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Gene and Protein Profiling of the Response of MA-10 Leydig Tumor Cells to Human Chorionic Gonadotropin

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ABSTRACT: Activation of the steroidogenic machinery by peptide hormones involves a number of steps for transmitting signals from the plasma membrane to mitochondria in a spatially and temporally coordinated manner. Although key proteins mediating the hormonal signal have been identified, recent data suggest that the pathway might involve more complex protein-protein and protein-lipid interactions. Genomic and proteomic methods of analysis, namely the Affymetrix Murine Genome U74A v2 GeneChip and the BD PowerBlot Western Array, were used to identify human chorionic gonadotropin (hCG)-induced changes in mRNA and protein of MA-10 Leydig tumor cells that parallel the increase seen in progesterone synthesis. To analyze the massive amount of data that was generated, a comprehensive protein information matrix summarizing the features of each gene or protein, including its known properties, as well as annotations derived by homology-based functional inference, was developed. Of the genes examined by Affymetrix array, approximately 79 were differentially expressed and of gene products examined by PowerBlot, 9 were differentially expressed (above twofold). Changes in the expression of selected transcripts of interest were confirmed using real-time quantitative polymerase chain reaction and immunoblot analyses. Collectively, these results indicate that hormonal regulation of steroidogenesis is a complex phenomenon, involving proteins that participate in various known and novel pathways, which are implicated in transmitting signals from the plasma membrane to mitochondria and nucleus.

Key words: Testis, gene ontology, genomics, proteomics, steroidogenesis.

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Steroid production by testicular Leydig cells is controlled by the pituitary gonadotropin, luteinizing hormone (LH). Although LH is the major physiological agonist in most animals, its action can be mimicked by the placental hormone, human chorionic gonadotropin (hCG). Hormone regulation of steroid synthesis can be thought as being either acute, which occurs within minutes and results in the rapid synthesis of steroids, or chronic, which occurs within hours and results in the continued synthesis of steroids reflecting increased synthesis of the components of the steroidogenic machinery.

Steroidogenesis is regulated by trophic hormones, which bind to their specific plasma membrane receptors and activate a stimulatory guanosine triphosphate (GTP)-binding protein that, in turn, stimulates adenylate cyclase. Activation of steroidogenesis by hCG occurs by several steps involving the plasma membrane and mitochondria. The stimulation results in an increase in cAMP, which is the major second messenger of this system. cAMP may trigger 3 responses: i) changes in the state of phosphorylation of specific proteins, via the cAMP-protein kinase (PKA), ii) induction of protein synthesis, and iii) stimulation of lipid synthesis (Simpson and Waterman, 1983; Hall, 1985; Kimura, 1986; Jefcoate, 2002). One or all of these cAMP-induced changes will trigger the transport of cholesterol from sites of storage or synthesis to the inner mitochondrial membrane, where C27 side-chain cleavage takes place via an enzymatic reaction. The P450scc enzyme, dependent on an electron-transport system comprising a ferredoxin and a flavoprotein, catalyzes this reaction. Detailed studies have shown that the reaction catalyzed by P-450scc is not the rate-limiting step in the synthesis of steroid hormones, but rather, it is the transport of the precursor, cholesterol, from intracellular sources to the inner mitochondrial membrane and the subsequent loading of cholesterol in the P450scc active site (Simpson and Waterman, 1983; Jefcoate, 2002). This hormone-dependent transport mechanism was shown to be mediated by cAMP and to be localized in the mitochondrion (Simpson and Waterman, 1983; Hall, 1985; Jefcoate, 2002). Pregnenolone formed then leaves the mitochondrion to undergo enzymatic transformation in the endoplasmic reticulum that will give raise to the final steroid products. Two proteins, steroidogenic acute regula-

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tory protein (StAR, actually a family of proteins), a cholesterol-transfer protein (Clark et al, 1994; Stocco, 2001), and the peripheral benzodiazepine receptor (PBR), an 18-kDa high-affinity drug ligand and cholesterol-binding protein (Papadopoulos et al 1990; Lacapere and Papadopoulos, 2003) are involved in this process. Cholesterol transfer across the membranes is dependent on de novo protein synthesis, which is the regulated step in the process (Simpson and Waterman, 1983; Jefcoate, 2002). It is also well established that steroidogenesis in Leydig cells is regulated by LH/hCG via a signal transduction pathway involving the second messenger cAMP. In addition to the identified proteins mediating this hormonal signal, recent data have indicated that many signal transduction pathways are activated and that complex protein-protein and protein-lipid interactions might also be involved in this process (Finkielstein et al, 1998; Seger et al, 2001; Hirakawa et al, 2002; Li et al, 2001).

In the present study, MA-10 Leydig tumor cells were used in an effort to identify new response targets of hCG and to gain further insight into the changes in gene expression that might underlie the regulation of steroidogenesis. We measured gene expression profiles using the Murine Genome U74A v2 GeneChip (Thimmulappa et al, 2002), which permits the screening of changes occurring in about 8000 combined genes and expressed sequence tag (EST) clusters, and the BD PowerBlot (Castedo et al, 2001; Malakhov et al, 2003), a high-throughput immunoblotting technique that permits analysis of the protein expression of about 800 gene products. We also used the real-time quantitative polymerase chain reaction (Q-PCR) and Western blotting to confirm findings obtained with the above-mentioned techniques. The data provided herein indicate that posttranscriptional events, such as regulated translation, protein stability, and posttranslational modifications, likely contribute to the acute stimulation of Leydig cell steroid formation by hCG.

Materials and Methods

Cell Culture and Treatments

MA-10 cells (a gift from Dr M. Ascoli, Department of Pharmacology, University of Iowa, Iowa City, Ia) were grown in Dulbecco modified Eagle medium (DMEM)/F-12, supplemented with 2.5% horse serum and 5.0% fetal bovine serum (FBS). Cells were plated onto 75-cm^2 flasks or 6-well plates and grown to subconfluence. Cells were then washed with serum-free medium and either incubated for 2 hours in the presence or absence of various concentrations of hCG (batch CR-125 of biological potency 11 900 IU/mg, a gift from NIDDK, NIH), for determining concentration-response effects, or incubated for various periods of time in the presence of 50 ng/mL hCG for time-course assays. Following incubation, media were collected for progesterone measurement, and cells were saved for protein determination, RNA isolation, or immunoblot (Western) analysis.

Preparation of Samples for Oligonucleotide Microarray Analysis

After incubation of MA-10 cells for 2 hours in the presence or absence of 50 ng/mL hCG, the cells were collected and RNA was isolated. The experiment was repeated 3 times, and total RNA collected from the replicates was pooled. Because of the variability of the cell response to hCG, RNA was collected only from cells showing a 50-fold increase in progesterone formation in response to hCG. Total RNA was amplified and biotinylated using the protocol detailed by Affymetrix (Santa Clara, Calif). Briefly, double-stranded cDNA was synthesized from 15 μg of total RNA using the SuperScript Choice System (InVitrogen Life Technologies, Carlsbad, Calif) and a T7-(dT)24 primer (Genset Corp, San Diego, Calif). In vitro transcription using double-stranded cDNA as a template in the presence of biotinylated UTP and CTP was carried out using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Affymetrix). Biotinylated cRNA was then purified, fragmented, and hybridized to the arrays. After washing, the arrays were stained with streptavidin-phycoerythrin (Molecular Probes, Inc, Eugene, Ore) and enhanced with anti-streptavidin antibody (Vector Laboratories, Burlingame, Calif) using a fluidics system and then scanned with a specifically designed confocal scanner (Affymetrix). Image data were analyzed using the MicroArray Suite v.5 Gene Expression analysis program (Affymetrix). The data were normalized, filtered, and subjected to cluster analysis.

