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Gonadotropin-Mediated Regulation of the Murine VEGF Expression in MA-10 Leydig Cells

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ABSTRACT: Presence of vascular endothelial growth factor (VEGF) is not only limited to cells directly involved in angiogenesis but has also been demonstrated in steroidogenic cells like testicular Leydig cells. Because Leydig cells are subjected to regulation by gonadotropic hormones and produce steroid hormones, we have investigated here the effects of human chorionic gonadotropin (hCG) or steroid hormones on VEGF expression in cultured mouse tumor Leydig cells (MA-10 cells) and have then analyzed the underlying molecular mechanisms. Northern blot analysis and enzyme-linked immunosorbent assays revealed increases in VEGF mRNA and protein levels, respectively, over 3–20 hours in MA-10 cells after stimulation with hCG or 8-Br-cAMP. Although MA-10 cells lack the classical progesterone receptor, progesterone was able to stimulate VEGF expression. Promoter analyses and antibody supershift experiments suggested that the proximal region is able to constitutively bind the transcription factors Sp1 and Sp3. Mutations of 2 potential Sp1 binding sites in the proximal region showed the requirement of these motifs for stimulation of VEGF by hCG and 8-Br-cAMP. The distal cytosine-rich sequence interacts with so far–unidentified faster migrating factors. Following stimulation with hCG or 8-Br-cAMP, the binding of these proteins was increased in the complexes formed in the proximal and distal regions. VEGF expression in Leydig cells is regulated by gonadotropin via a cAMP-dependent mechanism, and the transcription factors Sp1 and Sp3 appear to be involved in the activation of the promoter. Progesterone also appears to play a role in the regulation of VEGF, acting presumably via a nonconventional receptor that remains to be characterized yet.

Key words: Testes, angiogenesis, vascular permeability, growth factor, progesterone, nongenomic steroid action.

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Testicular functions, like steroidogenesis and spermatogenesis, are known to be primarily regulated by hypophyseal gonadotropins. Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) act on Leydig cells to promote steroid hormone synthesis and have been shown to exert their steroidogenic effects by binding to specific G protein–coupled receptors, which subsequently activate adenylate cyclase and increase intracellular cAMP levels (Ryan et al, 1977). In the testis, a close association between Leydig cells and capillaries has been demonstrated. The testicular microcirculation can be considered to be an important determinant for the regulation of both steroidogenesis and gametogenesis. The microvasculature allows a free exchange of nourishment, oxygen, and steroid hormones between the steroidogenic and gametogenic compartments of the testis (Ergün et al, 1994). It is therefore conceivable that vasoactive substances produced within the testis are involved in the maintenance of the permeability of testicular blood vessels helping to maintain an adequate perfusion of the testicular tissue.

Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis; in the formation of new capillaries and blood vessels in normal physiological processes, such as in growth and development; and in the progression of pathological conditions such as tumorigenesis (Folkman, 1995). VEGF stimulates endothelial cell proliferation, migration, and organization into tubules. Moreover VEGF, also called vascular permeability factor, can enhance permeability of existing blood vessels (Carmeliet, 2000), which ensures a thorough perfusion of the tissue, enabling the maintenance of an adequate supply of bloodborne factors like nutritious molecules, precursors, and hormonally active factors. Interestingly, in this context, it has recently been reported by Sone et al (2000) that VEGF plays a major role in the cellular transport of blood glucose. VEGF is a heparin-binding 46-kd disulfide-linked dimeric glycoprotein (Connolly et al, 1989; Ferrara and Davis-Smyth, 1997). The mouse VEGF gene is alternatively transcribed to produce at least 3 isoforms: $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$. The isoforms $VEGF_{120}$ and $VEGF_{164}$ are diffusible to the extracellular milieu, whereas the longer isoform binds to heparin sulfate proteoglycans at the cell surface (Connolly et al, 1989; Ferrara and Da-

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vis-Smyth, 1997). $VEGF_{164}$ has the most potent biological activity and is the most abundant subtype in vivo. VEGF exerts its biological function (Klagsbrun and D'Amore, 1996) by binding to tyrosine kinase receptor 1 (flt-1) and 2 (flk/KDR), which are also located on the surface of Leydig and Sertoli cells (Ergün et al, 1997). VEGF receptor knockout mice lack blood vessel formation (Shalaby et al, 1995) and inactivation of a single VEGF allele is lethal in the mouse embryo (Ferrara et al, 1996).

The physiological role of VEGF has been extensively investigated in the female reproductive tissues. VEGF mediates cyclical neovascularization in ovary and uterus (Cullinan-Bove and Koos, 1993; Shifren et al, 1996; Ferrara et al, 1998). In luteinized granulosa cells, VEGF expression is induced by gonadotropic hormones via activation of the cAMP-dependent second messenger system (Neulen et al, 1998). In rat uterus and human endometrial cells, both estrogen and progesterone have been reported to increase VEGF expression (Shifren et al, 1996; Karuri et al, 1998). In contrast, less is known about the role of this factor in the male reproductive system. VEGF has been demonstrated to be present in the epithelium of the prostate and seminal vesicle epithelium of the male genital tract and in semen (Brown et al, 1995). Also in human testis, the presence of VEGF has been reported (Ergün et al, 1997) in both Leydig and Sertoli cells. VEGF has been proposed to play a role in maintaining the permeability of testicular blood vessels; hence, it might regulate testicular functions (Ergün et al, 1997). Gonadotropin-induced production of VEGF from Leydig cells, isolated from both rat and mouse testes, has been reported recently (Anand et al, 2003). Leydig cells might thus be in a position to regulate the process of local vascular permeability through the production of angiogenic factors, which could have a regulatory effect on both gametogenesis and steroidogenesis. For example, an overexpression of VEGF in the testis and epididymis of transgenic mice resulted in aspermatogenesis and infertility (Korpelainen et al, 1998). Thus, VEGF could have profound effects on testicular functions. Specially, it appears that a finely regulated expression of VEGF is essential for the maintenance of testicular function.

To understand the role of VEGF in testicular physiology, we have initiated a study to investigate the regulation of the gonadotropin-induced expression of VEGF in Leydig cells with MA-10 cells as a model system. The focus in this study is on the analysis of the expression of the murine VEGF gene and the activation of its promoter in this cell line. At present, the molecular basis of the regulation of the mouse VEGF promoter remains obscure, although the human VEGF promoter has been a subject of extensive investigation. A number of factors stimulating human VEGF gene expression have been identified, including cAMP, steroid hormones, hypoxia, glucose, cobalt, iron, growth factors, and cytokines (Connolly et al, 1989). To extend these data, we have studied the effects of hCG, cAMP, and steroid hormones on the induction of VEGF and the transcription factors involved in the promoter regulation.

