THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 270, No. 21, Issue of May 26, pp. 12737–12744, 1995
Printed in U.S.A.

Members of the Sp Transcription Factor Family Control Transcription from the Uteroglobin Promoter*

(Received for publication, January 30, 1995, and in revised form, March 1, 1995)

Jörg Dennig, Gustav Hagen, Miguel Beato, and Guntram Suske‡

From the Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Straße 2, D-35037 Marburg, Federal Republic of Germany

Previous analyses of the uteroglobin promoter revealed seven distinct regions, which contribute to its overall activity in epithelial cells from endometrium and lung. Most significantly, a mutation of the promoter sequence around 65 base pairs upstream of the transcriptional start site severely impairs promoter activity. The transcription factor acting through this sequence has not been identified yet. Here, we report that members of the Sp transcription factor family specifically recognize this non-classical GC box, in addition to another functional motif located 230 base pairs upstream of the transcriptional start site. We have characterized in detail the interaction of recombinant Sp3 with both motifs by DNase I footprinting and methylation protection using the wild-type uteroglobin promoter and various linker scanning mutants as templates. Electrophoretic mobility shift analyses show that Sp1 and Sp3 both bind with similar affinity to these elements. We demonstrate that the DNA-binding proteins in the endometrial cell line Ishikawa which recognize these motifs are also Sp1 and Sp3. Gene transfer experiments into *Drosophila* Schneider cells that do not contain endogenous Sp factors revealed that both DNA motifs respond to transiently expressed Sp1 and Sp3. Our results show thus that the level of transcription from the uteroglobin promoter is controlled by members of the Sp transcription factor family through unusual Sp binding sites.

The level and pattern of expression of a gene is determined mainly by the combinatorial action of transcription factors binding to distinct promoter and enhancer elements. In the last few years, we have started to define the DNA elements and transcription factors responsible for the expression of the rabbit uteroglobin gene in several ontogenetically unrelated epithelial tissues. DNase I protection experiments (1), promoter deletion, and linker scanning analyses (2) revealed that at least seven distinct promoter regions (I–VII; see Fig. 1) contribute to the activity of the promoter in epithelial cells. Region VII is defined by the presence of an estrogen-responsive element (3). Region VI located 230 bp[1] upstream of the transcription start site contains two so-called CACCC boxes found in several other promoters and enhancers, including the tyrosine aminotransferase gene (4, 5), the tryptophan oxygenase gene (6, 7), the β-globin gene (8, 9), and the SV40 enhancer (10–12). Region V spans at least 60 nucleotides between −208 and −148 and probably contains several DNA elements that have not been characterized further. Regions III and IV contain degenerated octamer motifs, which are bound by Oct I *in vitro*. The strongest phenotypes are displayed by mutations affecting regions I and II, suggesting that these two regions determine mainly the activity of the uteroglobin promoter. A linker scanning mutation in region II (LS−64/−72; see Fig. 1) reduces the promoter activity 20-fold (Ref. 2, Table I). However, the transcription factor acting through this sequence has not been identified yet. Region I contains a noncanonical TATA box motif (TACA box), which is bound specifically by two factors, the TATA core factor TCF and the TATA palindrome factor TPF (13). Both are different from the TATA box-binding protein TBP.

Using an oligonucleotide containing region VI of the uteroglobin promoter, we have cloned previously two transcription factors (Sp3 and Sp4), which specifically bind to this element (14). Both proteins are paralogs of the human transcription factor Sp1. Sp1, Sp3, and Sp4 have similar structural features. Most significantly, the DNA binding domains of all three proteins are highly conserved and recognize the GC box and a CACCC motif present in region VI of the uteroglobin promoter (14, 15). Contrary to these factors, Sp2, another Sp1-related protein, seems to have different DNA binding specificities (16). Functional analysis of Sp3 and Sp4 in direct comparison with Sp1 revealed that Sp4, like Sp1, is a transcriptional activator, whereas Sp3 represses Sp1-mediated transcription (15).

In the present study, we have analyzed in greater detail the role of the Sp transcription factor family for the activity of the uteroglobin promoter. DNase I and dimethyl sulfate protection experiments as well as electrophoretic mobility shift analyses using the wild-type uteroglobin promoter and appropriate linker scanning mutants as templates revealed that, in addition to the proximal CACCC box of region VI, the transcriptionally essential region II of the uteroglobin promoter is specifically recognized by Sp3 and Sp1. We show that the proteins in nuclear extracts of the endometrial cell line Ishikawa which bind this motif are also Sp1 and Sp3. In addition, we demonstrate that the element is responsive to these two transcription factors in gene transfer experiments. The strong correlation between DNA binding data *in vitro* and our functional results suggests that (a) member(s) of the Sp multigene family control the activity of the uteroglobin promoter through binding to two non-GC box Sp binding sites.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Vectors for Sp1 and Sp3 expression in *Drosophila* Schneider cells (pPacSp1 and pPacSp3) have been described previously (15, 17). The β-galactosidase expression plasmid p97b was constructed by P. DiNocera and generously supplied by L. Lania. The plasmids $(EII)_2$-CAT, $(EII^*)_2$-CAT, and $(EVI)_2$-CAT are derivatives of BCAT-2 (17) and were generated as follows. First, we constructed the reporter plasmid BCAT-0, which lacks the two Sp1 binding sites of the

* This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Kempkes Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-6421-286697; Fax: 49-6421-285398.

[1] The abbreviations used are: bp, base pair(s); EMSA, electrophoretic mobility shift analysis; CAT, chloramphenicol acetyltransferase; HTLV, human T-cell lymphotrophic virus.