Oligonucleotide Microarray Analysis

The Affymetrix Murine Genome U74A v2 GeneChip Expression probe assay, which represents all gene sequences (~6000) in the mouse UniGene database (Build 74) and about 2000 EST clusters, was used to profile changes in gene expression and to characterize targets that respond to acute treatment with hCG. For control and treated cells, 3 separate preparations of RNA samples were pooled and submitted to array hybridization. This process was repeated twice, resulting in qualitatively identical results. hCG-treated samples and untreated control samples were compared using Affymetrix Microarray Suite software. This software, which is based on a decision matrix that includes the net change in intensity values, fold of change, and other parameters, enables one to determine whether a given gene is differently expressed. Genes for which expression changed twofold or more than twofold were considered to be hCG acute-response genes. The choice of a twofold difference limit was based on our previous experience with microarray data analysis and is also in general agreement with the limit used in other reported array experiments.

High-Throughput Western Blot Screening and Data Analysis

Primary screening was performed by BD Biosciences Transduction Laboratories (Lexington, Ky) using the PowerBlot™ assay (Castedo et al, 2001; Malakhov et al, 2003), which determined the expression level of 860 different signal-transducing proteins. Briefly, extracts containing total cellular protein from 3 separate

hCG-treated or control MA-10 cells, obtained from 3 independent experiments, were separated on 5%–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) linear gradients and transferred onto nitrocellulose membranes that were then divided into 40 lanes using a chamber-forming grid. Each individual lane was incubated with a blend of several specifically selected monoclonal antibodies (mAbs) that recognize proteins of nonoverlapping molecular weights. A total of 860 individual mAbs are used in the assay, some of which are different clones recognizing the same protein or phosphorylated derivatives of the same protein. Immunoblotting with specific mAbs was revealed by a secondary goat anti-mouse horseradish peroxidase followed by the capture of chemiluminescence data by a charge-coupled device camera-based system, and densitometric data by computerized processing. Data were normalized to the total intensity value of all pixels in an image multiplied by 1 000 000. Ratios were calculated in order to express increases or decreases in protein expression. Each sample was submitted 3 times in PowerBlot analysis.

Real-Time Quantitative PCR (Q-PCR)

Following incubation of MA-10 cells in the presence or absence of hCG, the cells were washed 3 times with 1× PBS (pH 7.4), and then total RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, Valencia, Calif) according to the manufacturer's specifications. Total RNA was submitted to On-Column DNase I digestion with RNase-free DNase to remove genomic DNA contamination. Q-PCR was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, Calif) as previously described (Li et al, 2002). Briefly, total RNA was reverse-transcribed into cDNA, and the resulting cDNAs were then processed for amplification of the genes of interest using specific primers. Each sample was run in triplicate. PCR products were detected directly by measuring the increase in fluorescence caused by the binding of SYBR® Green I Dye to double-stranded DNA. The Comparative C_T Method was used to analyze the data. mRNA expression corresponding to the target gene was normalized to the endogenous reference (18S rRNA). Target genes and endogenous reference genes were run separately. Results shown are means ± standard error of the mean (SEM) of four independent experiments carried out in triplicates.

Immunoblot (Western) Analysis

In order to validate the results of high-throughput Western blot analysis, we subjected proteins of interest, obtained from MA-10 cells, to immunoblotting using mAbs specific for StAR, IKKγ (inhibitor of kappaB kinase gamma), HSP 70 (heat-shock proteins of average weight 70 kD), PKARIIβ (cAMP-dependent protein kinase type II-beta regulatory chain), ChK2 (serine/threonine-protein kinase Chk2), ERK1 (extracellular signal-regulated kinase 1; also termed mitogen-activated protein kinase 3; MAPK3), and LCB1 (a component of serine palmitoyltransferase 1, which catalyzes the initial step in the biosynthesis of the long-chain base component of sphingolipids). Briefly, after exposing the cells to the above-described treatment protocol, whole-cell extracts were prepared in lysis buffer consisting of 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 6.8), 50% glycerol, and 0.1% bromophenol blue. Protein samples were resolved over 4%–20% Tris-Glycine Gel (InVitrogen) and transferred to a nitrocellulose membrane. Western blot analyses were performed according to the manufacturer's protocol. Proteins were visualized using an ECL kit (Amersham Health, North Arlington Heights, Ill) and analyzed using Fujifilm LAS-1000 (FUJIFILM Medical Systems USA, Inc, Stamford, Conn). For each antibody, Western blot was performed 3 times from 3 independent experimental treatments, and only 1 experiment is shown here.

Radioimmunoassay (RIA)

When MA-10 mouse Leydig tumor cells are stimulated by exposure to the trophic hormones LH and hCG, they respond by producing progesterone rather than testosterone as the major steroid (Ascoli, 1981). Therefore, we measured progesterone synthesis by RIA to provide a steroidogenic index of MA-10 Leydig cells, as previously described (Papadopoulos et al, 1990).

Bioinformatics and Functional Analysis of Differentially Expressed Genes and Proteins

Bioinformatic analysis of the Affymetrix gene expression results was conducted using the annotation information and Gene Ontology (GO) mining tools of the Affymetrix NetAffx Analysis Center (Liu et al, 2003). Annotation information for the probe sets includes gene chip and probe design information, gene and protein references (such as GenBank), and functional annotation (such as GO) (Ashburner et al, 2000). GO is a widely accepted standard vocabulary for annotating genes, consisting of terms for biological process, molecular function, and cellular component.

The BD PowerBlot protein expression results were analyzed using an integrated knowledge-base system developed at the Protein Information Resource (Wu et al, 2003). A protein information matrix was compiled for proteins affected by the hCG treatment, summarizing both known annotations and homology-based functional inference. Rich protein annotations were retrieved from iProClass, a value-added database of protein family, structure, and function information with links and summaries for over 50 molecular databases (Huang et al, 2003). Annotations were also inferred based on fully characterized protein homologs, with an evidence attribution indicating the sources and strength of the inference. The major function and pathway groups of differentially expressed proteins were identified using the mappings of the GO and KEGG (Kanehisa et al, 2002) and BioCarta (http://www.biocarta.com/) pathways. Specifically, the network structure of GO molecular function terms provided the basis for functional classification, while the metabolic, regulatory, or signal transduction pathway categories in KEGG and BioCarta were used for pathway categorization.

Statistics

Multiple means were compared using InStat's one-way analysis of variance (ANOVA) (Prism v. 3.0; GraphPad Inc, San Diego, Calif). All *P* values are provided in the text or figure legends.