Materials and Methods

Materials

MA-10 mouse Leydig tumor cells (Ascoli, 1981) were a gift from Dr Mario Ascoli (University of Iowa, Iowa). The VEGF reporter plasmids (Shima et al, 1996) were kindly provided by Dr Patricia A. D'Amore (Harvard Medical School, Boston, Mass). Dulbecco minimum essential medium and lipofectamine reagent were obtained from Gibco Life Technologies (Paisley, Scotland). The VEGF ELISA kit was from R&D systems (Minneapolis, Minn); the Galacto-Light Plus kit from PE Biosystems (Bedford, Ohio); and the luciferase reporter gene assay high-sensitivity kit from Roche Diagnostic GmbH (Mannheim, Germany). Bovine serum albumin was from Merck (Darmstadt, Germany) and 8-bromoadenosine 3′,5′-cAMP (8-Br-cAMP) from Boehringer (Mannheim, Germany). All other cell culture equipment and hormones were from Sigma (Steinheim, Germany). PeqGold RNA Pure was from Peq Lab Biotechnologie (Erlangen, Germany). [α-^{32}P]ATP and [γ-^{32}P]ATP were from Amersham (Freiburg, Germany). The positively charged nylon membranes were from Schleicher & Schuell (Dassel, Germany). The antibodies against Sp1, Sp3, and Egr-2 were from Santa Cruz (Dassel, Germany). The QuickChange site-directed mutagenesis kit was from Stratagene (Amsterdam, The Netherlands). All other chemicals were from Sigma.

Cell Culture

MA-10 mouse Leydig tumor cells were maintained in culture medium (a 1:1 mixture of Dulbecco minimum essential medium and Ham F-12 nutrient mixture supplemented with 7.5% horse serum, 2.5% fetal calf serum, 2 mM L-glutamine and 200 IU penicillin: 200 μg/mL streptomycin sulfate). At a 60%–80% confluence, MA-10 cells were cultured in serum-free medium plus 0.1% bovine serum albumin for 24 hours before stimulation. Fresh serum-free medium containing 50 ng/mL hCG; 1 mM of 8-Br-cAMP; and 3×10^{-5} M of testosterone, dexamethasone, or progesterone, as indicated in the text, was added to the MA-10 cells. After incubation for various periods of time, medium was removed and stored at -20°C for VEGF enzyme-linked immunosorbent assays (ELISAs). The cells were washed with Dulbecco phosphate-buffered saline, and the cell pellets were stored frozen at -80°C for RNA and nuclear extract preparations.

VEGF ELISA

The cells were seeded at a density of 1.5×10^5 cells/mL per well. After stimulation, 1 mL medium was collected and used for the VEGF immunoassay, using a commercial kit that measures $VEGF_{164}$ protein. ELISA was performed according to the manufacturer's protocol. Briefly, 50 μL of an assay diluent RD1N and 50 μL of each sample or standard were mixed into

the wells of a microplate coated with polyclonal antibody specific for mouse VEGF (both natural and recombinant protein). According to the information provided by the supplier, some minor cross-reactivity was observed with rhVEGF165 and rhVEGF165/PIGF. Following an incubation for 2 hours and several washing steps, 100 μL of solution containing antibody against mouse VEGF conjugated to horseradish peroxidase was added and incubated for a further 2 hours. After washing, 100 μL of substrate solution was added to the wells. The enzyme reaction was stopped with the addition of a diluted hydrochloric acid solution. The intensity of the color measured at 450 nm was in proportion to the amount of mouse VEGF bound in the initial step. The sample values were then read off the standard curve.

Total RNA Preparation and Northern Blot Analyses

Total RNA was isolated from 10^7 MA-10 cells with 2 mL PeqGold RNA Pure containing guanidinium thiocyanate and phenol. After extraction and precipitation, a total amount of 20 μg of RNA per lane was separated electrophoretically in a 1.3% (wt/vol) formaldehyde-agarose gel and subsequently transferred to a positively charged nylon membrane. As hybridization probes, α-^{32}P-labeled mouse VEGF cDNA coding from base pair 165 to 659 and GAPDH cDNA coding from base pair 341 to 539 were prepared by random priming using 125 ng template DNA, 250 ng random primer, 0.1 mM dNTPs, 6 μL α-^{32}P-dCTP, 200 ng/μL bovine serum albumin, and 5 U Klenow and incubating for 1.5 hours at 37°C. Hybridization was performed with 1–2 × 10^6 cpm/mL (25 ng) of α-^{32}P-labeled cDNA in 5 mL ULTRAhyb hybridization solution at 42°C overnight. After several washing steps with sodium dodecyl sulfate/sodium citrate, the membranes were usually incubated with ''Biomax MR'' film at −80°C overnight. Densitometric analysis of the RNA bands was performed using the NIH Image 1.62 software.

Preparation of Nuclear Extract for Electrophoretic Mobility Shift Assay

Nuclear extract was isolated from 1–2 × 10^6 MA-10 cells with 300 μL of a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. The suspension was incubated for 15 minutes on ice, and 20 μL of 10% Nonidet P-40 was added and subsequently centrifuged for 60 seconds at 10 000 × *g*. The nuclear pellet obtained was resuspended in 100 μL of a buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride and incubated for 20 minutes on a shaker at 4°C. After centrifugation for 5 minutes at 14 000 rpm and 4°C, the supernatant corresponding to the nuclear extract was collected.

For electrophoretic mobility shift assay (EMSA), 6–8 fmol of ^{32}P-end–labeled oligonucleotides were incubated with 4 μg of nuclear extract in 15 μL of a binding solution containing 4% Ficoll, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mg/mL bovine serum albumin, 2 mM spermine, and 0.4 μg of poly(dI/dC). After incubation for 15 minutes at room temperature, the binding reaction was separated on a native 6% polyacrylamide gel (29:1 cross-link) in 0.25 mM Tris borate-EDTA (TBE) buffer at 10 V/cm for 3 hours at room temperature. Competition experiments were carried out by mixing a 1000-fold molar excess of unlabeled competitor DNA to the binding reaction before adding the nuclear extract. For antibody supershift experiments, 4 μL of a monoclonal mouse antibody raised against an internal domain (amino acids 520–538) of SP1 of a rabbit polyclonal antibody against the carboxy terminus of Sp3 or of Egr-2 was added to the EMSA reactions.

The following oligonucleotides were synthesized for use in EMSA.

5′-TGGGGGTGGAGCTAGATTTCCTCTTTTTCT-3′ (base pair −52 to −23)
5′-AGACCGTCCCCGGGGCGGGTCTGGGCGGGGCTTGGG-3′ (base pair −84 to −48)
5′-CGAAAGGCGGTGCCTGGCTCCACCAGACC-3′ (base pair −108 to −80)
5′-ACTCCCCCCCCCCCGTAACCCCCTCCCCACA-3′ (base pair −723 to −693)
5′-GGATCCAGCGGGGGCGAGCGGGGGCGA-3′ (Egr consensus motif)
5′-GATCGAACTGACCGCCCGCGGCCCGT-3′ (AP2 consensus motif)

Site-Directed Mutagenesis

To generate the reporter plasmid pLuc MluI/Start*, site-directed mutations of both Sp1 binding sites in the region between −72 and −62 (Figure 1A) of the VEGF promoter were performed with the QuickChange site-directed mutagenesis kit according to manufacturer's instructions. The oligonucleotide primer containing mutations in small letters used to mutate both Sp1 sites was 5′-GACCGTCCCCGGttCGGGTCTGttCGGGGCTTGGGG-3′ (−84/−49) and complementary to opposite strands of the reporter plasmid pLuc MluI/Start.

