by transient transfection into Y1 cells (Fig. III-15). Surprisingly, none of these reporter gene constructs showed significant enhancer-like activity in this cell line. To eliminate the possibility that regulatory elements within this fragment did not work well with the heterologous minimal promoters, mutations were introduced directly into the -382/-321 region within the context of the original *Adcy4* promoter in p-404*Adcy*Luc (Fig. III-16A). Four blocks of 12-base-pair mutations were made to span this sequence, each destroying a subset of predicted transcription factor binding sites (Fig. III-16A). The effects of these mutations on luciferase reporter gene activity were analyzed in parent Y1 and mutant 10r9 and 10r6 cells (Fig.3-16B). Again, none of these mutations significantly affected promoter activity. These data together with the promoter truncation analyses suggested that the FP2 region did not contain essential regulatory elements for *Adcy4* gene expression. The apparent loss of differential expression of p-336*Adcy*Luc in parent and mutant cells appeared to reflect a type II statistical error as analyzed by two-way ANOVA (i.e. false negative) rather than the loss of an important regulatory element.
Fig. III-15. Assessment of enhancer-like activity in the FP2 region (-631/-290) in reporter gene assays

(A) The FP2 region (-631/-290) of Adcy4 was cloned in front of the SV40 core promoter element in both orientations to drive luciferase reporter gene expression. The resultant plasmids and their parental vector pGL3-promoter were transfected into Y1 and 10r6 cell as described in Fig. III-11. Results were compiled from 4 independent experiments each in triplicate and are expressed as mean percentages of the activity obtained with the pGL3-promoter control plasmid ± SEM. Statistical analysis, determined by ANOVA, showed no significant differences of activities among these plasmids in both cell lines. (B) The same FP2 region was cloned in front of the prolactin core promoter to drive luciferase expression. Reporter gene activity was compared to the control plasmid pPro36Luc in Y1 cells. Results were repeated 3 times each in triplicate and are expressed as mean percentages of the activity obtained with pPro36Luc ± SEM. No differential activity was observed as determined by Student t test.
Fig. III-16. Effects of the nuclear protein protected -382/-321 region on the Adcy4 promoter activity

(A) Blocks of four 12 bp mutations were introduced into the nuclear protein protected sequence (underlined) in the context of p-404_{Adcy}Luc. The location and nucleotide sequence of the block mutations are indicated and labeled M1 to M4. Each mutational block eliminates a subset of putative nuclear protein binding sites (boxed). Only the sequence from -404 to -321 in p-404_{Adcy}Luc is displayed. (B) The activities of p-404_{Adcy}Luc and its mutated derivatives were compared with the activity of the original plasmid following transfection into Y1 and mutant cells as described in Fig. III-11. Independent experiments were repeated 4 times each in triplicate in Y1 cells and 2 times each in triplicate in each mutant cell lines. Results were expressed as means ± SEM. The activities of p-404_{Adcy}Luc and its mutated plasmids were not significantly different as determined by ANOVA.
2.4 The Contribution of the Conserved Region from –135 to –19 to Adcy4 Promoter Activity

As described above, the highly conserved region from –135 to –19 may be essential for the activity of the Adcy4 promoter. Truncation or internal deletion of this region both significantly decreased reporter gene activity and abolished the differential activities seen between Y1 and mutant 10r6 cells. In addition, this region also contained multiple nuclear protein binding sites as revealed by DNase I footprinting assay and transcription factor binding site prediction. In an attempt to assess its enhancer- or promoter-like activity, the -142/-2 flanking DNA of Adcy4 was put in front of a luciferase reporter gene driven by the minimal TATA box motif from SV40. Reporter gene activity was then analyzed in both Y1 and mutant 10r6 cells. As shown in Fig. III-17A, this conserved region significantly increased luciferase expression 35-fold in Y1 cells but only 4.8-fold in mutant 10r6 cells (both with P<0.001); the relatively lower luciferase activity in 10r6 cells suggested that this region was functionally defective in the mutant. Replacing the TATA box with additional downstream Adcy4 sequence, including a predicted downstream promoter element (DPE) in exon I (i.e. p-142/+92AdcyLuc), yielded a similar pattern, but luciferase activity was much higher (Fig. III-17B). Truncation of this plasmid to –18 (i.e. p-18/+92AdcyLuc) abolished differential activity seen in Y1 and mutant cells (Fig. III-17B), suggesting that this conserved region alone contained important regulatory elements for Adcy4 promoter activity in Y1 cells which had compromised activity in the mutant. Adding additional downstream Adcy4 sequence, including exon I, intron I and the non-coding part of exon II, proportionally reduced the luciferase activities both in Y1 and 10r6 cells (Fig. III-17B), indicating that the first intron may have an inhibitory effect on the promoter activity in both
The conserved region from $-142$ to $-2$ (A) or from $-142$ to $-19$ (B) was cloned in front of the pTA-Luc reporter gene (A) or in front of the $Adcy4$ promoter with different 3' extensions (i.e., $-18$/$+92$ or $-18$/$+354$) (B) to drive a luciferase reporter gene expression as described in “Materials and Methods.” These reporter gene constructs were transfected into Y1 and 10r6 cells. Results were compiled from 5 independent experiments each in triplicate in both cell lines and are expressed as means ± SEM. All the plasmids containing the highly conserved $-142$/-2 region (A) or $-142$/-19 region (B) had activities significantly different from those of their corresponding vectors without the conserved insert in both Y1 and 10r6 cells ($P<0.001$); moreover, they also showed significantly different activities in Y1 and mutant cells ($P<0.01$). In contrast, the activities of all the vectors without the conserved region did not differ between Y1 and mutant cells.
parent and mutant cells. All these data are quite consistent with the results of the previously described promoter assays.

2.5 Discussion

I have dissected the promoter activity of the mouse Adcy4 5’-flanking DNA and defined potentially important regulatory regions for Adcy4 expression in both Y1 adrenocortical tumor cells and forskolin-resistant Y1 mutant cells. As mentioned before, the mouse adrenal Y1 cell line is a stable subclone originating from an adrenal tumor and maintains the ability to increase steroidogenesis upon the stimulation of ACTH via the cAMP signaling pathway (Yasumura 1968; Schimmer 1979). The forskolin resistant mutant cells were isolated from Y1 cells based on their resistance to the inhibitory effects of forskolin on cell proliferation and on the induction of cell rounding (Schimmer et al. 1984). These mutant cells lost their ACTH-stimulated steroidogenic ability, and their adenyl cyclase was resistant to activation by forskolin and by ACTH (Qiu et al. 1996). These cell lines therefore constitute an ideal cellular model to study the signaling mechanisms that regulate hormone-stimulated steroidogenesis. Previous research demonstrated that ACTH-resistance in the mutant clones results from an underlying defect that impairs the transactivation function of SF1 thereby preventing the expression of the gene encoding the ACTH receptor (Frigeri et al. 2000; Frigeri et al. 2002), and the resistance of the mutants to the growth-inhibitory and morphological effects of forskolin is due in large part to the loss of Adcy4 expression (Fig. III-8; Al-Hakim et al. 2004). Interestingly, even though the loss of ACTH receptor expression and the loss of Adcy4 are independent consequences of the underlying defect (Al-Hakim et al. 2004; Rui et al. 2004), this defect can be overcome by
transfection of mutant clones with expression vectors encoding Gβγ subunits (Qiu et al. 1998; Rui et al. 2004). These observations suggest that the expression of the ACTH receptor and Adcy4 in mouse Y1 adrenal cells may share similar regulatory mechanisms. Thus, Y1 cells and the forskolin-resistant mutants also provide useful tools to study the regulated expression of genes encoding the ACTH receptor and Adcy4.