Results

Profiling of hCG Acute-Response Genes by Oligonucleotide Array Analysis

Using the twofold difference limit to determine the number of genes that were induced or repressed by hCG treatment of MA-10 cells, we found that the expression of 79 genes was changed, 6 of these being decreased and 73 of these being increased in hCG-treated samples, as compared with control (see Table 1). Among the genes that were increased were those encoding Nuclear receptor subfamily 4 Group A Member 1 (NR4A1; also known as NGF1B), StAR, and JunB, an immediate early gene and transcription factor (AP-1 member).

High-Throughput Immunoblot Identification of hCG-Responsive Genes by BD PowerBlot

A semiquantitative value representing the general trend of the changes observed in protein detected by the BD PowerBlot analysis was used for assessing protein levels in hCG-treated cells relative to control cells. Based on the confidence level at which the identity of the protein could be deduced, we separated the bands into 5 groups (Table 2). As shown in the table, 9 of the 860 gene products examined by PowerBlot were differentially expressed in a significant manner (greater than twofold in triplicate from good-quality signals, confidence level 5), 10 genes were changed 1.5- to 1.9-fold in triplicate from good-quality signals (level 4), 8 genes were changed greater than twofold in triplicate from low signals (level 3), 28 genes were changed 1.25- to 1.5-fold in triplicate (level 2), and finally 38 genes were changed greater than twofold in duplicate from good-quality signals (level 1); proteins having multiple bands have observed molecular weights included in their names.

A protein information matrix categorizing the features of hCG acute-response genes identified by BD PowerBlot analysis into 8 Process categories, 11 pathway clusters, and 38 functional categories is provided in Table 3. Prominent groups of gene products that responded to hCG are involved in apoptosis (6 up-regulated, 7 down-regulated), cell-cycle regulation (3 up-regulated, 3 down-regulated), cell signaling (11 up-regulated, 10 down-regulated), cytokines/chemokines (6 up-regulated, 6 down-regulated), immunology (7 up-regulated, 3 down-regulated), and metabolism (10 up-regulated, 4 down-regulated) pathway. And prominent groups of gene products that responded to hCG are involved in hydrolase activity (6 up-regulated, 3 down-regulated), kinase activity (10 up-regulated, 5 down-regulated), nucleic acid binding (9 up-regulated, 8 down-regulated), nucleotide binding (15 up-regulated, 9 down-regulated), protein binding (5 up-regulated, 9 down-regulated), transferase activity (12 up-regulated, 5 down-regulated).

A comparison of the changes in gene expression shown by the Affymetrix array and Protein PowerBlot is summarized in Table 4. Affymetrix Murine Genome U74A v2 GeneChip permits the screening of changes occurring in about 8000 combined genes and EST clusters, and the BD PowerBlot Western Array only permits the identification and quantitation of proteins recognized by 860 distinct monoclonal antibodies. The lack of complete concordance between these 2 methods could be attributable either to false-positive signals in the array data or to discrepancies between transcript and protein expression.

Confirmation of Array Data by Q-PCR

Q-PCR was used to confirm changes of interest that were shown using the Affymetrix array. Results shown are means ± SEM of 4 independent experiments carried out in triplicate. As shown in Figure 1 and Table 1, changes in the expression of 3 genes that were detected by Q-PCR (ie, NR4a1, StAR and JunB), correlated well with the array data. Results obtained with the array showed that NR4a1 increased by about 17-fold, StAR increased by about 2- to 4-fold, and JunB increased by nearly 12-fold after hCG treatment. Results obtained with Q-PCR showed that NR4a1 was increased by 11.52- ± 1.43-fold, StAR increased by 5.41- ± 0.47-fold, and JunB increased by 1.98- ± 0.21-fold at the 50 ng/mL concentration of hCG. The expression levels of genes encoding NR4a1, StAR, and JunB all increased in a hCG concentration-dependent manner (Figure 1). Statistical analyses indicated that all of these values differed significantly from control values (see legend to Figure 1). Q-PCR results also indicated that the expression levels of NR4a1, StAR, and JunB increased with different time courses (Figure 2) and that these effects were statistically significant (see legend to Figure 2).

Verification of BD PowerBlot Data and Oligonucleotide Array Data Using Individual Antibodies

Western immunoblot analyses were performed with antibodies specific for proteins of interest that were obtained from hCG-treated MA-10 cells (ie, LCB1, ChK2, PKARIIβ, ERK1, HSP 70, and IKKγ; see Figure 3). LCB1 was increased 1.5- to 1.9-fold in triplicate from good-quality signals (confidence level 4) in PowerBlot (Table 2). LCB1 was confirmed increased 1.17- to 1.2-fold in cells treated with 50 and 100 ng/mL hCG (Figure 3). ChK2 and PKARIIβ were changed 1.25- to 1.5-fold in triplicate (confidence level 2) in PowerBlot (Table 2), PKARIIβ was confirmed increased by hCG treatment, but ChK2 was not confirmed to be by Western immunoblot (Figure 3). ERK1, HSP 70, and IKKγ were changed greater than twofold in duplicate from good-quality signals (confidence level 1) in PowerBlot (Table 2), and only IKKγ was confirmed to be decreased in Western immu-

Table 1. *Functional classification of MA-10 Leydig cell hCG response genes identified by Affymetrix array**

Fold	Description and GenBank No.
Antiproliferative protein	
3.43	B-cell translocation gene 1, antiproliferative/gb=Z16410
Apoptosis	
2.14	SH3-domain GRB2-like B1 (endophilin)/gb=AI842874
Blood coagulation factor	
2.91	Coagulation factor III/gb=M26071
Cell growth and/or maintenance	
3.06	Myelocytomatosis oncogene/gb=L00039
8.9	FBJ osteosarcoma oncogene/gb=V00727
Cell differentiation	
2.06	Gadd45b/gb=X54149
G-protein-coupled receptor protein signal pathway	
2.64	Chemokine orphan receptor 1/gb=AF000236
Integral membrane protein	
5.11	Immediate early response 3/gb=X67644
Metabolism/enzyme	
−2.07	Adenosine deaminase/gb=M10319
2.05	Nuclear-encoded mitochondrial acyltransferase/gb=L42996
2.23	YME1-like 1 (S. cerevisiae)/gb=AA276948
2.52	Cryptochrome 2 (photolyaselike)/gb=AB003433
Nucleic acid binding	
2.46	TIS11 primary response gene/gb=M58566
3.06	Sjogen syndrome antigen B/gb=L00993
Phospholipase inhibitor	
2.24	Annexin A1/gb=AV003419
Protein amino acid phosphorylation	
2.57	CDC-like kinase/gb=M38381
4.21	Clk4/gb=AF033566
Protein biosynthesis	
2.49	Eukaryotic translation initiation factor 4A2/gb=X12507
2.64	cDNA/gb=AV138783
2.69	eIF-1A (eIF-1A)/gb=AF026481
Regulation of cell cycle	
2.15	Cyclin E2/gb=AF091432
2.19	Zinc finger protein/gb=X95504
2.19	Cyclin G2/gb=U95826
2.92	Dusp1/gb=X61940
Regulation of transcription	
−2.48	Creb11/gb=AB010266
−2.28	Enhancer trap locus 1/gb=AV381829
2.15	Cyclin C/gb=U62638
2.22	Hex(prh) gene/gb=AB017132
2.38	Basic-helix-loop-helix protein/gb=Y07836
2.45	Musculin mRNA/gb=AF087035
2.71	cAMP responsive element modulator/gb=M60285
3.02	Activating transcription factor 3/gb=U19118
5.62	Gut-enriched Kruppel-like factor/gb=U20344
11.66	Transcription factor junB (junB)/gb=U20735
11.98	Transcription factor junB (junB)/gb=U20735
17.41	Nuclear receptor subfamily, Group A, Member 1 (Nr4a1)/gb=X16995
Response to heat	
2.01	Heat shock protein, 86 kDa 1/gb=J04633
2.39	Heat shock protein, 70 kDa 3/gb=M12571
2.41	Heat shock protein, 70 kDa 1/gb=AF109906