HTLV promoter but still contains the E1B TATA box fused to the CAT gene. For this, BCAT-2 was cut with *Hin*dIII and *Xba*I, filled in with Klenow polymerase, and religated. The oligonucleotides OEII, OEII*, and OEVI (Fig. 4*A*) were concatemerized via their *Hin*dIII and *Sal*I ends and cut with *Hin*dIII. The resulting dimeric oligonucleotides were then cloned into the *Xho*I site of BCAT-0, resulting in $(EII)_2$-CAT, $(EII^*)_2$-CAT, and $(EVI)_2$-CAT. The construction of all other plasmids, including the wild-type uteroglobin promoter construct pUG(−395)-CATSV as well as the appropriate linker scanning mutants fused to the CAT gene and the bacterial expression plasmids for Sp1 and Sp3, has been described (2, 14, 15).

Bacterial Extracts, Nuclear Extracts, and Electrophoretic Mobility Shift Analysis (EMSA)—Bacterial extracts containing recombinant Sp1 and Sp3 have been prepared according to Kadonaga *et al.*. (18). Nuclear extracts from Ishikawa cells were prepared from one 10-cm plate according to Andrews and Faller (19). Gel retardation assays were essentially performed as described (20, 21) with oligonucleotides containing the EII, EII*, and EVI motifs as binding sites, respectively. Annealing, labeling, and purification of oligonucleotides has been performed as described by Klug *et al.* (13). The sequences of the oligonucleotides are shown in Fig. 4*A*. For supershift assays, 1 μl of the appropriate antiserum was added to the binding reaction 10 min prior to the loading of the gel.

DNase I Protection Experiments—Fragments used for DNase I protection experiments were isolated from pUG(−395)CATSV and the appropriate linker scanning derivatives (2). The plasmids were cut with either *Xba*I (for the footprint on element II) or *Xho*I (for the footprint on element VI) and end-labeled. The subsequent restriction digest with *Xho*I and *Xba*I, respectively, released a 425-bp DNA fragment containing the uteroglobin promoter sequence from −395 to +14, which was purified further by polyacrylamide gel electrophoresis. The probe (30,000 cpm) was mixed with 10 μg of bacterial extract in a buffer containing 12.5 mM HEPES, pH 7.5, 6.25 mM $MgCl_2$, 9% glycerol, 5 μM $ZnSO_4$, 50 mM KCl, and 40 μg/ml poly(dI-dC), and incubated for 30 min at 4 °C. Then, 2.5 μl of DNase I (5 ng or 10 ng) in 50 mM $MgCl_2$ and 10 mM $CaCl_2$ was added and incubated for 30 s at 20 °C. The reaction was stopped by addition of 100 μl 100 mM Tris-HCl, pH 7.6, 20 mM EDTA, 0.5% SDS, 100 mM NaCl, 100 μg/ml proteinase K, and 20 μg of glycogen. After phenol extraction and ethanol precipitation, DNA fragments were resolved on 6.5% denaturing polyacrylamide gels. Sequencing ladders were prepared according to standard procedures (22).

Dimethyl Sulfate Protection Experiments—The following templates were used for methylation protection experiments. Element II, upper and lower strand, was a 150-bp *Xho*I-*Ssp*I fragment obtained from pUG(−395)CATSV and LS−68/−75, respectively (2). Element VI, upper strand, was a 250-bp *Xba*I-*Bgl*II fragment obtained from LS−142/−146 (2). Element VI, lower strand, was a 260-bp *Xba*I-*Ssp*I fragment obtained from pUG(−395)CATSV.

Bacterial extracts containing recombinant Sp3 (14) were preincubated for 10 min at 0 °C (total volume of 20 μl) in a buffer containing 12.5 mM HEPES, pH 7.5, 6.25 mM $MgCl_2$, 9% glycerol, 5 μM $ZnSO_4$, 50 mM KCl, 50 ng/μl poly(dI-dC), and 50 ng/μl bovine serum albumin. The appropriate end-labeled fragments (200,000 cpm) were added and incubated for 10 min. The methylation reaction was started by adding 2 μl of a 2% dimethyl sulfate solution. After incubation for 3 min at 20 °C, the reaction was stopped by adding 2 μl of 60 mM β-mercaptoethanol. Free and bound DNA were separated on 4% polyacrylamide gels and blotted onto DE81 ion exchange paper. After elution (2 h at 65 °C in 200 μl 10 mM Tris/Cl, pH 8.0, 1 mM EDTA, 1.5 M NaCl), the DNA fragments were ethanol-precipitated and treated with piperidine essentially as described (23). Finally, the reactions were analyzed on 7% denaturing sequencing gels along with sequencing reactions (G+A and C+T) obtained from the same DNA fragments.

Cell Culture and Transfections—Ishikawa cells were grown as monolayers in minimum essential medium supplemented with 10% fetal calf serum (routinely stripped from hormones by charcoal treatment), L-glutamate, and antibiotics. For the hormone response experiments, the cells were transferred to Dulbecco's minimal essential medium without phenol red (Life Technologies, Inc.) and the DEAE-dextran method was used for transfection (24). Every plate (9 cm) received 8 μg of reporter plasmid, 1 μg of RSVβgal, and 200 ng of human estrogen receptor expression plasmid HEGO (25). Hormonal response was tested 20 h post-transfection by addition of the synthetic estrogen diethylstilbestrol (10^{-8} M).

Schneider cells (26) were maintained in Schneider medium supplemented with 10% fetal calf serum at 25 °C. One day prior to transfection cells were plated onto 6-cm plastic dishes at a density of 4.5×10^6 cells/plate. Cells were transfected by the calcium phosphate method described by DiNocera and Dawid (27). Every plate received 14 μg of DNA including 4 μg of the β-galactosidase expression plasmid p97b as internal reference. Variable amounts of expression plasmids were compensated with the plasmid pPac. 24 h after addition of DNA, the medium was changed, and 24 h later the cells were washed twice with phosphate-buffered saline and harvested.