My luciferase reporter gene assays reveal that the optimal promoter activity of Adcy4 in Y1 cells requires 1459 bp of 5'-flanking DNA. This conclusion is supported by our data showing that progressively extending this fragment into the first intron of the upstream Ripk3 gene did not increase the reporter gene expression (Fig. III-11). We also found that extending Adcy4 promoter fragment to –5364 bp resulted in a total loss of reporter gene activity (Fig. III-11). This effect is most probably caused by promoter competition between Ripk3 and Adcy4 because this construct contained 576 bp of 5' flanking DNA in front of the potential major transcription start site of Ripk3. Transient transfection of the –5364 bp construct into cells could lead to competition between Ripk3 and Adcy4 for the limited sources of transcription factors, RNA processing and transport complexes and even translation machinery. In support of this hypothesis, I found that the expression of luciferase reporter genes driven by promoter elements from Adcy4 and other genes is frequently inhibited by empty reporter gene vectors—e.g., pcDNA 3.1, containing only a strong enhancer and core promoter. Another possibility is that the –5364/-4536 region bears a strong inhibitory element which abolishes the Adcy4 promoter activity, but this appears not the case in vivo because Adcy4 is effectively expressed in the wild type Y1 adrenal cells though with a lower level than in adrenal tissue as revealed by RT-PCR (Fig. III-8B).
Promoter truncation and deletion analyses suggest that two 5’ regulatory regions and one intron element may be involved in the Adcy4 promoter regulation. Both 5’ regulatory regions, located at -1459/-970 and -180/-18 respectively, behave as positive regulators of the promoter (Fig. III-12), whereas the first intron has an inhibitory effect on Adcy4 promoter activity that works both in Y1 and mutant cells (Figs. III-13 and III-17). Our results support the idea that the 5’-most –1459/-970 region may contain a minor regulatory element for Adcy4 promoter activity. Truncation of this region only reduces overall promoter activity by 25.4% and the differential luciferase activities between Y1 and mutant cells are still retained (Fig. III-12), indicating that the activity of this region is not compromised in the Adcy4 deficient mutant cells. The mechanism for the inhibitory effect of the first intron has not been systemically explored; however, negative influences of introns both on gene expression and on mRNA export from the nucleus have been reported previously and one of these mechanisms may be operative here (Luo et al. 1999; Maston et al. 2006).

Intriguingly, the –404/-336 region appeared to be another potential determinant of the Adcy4 promoter activity in both promoter truncation assays and DNase I footprinting experiments. Removal of this region significantly reduced the promoter luciferase activity in Y1 cells and abolished the statistically significant difference in promoter activity obtained between parent Y1 and mutant 10r6 cells (Fig. III-12). Moreover, a large part of the sequence in this region was protected by nuclear proteins isolated from Y1 cells (Fig. III-14). However, subsequent experiments revealed that the isolated promoter fragment containing this entire region had no enhancer-like activity when linked in either the forward or reverse direction to the basic promoter of SV40 which already has a fairly high basal activity, or linked to the prolactin core promoter which has almost no basal activity (Fig. III-
15). Mutating this region to eliminate subsets of the predicted transcription factor binding sites, had no effect on promoter activity in the context of the original $Adcy4$ luciferase reporter construct p–404$_{Adcy}$.Luc (Fig. III-16) and further truncation from –336 to –180 of $Adcy4$ proximal promoter region restored $Adcy4$ promoter activity and re-established the differential expression of the luciferase reporter gene between the wild type and mutant cells (Fig. III-12). In addition, the differential activity of p-336$_{Adcy}$Luc seen in Y1 and mutant cells was significant when analyzed by Student t Test rather than by a multivariate statistical analysis. Therefore, it appears that the apparent loss of activity of p-336$_{Adcy}$Luc in Y1 cells is caused by the abnormal behavior of this plasmid and reflects a type II statistical error rather than by the deletion of an important regulatory element.

As expected, the highly conserved 5’-flanking region, more specifically the –135/-19 region, is a critical determinant of the $Adcy4$ promoter activity in Y1 cells and may also be closely involved in the $Adcy4$-deficiency of the mutant cells. In support of this hypothesis, I found that 5’-truncation or internal deletion of this conserved region from the reporter gene constructs both abolished promoter activities in Y1 cells, and eliminated the differences of promoter activity between Y1 and mutant cells (Figs. III-12 and III-13). The isolated conserved sequence, when linked to the heterologous TATA box of SV40 or its own downstream fragment from $Adcy4$ which contains the major and minor transcription start sites in Y1 cells and a putative DPE element, can significantly increase reporter gene expression in Y1 cells but less effectively stimulate reporter gene expression in mutant 10r6 cells (Fig. III-17), strongly suggesting that this sequence alone is functional in Y1 cells but has impaired activity in mutant cells. Interestingly, adding more downstream $Adcy4$ sequence, including intron I, into the latter construct significantly and proportionally
reduced the luciferase activities both in Y1 and mutant cells (Fig. III-17). These results confirm the inhibitory effect of intron I on Adcy4 promoter activity and at the same time indicate that intron I may, to some degree, contribute to the absence of Adcy4 expression in mutant cells. Finally, the highly conserved sequence that harbors the predicted Sp1-like and SF1-like motifs is also protected by nuclear proteins from Y1 cells as revealed by DNase I footprinting assay (Fig. III-14). Together, these observations support the idea that the conserved sequence, particularly the putative transcription binding sites therein, are key elements in Adcy4 promoter regulation in Y1 cells and are related to the Adcy4 deficient phenotype in mutant cells.

Similar to the mechanism of ACTH receptor deficiency, Adcy4 deficiency in the mutant cells is not caused by gene mutation or large deletion; instead, it is more likely caused by some defect impairing the transcription of the gene encoding Adcy4. These conclusions were supported by the findings that the Adcy4 gene is present and intact as judged from Southern blot hybridization analysis (Fig. III-7); that transfection of these mutants with reporter genes driven by promoter fragments from a normal Adcy4 gene generated luciferase activities that were statistically different from a promoter-less vector in contrast to their behavior in Y1 cells (Fig. III-12); and that Adcy4 expression could be restored by transfecting mutant cells with Gβγ expression vectors (Rui et al. 2004). It is tempting to hypothesize that the underlying defect in the mutant cells may affect the trans-acting functions of some transcription factors required for the promoter activity of Adcy4. Although the identities of these factors are not clear yet, the target sites for these factors in the mutant cells could well be located within the above-mentioned critical regulatory regions, especially the -135/-19 region.
3. Contributions of Sp1, Sp3, and SF1 to Adcy4 Promoter Activity

3.1 Sp1, Sp3, and SF1 Binding Sites in the Proximal Region of the Adcy4

Promoter

Seven overlapping, double-stranded oligonucleotides were labeled and used as probes in EMSA to identify the transcription factor binding sites in the highly conserved region of Adcy4 (Fig. III-18A). Probes corresponding to the oligonucleotides 2, 3, 4 and 7 were found to bind specific nuclear proteins extracted from parent Y1 and mutant 10r6 adrenal cells. As shown in Fig. III-18B and C, the oligonucleotide 2 and 3 probes formed very similar DNA-protein complexes; the complexes common to both probes were efficiently displaced by either unlabeled oligonucleotide, each of which contained a sequence that perfectly matched the Sp1 binding sites from several genes, or were less efficiently displaced by the unlabeled oligonucleotide 4, which included a weak consensus binding site for Sp1. The oligonucleotide 3 probe also formed an additional complex of faster mobility. This latter complex was only competed away with unlabeled oligonucleotides 7 and 3, both of which had the same putative SF1 binding site, or with a bona fide SF1-binding oligonucleotide. Oligonucleotides 2 and 4, both with no putative SF1 sites, or a mutated SF1 oligonucleotide failed to compete away the faster moving complex (Fig. III-18C).

When the authentic Sp1 oligonucleotide was used as competitor, the common complexes formed with oligonucleotide probes 2 and 3 were completely abolished; when the
Fig. III-18. Identification of nuclear protein interactions in the proximal promoter region of *Adcy4* (Adapted from Rui *et al.* 2008)

(A) The sequence of *Adcy4* proximal promoter region and the major transcription start site (*arrow*) in Y1 cells are shown. The locations and nucleotide sequences of seven partially overlapping oligonucleotide probes used for EMSA experiments are indicated and labeled as Oligo1 to Oligo7. The three putative Sp1 sites (*underlined*) and one putative SF1 site (*boxed*) and their relations to corresponding oligonucleotide probes also are shown. (B) and (C) Radiolabeled double-stranded oligonucleotide probes Oligo2 and Oligo3 were evaluated for interaction with BSA or with nuclear extract (NE) from Y1 adrenal cells by EMSA. Where indicated, unlabeled oligonucleotide competitors were included in the binding reactions at 50- and 100-fold molar excess over probe (B), or at 100-fold molar excess over probe (C). *Arrows* designate the positions of specific shifted complexes.
authentic Sp1 and SF1 oligonucleotides were added together, all the DNA-protein complexes formed by oligonucleotide probe 3 were displaced (Fig. III-19A and B). Furthermore, mutating the putative Sp1 binding sites in oligonucleotide probes 2 and 3 disrupted their ability to form the common complexes with nuclear proteins from Y1 cells, whereas disrupting the putative SF1 site in oligonucleotide probe 3 specifically abolished the formation of the faster complex unique to oligonucleotide 3 (Fig. III-19A and B). Interestingly, the putative Sp1B site and the overlapping putative SF1 site of oligonucleotide probe 3 showed a mutually exclusive binding pattern: when the Sp1B site was disrupted, the binding of the unique complex to oligonucleotide probe 3 increased whereas when the SF1 site was mutated the formation of the DNA-protein complexes common to oligonucleotide probes 2 and 3 increased (Fig. III-19B).