Table 1. *Continued*

Fold	Description and GenBank No.
Steroid biosynthesis	
2.04	Star/gb=AV362816
4.69	Star/gb=L36062
Transferase	
2.06	RAB geranylgeranyl transferase, b subunit/gb=U12922
2.08	Deoxycytidine kinase/gb=X77731
2.24	Rock2/gb=U58513
2.51	Phosphoribosyl pyrophosphate synthetase 1/gb=AV107396
tRNA processing	
2.1	tRNA nucleotidyl transferase, CCA-adding, 1/gb=AI845321
cDNA and EST	
−2.55	cDNA, 3 end/gb=AV370769
−2.24	cDNA, 3 end/gb=AV334517
2.01	cDNA, 3 end/gb=AW049254
2.02	cDNA, 3 end/gb=AI854581
2.04	cDNA, 5 end/gb=AA717740
2.05	cDNA, 3 end/gb=AI461631
2.06	cDNA, 3 end/gb=AW047929
2.1	cDNA, 3 end/gb=AI840450
2.12	cDNA, 5 end/gb=AA733594
2.14	cDNA, 3 end/gb=AI835436
2.14	cDNA, 5 end/gb=AI464596
2.15	cDNA, 3 end/gb=AI785289
2.15	cDNA, 3 end/gb=AI848056
2.17	cDNA, 5 end/gb=AI156731
2.22	cDNA, 3 end/gb=AW124599
2.26	cDNA, 3 end/gb=AI450597
2.34	cDNA, 3 end/gb=AI848222
2.35	cDNA, 3 end/gb=AW124835
2.46	cDNA, 5 end/gb=AW228316
2.53	cDNA, 3 end/gb=C85523
2.55	cDNA, 5 end/gb=AA711516
6.18	cDNA, 5 end/gb=AI464596
6.32	cDNA, 3 end/gb=AW120868
2.04	cDNA, 5 end/gb=AA032310
2.15	cDNA, 5 end/gb=AA929348
2.2	Zinc finger protein (Rzf)/gb=AF037205
2.2	cDNA, 5 end/gb=AA710439
3.68	cDNA, 3 end/gb=AI853294
Others	
−2.23	Histone H3 (H3.2-221)/gb=M32459
2.06	Neoplastic progression 3/gb=Z31362
2.33	F-box and leucine-rich repeat protein 3a/gb=AW123223
2.84	Interferon beta, fibroblast/gb=V00756
3	Angiomotin like 2/gb=AI854404″

* − indicates decrease with hCG treatment (50 ng/mL for 2 h); +, increase with hCG treatment (50 ng/mL for 2 h), as compared with control; two-fold change selected.

noblot (Figure 3), while ERK1 and HSP 70 showed no significant change by hCG stimulation. Western immunoblot was also performed with antibody specific for StAR (Figure 3), which confirmed again the oligonucleotide array data (Table 1). Compared with control, StAR protein level was increased by 1.29-, 1.87-, and 2.29-fold in cells treated with 10, 50, and 100 ng/mL hCG for 2 hours, respectively. The graph represents 1 of the 3 independent experiments.

Effect of hCG on Progesterone Production by MA-10 Cells

As shown in Figure 4, incubation of MA-10 Leydig cells for 2 hours with increasing concentrations of hCG (1.0–100 ng/mL) led to a highly significant concentration-dependent increase in progesterone production. Progesterone production was increased even at the low concentration of 0.5 ng/mL hCG.

Table 2. *High-throughput immunoblot identification of hCG response proteins by BD PowerBlot**

Protein Name	Change	Protein ID
Confidence level 5		
AKAP220	−	NP_057332.1-human
espin cl. 31	+	NP_062531.1
hsORC4	+	NP_036088.2
IRS-1	+	NP_034700.2
JAK 1-116	+	P52332
KIF1A-180.9	−	A56921
Ninjurin-108	+	NP_038638.1
NTF2	−	NP_080808.1
TFII-I	+	Q9ESZ8
Confidence level 4		
Caveolin 1	−	P49817
ChK1	+	NP_031717.2
hHR23B	+	NP_033037.1
KAP3A	−	NP_034759.1
KIF1A-126	−	A56921
LCB1	+	NP_033295.2
mEPHX	+	NP_034275.1
MSH2	−	NP_032654.1
p53	+	NP_035770.1
UbcH7	−	NP_033482.1
Confidence level 3		
Bax	−	NP_031553.1
EBP 50-52	−	AAC52084-human
Gelsolin	−	NP_666232.2
KAP-45.4?	+	Q16667-human
Paxillin cl. 165–64.6	−	NP_035353.1
PKA RII beta-34	+	AAA40057
PNUTS	−	NP_075240-rat
RPTP beta	+	NP_084204.1
Confidence level 2		
4.1N	+	NP_038538.1
4F2hC	−	P10852
AIP1	+	NP_035182.1
AKAP121	+	NP_033778.1
APP-BP1	+	NP_659180.1
Brm	+	S39580-human
CART1	+	CAA63103
Chk2	+	NP_057890.1
COMT-28.3	+	NP_031770.1
Csk	+	NP_031809.2
eps8-92.5	+	NP_031971.1
FLAP-92.6?	−	AAC40072
G beta-33?	−	S29121
Glucocorticoid R	−	P06537
HEC	+	NP_075783
Kip1/p27	−	NP_034005.2
KSR-1	+	NP_038599.1
LDLB	−	NP_038609.2
Metaxin cl.28-22.2	+	NP_038632.1
panERK-83.6	+	P27703
PKA RII beta	+	AAA40057
PKB alpha/Akt-68	+	NP_033782.1
PKC delta	−	NP_035233.1
Plectin-226	−	AAK63129
pp120-99	+	A56357
RANBP1	−	NP_035369.1
SMN	+	NP_035550.1
ZBP-89	−	Q61624
Confidence level 1		
Bid cl.7	−	NP_031570.2
c-Cbl	+	CAA40394
Cam Kinase IIB	−	P28652
Casein Kinase IIa	+	NP_031814.2
CDC27	+	NP_663411.1
CHD3	+	AAB87383-human
CtBP1-31.2	+	NP_038530.1
CtBP1-46	+	NP_038530.1
Cyclin D3	−	NP_031658.1
ERK1	+	NP_036082.1
ERK1-29.7	+	NP_036082.1
FLAP	−	AAC40072
HSF4-38?	−	Q9R0L1
Hsp70-78.1	−	P17879
IAK1-43	+	AAB63205
IKK gamma	−	NP_034677.1
IRAK-117?	−	NP_032389.1
KAP	+	Q16667-human
KAP-27	−	Q16667-human
Karyopherin B	−	NP_032405.2
Katanin p80	+	AAC09328-human
Lap2	−	AAC52209-rat
LRP	−	NP_032538.1
Neurotensin R 3	−	NP_064356.1
Ninjurin-23	+	NP_038638.1
p160-176.8	−	NP_058056.1
panERK-45.3	−	P27703
Paxillin cl. 177	−	NP_035353.1
Phosphotyrosine PY20	−	A38740
Rab8	−	P55258
ras-GAP-137.1?	+	NP_038860.1
Rin 1-43	+	NP_663470.1
Rsk-92	+	NP_033123.1
SIII p15	−	NP_035673.2
Sos-1	+	NP_033257.1
SQS	+	NP_034321.1
TLS-79	−	NP_631888.1
UBA2-84?	−	NP_057891.1