Mouse VEGF Promoter-Luciferase Constructs

As shown in Figure 1A, the reporter plasmid pLuc 1.6 kb contains a 1.6-kb fragment of VEGF DNA, which encompasses 1.2 kb of the 5′ flanking sequence, the transcription start site, and 0.4 kb of the corresponding 5′ untranslated region (UTR). 5′ deletions were made from the 5′ terminus of the 1.6-kb fragment to the ApaI site at −772 bp (pLuc Apa/Start) and to the Mlu site at −449 (pLuc Mlu/Start) (Shima et al, 1996). The plasmid pLuc Mlu/Start* contains 2 mutated Sp1 binding sites in the −72/−62 region 5′ to the single Sp1 site and 3′ to the overlapping Sp1 site (Figure 1A). The promoterless plasmid pLuc-basic served as a negative control.

Transient Transfections and Luciferase Assays

An aliquot of 4.5 × 10^5 cells per well was transiently transfected with 10 μL of Lipofectamine reagent and 6 μg of plasmid and incubated for 6 hours at 37°C. As a control for transfection efficiency, 1.5 μg of pCMV LacZ coding the β-galactosidase enzyme were cotransfected with the VEGF promoter constructs. After stimulation for 6 hours, the cells were lysed and 50 μL lysate was used to measure luciferase activity with the use of the luciferase reporter gene assay high-sensitivity kit. For the β-galactosidase activity measurement with the Galacto-Light Plus kit, an aliquot of 2 μL lysate was used. The samples were mea-

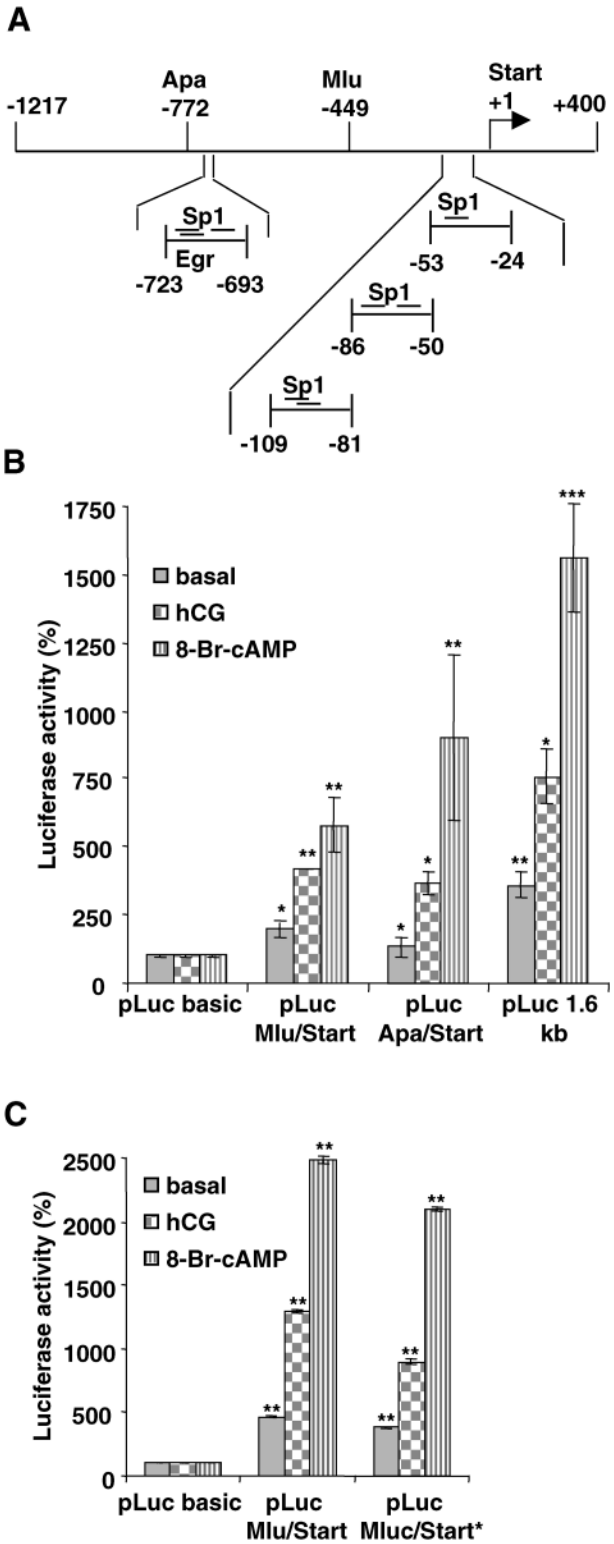

Figure 1. Activation of the vascular endothelial growth factor (VEGF) promoter by human chorionic gonadotropin (hCG) and 8-Br-cAMP. **(A)** Mouse VEGF 1.6 kb promoter region. Restriction sites are marked for the enzymes Apa and Mlu. The locations of the probes containing the putative binding sites for Sp1 and Egr used for electrophoretic mobility shift assays (EMSAs) are indicated. Transient transfection assays in MA-10 cells were performed with the reporter plasmids: pLuc basic, pLuc Mlu/Start, pLuc Apa/Start, pLuc 1.6 kb **(B)** and pLuc Mlu/Start* **(C)**. The reporter plasmids were cotransfected with a reference plasmid pCMV LacZ coding for β-galactosidase enzyme. Subsequently, the transfected cells were stimulated with 50 ng/mL hCG and 1 mM 8-Br-cAMP for 6 hours. The signals derived from the reference were used to normalize the variability in transfection efficiency. The quantified data were verified in several independent experiments. The activities are shown relative to the activity of the pLuc basic plasmid, which was arbitrarily set to 100%. *** $P < .01$, ** $P < .01$, and * $P < .05$ in comparison to the basal values within every treatment **(B)** and ** $P < .01$ in comparison of pLuc Mlu/Start* to pLuc Mlu/Start **(C)**.

sured in a Lumat LB 9501 luminometer. The β-galactosidase activity was used to normalize luciferase assay values.

Statistics

The results were analyzed by NIH Image software. Values have been expressed as mean ± SD. The statistical significance of differences between treated and control groups has been assessed on the basis of a post hoc (Dunnett) test with the program GraphPad Prism.