To confirm the identity of these binding proteins, gel mobility shift assays were performed in the presence of specific antibodies against Sp1, Sp3, and SF1. As shown in Fig. III-20A and B, the complex of slowest mobility common to oligonucleotide probes 2 and 3 were partially super-shifted or displaced by the Sp1 antibody; the complexes of faster mobility common to both probes were displaced by the Sp3 antibody. These complexes were completely displaced by adding Sp1 and Sp3 antibodies together, but were not displaced by a control rabbit IgG. The complex unique to probe 3, however, was only displaced by a SF1 antibody. When anti-Sp1, -Sp3 and –SF1 antibodies were used in combination, all of the complexes obtained with oligonucleotide probe 3 were markedly diminished (Fig. III-20A and B). All these data suggested that the common complexes seen with oligonucleotides 2 and 3 are formed by the binding of members of the Sp1 family, Sp1
Fig. III-19. Identification of Sp1 and SF1 binding sites in the Oligo2 and Oligo3
(Adapted from Rui et al. 2008)

(A) The sequences of oligonucleotides 2 and 3 and their putative Sp1 (underlined) and SF1 (boxed) binding sites are shown. Mutated oligonucleotides with their putative Sp1 and SF1 sites disrupted by nucleotide substitutions were designed using the online PATCH™ algorithm (http://www.gene-regulation.com/pub/programs.html#pmatch) to make sure that novel transcription factor binding sites were not introduced. (B) Double-stranded wild-type and corresponding mutant oligonucleotides described in (A) were radiolabeled and evaluated for interaction with BSA or with nuclear extracts (NE) from Y1 cells by EMSA. Where indicated, competitions for binding were conducted in the presence of a 100-fold molar excess of the corresponding unlabeled oligonucleotides, or bona fide Sp1 and SF1 binding-site-containing oligonucleotides. The positions of the displaced complexes (arrows) are indicated.
Double-stranded oligonucleotide probes containing the Sp1A site (Oligo2) (A) or the overlapping Sp1B/SF1 sites (Oligo3) (B) were radiolabeled and subjected to the binding reaction in the presence of BSA or nuclear extracts (NE) from Y1 adrenal cells in EMSA. Where indicated, a rabbit IgG control, IgG antibodies (α-) against Sp1 or Sp3 (1 µg), and/or SF1 antiserum (1 µl) were added to the reaction to identify the specificity of the interactions produced by Y1 nuclear extracts. Arrows designate the positions of the complexes displaced by the Sp1, Sp3 and SF1 antibodies.
and Sp3, and that the complex unique to oligonucleotide 3 is generated by the binding of the orphan nuclear receptor SF1.

As mentioned above, oligonucleotide 4 contained a weak consensus Sp1 binding site. When this oligonucleotide was labeled and used as a probe in EMSA experiments, it formed at least two groups of specific complexes (Fig. III-21A). The slower group of complexes was efficiently displaced by unlabeled oligonucleotides 2, 3 and 4, all of them containing potential Sp1 binding sites, or by an authentic Sp1 oligonucleotide (Fig. III-21A), suggesting these complexes were formed by the binding of Sp1 family factors. In addition, oligonucleotide probe 4 formed another faster migrating group of complexes that were competed away only with its own unlabeled oligonucleotide, not with oligonucleotides 2, 3 or Sp1 (Fig. III-21A), indicating its binding to a different nuclear protein(s). When the putative Sp1C site of oligonucleotide 4 was mutated, the Sp1-like complexes were abolished, but the formation of unknown complexes was markedly increased (Fig. III-21A). The identity of nuclear protein(s) in the latter complexes was not pursued further. When oligonucleotide 7 was used as a probe in gel shift assays, a single complex was formed which was uniquely competed away with unlabeled oligonucleotide 3, or with the SF1-binding oligonucleotide and displaced by anti-SF1 antibody, suggesting this complex was formed by SF1 (Fig. III-21B).

Nuclear proteins extracted from mutant 10r6 or 10r9 cells were also used in these gel mobility shift assays. These nuclear proteins from the mutant cells produced the same shifted profiles as did extracts from the parent Y1 cells with all the seven labeled oligonucleotide probes except for oligonucleotides 3 and 7. These two oligonucleotides, which contained the same putative SF1 site, produced shifted complexes associated with
Fig. III-21. Identification of a Sp1 binding site in oligonucleotide 4 and a SF1 binding site in oligonucleotide 7

(A) The wild type oligonucleotide probe 4 (Oligo4) and its mutated probe (Oligo4mut), designed as described in Fig. III-19, were radiolabeled and subjected to EMSA to evaluate their interactions with BSA or nuclear extracts (NE) from Y1 adrenal cells. Where indicated, a 100-fold molar excess of unlabeled oligonucleotide competitors were added to the reactions except for the unlabeled Oligo4, which was added at 25-, 50- and 100-fold molar excess. The positions of specific shifted complexes are indicated (arrows); NS refers to non-specific DNA-protein complexes which are not competed away with unlabeled probe. (B) The oligonucleotide probe containing the putative SF1 site, Oligo7, was radiolabeled and subjected to EMSA as described in (A) with nuclear extracts (NE) from mutant adrenal 10r6 cells. Where indicated, a 100-fold molar excess of unlabeled oligonucleotide competitors, or antibodies (α-) against Sp1, Sp3 and SF1 were added to the reaction. The position of complex displaced by unlabeled oligonucleotides containing SF1 binding sites or by the SF1 antibody is indicated.
SF1 binding that were more prominent in mutant cells than in Y1 cells (Fig. III-22A and B)—a finding that is consistent with earlier observations that SF1 is overexpressed in the 10r6 mutant (Schimmer et al. 2002; 2003).

To confirm the binding of SF1 to the proximal promoter region of Adcy4 in intact cells, a chromatin immunoprecipitation assay was performed using cross-linked chromosomal fragments of Y1 cells expressing a hemagglutinin-tagged SF1. As shown in Fig. III-23, the genomic fragments precipitated by the anti-SF1 and anti-hemagglutinin antibodies each contained DNA that was amplified by primers specifically complementary to the proximal promoter region (from -199 to +4) of Adcy4. In contrast, primers targeted to a more distal region of adcy4 (from +2548 to +2744) within intron 4 of the gene that did not have a potential SF1 site or control IgG-precipitated chromosomal DNA did not have target fragments for either of two Adcy4 primer sets. These data indicated that SF1 interacts with the proximal promoter region of Adcy4 in living adrenal cells.

Taken together, results from the EMSA and ChIP assays demonstrate that the highly conserved proximal promoter region of Adcy4 contains binding sites for Sp1 family and SF1 transcription factors, and suggest that Sp1, Sp3 and SF1 may regulate the Adcy4 promoter through actions within this region.

3.2 Contributions of the Sp1, Sp3, and SF1 Binding Sites to Adcy4 Promoter Activity

A series of luciferase reporter gene plasmids were constructed with mutations that disrupted the Sp1A, Sp1B and SF1-binding sites (Fig. III-18) and were tested for activity following transfection into Y1 and mutant 10r6 cells (Fig. III-24). These mutations were
Double-stranded oligonucleotide 3, which contained the overlapping Sp1B/SF1 sites (A), and oligonucleotide 7, which contained only the putative SF1 site (B) were radiolabeled. The interactions of these labeled probes with nuclear extracts (NE) from Y1 and its mutant adrenal cells (10r6, 10r9 and OS3) were compared in EMSA. Where indicated, a 100-fold molar excess of the corresponding unlabeled probe was added to the reactions. The identities of the shifted complexes are based on their displacement with specific antibodies (e.g., Fig. III-20).
Cross-linked genomic fragments were prepared from Y1 cells which expressed a myc- and hemagglutinin-tagged SF1 and were immunoprecipitated with antibodies as indicated. Purified DNA was amplified by PCR and separated on a 2% agarose gel. Input DNA represents a portion (1%) of the sonicated chromatin before immunoprecipitation, used as the positive control for PCR. Primer pairs BS773/BS774 were used to amplify the proximal promoter region of Adcy4 from –199 to +4, which contains the SF1 binding site (A). The control pairs BS775/BS776 were used to amplify a region from +2548 to +2744 downstream of the transcription start site, which was within intron 4 and did not contain SF1 site (B).