Table 2. *Continued*

* Fold change = a semiquantitative value that represents the general trend of protein changes for the hCG treatment relative to control. − indicates a decrease in protein level in the hCG (50 ng/mL)-treated sample relative to control; and +, an increase in protein level in the hCG-treated sample relative to control. Confidence levels for protein changes are listed in order of confidence, 5 being the highest confidence. Changes greater than twofold in triplicate from good-quality signals are of highest confidence (level 5), followed by changes 1.5- to 1.9-fold in triplicate from good-quality signals (level 4), changes greater than twofold in triplicate from low signals are listed next (level 3), changes 1.25- to 1.5-fold in triplicate (level 2), and finally changes greater than twofold in duplicate from good-quality signals (level 1); proteins having multiple bands have observed molecular weights included in their names. Protein ID with additional label human or rat means no mouse protein sequence is available and the protein ID comes from either human or rat protein sequence; otherwise, the protein ID is for the mouse protein sequence.

Use of the Gene Ontology Mining Tool

As mentioned above, results obtained with the Affymetrix Array MG-U74A v2 showed that the expression of 79 genes was altered by twofold or more than twofold by hCG treatment, as compared with controls (see also Table 1). Up-loading of these 79 probe sets (Genes) in the GO Mining Tool in the Affymetrix NetAffx Analysis Center (Liu et al, 2003) showed that 48 probe sets (genes) have annotations for GO biological process, 42 probe sets (genes) have annotations for GO cellular component, and 53 probe sets (genes) have annotations for GO molecular

Table 3. *Protein information matrix summarizing the features of hCG response gene products identified by BD PowerBlot analysis**

Category	ID	Total	+	−
Process				
Cell adhesion	GO:0007155	1	0	1
Cell cycle	GO:0007049	2	1	1
Cell growth	GO:0016049	2	1	1
Host-pathogen interaction	GO:0030383	2	1	1
Metabolism	GO:0008152	3	1	2
Response to external stimulus	GO:0009605	3	1	2
Signal transduction	GO:0007165	3	1	2
Virus-host interaction	GO:0019048	2	1	1
Pathway cluster				
Adhesion		6	3	3
Apoptosis		13	6	7
Cell activation		1	1	0
Cell cycle regulation		6	3	3
Cell signaling		21	11	10
Cytokines chemokines		12	6	6
Developmental biology		2	2	0
Hematopoiesis		2	1	1
Immunology		10	7	3
Metabolism		14	10	4
Neuroscience		3	2	1
Function				
GTPase regular activity	GO:0030695	3	2	1
Apoptosis activator activity	GO:0016506	1	0	1
Apoptosis regulator activity	GO:0016329	2	0	2
Catalytic activity	GO:0003824	3	2	1
Chaperone activity	GO:0003754	1	1	1
Enzyme activator activity	GO:0008047	2	0	1
Enzyme inhibitor activity	GO:0004857	1	0	1
Heat shock protein activity	GO:0003773	2	0	2
Helicase activity	GO:0004386	2	2	0
Hydrolase activity	GO:0016787	9	6	3
Kinase activity	GO:0016301	15	10	5
Kinase regulator activity	GO:0019207	4	1	3
Ligase activity	GO:0016874	1	0	1
Lipid binding	GO:0008289	1	0	1
Metal ion binding	GO:0046872	4	4	0
Microtubule motor activity	GO:0003777	2	0	2
Motor activity	GO:0003774	1	0	1
Nucleic acid binding	GO:0003676	17	9	8
Nucleotide binding	GO:0000166	24	15	9
Oxidoreductase activity	GO:0016491	2	2	0
Peptide binding	GO:0042277	1	0	1
Protein binding	GO:0005515	14	5	9
Protein transporter activity	GO:0008565	6	1	5
Receptor activity	GO:0004872	5	2	3
Receptor binding	GO:0005102	1	1	0
Receptor signaling protein activity	GO:0005057	1	1	0
Signal transducer activity	GO:0004871	2	1	1
Small protein-activating enzyme activity	GO:0008641	1	0	1
Small protein-conjugating enzyme activity	GO:0008639	1	0	1
Steroid binding	GO:0005496	1	0	1
Structural molecule activity	GO:0005198	1	1	0
Transcription cofactor activity	GO:0003712	1	1	0
Transcription factor activity	GO:0003700	3	1	2
Transcriptional elongation regulator activity	GO:0003711	1	0	1
Transferase activity	GO:0016740	17	12	5
Translation factor activity (nucleic acid binding)	GO:0008135	1	0	1
Translation regulator activity	GO:0045182	3	2	1
Transporter activity	GO:0005215	1	0	1

* Total indicates the total number of genes for which the protein level was increased or decreased in the hCG (50 ng/mL)-treated sample relative to control; +, the total number of genes that were increased in protein level in the hCG-treated sample relative to control; and −, the total number of genes that were decreased in protein level in the hCG-treated sample relative to control. Pathway cluster includes both Biocarta and KEGG pathways. Function was organized based on major functional categories.

Table 4. *Common hCG response gene and gene products identified by both Affymetrix array and Protein PowerBlot**