Results

Regulation of VEGF Expression by hCG via Activation of the cAMP-Dependent Signaling Pathway

In our previous studies, we reported the presence of VEGF and its receptors in the human testis (Ergün et al, 1997). Using reverse-transcriptase polymerase chain reaction (RT-PCR), we have observed that VEGF is expressed also in mouse and rat Leydig cells (Anand et al, 2003). Additionally, immunohistochemical (unpublished) data confirms this observation. However, in the current study, which requires a relatively large supply of cells, we have used MA-10 cells, a mouse tumor Leydig cell line (Ascoli, 1981) that retains many characteristics of their nontransformed counterparts, including the steroidogenic response to stimulation with gonadotropic hormones. These cells produce progesterone as a main steroid hormone upon stimulation with LH/hCG and cAMP. The progesterone production of the MA-10 cells was measured by an ELISA for progesterone developed in our institute (Bathgate et al, 1999), and a stimulation of MA-10 cells for 5 hours with hCG resulted in a fivefold increase in progesterone production (Schwarzenbach et al, 2003). In order to analyze the regulation of the VEGF gene in MA-10 cells, the cell line was exposed to hCG (50 ng/mL) and the cAMP analog, 8-Br-cAMP (1 mM), for various periods of time. The detection of the 2 bands at 1.8 kb (VEGF120) and 2.4 kb (VEGF164) in the Northern blot analysis (Figure 2A) is in agreement with the reported lengths of the RNA transcripts. VEGF164 is also the most abundant isoform in MA-10 cells. Interestingly, an additionally distinct band at 2.2 kb could be detected

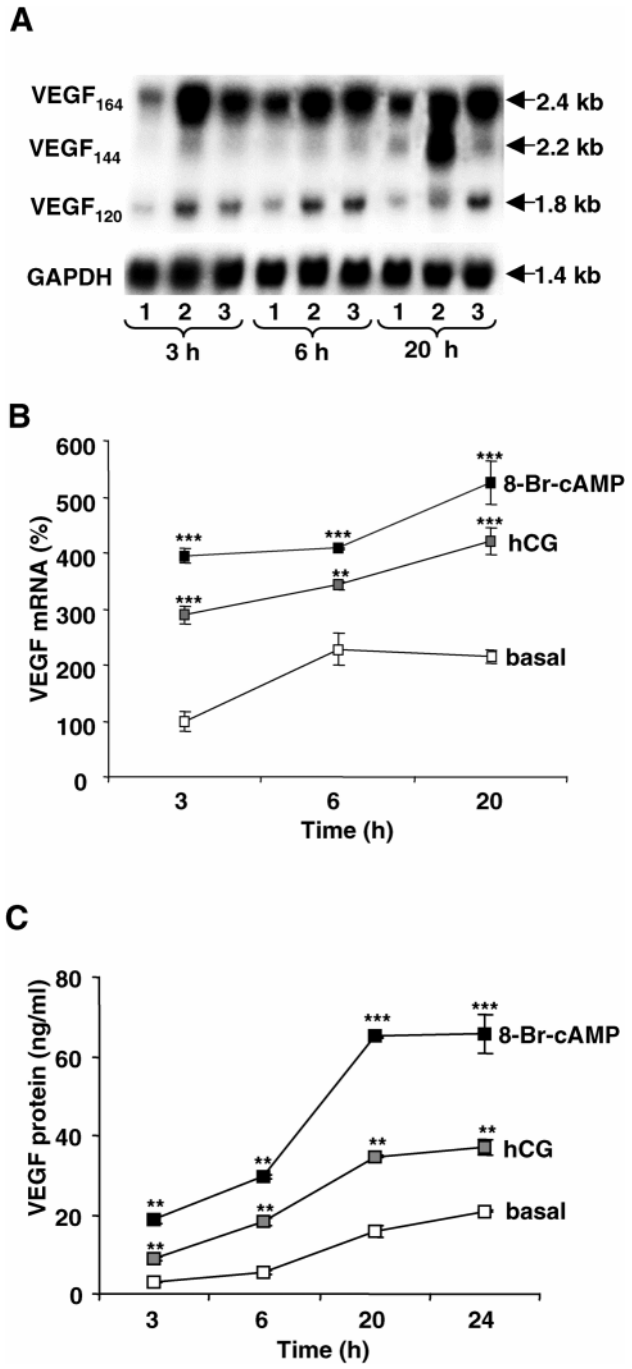

Figure 2. Time course of human chorionic gonadotropin (hCG)– and 8-Br-cAMP–stimulated vascular endothelial growth factor (VEGF) expression. MA-10 cells were incubated with 50 ng/mL hCG or 1 mM 8-Br-cAMP for 3, 6, 20, and 24 hours (h). **(A)** Total RNA was prepared for Northern blot analysis. After gel electrophoresis and transfer to a positively charged nylon membrane, 20 μg mRNA per lane was hybridized with a radioactive-labeled VEGF and GAPDH cDNA probes. Lane 1, basal; lane 2, 8-Br-cAMP; lane 3, hCG. **(B)** Densitometric analysis of the RNA band at 2.4 kb was performed by quantification of 3 independent experiments. The hybridization signals derived from the reference (GAPDH) transcripts were used to normalize the VEGF signals. **(C)** The concentrations of VEGF production in the culture medium were determined with an enzyme-linked immunosorbent assay (ELISA). Values are means ± SD (n = 3). ** $P < .01$ and *** $P < .001$ in comparison to the basal values.

at 20 hours of incubation. This RNA transcript might correspond in size to a mRNA encoding the low-abundance VEGF splice variant of 144 amino acids. As shown in the densitometric evaluation (Figure 2B) of the Northern blot (Figure 2A), significantly increased mRNA levels were evident between 3 and 20 hours after addition of hCG or 8-Br-cAMP.

To confirm that the increase in VEGF mRNA levels in MA-10 cells in response to hCG/cAMP stimulation is coincident with an increase in VEGF protein synthesis, we have measured $VEGF_{164}$ protein secretion into the culture medium. Treatment of MA-10 cells with hCG or 8-Br-cAMP progressively enhanced $VEGF_{164}$ production from 3 through 20 hours. At 20 hours, hCG and 8-Br-cAMP stimulated $VEGF_{164}$ synthesis about 2.5- and 4-fold, respectively, and after 20 hours, a plateau was gradually reached (Figure 2C). These data show that the major 2.4-kb RNA transcript is subsequently translated into $VEGF_{164}$ protein.

Progesterone Induces VEGF mRNA and Protein Expression

In Leydig cells, cAMP is the main intracellular regulator of steroidogenesis. In order to investigate whether steroid hormones also have an influence on VEGF expression, MA-10 cells were exposed to 3×10^{-5} M testosterone, dexamethasone or progesterone for 6 (data not shown) or 20 hours (Figure 3A). In contrast to testosterone and dexamethasone having no effect on VEGF expression, progesterone was able to induce VEGF mRNA transcription (Figure 3A). These results are in agreement with a report by Sone et al (1996), showing that VEGF expression can be up-regulated in retinal epithelial cells by micromolar concentrations of progesterone. An approximately 1.5-fold and 2-fold increase over the basal level of VEGF mRNA could be observed in MA-10 cells treated with progesterone for 6 and 20 hours, respectively (Figure 3B and C). Again, the 2.2-kb band, possibly corresponding to the $VEGF_{144}$ variant, could be detected on 20 hours stimulation with progesterone (Figure 3B). Treatment of MA-10 cells with 3×10^{-6} M progesterone caused only a weak effect on VEGF expression (data not shown). The data of the VEGF ELISA in Figure 3D showed that the amounts of VEGF secreted in the culture medium were twofold enhanced after treatment of MA-10 cells with progesterone for 20 hours.