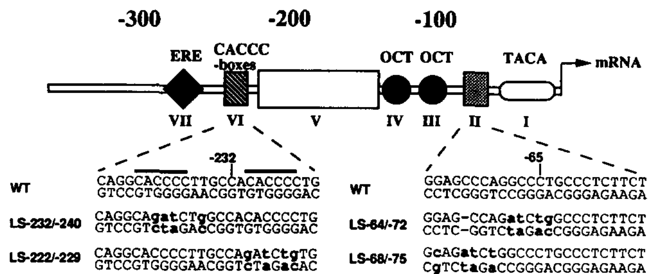

FIG. 1. **The rabbit uteroglobin promoter structure and nucleotide sequences of elements II and VI in the wild-type promoter and the corresponding linker scanning promoter mutants LS−64/−72, LS−68/−75, LS−232/−240, and LS−222/−229.** Seven regions (*I–VII*) mapped by DNase I footprinting (1), deletion, and linker scanning analyses (2) contribute to the overall activity of the promoter. The two CACCC sequences present in region VI are depicted by *solid lines*. *TACA*, TACA box (noncanonical TATA element; Ref. 13); *OCT*, octamer factor binding sites; *ERE*, estrogen-responsive element.

CAT and β-Galactosidase Assays—For CAT assays, cells were suspended in 250 mM Tris/Cl, pH 7.8, and lysed by three rounds of freezing and thawing. CAT assays were carried out according to Gorman *et al.*, (28). Protein concentrations in the CAT assay and reaction times were adjusted to bring the extent of CAT conversion into a range that is linear with the CAT enzyme concentration. CAT conversion was assayed by thin layer chromatography and quantitation of the acetylated and non-acetylated forms of [^{14}C]chloramphenicol performed with an automated Imaging Scanner (United Technologies Packard). The ratio of acetylated to total chloramphenicol is displayed as percentage of conversion. The β-galactosidase assays were performed according to Hall *et al.*. (29). The values were used to normalize the CAT conversion data for plate to plate variations in transfection efficiency.

RESULTS

The Uteroglobin Promoter Does Contain Two Binding Sites for Members of the Sp Transcription Factor Family—Region VI of the uteroglobin promoter contains two adjacent CACCC motifs (Fig. 1). Previously, we have shown that an oligonucleotide containing the proximal CACCC box is bound specifically by Sp1, Sp3, and Sp4 (14, 15). To see whether the distal CACCC motif is also recognized by members of the Sp family, we performed DNase I protection experiments with recombinant Sp3 using the wild-type uteroglobin promoter (*WT*) and the appropriate linker scanning mutants LS−232/−240 and LS−222/−229 (Fig. 1) as templates. In LS−232/−240 and LS−222/−229 either the distal or the proximal CACCC sequence is replaced by a *Bgl*II linker sequence (Fig. 1). A footprint over element VI was obtained with the wild-type uteroglobin promoter (*WT* in Fig. 2*A*; compare *lane 8* with *lanes 9* and *10*) and the linker scanning mutant LS−232/−240 containing the mutated distal CACCC motif (Fig. 2*A*, compare *lane 5* with *lanes 6* and *7*). With the promoter variant in which the proximal CACCC motif is mutated (LS−222/−229), no protection from DNase I cleavage is visible (Fig. 2*A*, compare *lane 11* with *lanes 12* and *13*). These results show that region VI of the uteroglobin promoter contains only a single Sp binding site and that only the proximal CACCC box is part of the Sp recognition sequence.

In the upper part of the gel shown in Fig. 2*A* another protection from DNase I cleavage (around −65) is visible. This region was protected also when footprinting experiments were performed with nuclear extracts from Ishikawa and HeLa cells (1). To further delineate the sequence protected by Sp3, we labeled the uteroglobin promoter close to the transcriptional