Protein ID	Description	Protein			Array Fold	GeneBank ID
		Mean	STD	SEM		
Protein changes in triplicate, confidence level 5						
hsORC4	Origin recognition complex subunit 4	2.71	0.50	0.35	1.130	gb=Y16386
JAK 1-116	Tyrosine-protein kinase JAK1 (Janus kinase 1)	2.71	0.72	0.51	1.204	gb=AI837528
Ninjurin-108	Ninjurin	3.79	1.18	0.84	1.056	gb=U91513
TFII-I	General transcription factor II-I (GTFII-I) (TFII-I)	4.71	2.25	1.59	1.258	gb=AV146566
Protein changes in triplicate, confidence level 4						
ChK1	Checkpoint kinase Chk1	1.82	0.16	0.11	1.285	gb=AF016583
UbcH7	Ubiquitin-conjugating enzyme E2-18 kDa UbcH7	−2.19	0.46	0.32	−1.295	gb=AJ130961
Protein changes in triplicate, confidence level 3						
Bax	Bc12-associated X protein	div/0	…		−1.131	gb=L22472
Gelsolin	Gelsolin	div/0	…		−1.625	gb=AV369888
Protein changes in triplicate, confidence level 2						
AKAP121	A kinase anchor protein 1	1.70	0.61	0.43	1.456	gb=U95146
APP-BP1	Amyloid beta precursor protein-binding protein 1	1.84	0.71	0.71	1.457	gb=AI846393
eps8-92.5	Epidermal growth factor kinase substrate EPS8	4.73	3.98	2.82	1.373	gb=L21671
PKC delta	PKC delta	−2.39	1.17	0.83	−1.012	gb=AB011812
Plectin-226	Plectin	−4.28	5.14	3.64	−1.040	gb=AW123286
Protein changes in duplicate, confidence level 1						
Bid cl.7	BH3 interacting domain death agonist (BID)	div/0	…		−1.416	gb=U75506
Casein Kinase IIa/a-38	Casein kinase II alpha subunit	3.28	0.31	0.31	1.438	gb=U51866
Cyclin D3	Cyclin D3	−2.53	0.36	0.36	−1.063	gb=M86183
HSF4-38?	Heat-shock factor protein 4 (HSF 4)	−3.24	1.22	1.22	−1.138	gb=AB029349
p160-176.8	Myb-binding protein p160	−3.33	1.32	1.32	−1.229	gb=AI506202
Rsk-92	Ribosomal protein S6 kinase	5.86	2.62	2.62	1.042	gb=AV321321

* Comparison of changes in gene expression detected by array and Western blots were analyzed. Values represent treatment : control ratios. − indicates a decrease in protein level in the experimental sample relative to control; +, an increase in protein level in the experimental sample relative to control. Only genes which showed consistent changes in both the Affymetrix array and the Protein PowerBlot are shown.

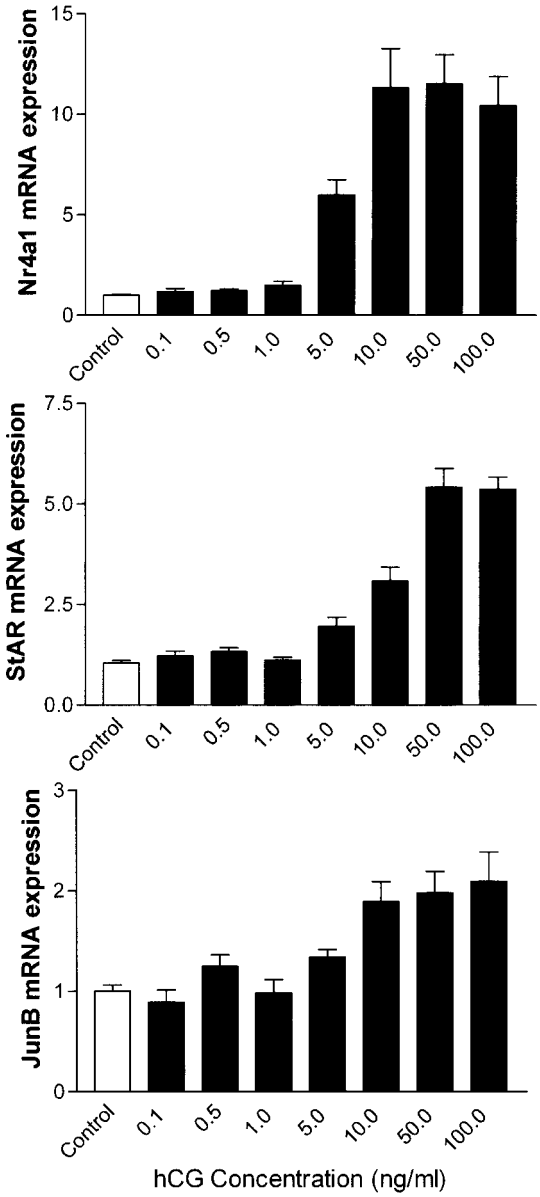

Figure 1. Effect of hCG on mRNA levels of specific genes. MA-10 Leydig tumor cells were treated with different concentrations of hCG in serum-free medium for 2 hours. After incubation, cells were washed, RNA was isolated, and the mRNA levels of the indicated genes were determined by Q-PCR. Results shown are means ± SEM of 4 independent experiments carried out in triplicate. The following forward and reverse primers (5′–3′) were used for Q-PCR: CCAGTAAGTGCGGGTCAT and CCAATCGGTAGTAGCGACGG for 18S rRNA, CTCACAGGAAGCCTGCAAGTC and CCTCCCGATGCTGTTAGCTG for StAR, CCGTGGCACCGGTCAG and GGTCTTGGCACCAGGGACTT for JunB, TTCTTTCTGAACCGTGCTGGA and TGGCCATTTCCTTCAGCAAC for NR4a1. Analysis of the data by one-way ANOVA indicated that the effect of different concentrations of hCG on the expression of NR4a1, StAR, and JunB mRNAs in MA-10 cells was highly significant ($P < .0001$), by one-way ANOVA or by Bartlett's test for equal variances, as compared with control.

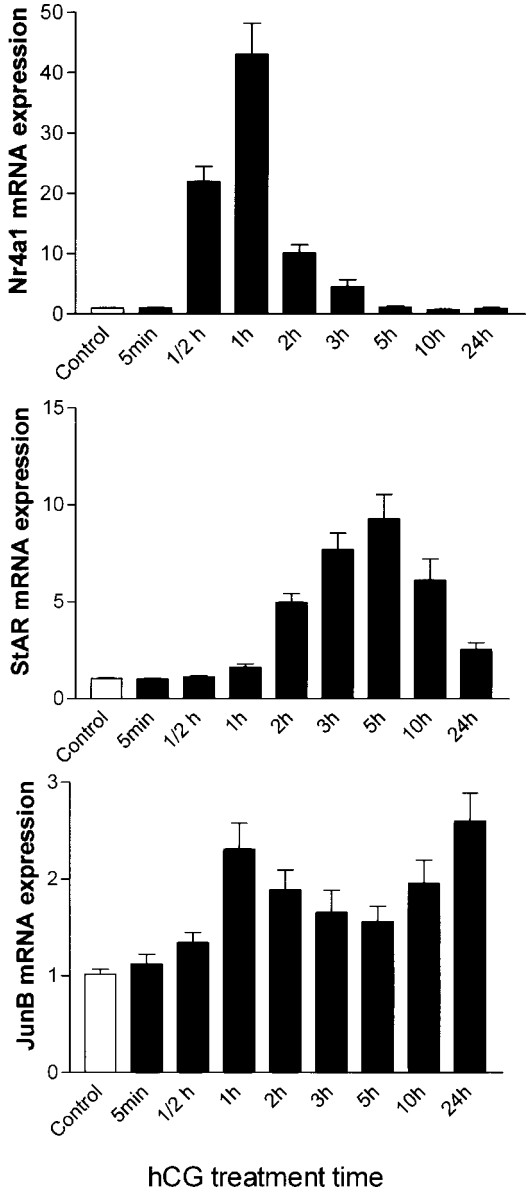

Figure 2. Effects of hCG on mRNA levels of specific genes. MA-10 Leydig tumor cells were treated with 50 ng/mL hCG in serum-free medium for periods ranging from 5 minutes to 24 hours. After incubation, cells were washed, RNA was isolated, and mRNA levels corresponding to the indicated genes were determined by Q-PCR. Primers used for Q-PCR are indicated in the legend to Figure 1. Analysis of the data by one-way ANOVA indicated that the effects of 50 ng/mL hCG on the expression of NR4a1, StAR, and JunB mRNAs in MA-10 cells differed significantly with respect to treatment time ($P < .0001$), by one-way analysis of variance or by Bartlett's test for equal variances. Results shown are means ± SEM of 5 independent experiments carried out in triplicates.