Functional Analysis of the Mouse VEGF Promoter

Having demonstrated that both hCG and progesterone can up-regulate VEGF expression in MA-10 cells, it was of interest to further characterize the underlying transcription regulatory mechanism. Because the effect of progesterone was observed at a high concentration and a classical progesterone receptor is absent from these cells (Schwarzen-

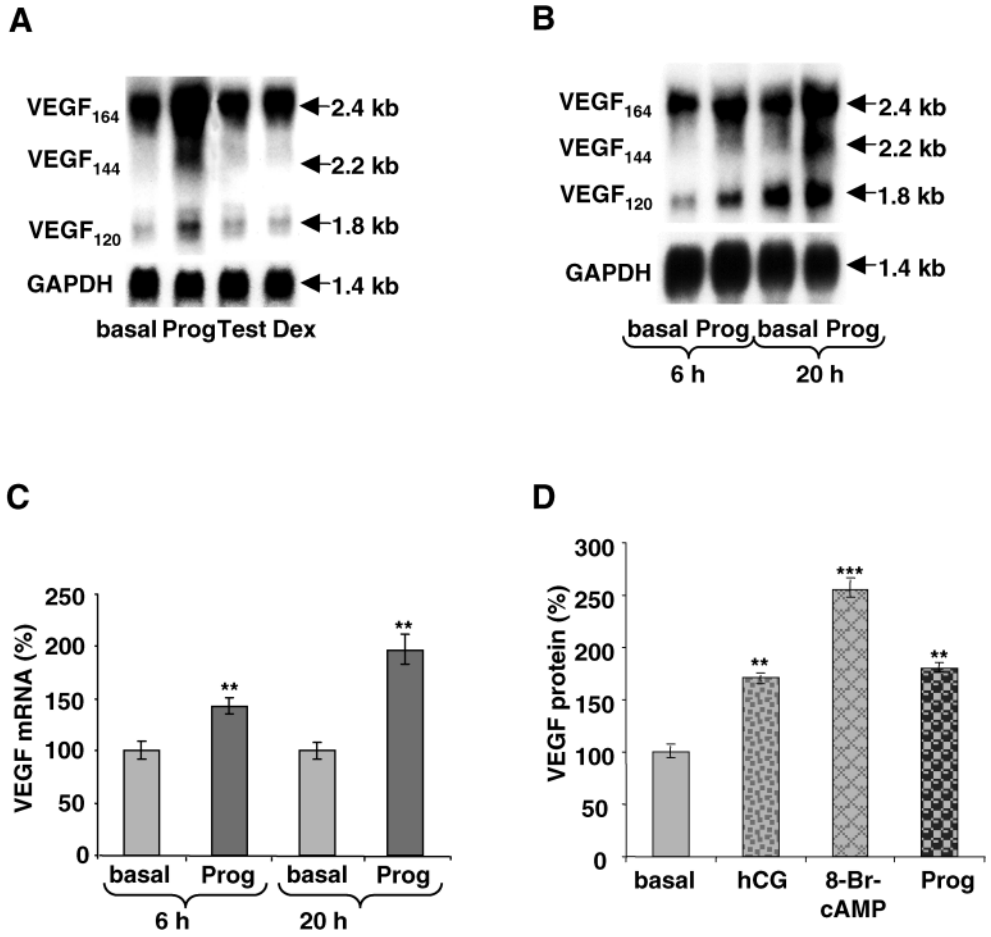

Figure 3. Kinetics of progesterone-induced vascular endothelial growth factor (VEGF) mRNA and protein expression. MA-10 cells were incubated with 3×10^{-5} M progesterone (Prog), testosterone (Test), or dexamethasone (Dex) for 20 hours **(A)** and with 3×10^{-5} M Prog for 6 or 20 hours **(B)**. Total RNA was prepared for Northern blot analysis. After gel electrophoresis and transfer to a positively charged nylon membrane, 20 μg of mRNA per lane were hybridized with a radioactive-labeled VEGF and GAPDH cDNA probes. **(C)** Densitometric analysis of the RNA bands was performed by quantification of 3 independent experiments. The hybridization signals derived from the reference (GAPDH) transcripts were used to normalize the VEGF signals. **(D)** MA-10 cells were incubated with 50 ng/mL human chorionic gonadotropin (hCG), 1 mM 8-Br-cAMP, or 3×10^{-5} M progesterone (Prog) for 20 hours. The concentrations of VEGF in the culture medium were determined with an ELISA specific for VEGF. Values are means ± SD (n = 3). ** $P < .01$ and *** $P < .001$ in comparison to the basal values.

bach et al, 2003), its physiological relevance remains rather obscure. Hence, for further experiments, attention has been focused solely on hCG/cAMP-mediated mechanisms.

To define the regions that confer hCG/8-Br-cAMP–induced promoter activity, a 1.6-kb fragment extending from −1217 to +400 (relative to the transcriptional start site) and 5′ deletions of the 1.6-kb fragment were inserted into a promoterless luciferase reporter plasmid, pLuc-basic (Shima et al, 1996). As shown in Figure 1A, the 1.6-kb fragment encompasses 1.2 kb of the 5′ flanking sequence, the transcription start site and 0.4 kb of the 5′ UTR (pLuc 1.6 kb). The plasmids pLuc Apa/Start and pLuc Mlu/Start contain the promoter fragments extending from −772 to +400 and from −449 to +400, respectively. The reporter plasmid pLuc Mlu/Start* contains both Sp1 binding sites mutated in the region between −72 and −62 (Figure 1A). The plasmids were transiently transfected into MA-10 cells, and cell lysates were assayed for luciferase activity 48 hours posttransfection. pLuc Mlu/Start and pLuc 1.6 kb usually produced a 2- and 3.5-fold basal increase in promoter-driven luciferase activity, respectively, when compared to promoterless luciferase construct (pLuc basic), whereas pLuc Apa/Start resulted in a 50% reduction of the activity of pLuc Mlu/Start (Figure 1B). These data indicate that the Mlu/Start and 1.6 kb/Apa regions are responsible for basal transcriptional activity and the Apa/Mlu region for basal repression in MA-10 cells. When MA-10 cells were treated for 6 hours with 50 ng/mL hCG (2.5, 3.5, and 2-fold) or 1 mM 8-Br-cAMP (3-, 7-, and 4.5-fold), both of the agonists stimulated promoter activity of all 3 reporter plasmids in comparison to basal values. A continuous increase of the activation paralleled by the lengths of the promoter frag-

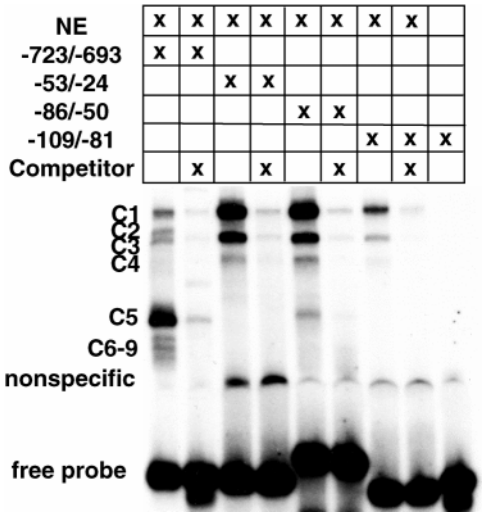

Figure 4. Specific protein complexes bind to the murine vascular endothelial growth factor (VEGF) promoter. An electrophoretic mobility shift assay (EMSA) was performed with ^{32}P–labeled probes covering various promoter fragments (−723/−693, −53/−24, −86/−50, and −109/−81) and 8 μg nuclear extract (NE) prepared from MA-10 cells. Competitions were carried out by adding 2500 fmol of unlabeled cognate oligonucleotides to the reactions. Specific DNA/protein complexes are designated C1, C2, C3, C4, C5, and C6–9 on the left.