Fig. III-23. Assessment of SF1 binding in vivo by chromatin immunoprecipitation (ChIP) assay (From Rui et al. 2008)
evaluated in the context of the *Adcy4* promoter-regulatory sequences from −142 to +92 to obviate the inhibitory influences of the first intron. The −142/+92 construct had 25-times the activity of the truncated −18/+92 reporter construct in parent Y1 cells. Mutating the Sp1 B site, the SF1 site or both the Sp1B and SF1 sites progressively increased reporter gene activity up to an additional 2.8-fold (p < 0.001; Fig. III-24), suggesting possible repressor activities at these sites. Mutating the Sp1A site, either alone or in combination with the Sp1B and/or SF1 sites decreased reporter gene activity relative to the activity of p−142/+92*AdcyLuc* (p < 0.001; Fig. III-24). All of the constructs harboring the Sp1A-site mutation had activities that were greater than the minimal promoter construct −18/+92*AdcyLuc* (Fig. III-24), possibly due to the contribution of the most downstream Sp1C site (Fig. III-18). Interestingly, the repressor-like activities of the Sp1B and SF1 sites on *Adcy4* promoter activity seemed to be dependent on the integrity of the Sp1A site since mutating the Sp1B and SF1 sites did not compensate very much for the loss in activity due to mutation of the Sp1 A site (Fig. III-24).

In the absence of the inhibitory influence of intron I, the p−142/+92*AdcyLuc* reporter gene had appreciable activity in the 10r6 mutant (approximately 12-times the activity of the truncated p−18/+92*AdcyLuc*) that was, nevertheless, significantly lower than the activity observed in parent Y1 cells (p < 0.001; Fig. III-24). All of the constructs with an intact SF1-binding site had activities that were greater in parent Y1 cells than in the 10r6 mutant, whereas all the constructs with the SF1 site disrupted had markedly enhanced activities in the mutant such that their activities in parent and mutant cells were equivalent (Fig. III-24). These latter observations suggested that the AC4 deficiency seen in the 10r6 mutant might be secondary to the defect that impaired SF1 function.
Fig. III-24. Contributions of the Sp1 and SF1 binding sites to Adcy4 promoter activity
(From Rui et al. 2008)

The luciferase reporter construct p-142/+92\textsubscript{AdcyLuc}, which contained exon I of \textit{Adcy4} and 142 bp of 5’ flanking DNA, was mutated to disrupt the Sp1A, Sp1B, and/or SF1 sites as described in Fig. III-19. The transcription start site (arrow) and the first exon (filled box) also are shown. The resultant mutated plasmids and the parental p-142/+92\textsubscript{AdcyLuc} were transfected into Y1 and mutant 10r6 cells. The activities of these constructs were compared to that of a control plasmid driven by a truncated promoter (p-18/+92\textsubscript{AdcyLuc}). Results were compiled from three independent experiments each in triplicate and are expressed as means ± SEM. Differential activities between Y1 and mutant 10r6 cells were only seen in plasmids without a mutation in the SF1 binding site (p < 0.05 to 0.001). All the mutant plasmids and the control p-18/+92\textsubscript{AdcyLuc} had activities in Y1 cells that were significantly different from the value obtained using the wild-type p-142/+90\textsubscript{AdcyLuc}. *, Significant differences of activities in Y1 cells between the indicated plasmids (P<0.001).
3.3 Inhibitory Effects of SF1 on Adcy4 Promoter Activity

Site-directed mutagenesis suggested that SF1 exerts an inhibitory effect on Adcy4 promoter activity. To test if overexpression of SF1 could suppress the activity of wild type Adcy4 promoter constructs, SF1-negative 3T3 cells and SF1-containing Y1 cells were both co-transfected with Adcy4 promoter-luciferase reporter plasmids and a His-tagged-SF1 expression vector. An expression plasmid containing His-tagged SF1 in the antisense orientation and the empty expression vector pcDNA3.1 served as controls. As shown in Fig. III-25, epitope-tagged SF1 suppressed the luciferase activity of p-970\textsubscript{Adcy}Luc by 52% in 3T3 cells and 35% in Y1 cells (both with p < 0.001), when compared to the empty vector, pcDNA3.1. In contrast, the anti-sense, epitope-tagged SF1 did not have such effect. According to previous observations, usually less than 50% of Y1 cells express both genes when co-transfected with two different plasmids (Schimmer et al. 1995). Thus the 35% reduction in Adcy4 promoter activity seen in Y1 cells transfected with the His-tagged SF1 implies a substantial suppressive effect of SF1 on the activity of Adcy4 promoter. In contrast, expression of the same epitope-tagged SF1 in both cell lines did not show significantly different inhibitory effects on the truncated reporter construct, p-18\textsubscript{Adcy}Luc, when compared to the expression of the anti-sense epitope-tagged SF1 or the empty vector pcDNA3.1 (Fig. III-25). The stronger suppressive effect of SF1 seen in 3T3 cells may reflect a higher transfection efficiency of these cells than Y1 cells. Transfection of these two cell lines with a β-Gal reporter vector followed by X-Gal staining revealed that transient transfection efficiency of Y1 cells using the calcium phosphate procedure as described in “Materials and Methods” was ~10-20%, whereas this efficiency of 3T3 cells, when transfected with Lipofectamine 2000, was higher, reaching ~20-30%.
Fig. III-25. Effect of transient SF1 expression on Adcy4 promoter reporter activity in Y1 and 3T3 cells (Adapted from Rui et al. 2008)

Two cell lines, 3T3 and Y1 without and with expression of endogenous SF1 (panel A and panel B), were transfected with luciferase reporter constructs p-970AdcyLuc and p-18AdcyLuc, together with equimolar amount of expressing vectors for a His-tagged SF1 (SF1 sense), a His-tagged SF1 antisense control (SF1 antisense), or the empty expression plasmid, pcDNA3.1. 3T3 cells were transfected with Lipofectamine™ 2000 according to the manufacturer’s instructions (Invitrogen Canada, Burlinton, ON) and Y1 cells were transfected with the procedure described in “Materials and Methods.” In both cell lines luciferase activities were compiled from 6 independent experiments for His-tagged SF1 and from 3 independent experiments for two control plasmids. Results were expressed as means ± SEM as indicated in Fig. III-11. The effect of His-tagged SF1 expression on p-970AdcyLuc was significantly different (p<0.001) from that of pcDNA3.1 or of the His-tagged antisense SF1 in both cell lines.
3.4 Effects of SF1 Knockdown on the Expression of Endogenous Adcy4 in Adcy4 Deficient Mutant Cells

To further test the contribution of SF1 to Adcy4 expression, mutant 10r6 cells were transfected with plasmids encoding hsRNAs targeted to three different parts of the SF1 transcript. In stable transformants isolated from each transfection, SF1 transcripts were reduced by 68% (range from 60% to 76%; Fig. III-26A). The stable knockdown of SF1 expression was also confirmed by Western-blot analysis of SF1 proteins in these clones (Fig. III-26B). In the same transformants, Adcy4 transcripts increased by 178% (range from 157% to 189%). In contrast, a hsRNA plasmid targeted to an unrelated nuclear receptor—i.e., germ cell nuclear factor (GCNF, Nr6a1)—did not affect the levels of SF1 and Adcy4 transcripts in 10r6 cells (Fig. III-26A) although it reduced the GCNF transcripts by 58.6 ± 7.8% (P<0.01, n=3). These results demonstrate that hsRNA-mediated knockdown of SF1 level specifically induces the expression of the endogenous Adcy4 in mutant cells.

3.5 Discussion

In Section 2, I demonstrated that Adcy4 promoter activity in Y1 adrenocortical tumor cells is determined by at least two elements in the 5’ flanking region and one intron element. Among these, the highly conserved proximal promoter region from −135 to −19, containing putative Sp1 and SF1 binding sites, is essential for Adcy4 promoter activity in Y1 cells and is involved in the differential expression of Adcy4 in parent and Adcy4-deficient mutant cells. In this section, I further demonstrated that the predicted Sp1-like and SF1-like motifs of this conserved region bind transcription factors Sp1, Sp3 and SF1, and exert an essential but complicated role in the regulation of Adcy4 promoter activity within these cells.
Fig. III-26. Effect of SF1 knockdown on Adcy4 mRNA levels in mutant 10r6 cells
(Adapted from Rui et al. 2008)

(A) 10r6 cells were stably transfected with hsRNAs targeted to three different regions of the SF1 mRNA (accession no. AF511594): i.e., nucleotides 151/171 (SF1-R1), 565/585 (SF1-R2) and 1375/1395 (SF1-R3). As a negative control, 10r6 cells also were stably transfected with hsRNA targeted to the nuclear receptor, GCNF (NR6a1). RNA samples extracted from early passages of stable clones were examined for SF1 and Adcy4 mRNA levels by quantitative RT-PCR using primer pairs BS425/BS426 and BS577/BS578, respectively. Depending on genes, results were compiled from 3-4 independent experiments each assayed in quadruplicate, and are expressed as mean percentages of transcript levels obtained in control 10r6 cells ± SEM. The levels of SF1 and Adcy4 transcripts in all three SF1 knockdown clones are significantly different from the corresponding levels obtained in GCNF knockdown control clone (p < 0.001). (B) The effect of SF1 knockdown on SF1 protein levels was determined by immunoblot assay of cell lysates from the indicated clones and control 10r6 cells. The same membrane was stripped and reprobed with an antibody against GAPDH to check for equal sample loading.
The proximal portion of the *Adcy4* 5'-flanking DNA does not contain a TATA box and, like many other TATA-less promoters, is GC-rich. This region contains three putative Sp1 binding sites (designated as Sp1A, Sp1B, and Sp1C) and a SF1 motif, overlapping the Sp1B site. Systematic analysis of DNA-protein interaction by EMSA revealed that Sp1, Sp3 and SF1 were the major transcription factors working in this conserved region of *Adcy4*. Nuclear extracts from Y1 and mutant cells produced similar protein binding profiles at the Sp1 sites, whereas the binding profiles at the SF1 site were more prominent using nuclear extracts from mutant cells (Fig. III-22), consistent with the overexpression of SF1 in the mutants (Schimmer et al. 2002; 2003). This observation suggests that the highly conserved region of *Adcy4* may be subject to a similar regulation by the general transcription factors Sp1 and Sp3 in Y1 and mutant cells but to a differential modulation by the tissue-specific factor SF1.