function. Each gene may belong to more than one of the following categories. In GO biological processes, many of the genes are involved in cellular processes (n = 22) and physiological processes (n = 47), which include cell communication (n = 5), death (n = 5), cell growth and/or maintenance (n = 16), metabolism (n = 34), response to external stimuli (n = 5), and response to stress

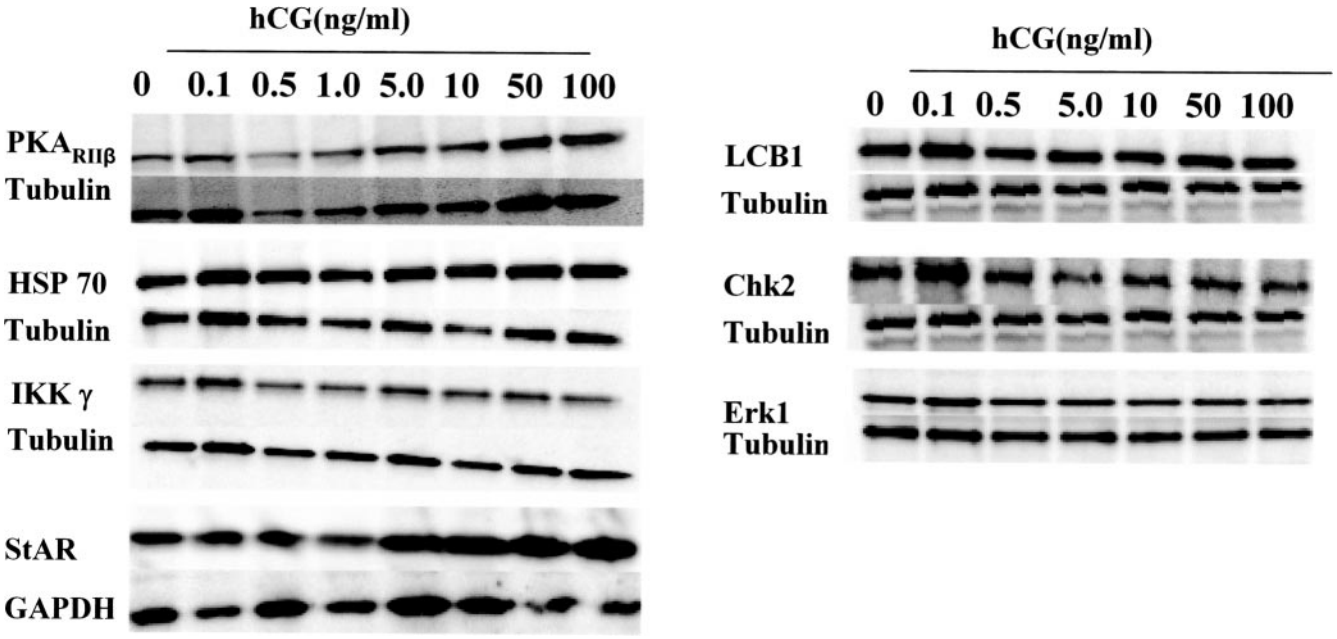

Figure 3. Confirmation of array data by Western blot analysis. MA-10 cells were incubated in the presence or absence of the indicated concentrations of hCG for 2 hours. After incubation, cells were washed, cell extracts were prepared, and Western blot analysis was performed to detect specific protein levels. Proteins from MA-10 cells were subjected to immunoblotting using mAbs specific for IKKγ (48 kDa), HSP 70 (70 kDa), PKARIIβ (53 kDa), ERK1 (44 kDa), ChK2 (60 kDa), LCB1 (53 kDa), and 30 kDa StAR. Signals were normalized to those for GAPDH or tubulin (internal controls) to control for loading variation. The graph represents 1 of the 3 independent experiments.

(n = 4). In the GO cellular component, genes belong to the cell (n = 41) component and extracellular (n = 2) components. For the 41 genes belonging to cell, 37 genes are intracellular and 8 genes are located in the membrane. GO molecular function shows genes involved in enzyme regulator activity (n = 4), structural molecule activity (n = 3), binding (n = 37), catalytic activity (21), signal transducer activity (n = 5), transporter activity (n = 4), translation regulator activity (n = 6), and transcription regulator activity (n = 11).

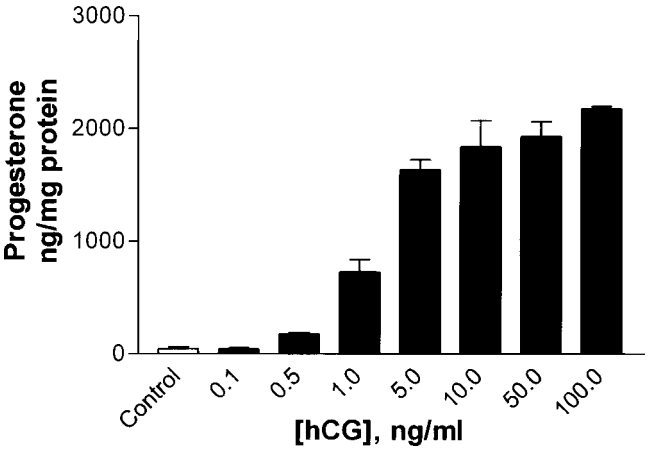

Figure 4. Concentration-response effect of hCG on progesterone biosynthesis in MA-10 cells. The cells were incubated in the presence or absence of different concentrations of hCG for 2 hours. Following incubation, media were collected for determination of progesterone levels by RIA. Results shown are means ± standard deviation (SD) of 3 independent experiments carried out in triplicate. Analysis of the data by one-way ANOVA indicated that this effect of hCG of increasing progesterone concentration in the medium was highly significant ($P < .0001$).

Discussion

The results presented herein have led to the identification of certain new hCG-modulated targets and have increased our understanding of the changes in gene expression that underlie the regulation of steroidogenesis. Our experiments enabled us to gather a massive amount of descriptive data, which indicated that many genes are indeed responsive to acute hormone treatment. Of the approximately 8000 genes represented in Affymetrix GeneChip array, approximately 79 were differentially expressed above twofold, and of the 860 gene products examined by PowerBlot, 9 were differentially expressed above twofold. Results obtained using Q-PCR and Western blotting verified some of the changes in gene expression seen in selected transcripts of interest. However, although differential protein expression correlated well with differential mRNA expression in some cases, this was not uniformly observed for many signaling molecules and transcription factors, in agreement with previously reported studies (Cham et al, 2003). From the array data and by grouping the identified genes on the basis of their known functions, we were able to develop an integrated scheme of signal-

ing pathways that indicate the pleiotropic action of hCG and might explain the action of hCG in stimulating steroid synthesis in Leydig cells. It should be noted, however, that the fact that PowerBlot analysis allowed for the identification of only a subset (10%) of the genes present in the GeneChip array brings a limitation of the method. In some cases, such as StAR, we were able to overcome this using available antisera. Nevertheless, considering that, for many of the genes there are no antibodies available or other means for high throughput screening of protein expression, this is the first attempt to integrate genomic and proteomic data in steroidogenic cells.