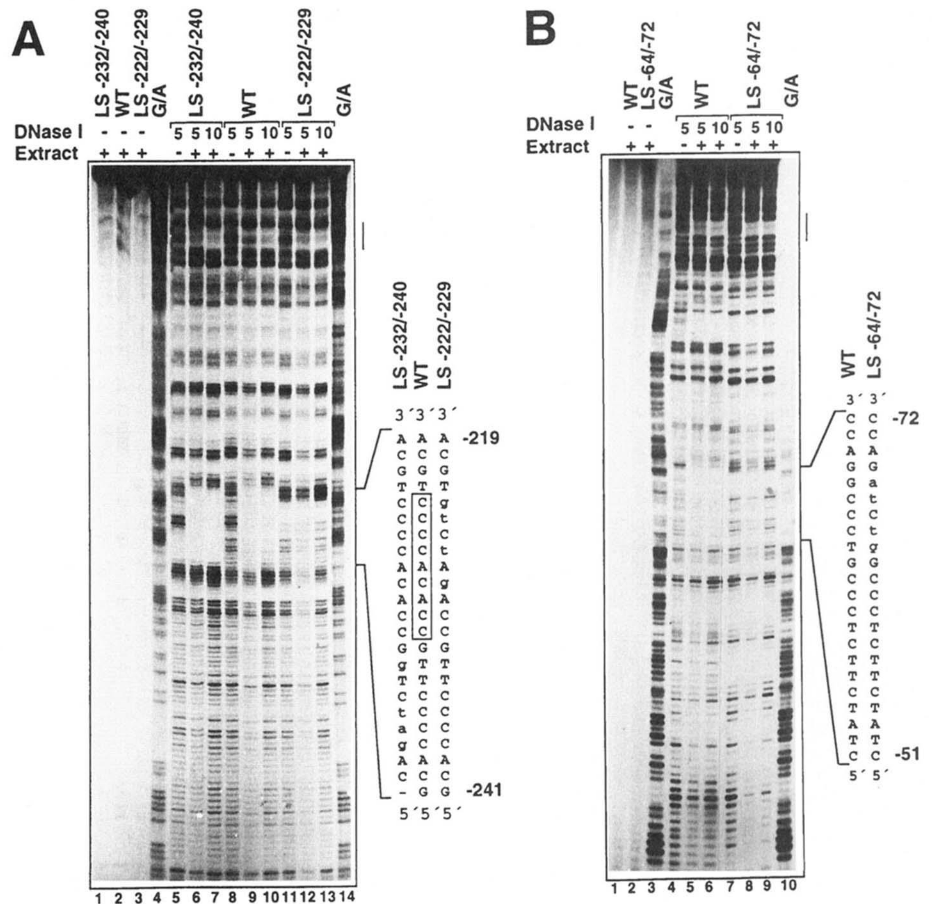

FIG. 2. **DNase I protection of the uteroglobin promoter by recombinant Sp3.** Reactions contained 10 μg of bacterial protein with (+) or without (−) Sp3 and no (−), 5, or 10 ng DNase I, respectively, as indicated. *Lanes G/A* contain Maxam-Gilbert sequencing ladders prepared from the wild-type uteroglobin promoter fragment. *A*, DNase I protection experiments using the wild-type uteroglobin promoter (*lanes 2* and *8-10*) and the linker scanning promoter mutants LS−232/−240 (*lanes 1* and *5-7*) and LS−222/−229 (*lanes 3* and *11-13*) labeled approximately 400 bp upstream of the transcriptional start site. The sequences on the *right* show the Sp3 binding region of the wild-type promoter (*WT*) and the corresponding linker scanning mutants LS−232/−240 and LS−222/−229. The mutations present in the linker scanning mutants are indicated by *lowercase letters*. The proximal CACCC motif region is *boxed*. The *bar* (*top right*) indicates the second protected region around −65. *B*, DNase I protection experiment with the wild-type uteroglobin promoter (*WT*, *lanes 1* and *4–6*) and the linker scanning promoter mutant LS−64/−72 (*lanes 2* and *7-9*) labeled close to the 3′-end of the promoter. The sequences on the *right* show the Sp3 binding region of the wild-type promoter (*WT*) and the corresponding sequence of the linker scanning mutant LS−64/−72. The mutations in the linker scanning mutant are indicated by *lowercase letters*.

start point and performed footprinting experiments with the wild-type promoter and a linker scanning mutant (LS−64/−72, see Fig. 1), which reduced the promoter activity almost to background levels (at least 20-fold) in gene transfer experiments (2). A footprint over element II was obtained with the wild-type uteroglobin promoter but not with the LS−64/−72 mutant (Fig. 2*B*, compare *lane 4* with *lanes 5* and *6* and *lane 7* with *lanes 8* and *9*). This result suggests that the absolute requirement of region II for the activity of the uteroglobin promoter might be due to an unusual Sp binding site.

Guanine Contacts of Sp3 with the Non-classical GC Box of Element II and the CACCC Box Binding Site—In order to obtain a more detailed picture of the interaction between Sp3 and the unusual binding site of element II, we performed dimethyl sulfate methylation protection experiments (Fig. 3). Sp3-DNA complexes were allowed to form at 4 °C and subjected subsequently to partial methylation by dimethyl sulfate. Bound and free DNA were separated through native polyacrylamide gels and analyzed on denaturing sequencing gels.

The results obtained with both strands of element II are shown in Fig. 3 (*A* and *B*). In the presence of recombinant Sp3, four guanine residues (at positions −58, −60, −61, and −62) of the lower strand (compare *lanes 3* and *4* in Fig. 3*A*) and two residues of the upper strand (positions −63 and −68; compare *lanes 9* and *10* in Fig. 3*B*) of element II showed specific and almost complete protection from methylation. In addition, three guanine residues of the lower (positions −65 to −67) and one of the upper (position −69) strand are partially protected. In the linker scanning mutant LS−68/−75, guanine residues at positions −72, −73 (lower strand), and −76 (upper strand) are substituted by adenine, thymidine, or cytidine residues. A promoter fragment containing these mutations showed essentially the same protection pattern and the same extent of protection from methylation as the wild-type promoter sequence (Fig. 3, *A* and *B*, compare *lane 4* with *lane 8* and *lane 10* with *lane 16*), indicating that no interactions occur between Sp3 and the guanine-rich sequence immediately upstream of the protected area. A promoter fragment that contains the linker scanning mutation of LS−64/−72 (Fig. 1) did not bind to Sp3; therefore, no protection from methylation could be observed (data not shown). In LS−64/−72, the strong protected G residue at position −68 of the upper strand and the two partially protected G residues at positions −65 and −67 of the lower strand are replaced by thymidine or adenine residues, respectively (see Fig. 1), indicating that at least one of these three guanine residues is required for the interaction of Sp3 with element II.

To show also how Sp factors interact with the CACCC box, methylation protection experiments were performed with promoter fragments containing element VI. Seven guanine residues of the lower strand (positions −223 to −226, −228, −230, and −231) are protected completely from methylation by Sp3 (Fig. 3*C*, compare *lane 19* with *lane 20*). The guanine residue at position −232 of the upper strand is only partially protected. No guanine residue was hypermethylated.

In the linker scanning mutant LS−222/−229, the four protected guanine residues at positions −223, −224, −226, and −228 are replaced by non-guanine bases, which explains why Sp3 could not confer DNase I protection to this promoter variant (see Fig. 2). Moreover, the exchange of important contact sites for Sp factors in the linker scanning mutants LS−64/−72 and LS−222/−229 could also explain why promoter activity is reduced in these two promoter variants.