My reporter gene assays demonstrate that the activity of the proximal *Adcy4* promoter is governed by a complex interplay of proteins of the Sp1 family interacting with activating and repressing GC-rich elements. In detail, the Sp1A, and by inference, the Sp1C sites appear to have a positive effect on *Adcy4* promoter activity, whereas the Sp1B site appears to have an inhibitory influence. These conclusions are supported by the findings that the Sp1A and Sp1B sites bind to Sp1 and Sp3 with similar efficiency (Fig. III-20) but disrupting the Sp1A or Sp1B site in reporter constructs has opposite effects on luciferase activity (Fig. III-24), and by the observation that mutating the Sp1A, Sp1B, and SF1 sites, leaving the Sp1C site intact in the same construct, still leaves a considerable activity compared to the control plasmid (Fig. III-24). However, it should be advised that the inferred stimulatory effect of the Sp1C site might reflect another layer of complicated
regulation because this site appears to contain a composite *cis* element. Mutating the Sp1C site leads to the increased binding of an unknown factor. Thus, future experiments to identify this unknown factor and its function are needed in order to effectively evaluate the contributions of Sp1C and this unknown element to *Adcy4* promoter activity.

In fact, single or multiple Sp1 binding sites have been extensively documented in promoters and enhancers of many genes where they participate in a variety of complicated regulations to modulate almost all cellular processes. These sites are recognized by a large transcription factor family containing three conserved Cys2His2 zinc fingers, which form the DNA binding domains (Philipsen et al. 1999; Suske 1999). Within this family of factors, Sp1 and Sp3 are expressed ubiquitously and regulate a wide range of cellular functions including cell growth, differentiation, apoptosis, and oncogenesis (Bouwman et al. 2002; Li et al. 2004a). These two factors can act either as activators or repressors, or both, depending on a number of variables, including: the relative abundance of Sp1 and the long and short (inhibitory) forms of Sp3 (Xu et al. 2000; Le Goff et al. 2003); the posttranslational modifications of Sp1 and Sp3 (Armstrong et al. 1997; Du et al. 2000; Samson et al. 2002); the levels of coregulators and the promoters with which they interact (Doetzlhofer et al. 1999; Suzuki et al. 2000; Jang et al. 2002). Based on these reports, the differential activities of the Sp1A and Sp1B sites in the *Adcy4* promoter are not very surprising. Similar modes of regulation in which distinct Sp1 sites on the same promoter have opposite effects on the transcription of the same gene, such as human *Hand1* (Vasicek et al. 2003), have been reported previously. In this latter case, the genomic context of the recognition sequences seemed very important even though other possibilities, for example, differential CpG methylation could not be excluded (Zhu et al. 2003).
Intriguingly, SF1 appears to have a negative regulatory effect on mouse *Adcy4* expression through a site that overlaps with the Sp1B site in the proximal region of the promoter. In support of this suggestion, my EMSA and ChIP experiments demonstrated that SF1 bound to this site both *in vitro* and *in vivo* (Figs. III-22 and III-23). Disruption of the SF1 site by mutation significantly increased the luciferase activity of *Adcy4* promoter constructs in Y1 and mutant adrenal cells (Fig. III-24). Knockdown of SF1 using specific hsRNA increased Adcy4 transcript accumulation in the Adcy4 deficient mutant cells (Fig. III-26) and overexpression of SF1 in Y1 adrenal cells or 3T3 fibroblast cells reduced reporter gene activity driven by *Adcy4* 5’ flanking DNA (Fig. III-25). Moreover, silencing SF1 in parent Y1 cells by hsRNA also increased accumulation of Adcy4 transcript but at the same time decreased mRNA levels of multiple SF1-dependent genes such as *Mc2r* (Rui et al. 2008). These observations, together, support the conclusion that SF1 is an important regulator of *Adcy4* expression in SF1-expressing cells. SF1 is a member of the nuclear receptor superfamily that has been shown to regulate all the major genes involved in the steroidogenic pathways, including those encoding various steroidogenic enzymes (Cyp11A1, 3β-HSD, Cyp21, Cyp11, Cyp19, and Cyp17), peptide hormones (α- and β-subunits of gonadotropins), membrane-bound hormone receptors (MC2R), and the trans-mitochondrial cholesterol carrier (StAR) (Parker et al. 1997; Hammer et al. 1999a; Sewer et al. 2007). Although SF1 has the ability to interact with transcriptional corepressors (Crawford et al. 1998; Nachtigal et al. 1998; Hammer et al. 1999b) and interact with inhibitory ligands (Urs et al. 2006), SF1 is generally regarded as an activator for the expression of these target genes. My findings that SF1 regulates *Adcy4* promoter activity, a gene never listed as a target of SF1, and exerts a repressor function at this promoter in Y1.
and mutant cells were therefore unexpected. Inspection of SF1 site in *Adcy4* reveals that its sequence is more like the SF1 binding sites found in the promoters of the horse and rat *LHB* gene (TGGCCTTG) (Wolfe 1999; Kaiser et al. 2000) and the human *MC2R* (CCAAGTCC on the complementary strand) (Marchal et al. 1998); however, it differs from each by a single nucleotide. Since slight variations in the sequence of the DNA binding elements can have profound allosteric effects on transcription factor activity for some nuclear receptors such as estrogen receptor (Lefstin et al. 1998; Hall et al. 2002a; Hall et al. 2002b), we propose that this variant binding sequence may facilitate the SF1 interacting with corepressors (e.g. DAX-1 and NcoR) (Crawford et al. 1998; Nachtigal et al. 1998), or with inhibitory ligands (e.g. sphingosine) (Urs et al. 2006), and that one or both of these interactions in turn contribute to the repressor activity of SF1 at the *Adcy4* promoter. Alternatively, other mechanisms such as the helical phasing and relative distance of SF1 site to other regulatory elements, especially to the transcription start complex may also influence the activity of this SF1 motif. These kinds of effects have been reported in both prokaryotic and eukaryotic promoters for other transcription factors (Ushida et al. 1990; Yu et al. 1997).

Sp1 and SF1 have been reported to interact cooperatively in the regulation of a number of genes derived from the pituitary and adrenal glands and from the gonads, including those encoding rat LHβ (Kaiser et al. 2000; Horton et al. 2004), bovine CYP11A (Liu et al. 1999), and human StAR (Sugawara et al. 2000). But in the mouse *Adcy4* promoter, even though these two binding sites are partially overlapping in the 5’ flanking region of this gene, SF1 does not show any cooperative interaction with Sp1 or Sp3 at this site in Y1 adrenal cells. In contrast, SF1 seems to act competitively with Sp1 and Sp3 and forms an independent complex. In support of these conclusions, I showed that SF1 binding
to this site cannot be displaced by Sp1 and Sp3 competitor oligonucleotides or by Sp1 and Sp3 antibodies; similarly, Sp1- and Sp3-formed complexes at this site are not displaced by SF1 competitor oligonucleotides and antibodies (Figs. III-19 and III-20). In addition, the exclusive binding to this site by SF1 and Sp1 family members is further evidenced by the finding that disruption of the Sp1 binding site or displacement of Sp1 and Sp3 binding by competitor oligonucleotides both increase the amount of SF1 bound (Fig. III-19). These observations support the idea that SF1 and the Sp1 family work independently at this site in the *Adcy4* promoter regulation. However, it is noteworthy that both the SF1 and Sp1B sites exert represssive effects on the *Adcy4* promoter activity and that their repressor functions seem to be partially dependent on the integrity of Sp1A site in Y1 cells. When the Sp1A site is inactive, no further increase of promoter activity occurs after the Sp1B or SF1 site is mutated alone or in combination, even though the promoter still has considerable residual activity (Fig. III-24). These distinct inhibitory functions of the Sp1B and SF1 sites could be explained by the allosteric effects of the binding sequences as discussed above; but, they also raise the possibility that this overlapping Sp1B and SF1 site plays an interfering role on the communication between Sp1A site and the basal transcription machinery. Further investigation is needed to confirm the latter hypothesis.
SECTION IV: GENERAL DISCUSSION AND FUTURE DIRECTIONS