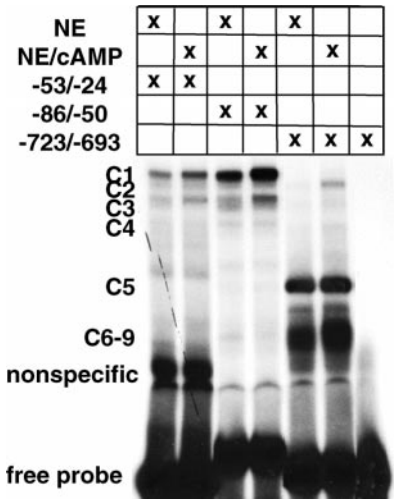

Figure 5. 8-Br-cAMP induces the vascular endothelial growth factor (VEGF) promoter activity. An electrophoretic mobility shift assay (EMSA) was performed with ^{32}P–labeled probes covering various promoter fragments (−723/−693, −53/−24, and −86/−50) and 8 μg nuclear extract (NE) prepared from MA-10 cells stimulated with 1 mM 8-Br-cAMP (NE/cAMP) for 6 hours, as indicated above each lane. Specific DNA/protein complexes are C1, C2, C3, C4, C5, and C6–9 on the left.

ments was caused by 8-Br-cAMP. The basal repression of the Apa/Mlu region was abrogated by the stimulatory effect mediated by 8-Br-cAMP but not by hCG (Figure 1B). These data show that hCG and its intracellular signaling molecule cAMP are able to activate the VEGF promoter. To determine whether the putative Sp1 sites play any role in the regulation of the VEGF promoter by hCG/cAMP, we performed transfection assays with the reporter plasmids pLuc Mlu/Start and pLuc Mlu/Start* followed by a stimulation of the transfected MA-10 cells with hCG or 8-Br-cAMP. Although only 2 Sp1 motifs out of 5 Sp1 sites were mutated (pLuc Mlu/Start*), the mutations in the region −86/−50 (Figure 1A) under basal conditions reduced the activity of the intact promoter (pLuc Mlu/Start) by 20%. After addition of hCG or 8-Br-cAMP, pLuc Mlu/Start* repressed the luciferase activity of pLuc Mlu/Start by 30% and 25%, respectively (Figure 1C). The mutational analysis data suggest that the Sp1 binding sites seem to allow hCG/cAMP stimulation of the VEGF promoter.

Binding Analysis of the Mouse VEGF Promoter

The promoter region of the mouse VEGF gene contains potential consensus binding sites for the transcription factors Sp1, AP2, NF-κB, GCN-4, RAP-1, Egr-2, and Gata-1. To determine the binding activity of various regions, we analyzed 4 sequences, −53/−24, −86/−50, −109/−81, and −723/−693 of the murine VEGF promoter. We chose these fragments because they all contain putative binding sites for the transcription factors Sp1 and Egr (Figure 1A). When the ^{32}P-end–labeled oligonucleotides were incubated with nuclear extracts prepared from MA-10 cells, several specific complexes were detected by EMSA, as shown in Figure 4. Binding competition occurred with the addition of an excess of cold cognate oligonucleotides to indicate specificity of the binding. Two prominent complexes, C1 and C3, formed with the sequences −53/−24 and −86/−50 could be observed. In comparison to these 2 regions, the sequence −109/−81 only demonstrated a weak binding of the proteins in the complexes C1 and C3. An explanation for the weak binding affinity might be that the sequence from −109 to −81 contains an overlapping Sp1 binding site, in contrast to the 2 other sequences containing together 3 single Sp1 sites (Figure 1A). With the use of the distal sequence −723/−693, a prominent band C5 and 4 faster migrating complexes C6–C9 were obtained in addition to 3 weaker complexes (Figure 4).

hCG and 8-Br-cAMP Can Stimulate the VEGF Promoter Activity

The mouse VEGF promoter contains no cAMP response elements. To investigate the region that shows hCG- and 8-Br-cAMP–stimulated binding activity, we set up a binding reaction between the various promoter fragments (−53/−24, −86/−50, and −723/−693) and nuclear extracts prepared from MA-10 cells untreated or treated with hCG (data not shown) or 8-Br-cAMP (Figure 5). Compared with the basal nonstimulated cell extract, amounts of the complexes C1 and C3 formed with the sequences −53/−24 and −86/−50 as probes were mark-

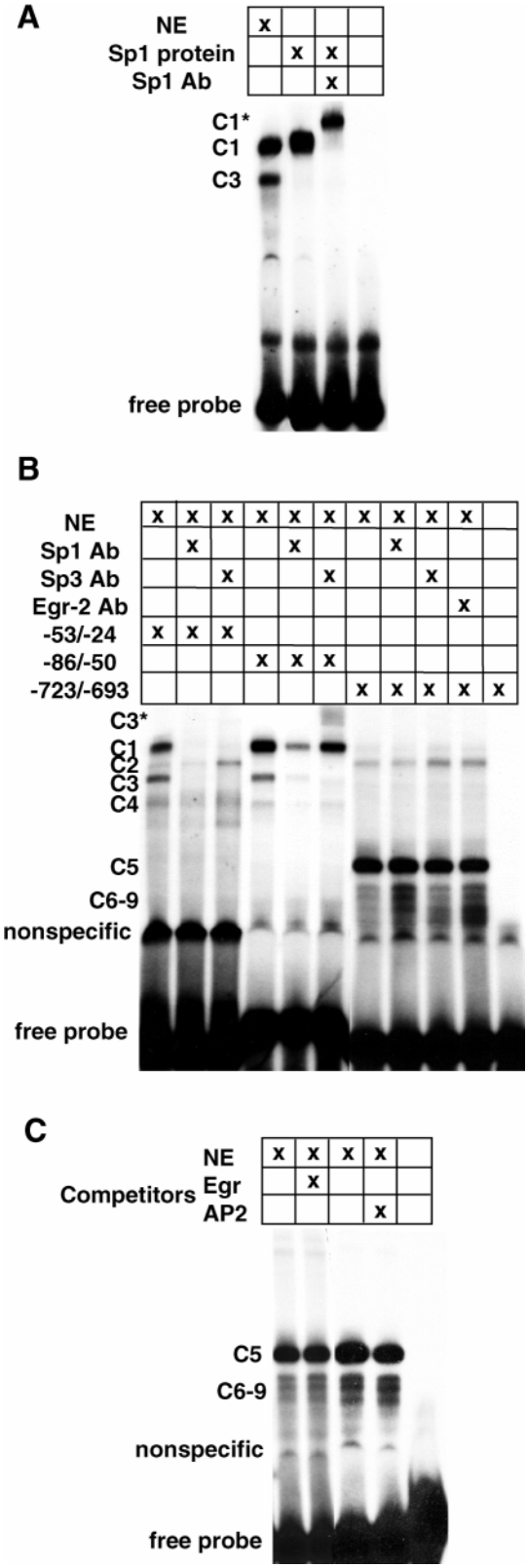

Figure 6. Identification of C1 and C3 binding activity as Sp1 and Sp3, respectively. **(A)** An electrophoretic mobility shift assay (EMSA) was performed with ^{32}P–labeled probes covering the promoter fragment −53/−24 and 8 μg nuclear extract prepared from MA-10 cells or 2 μl recombinant Sp1 proteins. Mouse monoclonal antibody against a peptide consisting of amino acids 520–538 of Sp1 was added to the EMSA reaction. **(B)** EMSA was performed with various promoter fragments (−723/−693, −53/−24, and −86/−50) and 8 μg nuclear extract. Mouse monoclonal antibody against Sp1 and rabbit polyclonal antibody against the carboxy terminus of Sp3 or of Egr-2 were added to the EMSA reaction as indicated above each lane. **(C)** EMSA was performed with the promoter fragment −723/−693. Competitions were carried out by adding 2500 fmol of unlabeled consensus motifs for the transcription factors Egr and AP2 to the EMSA reactions. Specific DNA/protein complexes are designated C1, C2, C3, C4, C5, and C6–9 on the left. * refers to the supershifted band.