Results obtained with the GO method and other methods indicated that a prominent group of genes, the expression of which was altered by hCG treatment, is implicated in regulating transcription as well as the cell cycle, cell growth, and cell maintenance. Many positive cell-cycle regulators were induced by hCG, among which were JunB (Andrecht et al, 2002; Finch et al, 2002; Passegue et al, 2002) and cyclin C (Tassan et al, 1995; Liu et al, 1998). Changes in the expression of these genes could modify cell-cycle progression. The expression of certain genes that are involved in steroid biosynthesis, general metabolism, and protein biosynthesis was also altered by exposure of MA-10 cells to hCG. In addition, hCG up-regulated the expression of StAR, transferase, nucleic acid-binding protein, and heat-shock protein. The results provided in Table 1 show that some signal transduction genes were also responsive to hCG. Also, hCG modulated a large group of transcription factors (*trans*-acting factors), in addition to JunB and NR4a1, which play critical roles in regulating cell-cycle progression and of which altered expression could lead to modifications in the transcription of other genes.

Some of the observed changes seem deserving of special comment because they relate to the primary purpose of the experiments that were conducted. First, our results showed that hCG can increase the steady-state level of StAR transcripts and protein in MA-10 cells in a time- and concentration-dependent manner, which confirms previous results (Clark et al, 1994, 1995; Stocco, 2001; Jefcoate, 2002). StAR, which is synthesized in response to LH stimulation and the expression of which, in the absence of hormone stimulation, is sufficient to induce steroid production (Bose et al, 2002), is a candidate for the role of a newly synthesized, acute regulatory protein involved in regulating cholesterol transfer across mitochondrial membranes. StAR plays a crucial role in regulating steroid hormone biosynthesis in the gonads and adrenal glands. Two hours of exposure to hCG did not significantly affect the 18-kDa transcript and monomeric PBR protein levels (data not shown), in agreement with previous reports (Boujrad et al, 1994).

The up-regulation of JunB by exposure of MA-10 cells to hCG is also of special interest. It is known that JunB controls cyclin A during cell-cycle regulation in mouse and that its antagonism of the action of c-Jun in transcriptional regulation indicates that it might be a negative regulator of cell proliferation. Cyclin A is a direct transcriptional target of JunB in relation to cell proliferation (Andrecht et al, 2002). At the clinical level, it is of interest that the expression of JunB protein has a negative effect on malignant tumor cell proliferation in part through its ability to inhibit AP-1 transactivation (Finch et al, 2002).

Steroid or nuclear hormones receptors were also altered in the present study. These proteins constitute an important superfamily of transcription regulators that are involved in diverse physiological functions, including control of embryonic development, cell differentiation, and homeostasis. Nuclear receptors are extremely important in medical research, a large number of them being implicated in diseases such as cancer, diabetes, and hormone-resistance syndromes. More specifically, NR4A1 was up-regulated by exposure of MA-10 cells to hCG (see Table 1), in agreement with a published report (Song et al, 2001). It might be noted that NR4A1 (also termed Nur77) is an orphan member of the nuclear hormone-receptor superfamily. NR4A1 and its close relatives Nurr1 and NOR-1 bind as monomers to a consensus binding site, the nerve growth factor-induced protein I-B-binding response element. These nuclear receptors are classified as immediate early response genes, which are induced through multiple signal-transduction pathways. They have been implicated in cell proliferation, differentiation, and apoptosis (Wansa et al, 2002).

Cyclin C, which was also up-regulated by exposure of MA-10 cells to hCG, may play a dual role within the cell in that it can regulate both cell-cycle progression and gene transcription. Cyclin C associates with Cdk8 (Tassan et al, 1995), forming a complex that can induce gene transcription of Cdc2 (Cdk1) (Liu et al, 1998). Because Cdc2 kinase is important for cell entry into the mitotic phase of the cell cycle, the capacity of cyclin C to regulate cell-cycle progression may be attributed, in part, to its modulation of Cdc2 protein expression (Liu et al, 1998).

Checkpoint kinase Chk1 was increased at both the mRNA and protein levels in response to hCG treatment. Chk1 is required for normal cell division and Chk-1-dependent processes are participating in tumor suppression (Bartek and Lukas, 2003; Zachos et al, 2003), suggesting that this protein may be part of the hormone-induced mechanism reducing cell proliferation of steroidogenic tumor cells (Morera and Saez, 1980).

Among the hCG-acute response genes identified by both the genomic and proteomic analyses, the general transcription factor TFII-I and the A kinase anchor protein 121 (AKAP121) are of distinct interest for Leydig cell function: TFII-I, because it is a multifactorial transcrip-

tional activator induced in response to various extracellular signals. TFII-I upon activation translocates to the nucleus to initiate signal-induced gene expression (Roy, 2001). AKAP121 targets the cAMP-dependent protein kinase type II to the outer mitochondrial membrane (Cardone et al, 2002). AKAP121 induction was shown to stimulate the cAMP-dependent protein kinase A-induced phosphorylation of proteins associated with the mitochondria, such as BAD (Affaitati et al, 2003), and its expression in HeLa cells induced the translocation of the manganese superoxide dismutase mRNA from the cytosol to the mitochondria, in a cAMP-dependent manner (Ginsberg et al, 2003). Translation at the mitochondrial level was suggested as a mechanism facilitating the import of mitochondrial proteins (Ginsberg et al, 2003). This may be one of the hCG and cAMP-induced mechanisms, in the first minutes upon stimulation, by which StAR mRNA may be targeted to mitochondria for local translation and import.

Akt1/PKB was increased 1.25- to 1.5-fold in triplicate (level 2) among hCG response proteins identified by BD PowerBlot (Table 2). PKB is activated in response to growth factors through the activation of PI3-kinase and Ras (Marte and Downward, 1997). Cellular stress leading to the activation of the p38 MAP kinase also induces PKB activation, indicating a multiplicity of signaling pathways that activate PKB. However, Taylor (2002) failed to show an involvement of PI3K in the hormone-induced steroid formation by MA-10 cells. In preliminary studies, we also failed to show a role of PKB in Leydig cell steroidogenesis after silencing the PKB mRNA (data not shown).

In conclusion, although this study demonstrates the power of the genomic, proteomic, and bioinformatic methodology in unveiling novel pathways activated by hCG in Leydig cells, the results presented herein raise a number of issues to be taken into consideration: i) changes at the mRNA level do not always translate into changes at the protein level, ii) changes at the protein level may occur in a manner independent of changes at the mRNA level, iii) a minor change at the mRNA level may result in major change at the protein expression level, iv) a major level at the mRNA level does not necessarily translate into a major change at the protein level, and v) hormones may induce changes at either the mRNA or protein levels of genes not linked to the steroidogenic response.

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