1. General Discussion

Although adenylyl cyclase has been defined as the major signaling mediator of ACTH action on steroid hormone production in the adrenal cortex (Gallo-Payet et al. 2003), the expression profile and functions of adenylyl cyclase isozymes in each layer of the adrenal cortex have not been well defined and the factors controlling the expression of these Adcy enzymes are largely unknown. Our previous studies on the Y1 mouse adrenal cell model showed that among the five Adcy isozymes detected in this cell line, Adcy4 is the least abundant but is the preferred target for forskolin as evidenced by the resistance to forskolin that results from Adcy4 deficiency in mutant cells (Al-Hakim et al. 2004). Moreover, Adcy4 deficient mutants have an impaired SF1 transactivation activity, fail to express several SF1-dependent genes involved in steroidogenesis and have dysfunction in steroid hormone biosynthesis (Al-Hakim et al. 2004), suggesting that SF1 may be involved in the regulation of Adcy4 expression in Y1 cells. This thesis presents the systematic investigation of the factors that govern the expression of Adcy4 in Y1 adrenal cells and its forskolin-resistant mutants. Understanding the regulatory mechanism of Adcy4 expression in these cells can contribute to the study of the whole signaling system that regulates steroidogenesis in these cell models.

In this study, I have determined the cDNA sequence and gene structure for the mouse Adcy4 gene and have characterized its promoter regulatory elements. My data demonstrate that Adcy4 is regulated in a complicated manner by the tissue-specific factor SF1 and by the widely expressed Sp1/Sp3 proteins in both the Y1 mouse adrenal cell line
and its mutant counterparts. Although the precise mechanism is still elusive, a working model for Adcy4 promoter regulation is proposed and presented in Fig. IV-1. Mouse Adcy4 initiates transcription from one major and two minor start sites in Y1 cells. The promoter activity of Adcy4 is mainly driven by the highly conserved \(-135/-19\) region and is inhibited by the first intron (by one or more of several potential mechanisms). Within the conserved region, three Sp1/Sp3 – and one SF1-binding sites, via interacting with their corresponding transcription factors, are all involved in the promoter regulation of Adcy4, but exhibit quite different activities. The Sp1C site and/or an unidentified motif in its vicinity are closest to the major transcription start site, and thus are probably part of the core promoter that helps to assemble the preinitiation complex (PIC) and promote basal Adcy4 transcriptional activity. The Sp1A and Sp1B sites, though binding the transcription factors Sp1 and Sp3 similarly in EMSA experiments, exert opposite effects on this promoter activity, perhaps reflecting a subtle difference in their interaction with various cofactors: Sp1A may favor the recruitment of co-activators, mediators and chromatin remodeling complexes, or even contact directly to the basal transcription machinery, thereby stimulating Adcy4 promoter activity; whereas, Sp1B probably tends to recruit co-repressors and inhibitory chromatin remodeling complexes such as Sin3/NuRD, SMRT and NcoR, and thus prevents the Sp1A activation complex from interacting with the PIC (Li et al. 2004a). Surprisingly, SF1, generally regarded as a master activator for steroidogenic gene expression, actually works as a repressor on Adcy4 promoter activity in Y1 and its mutant cells. The mechanisms for this unexpected effect of SF1 are still not clear and may involve several possible mechanisms,
Fig. IV-1. Schematic model of Adcy4 promoter regulation in Y1 and its forskolin-resistant mutant cells.

The highly conserved 5’-flanking region (shaded line) and 5’ partial genomic fragment including exon I, intron I and exon II of Adcy4 are presented. The transcription factor binding sites and their centered positions within the conserved region, the major and minor transcription start sites in Y1 cells (Bent arrows), and the presumed preinitiation complex (PIC) are also shown. In both cell lines, the Sp1A site directly or indirectly stimulates Adcy4 promoter activity by potentially different mechanisms (black curved arrow); the Sp1C site and/or the unknown motif (TF?) in the vicinity may directly contribute to the formation of the PIC; whereas Sp1B and SF1 sites each suppress promoter activity probably by interacting with co-repressors (the dashed, curved arrows). The first intron also exhibits an inhibitory effect on Adcy4 expression via several possible mechanisms. However, in the mutant cells, SF1 is overexpressed and its transactivation function is impaired, which together may compromise the activity of the whole conserved region and account at least in part for the Adcy4 deficiency in these cells.
such as recruitment of co-repressors and/or binding to inhibitory ligands. However, other explanations regarding the effect of this transcription factor on Adcy4 promoter activities are still exist. For example, the inhibitory effects of both the SF1 site and the Sp1B site may just arise from an mechanism that interferes with communication between the Sp1A site and PIC.

The finding of SF1 acting as a repressor at the Adcy4 promoter has clearly enriched our knowledge of SF1 as a master regulator of steroidogenesis in both our cell system and in adrenal tissue. SF1 is usually thought to stimulate the cell-selective expression of multiple genes associated with steroidogenesis and reproduction. This study thus presents the first example of the repressor-like activity of SF1 that suppresses Adcy4 expression in Y1 adrenal cells, leading to potential changes of the Adcy isozyme composition in these cells. This observation may explain why Adcy4 is the least abundant of the five Adcy isozymes expressed in cultured Y1 cells (Al-Hakim et al. 2004). Because stimulation of Y1 adrenal cells with ACTH or other agents that increase cAMP levels significantly increases both SF1 mRNA and protein (Lehmann et al. 2005), it is possible that SF1 can serve as a feedback inhibitory regulator, suppressing Adcy4 expression, and eventually reducing the sensitivity of the adenylyl cyclase system to Gßγ. In addition, the findings of this study may also help explain some of the phenotypes observed in the forskolin-resistant mutant cells. In these cells, SF1 is overexpressed due to the presence of multiple copies of the SF1 gene on an acentric chromosomal fragment from chromosome 2 (Frigeri et al. 2002; Schimmer et al. 2002; 2003) and the transactivation function of SF1 is impaired due to a mutation residing in an unidentified gene (Frigeri et al. 2002). Thus, either SF1 overexpression or loss of its transactivation activity may result in the specific Adcy4 deficiency, which in turn leads to
the forskolin-resistance in these mutant cells (Al-Hakim et al. 2004). Interestingly, the expression of other Adcy isozymes, Adcy1, Adcy3, Adcy5 and Adcy6, are not affected in both Y1 and Adcy4-deficient mutant cells (Al-Hakim et al. 2004), suggesting they are not negatively regulated by SF1. Inasmuch as Adcy4 is regulated by Gβγ (Simonds 1999), whereas the other isozymes expressed in Y1 cells are affected by calcium (Al-Hakim et al. 2004), the net effect of SF1 may be to decrease the influence of Gβγ on cAMP signaling while increasing the influence of calcium. Previously, we have demonstrated that loss of SF1 transactivation function in the mutant cells contributes to the decreased expression of many other SF1-regulated genes, such as Mc2r, Cyp11a1, Cyp11b1 and Star (Frigeri et al. 2000). These findings, together, support the idea that SF1 plays a central role in the mutant phenotype.

How overexpressed, functionally defective SF1 represses Adcy4 promoter activity in mutant cells has not been defined. Previously, we found that SF1 in these cells bears a polymorphism site at S172 (Frigeri et al. 2002); however, this polymorphic SF1 did not show reduced activity compared to the wild type SF1 when both were tested on reporter gene assays after transient transfection into Y1 cells (Schimmer et al. 2003). As a member of the nuclear hormone receptor family, SF1 transcriptional activity can be modulated by regulatory ligands, posttranscriptional modification, and interaction with different coregulatory proteins or protein complexes (Parker et al. 2002). It is quite possible that in the mutant Y1 cells, SF1 is differentially modulated by the above-mentioned factors, resulting in binding of potential inhibitory ligands (Urs et al. 2006) or recruitment of corepressors (Parker et al. 2002) that lead to the loss of expression of many SF1-regulated genes, including Adcy4. However, such a hypothesis cannot fully explain the repressor-like
activity of SF1 on Adcy4 promoter activity in parent Y1 cells, where SF1 simultaneously stimulates many steroidogenic genes, such as Star, Cyp11a1 and Cyp11b2 (Frigeri et al. 2000; Sewer et al. 2007). This observation raises another possibility that the SF1 site at the Adcy4 promoter is different from those at the steroidogenic genes and exerts a unique allosteric effect for SF1, favoring its interaction with the inhibitory coregulators. Indeed, the SF1 site in Adcy4 differs from the SF1 binding sites in the promoters of the horse and rat LHB and human MC2R, by one nucleotide (Marchal et al. 1998; Wolfe 1999; Kaiser et al. 2000). Such slight variations in the sequences of DNA binding elements have been reported to have profound allosteric effects on several other transcriptional regulators (Lefstn et al. 1998; Hall et al. 2002b). Finally, it cannot be ruled out that the helical phasing and special position of this SF1 site influenced its effect on Adcy4 promoter activity (Ushida et al. 1990; Yu et al. 1997). For example, the binding of SF1 to this site may interfere with the communications between the Sp1A site and the core promoter elements and reduce the Sp1A-mediated stimulatory activity on Adcy4 promoter. This is partially supported by the finding that the repressor-like activity of the SF1 site in Adcy4 seems dependent on the integrity of the Sp1A site (Fig. III-24).