edly increased when nuclear extract from 8-Br-cAMP–treated cells were used. 8-Br-cAMP was also able to increase the amount of the complexes C5 and C6–C9 bound to the sequence −723/−693 (Figure 5). Furthermore, 50 ng/mL hCG was also able to increase the binding of the complexes C1, C3, C5, and C6–C9 (data not shown). For all these experiments, nuclear extracts derived from MA-10 cells stimulated for 6 hours were assayed. If nuclear extract stimulated for 20 hours was used, no stimulatory effect by 8-Br-cAMP or hCG could be observed (data not shown).

Sp1 and Sp3 Bind to the Proximal Promoter Region

Previous studies on the human promoter (Finkenzeller et al, 1997; Gille et al, 1997; Yoshida et al, 1997; Milanini et al, 1998; Stoner et al, 2000; Zhang et al, 2000) showed that the transcription factors Sp1 and Sp3 are able to bind to the proximal promoter region, and we wanted to confirm and extend these original data for the murine promoter. To provide evidence that the upper complex C1 contains Sp1 and the lower complex C3 Sp3 protein, we compared the electrophoretic mobility of the upper complex with that formed by recombinant Sp1 protein. Figure 6A demonstrates that recombinant Sp1 protein incubated with the sequence −53/−24 gave a retarded complex comigrating exactly with the upper complex C1 identified with MA-10 cell extract. For confirmation that complexes C1 and C3 formed by the sequences −53/−24 and −86/−50 contain Sp1 and Sp3, we used antibodies (Ab) specifically against Sp1 and Sp3 protein. When added to the EMSA reaction, Sp1 Ab and Sp3 Ab caused the disappearance of C1 and C3 complexes with the use of the −53/−24 sequence. Similarly, both caused disappearance of C3 and decrease of C1 complexes with the use of the −86/−50 sequence (Figure 6B). Because of their size, the supershifts were partly not obvious because they were caught by the slots of the gel. The transcription factor Sp1 and its phosphorylated form have a molecular mass of 95 and 105 kd, respectively, whereas Sp3 has a mass of 90 kd. These findings suggest that the slower migrating complex C1 might contain Sp1 and the faster migrating complex C3 contains Sp3. Both proteins seem to consti-

tutively bind to the proximal region, and this binding appears to be up-regulated by hCG and 8-Br-cAMP.

The complex C2 migrating between the complexes C1 and C3 does not appear to contain Sp1 or Sp3 protein because no supershift with the antibodies against Sp1 and Sp3 could be seen. Further studies are required to identify this factor. Also the weak complex C4, in particular, has to be analyzed.

Binding Analyses of the Cytosine-Rich −723/−693 Sequence

The fragment −723/−693 contains 75% of cytosine residues covering 2 putative binding sites for Sp1 and a site for the transcription factor Egr overlapping with the 5′ Sp1 site. The cytosine residues form 2 stretches separated by the nucleotides GTAA. EMSA revealed a prominent C5 complex and 4 smaller and faster migrating complexes C6–C9. To characterize the nature of the factors giving rise to these complexes, we performed competition experiments with oligonucleotides containing the consensus motif for the Egr protein family or for the transcription factor AP2, whose molecular weight is about 50 kd. As shown in Figure 6, competition for binding of the complexes did not occur with an excess of the consensus motifs, indicating that complexes C5 and C6–C9 contain no Egr or AP2 protein. Also, antibodies specific for the Egr-2 (Figure 6B) and AP2 proteins (data not shown) did not react with the complexes C5 and C6–C9, confirming the results obtained from the competition experiments. Further investigations are needed to identify the factors binding to the cytosine-rich sequence, which appear to be up-regulated by hCG and 8-Br-cAMP.

Discussion

We report here that hCG induces the expression of VEGF transcripts in MA-10 Leydig tumor cells. The unstimulated cells express mostly 2.4-kb ($VEGF_{164}$) and a minor amount of 1.8-kb ($VEGF_{120}$) transcripts, in accordance with the reported lengths of the RNA transcripts of VEGF. The steady-state levels of these transcripts increase markedly following gonadotropic stimulation. $VEGF_{164}$ appears to be the most abundant isoform in both unstimulated and stimulated MA-10 cells. However, an additional transcript of 2.2 kb becomes detectable at 20 hours after stimulation, which might correspond to the $VEGF_{144/145}$ isoform. It is not clear why this transcript arises at a late time point after gonadotropic stimulation, although it is possible that because it is relatively less abundant, it becomes detectable only after a sufficient amount of the transcript has accumulated. Charnock-Jones (1993) detected the $VEGF_{144/145}$ isoform in human placental and uterine tissues with RT-PCR experiments. As the shorter forms of VEGF, $VEGF_{144/145}$ is also secreted from producing cells. However, in contrast to $VEGF_{120}$ and $VEGF_{164}$, $VEGF_{144/145}$ binds efficiently to the extracellular matrix. Compared with several cell types producing $VEGF_{120}$ and $VEGF_{164}$, the number of cell types that express $VEGF_{144/145}$ appears to be rather limited (Burchardt et al, 1999).