Electrophoretic Mobility Shift Analyses (EMSA) with Recombinant Sp1 and Sp3—To examine further the interaction between Sp factors and element II of the uteroglobin promoter and to determine the relative binding affinity of this site, we

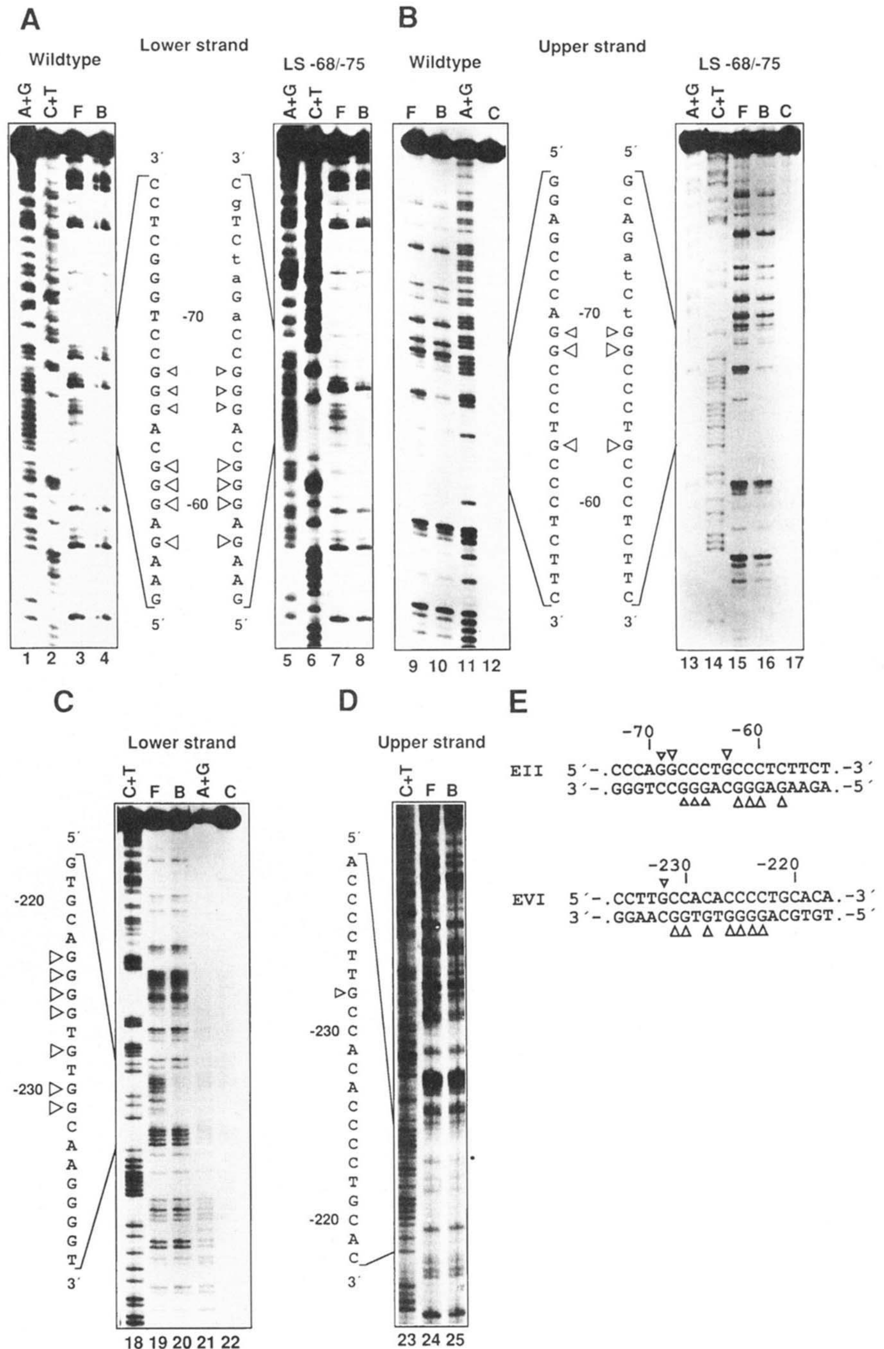

FIG. 3. **Methylation protection pattern of the uteroglobin promoter obtained with recombinant Sp3.** Appropriate end-labeled uteroglobin promoter fragments were incubated with recombinant Sp3. After methylation, bound DNA (*lanes B*) was separated from free DNA (*lanes F*) by native polyacrylamide electrophoresis, eluted, and applied to denaturing polyacrylamide gels. *Lanes C* contain non-methylated free DNA, *lanes A+G* and *C+T* Maxam-Gilbert sequencing ladders. Specific guanine residues protected from dimethyl sulfate (*DMS*) methylation by Sp3 are depicted by *large* (completely protected residues) and *small* (partially protected residues) *triangles*. *A* and *B*, DMS methylation protection in region II (lower (*A*) and upper (*B*) strand) obtained with the wild-type promoter (*lanes 1–4* and *9–12*) and the linker scanning mutant LS−68/−75 (*lanes 5–8* and *13–17*). *C* and *D*, DMS methylation protection in region VI (*lanes 18–25*; lower (*C*) and upper (*D*) strand). *E*, summary of the protection pattern of guanine residues in elements II and VI.