The extent to which SF1 mediates gene repression globally has not been evaluated extensively. However, Adcy4 seems not to be the only gene repressed by SF1. In H295R adrenal tumor cells (Doghman et al. 2007), increased SF1 dosage triggered both up-regulation and down-regulation of distinct sets of transcripts that accompanied an increase in cell proliferation, and decreases in apoptosis and glucocorticoid biosynthesis. Whether the changes in transcript accumulation seen upon over-expression of SF1 reflect inductive and suppressive effects of SF1 on gene expression or are secondary to effects on growth,
apoptosis or steroidogenesis is uncertain. But at least some changes, such as the increased accumulation of FATE1 transcripts, encoding the fetal and adult testis expressed-1, is mediated by direct interaction of SF1 with its consensus binding site at the –78/-71 proximal promoter region (Doghman et al. 2007). In addition, preliminary studies indicate that over 1,000 transcripts are up regulated following hsRNA-mediated knockdown of SF1 in Y1 adrenal cells (Schimmer and Tsao, unpublished). Studies that correlate genome-wide SF1 binding with genome-wide expression data, followed by detailed promoter analysis, will be required to determine the global influence of SF1 on gene repression.

As an addition to the global role of SF1 in the regulation of steroidogenesis, gene repression by SF1 might contribute to the functional zonation of the adrenal cortex. It has been reported that SF1, in transient transfection experiments, suppresses the expression of CYP11B2 by interfering with the effects of NURR1, NGFI-B and COUP-TF (Bassett et al. 2004b; Shibata et al. 2004) and suppresses the expression of HSD3B2 by interfering with the effects of NGFI-B in co-transfection experiments using H295R adrenocortical tumor cells (Bassett et al. 2004a). Over-expression of SF1 alone in this cell line resulted in significant decreases in the accumulation of HSD3B2 and CYP21A2 transcripts (Doghman et al. 2007). CYP11B2 is essential for the biosynthesis of aldosterone in zona glomerulosa; thus, these suppressive effects might influence the relative expression of these genes in the three zones of the adrenal cortex and consequently impact on the zone-specific synthesis of aldosterone, cortisol and dehydroepiandrosterone, the major steroid products of the human adrenal gland.

In conclusion, this study has extended our understanding of the central role of SF1 in the regulation of steroidogenesis in the Y1 adrenal cell model. The overexpression of SF1 coupled with its functional defect in Y1 mutant cells may be the leading cause of a complex
phenotype characterized not only by the deficiencies in SF1-regulated genes such as Mc2r, Cyp11a1, Cyp11b1 and Star (Frigeri et al. 2000; Frigeri et al. 2002), but also by the loss of Adcy4 expression (Al-Hakim et al. 2004). Thus, further characterization of Adcy4 regulation in these cells is likely to lead to a better understanding of SF1 function at different promoters in mice.

2. Future Directions

The findings of this thesis have suggested several exciting avenues of experimentation that will further our understanding of Adcy4 gene regulation and global repressive effects of SF1 in Y1 adrenal cells. These experiments focus upon the following three major areas:

2.1 Characterize the Regulatory Mechanisms of Sp1 and SF1 Binding Sites and Intron I Element on Adcy4 Promoter Activity

2.1.1 Define the Mechanisms Behind the Repressive Effects of the Sp1B and SF1 Sites on Adcy4 Promoter Activity

The Sp1B site and its overlapping SF1 motif each repress Adcy4 promoter activity. As we discussed before, several potential mechanisms may be behind the Sp1B repressive effect. First, the Sp1B site may have a different selectivity for Sp1 and the different Sp3 isozymes compared to the Sp1A site in the context of chromatin, even though their in vitro binding to Sp1 and Sp3 are similar (Li et al. 1994). To determine the affinities of Sp1 and the Sp3 isozymes to the A and B sites, I would introduce a β-gal reporter cassette driven by
the 5’ flanking DNA of *Adcy4* containing the intact Sp1A and Sp1B sites but a mutated Sp1C site into the endogenous *Adcy4* site together with a selective marker by gene targeting (homologous recombination) (Toledo et al. 2006). I would then cross-link proteins bound to the Sp1A and Sp1B sites and shear the chromatin DNA into smaller pieces by sonication. I would then precipitate all the Sp1/Sp3 bound genomic fragments using a standard ChIP assay with saturating amounts of Sp1 and Sp3 antibodies, digest these fragments with BstU1, which cuts between the Sp1A and Sp1B sites (assuming this restriction site is accessible because promoter regions usually are free of nucleosome binding) and repeat the immunoprecipitation with Sp1 and/or Sp3 antibodies. I would then measure the precipitated Sp1A and Sp1B containing fragments by qPCR to indirectly assess the relative ratios of Sp1 versus Sp3 binding to these two Sp/Sp3 sites. Second, the DNA sequence of the Sp1B site can have profound allosteric effects on transcription factors (Lefstin et al. 1998; Hall et al. 2002a; Hall et al. 2002b). This can be tested by substituting the Sp1B site with consensus binding site sequence to see if I can reverse the repressive effect. Finally, the binding of cognate transcription factors to the Sp1B site can have an interfering effect on the communication between the Sp1A site and the basal transcription machinery (Zhang et al. 2003). Switching the position of the Sp1B and SF1 sites with the Sp1A site, or moving the Sp1B and SF1 sites further upstream in the context of *Adcy4* promoter reporter constructs may give some clues on this effect.

Similar mechanisms could explain the inhibitory effect of the SF1 motif. For example, the variation in the SF1 binding sequence could influence the interactions of SF1 with different coregulators and the helical position of SF1 motif could interfere with the activity of Sp1A site or the function of basal transcription machinery. Thus, some of the
experiments designed for testing the Sp1B function also could be employed to investigate the regulatory effect of the SF1 site, such as mutating the SF1 motif to a *bona fide* consensus or moving this site to a different location to see the potential reverse of its repressive effect. Another way to examine the inhibitory effect of the SF1 site is to compare the activity between two different concatemers made from this site or from a consensus SF1 sequence. However, the negative regulation of SF1 on *Adcy4* promoter could be more complicated, particularly in Y1 mutant cells, in which SF1 is overexpressed but its transactivation activity is impaired (Frigeri et al. 2000; Schimmer et al. 2003). As discussed above, loss of SF1 function in mutant cells could be caused by changes of SF1 in its posttranslational modification, ligand binding, or interaction with coregulators. Experiments designed to check the modification status on the reported phosphorylation (Hammer et al. 1999b; Desclozeaux et al. 2002), acetylation (Chen et al. 2005) or sumoylation (Komatsu et al. 2004) sites or mutating these sites to see their effects on SF1 function in reporter gene assays will possibly give some answers to this question. Investigation of SF1 ligand binding profiles using a combined approach such as immunoprecipitation of SF1 from Y1 and its mutant cells followed by mass spectrometry assays may be helpful. In addition, investigation of the recruitment of coactivators or corepressors to the SF1 site may be necessary. But the final resolution of this question may largely depend on the defining of the underlying mutations in Y1 mutant cells, for which only very limited information is available so far (Frigeri et al. 2000).

2.1.2 Define the Mechanisms Behind the Inhibitory Effect of the First Intron on *Adcy4* Promoter Activity
My data indicate that the first intron of Adcy4 exerts an inhibitory effect on Adcy4 promoter activity. This inhibitory effect could have resulted from a negative regulatory element within the intron sequence (Maston et al. 2006) or from a reduced efficiency of mRNA splicing and export from the nucleus (Luo et al. 1999). To explore these possibilities, I would put this intron into the transcript of another reporter gene to see if its inhibitory effect is gene-specific or universal and then mutate the splicing sites and splicing regulatory elements of the first intron to eliminate the necessity of splicing for the reporter gene transcripts. Alternatively, I would make serial deletions and site-directed mutations to define the potential inhibitory element within the intron. Finally, I would compare the mRNA splicing and transport of the reporter gene transcripts from the nucleus to the cytoplasm in the presence or absence of the first intron to see the effect of the first intron on these processes.