The notion that the stimulatory action of the gonadotropin in the induction of VEGF is mediated by cAMP as the signaling molecule is strengthened by the fact that 8-Br-cAMP could mimic the action of hCG. That cAMP is a physiologically important regulator of VEGF gene expression has been previously demonstrated in a variety of nontransformed cells like bovine retinal pericytes, endothelial cells (Takagi et al, 1996), and human endometrial stromal (Popovici et al, 1999) and granulosa (Christenson and Stouffer, 1997) cells. Apart from cAMP, steroids also have been reported to be able to regulate VEGF transcription. For example, progesterone and estrogens at micromolar concentrations significantly elevate VEGF in retinal pigment epithelial cells (Sone et al, 1996). In contrast, VEGF induction in endometrial cancer cells has been reported (Stoner at al, 2000) to be inhibited by estrogen. Androgens have been shown to regulate VEGF expression in prostatic cells (Levine et al, 1998). Because steroids play a role in the regulation of VEGF transcription, it is possible that progesterone produced by MA-10 cells following hCG/cAMP stimulation might lead to the up-regulation of VEGF expression, perhaps in parallel to the direct action of the cyclic nucleotide. Our results further indicate that VEGF mRNA was induced by progesterone itself in the absence of added gonadotrophin or cAMP in MA-10 cells, although these cells lack a classical nuclear progesterone receptor (Schwarzenbach et al, 2003). In addition to the known mechanism of action of steroid hormones at the level of gene expression via nuclear receptors, a large number of publications suggest that the steroids can also exert physiological effects by interacting with a novel class of extranuclear receptors responsible for rapid nongenomic effects (Valverde and Parker, 2002). The induction of VEGF might be mediated by such a novel membrane receptor for progesterone as proposed by El-Hefnawy et al (1998, 2000) for tumor Leydig cells. Also in MA-10 cells, we have recently reported an up-regulation of StAR gene expression in response to micromolar concentrations of progesterone, presumably acting via nonconventional receptors (Schwarzenbach et al, 2003). Moreover, such nonconventional extranuclear receptors for steroids have been proposed for several other types of cells (reviewed in Wehling, 1997; Revelli et al, 1998). In a number of studies with rat Leydig cells (Rossato et al, 1999), murine Leydig tumor cells (El-Hefnawy, 1998), a human ovarian cancer cell line (McDonnel and Murdoch, 2001), spermatozoa (Cheng et

al, 1998), and retinal pigment epithelial cells (Sone et al, 1996), micromolar concentrations of progesterone have been reported to effect rapid nongenomic cellular responses in cells, where progesterone presumably acts via nonconventional extranuclear receptors. Also in the case of MA-10 cells, high concentrations of progesterone were needed to induce the expression of VEGF. Although the concentration of 3×10^{-5} M progesterone for the induction of VEGF expression seems to be high, such levels can be observed in the testis in vivo after LH/hCG stimulation (El-Hefnawy et al, 1998), albeit only when supraphysiological doses of hCG are used. It can be noted that progesterone added to MA-10 cells is rapidly metabolized to other steroids (Rommerts et al, 2001), thus reducing its effective concentration in the medium, which might explain why micromolar concentrations of this steroid are required for an effect to be seen.

The regulation of steroidogenesis in Leydig cells can be affected by a number of endocrine/autocrine/paracrine factors (Rommerts et al, 2001), but the gonadotropin-mediated cAMP-dependent pathway can undoubtedly be considered as the primary mechanism involved. Therefore, for gonadotropin-induced VEGF expression, we have examined the role of cAMP for VEGF promoter regulation. Studies on the human promoter have shown that the proximal region is constitutively bound by the proteins Sp1, Sp3, and AP2 and that activation of the promoter with cytokines, steroids, and MAP kinase is mediated by these transcription factors (Forsythe et al, 1996; Finkenzeller et al, 1997; Gille et al, 1997; Yoshida et al, 1997; Milanini et al, 1998; Diaz et al, 2000; Stoner et al, 2000; Zhang et al, 2000). Furthermore, it has been demonstrated that LH/hCG induces VEGF transcription in granulosa cells (Christenson et al, 1997; Neulen et al, 1998) and in the rat ovary (Koos, 1995). Popovici et al (1999) reported that 8-Br-cAMP increased levels of VEGF in endometrial cells. In addition, we demonstrated that the promoter had a high hCG/cAMP inducible activity with 5′ deletions of the VEGF promoter in transient transfection assays. However, there is no canonical cAMP responsive element (CRE) in the VEGF promoter. We speculate that the cAMP/protein kinase A-dependent signaling transduction might stimulate de novo protein synthesis of a transcription factor involved in the VEGF transcription. Gel shift studies show that the regions −53/−24, −86/−50, and −109/−81 are essential for basal as well as hCG/cAMP-induced expression. The GC-rich region from −42 to −78 of the murine VEGF promoter has a homology of 70% to that from −49 to −90 of the human promoter and also contains 3 binding sites for the transcription factor Sp1 but not for AP2. In accordance with the analysis of the human VEGF promoter, supershift studies demonstrated that Sp1 and Sp3 constitutively bound to the mouse VEGF promoter in unstimulated MA-10 cells. Moreover, we could extend these data by the fact that hCG/cAMP could increase the level of binding of complexes C1/Sp1 and C3/Sp3 to the putative Sp1 sites in a transient fashion because within 6 hours, stimulation binding was markedly enlarged. Following 20-hour stimulation, however, no difference in binding could be detected between quiescent and stimulated cells. With the use of a reporter plasmid containing mutated Sp1 binding sites in a transfection assay, we also demonstrated that hCG/cAMP may stimulate the promoter activity via these sites. It seems that Sp1/Sp3 is involved in regulation of the VEGF promoter by hCG/cAMP. Whether hCG stimulates the increase in the binding affinity of Sp1 and Sp3, which might be regulated by phosphorylation, or in the amounts of both factors will require further investigations. Moreover, we cannot completely exclude that hCG/cAMP might activate other transcription factors binding to the Sp1 sites that could be components of the C1 and C3 complexes.

The transfection assays also showed a weaker basal activity of the reporter plasmid pLuc Apa/Start compared with the pLuc Mlu/Start in MA-10 cells. From these results, we conclude that the Apa/Mlu (−772/−449) region is involved in the basal repression. The cytosine-rich sequence −723/−693 could be responsible for the reduction in the murine VEGF promoter activity. It contains 2 potential recognition sites for the transcription factors Sp1/Sp3 and a binding site for Egr, which overlaps one of the Sp1 sites. Gel shift studies show several prominent complexes, C5 and C6–C9, which are also involved in the cellular response to cAMP. The Egr protein family could be attractive candidates for the factor C5 and for the repression. Huang et al (1997) reported that the function of Egr-1 might be to down-regulate certain mammalian gene promoters by competing with Sp1 for binding an overlapping binding motif. In the murine VEGF promoter, the complex C5, as well as the complexes C6–C9, could displace the binding of Sp1 and Sp3 to the cytosine-rich sequence and therefore cause a down-regulation of the mouse VEGF promoter. The members of the Egr protein family can probably be eliminated, partly because of their different molecular weights and by the lack of competition and supershift. AP2 has a molecular weight of 50 kd and could be contained in the complexes C5 or C6–C9. However, competition and supershift studies showed that a nuclear extract of the complexes C5 and C6–C9 contains neither Egr-2 nor AP2 protein. Further analyses are necessary to identify the complexes C5 and C6–C9 and to determine whether the cytosine-rich sequence is actually involved in the repression of VEGF gene.

In this study, we present novel information on the activation of the VEGF promoter, demonstrating that hCG/8-Br-cAMP and progesterone are able to stimulate VEGF gene expression. The gonadotropin, hCG, regulates ste-

roidogenesis and consequently increases the progesterone production in MA-10 cells. The direct effect of the cAMP-dependent signaling pathway on the induction of the VEGF gene appears to be a major mechanism in gonadotropin-stimulated VEGF production in MA-10 cells. The complexes C1/Sp1 and C3/Sp3 seem to mediate cAMP-induced transcriptional activation of the mouse VEGF gene. In this process, the distal cytosine-rich sequence −723/−693 contributes to the regulation of the murine VEGF promoter and is also activated by cAMP. The repression of the sequence −772/−449 seems to control the basal expression.

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