performed EMSA experiments with bacterially expressed Sp1 and Sp3 and oligonucleotides containing the wild-type sequence of element II (OEII oligonucleotide) and the sequence of the corresponding linker scanning mutant LS−64/−72 (OEII* oligonucleotide). As reference probes, we used oligonucleotides containing a classical GC motif (OGC oligonucleotide) or the proximal CACCC box of element VI (OEVI oligonucleotide) (Fig. 4*A*). The element II-containing oligonucleotide was labeled and incubated with recombinant Sp1 and Sp3 fragments. A predominant complex was generated with the 263 C-terminal amino acids encoding Sp3 fragment, whereas full-length Sp1 (14) produced two specific complexes (Fig. 4*B*). The two Sp1-containing complexes have been observed previously. Both are very likely degradation products of the complete Sp1 protein (14). All three complexes were competed equally with increasing amounts of unlabeled oligonucleotides containing the EII, EVI, and GC motif showing that Sp1 and Sp3 do have the same binding specificity for all three motifs. In contrast, the EII* oligonucleotide, which contains the corresponding sequence of the linker scanning mutant LS−64/−72, did not at all compete the labeled oligonucleotide (Fig. 4*B*, compare *lane 3* with *lane 15*). Obviously, the GC box-containing oligonucleotide (OGC) competed significantly better than the EVI and the EII oligonucleotides. Quantitative evaluations of the band shift data show also that the affinity of the element II-containing oligonucleotide for Sp1 and Sp3 is approximately 2-fold lower compared with the CACCC box-containing oligonucleotide.

Sp1 and Sp3 Are the Nuclear Proteins Binding to Element II of the Uteroglobin Promoter—Next, we asked whether the nuclear proteins binding to element II are indeed Sp1 and Sp3 or whether other additional transcription factors are able to recognize this element. To address this question, we performed EMSA experiments with crude nuclear extracts from Ishikawa cells. As labeled DNA probe, we used the EII oligonucleotide. In

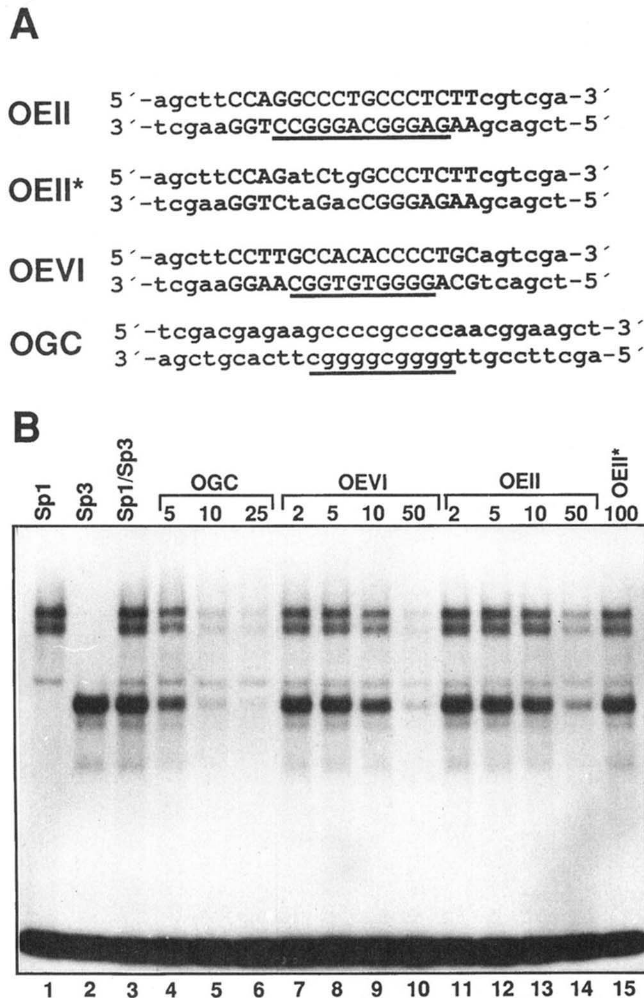

FIG. 4. **Electrophoretic mobility shift analysis with uteroglobin promoter elements II and recombinant Sp1 and Sp3.** *A*, oligonucleotides used for the EMSA of this and the following figure. Sequences present in the wild-type uteroglobin promoter are written in *uppercase letters*. The Sp binding sites in the oligonucleotides OEII, OEVI, and OGC are *underlined*. *B*, the oligonucleotide OEII shown in *panel A* was end-labeled and incubated with bacterially expressed Sp1 (*lane 1*), Sp3 (*lane 2*), or a mixture of both proteins (Sp1/Sp3; *lanes 3–15*). Competitions were performed with *x*-fold molar excess of cold oligonucleotides OGC, OEVI, OEII, and OEII* as indicated.