2.1.3 Investigate the Recruitment of Coactivators or Corepressors by the Sp1A, Sp1B and SF1 Sites

The Sp1A, Sp1B and SF1 sites are all involved in Adcy4 promoter regulation in Y1 adrenal cells, but exhibit quite different effects on activity. It has been well documented that the regulatory effect of a cis-element on promoter activity is largely dependent on its ability to interact with the basal transcription machinery or associate with different coregulators. For example, Sp1-mediated transactivation from GC-rich promoters involves complex interactions with several TBP-associated factors (TAFs) that form part of the transcriptional machinery (Pugh et al. 1990; Chiang et al. 1995; Emami et al. 1998) or recruitment of multiprotein nuclear complexes, such as: cofactors required for Sp1 coactivation (CRSP),
vitamin D interacting proteins (DRIPs), thyroid hormone receptor associated proteins (TRAPs), Mediator complexes (Med), SRB/MED containing cofactor complex (SMCC), and positive cofactor 2 (PC2) (Ryu et al. 1999; Safe et al. 2004). In both scenarios, Sp1/Sp3 help direct the formation of a preinitiation complex within a loosely defined start site window. Similarly, SF1-mediated promoter regulation also depends on its interactions with other transcription factors, or various coactivators and corepressors (Parker et al. 2002). The different functions of these Sp1/Sp3 and SF1 sites quite possibly reflect their different recruitment of these coregulators. It would be interesting to explore the proteins or protein complexes associated with Sp1/Sp3 and SF1 in these local sites. A specialized immunoprecipitation method with biotin-labeled Sp1/Sp3 or SF1 oligo probe and avidin-beads can be used to precipitate these proteins and their complexes (Zhang et al. 2003), followed by immunoblot assay or mass spectrometry analysis to determine their identities. ChIP assays using specific antibodies against the components of these complexes can be performed to confirm their in vivo interactions with these Sp1/Sp3 and SF1 sites. Investigation of these interactions will help to understand the complicated regulatory function of these sites on Adcy4 transcription regulation.

2.1.4 Evaluate the Contributions of the Sp1C Site and its Neighboring Unidentified Motif to Adcy4 Promoter Activity

My site-directed mutagenesis and reporter gene assays imply that the Sp1C site contributes to the promoter activity of Adcy4. However, this site seems to bind other unknown nuclear proteins in EMSA experiments. Mutating the site to disrupt Sp1/Sp3 binding increased the binding of the other nuclear proteins. Moreover, this composite site is
located at –23, very close to the major transcription start site in Y1 cells. Thus, it is quite possible that the other nuclear proteins bound in this site are part of the basal transcription machinery. To test this hypothesis and further characterize the unknown nuclear proteins, EMSA experiments using more specifically mutated probes can be performed together with supershift assays with antibodies against different components of basal transcription machinery, such as TFIIB, TFIID and different TAFs. These antibodies can also be used for ChIP assays to confirm the in vivo binding of the corresponding nuclear proteins. Next, site-directed mutagenesis and reporter gene assays can be done to define the function of this composite site.

2.2 Explore the Inhibitory Effect of SF1 on Global Gene Expression in Y1 Adrenal Cells

2.2.1 Define the Signature Genes Repressed by SF1

My study suggested that SF1 works as a repressor and directly suppresses Adcy4 expression in both adrenal Y1 and its mutant cells. Although such direct repression of gene expression by SF1 has not been reported, overexpression of SF1 in H295R human adrenal cells clearly showed both up-regulation and down-regulation of different sets of transcripts (Doghman et al. 2007). Thus, it is quite possible that SF1, as a master regulator of steroidogenic function, can either stimulate or inhibit the expression of different sets of genes to optimize steroid hormone production within these cells. To explore the global, inhibitory effect of SF1, I would knockdown the level of SF1 in Y1 cells and profile the potential target genes using microarray analysis with scrambled hsRNA as a control. Then I
would use a rescue strategy, by mutating the cDNA, which can obviate the effect of SF1-targeting hsRNA and restore the expression of SF1, to confirm the findings. If necessary, overexpression of SF1 in Y1 cells could be used as another control to validate the defined target genes. Representative genes inhibited by SF1 would be further confirmed by real-time qPCR and by analysis of SF1 effects on promoter activity. Grouping these genes using hierarchical clustering analysis might reveal enrichment in processes that reflect effects on the coordinated activities of certain pathways (Schimmer et al. 2006).

2.2.2 Explore Potential SF1 Binding Sites in the Promoter Regions of SF1-Repressed Target Genes

Potential SF1 binding sites in the 5’-flanking regions of SF1-targeted genes could be explored using *in silico* analysis and confirmed using ChIP-Chip or ChIP-Seq assays (Ren et al. 2000; Robertson et al. 2007). Of particular interest would be the identification of SF1 binding sites in the 5’-flanking regions of the SF1-repressed genes. The ability of SF1 binding to these potential sites *in vitro* and *in vivo* would be validated using EMSA and regular ChIP assays, respectively.

2.2.3 Validate the Direct Repressor Function of SF1 on the Promoters of SF1-Repressive Genes

The repressor activity of SF1 on the promoters of SF1-repressed genes would be evaluated using reporter gene assays. The 5’-flanking regions of the SF1-repressed genes placed in front of a luciferase reporter gene would be cotransfected into Y1 cells together with SF1 expression vector to test the repressive effect of SF1 on these promoters. If
necessary, the SF1 binding sites on these promoters would be disrupted using site-directed mutagenesis to test the ablation of SF1 repression on reporter gene activity. Alternatively, the SF1 binding sites in their corresponding promoters can be retrieved and placed in front of another reporter gene to examine their isolated function and responses to SF1.

2.3 Investigate the Regulatory Role of Gβγ Subunits in the Gene Expression of Mc2r and Adcy4

As mentioned in the Introduction, Mc2r deficiency and Adcy4 deficiency in mutant cells are independent consequences of the same underlying mutation (Rainey et al. 2004). Introducing the Mc2r expression vector into these mutant cells restores the levels of the ACTH receptor and the sensitivity of these cells to ACTH stimulation, but not the expression of Adcy4 and responsiveness to forskolin (Qiu et al. 1996); expression of Adcy4 in the mutant cells only rescues the sensitivity of adenylyl cyclases to forskolin, but does not affect the expression of the Mc2r and the responses to ACTH (Al-Hakim et al. 2004). However, results from this study and our previous report (Frigeri et al. 2000) suggest that these two phenotypic changes are closely related to the functional defect in SF1 caused by the common underlying mutation and are partially suppressible by overexpressing Gβγ subunits in the mutant clones (Rui et al. 2004). These findings prompt us to hypothesize that the common underlying mutation is probably linked to the function of Gβγ subunits, which is also impaired in the mutant cells, and which in turn orchestrates all of the phenotypic changes in these cells including the concurrent loss of Mc2r and Adcy4 expression through effects on SF1. To test this hypothesis, overexpression of Gβγ subunits in the mutant cells could be performed to see its effect on the activity of reporter genes driven by the Mc2r and
Adcy4 promoters. If necessary, the SF1 binding sites on these two promoter regions could be mutated to check the target sites of the Gβγ signaling pathway. However, it cannot be excluded that the Gβγ subunits may exert their regulatory effect on the Mc2r and Adcy4 promoters through pathways other than SF1.

Through these studies we will certainly get more knowledge on the whole story about Adcy4 expression regulation and global repressive activity of SF1 in Y1 adrenal cells and its forskolin-resistant mutants. However, extrapolation of our data to the adrenal cortex tissues will require more studies involving transgenic animal models. For example the generation of a transgenic mouse expressing a β-galactosidase reporter gene driven by the 5’-flanking DNA of Adcy4 with wild-type and mutated Sp1/Sp3 or SF1 sites would be extremely useful in determining the in vivo pattern of Adcy4 gene regulation. These types of studies could directly examine the effects of these regulatory factors in the context of genomic organization and in the whole animal environment, which will more accurately reflect their true regulatory functions.


Brock, B. J. and M. R. Waterman (1999). "Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species." Biochemistry 38(5): 1598-1606.


Kaiser, U. B., L. M. Halvorson and M. T. Chen (2000). "Sp1, steroidogenic factor 1 (SF-1), and early growth response protein 1 (egr-1) binding sites form a tripartite


"The crystal structures of human steroidogenic factor-1 and liver receptor homologue-1." Proc Natl Acad Sci U S A 102(21): 7505-7510.