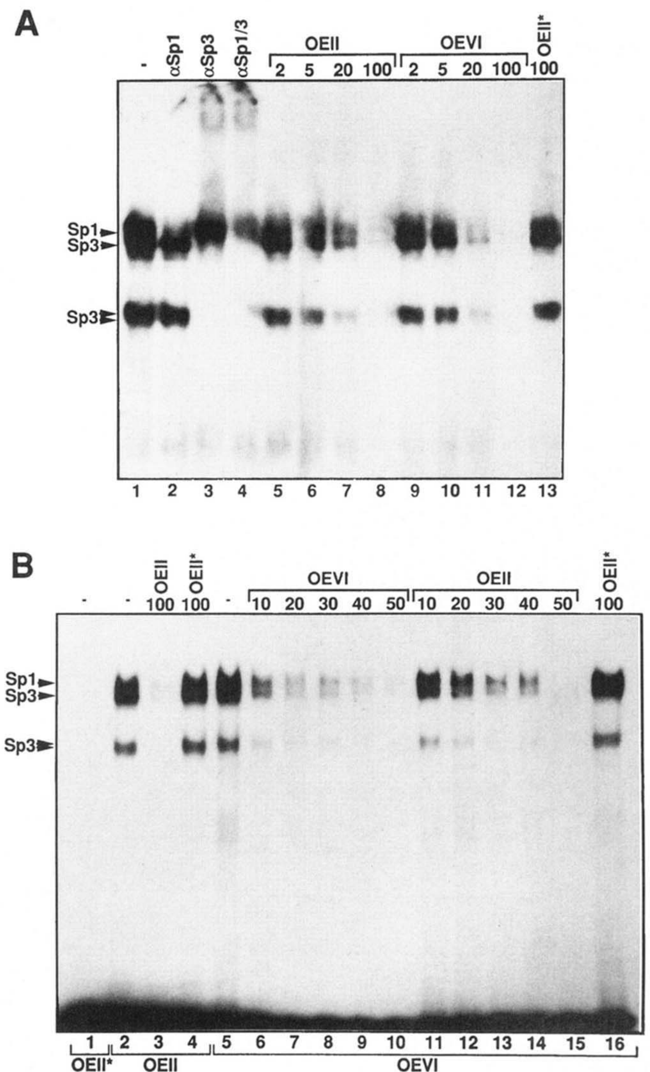

FIG. 5. **Nuclear proteins binding to uteroglobin promoter elements II and VI are Sp1 and Sp3.** *A*, sequences of the oligonucleotides OEII, OEII*, and OEVI are shown in Fig. 4*A*. The oligonucleotide OEII was labeled and incubated with nuclear extracts from Ishikawa cells. One microliter of protein A-Sepharose-purified sera against Sp1 (*αSp1*), Sp3 (*αSp3*), or a mixture of both (*αSp1/3*) were included in the binding reactions as indicated at the *top*. Specific complexes for Sp1 and Sp3 are indicated on the *left*. In the competition experiments, an *x*-fold molar excess of oligonucleotides OEII (*lanes 5–8*), OEVI (*lanes 9–12*), and OEII* (*lane 13*) was included in the binding reactions as indicated. *B*, the oligonucleotides OEII* (*lane 1*), OEII (*lanes 2–4*), and OEVI (*lanes 5–16*) were end-labeled and used for EMSA experiments with nuclear extracts from Ishikawa cells. Competitions with cold oligonucleotides OEII (*lanes 3* and *11–15*) and OEVI (*lanes 6–10*) were performed with 10–50-fold molar excess, respectively. In the competition experiment with OEII* (*lane 16*), a 100-fold molar excess of the oligonucleotide was used. Complexes specific for Sp1 and Sp3 are indicated on the *left*.

high resolution gels, two slow migrating and two fast migrating complexes were observed (Fig. 5*A*). Essentially, the same migration pattern is observed with the element VI-containing oligonucleotide (Fig. 5*B*). All four bands were specifically competed with oligonucleotides containing elements II or VI, but not with the EII* oligonucleotide (Fig. 5, *A* and *B*). In the presence of a Sp1 antiserum, the slowest migrating complex disappeared. When the anti-Sp3 serum was present in the binding reaction, the three other complexes were shifted but the slowest migration complex was unaltered. If antisera against Sp1 and Sp3 were present in the binding reaction, all four bands disappeared. This experiment demonstrates that other factors distinct from Sp1 and Sp3, which may bind to element II (and VI), are not present in the nuclear extract from Ishikawa cells, suggesting that Sp1 and/or Sp3 are responsible for the transcriptional activity of both promoter elements.

Element II of the Uteroglobin Promoter Mediates Activation by Sp1 and Repression by Sp3 in SL2 Cells—To further substantiate the conclusion that members of the Sp family control the level of transcription from the uteroglobin promoter, we asked whether Sp1 and Sp3 are able to act as transcriptional regulators through binding to element II. We performed gene transfer experiments into the *Drosophila melanogaster* Schneider cell line (SL2 cells) that lacks endogenous Sp factors (30, 15). The CAT reporter constructs designed for these experiments contain the oligonucleotides OEII and OEII* (Fig. 4*A*) as dimers upstream of the E1B TATA box (Fig. 6*A*). For direct comparison, we also used $(EVI)_2$-CAT and BCAT-2 as reporter plasmids. In $(EVI)_2$-CAT two copies of the proximal CACCC motif-containing oligonucleotide (OEVI in Fig. 4*A*) is fused to the E1B TATA box. BCAT-2 contains two Sp1 binding sites from the HTLV-III promoter in a tandem array fused to the E1B TATA box. This construct has been used previously to

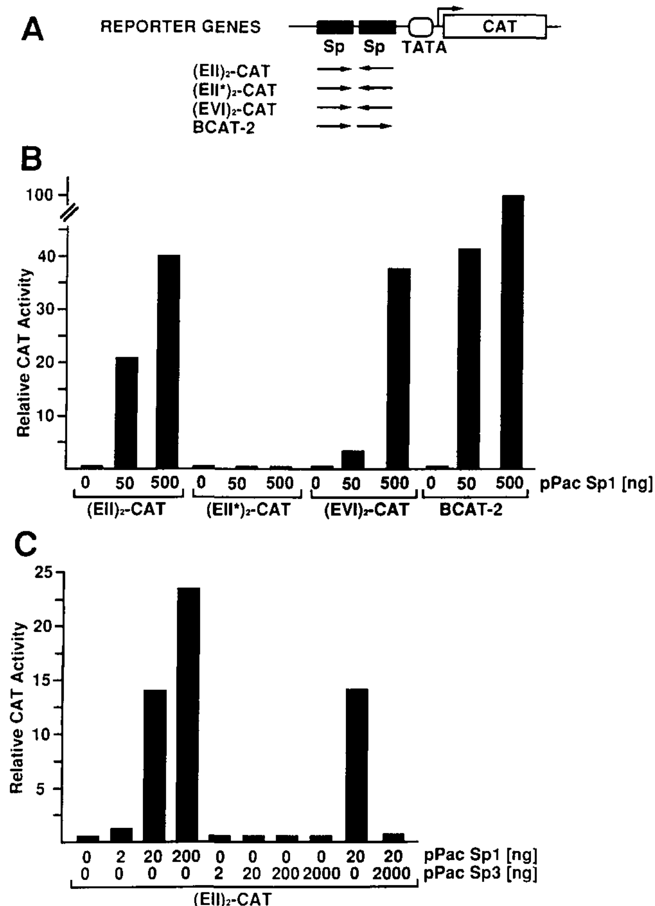

FIG. 6. **Functional analysis of promoter elements II and VI in SL2 cells.** *A*, schematic representation of the reporter plasmids $(EII)_2$-CAT, $(EII^*)_2$-CAT, $(EVI)_2$-CAT, and BCAT-2. *B*, 8 μg of the reporter plasmids $(EII)_2$-CAT, $(EVI)_2$-CAT, $(EII^*)_2$-CAT, and BCAT-2 were transfected in SL2 cells along with 50 or 500 ng of pPacSp1 as indicated. The cells were subsequently lysed and CAT activities determined as described under "Experimental Procedures." The values represent the average of two independent determinations. *C*, Sp3 represses Sp1-mediated transactivation through uteroglobin promoter element II. Eight micrograms of $(EII)_2$-CAT was transfected along with various amounts of pPacSp1 (2, 20, and 200 ng) and pPacSp3 (2, 20, 200, and 2000 ng) as indicated. The cells were subsequently lysed and assayed for CAT activity.

characterize transcriptional properties of Sp1 and Sp3 (15, 17).

A constant amount of the reporter constructs $(EII)_2$-CAT, $(EII^*)_2$-CAT, $(EVI)_2$-CAT, and BCAT-2 was transfected into SL2 cells along with 50 or 500 ng of the expression plasmid pPacSp1 (30). The three reporter constructs $(EII)_2$-CAT, $(EVI)_2$-CAT, and BCAT-2 were activated by Sp1 (Fig. 6*B*). In contrast, the activity of the construct containing the linker scanning mutation of LS−64/−72 ($(EII^*)_2$-CAT) was not at all influenced by Sp1. Sp3 did not activate any of the promoter constructs (Fig. 6*C* and data not shown), although Sp3 is efficiently expressed in transfected SL2 cells (15). However, when we cotransfected $(EII)_2$-CAT with 20 ng of Sp1 expression plasmid and an excess of the Sp3 expression plasmid, Sp1-mediated activation was strongly repressed by Sp3 (Fig. 6*C*). Similar results were obtained previously with BCAT-2. Sp3 repressed Sp1-mediated activation of BCAT-2 due to the competition of both factors for their common binding sites (15). Thus, our results obtained with the element II-containing reporter construct demonstrate that Sp1 and Sp3 also bind to element II *in vivo*, thereby acting as transcriptional regulators.

Elements II and VI of the Uteroglobin Promoter Mediate Basal Activity but Not Estrogen Inducibility—The estrogen-responsive element of the uteroglobin gene is located immediately upstream of the CACCC motifs of region VI (Fig. 1). This is reminiscent of the situation found in the tyrosine aminotransferase gene (4) and the tryptophan oxygenase gene (6) where the CACCC boxes are also close to hormone-responsive elements. Moreover, the hormone inducibility of the tryptophan oxygenase gene is dependent on the integrity of the CACCC box (6). Consequently, we raised the question whether the enhancement of the uteroglobin promoter activity by estrogens (3) is also dependent on the integrity of the adjacent CACCC sequences. Since binding of Sp factors is common to element VI and element II, we also tested the linker scanning mutant LS−64/−72 that destroys the latter element (Fig. 1). Constructs containing the CAT gene driven by the uteroglobin wild-type promoter or the promoter linker scanning mutants LS−232/−240, LS−222/−229, and LS−64/−72 were transfected along with an expression vector for the human estrogen receptor into Ishikawa cells. The result of these experiments is summarized in Table I. The mutations in LS−222/−229 and LS−64/−72 but not those in LS−232/−240 reduced significantly the basal activity of the uteroglobin promoter, but the inducibility by diethylstilbestrol, a synthetic estrogen, is in no case affected. The integrity of element II and the proximal CACCC box of region VI is thus necessary to retain the wild-type promoter activity. However, mutations in these regions do not interfere with the inducibility of the uteroglobin promoter by estrogens.

TABLE I
Effects of uteroglobin promoter linker scanning mutations in the absence and presence of diethylstilbestrol (DES)

The values represent mean values of two independent transfection experiments and are expressed relative to the wild type, which have been given the arbitrary value of 100. The values were normalized for β-galactosidase activity for variations in transfection efficiency.

	Relative CAT activity	
	−DES	+DES
Wild-type	100	370
LS−232/−240	80	314
LS−222/−229	30	135
LS−64/−72	5	19

DISCUSSION

The Proximal but Not the Distal CACCC Motif of Uteroglobin Promoter Element VI Interacts Specifically with Sp Factors—Our footprinting analyses revealed that the proximal but not the distal CACCC box of the uteroglobin promoter region VI is bound by Sp factors. This finding is corroborated by the methylation protection experiments, which showed that additional guanine residues flanking the GGGTG core sequence are protected from methylation, which are not present in the distal CACCC sequence. These *in vitro* DNA binding data correlate with our functional results. Substitution of the proximal CACCC box reduced the promoter activity more than 3-fold, whereas a mutation of the distal CACCC box did not impair promoter activity significantly. Thus, the CACCC core sequence alone is not sufficient to indicate a functional Sp binding site.

Close inspection of CACCC boxes of various promoters and enhancers revealed that six of the seven protected guanine residues are present in the CACCC boxes of the tryptophan oxygenase gene, the tyrosine aminotransferase gene, and the β-globin gene, as well as in the SV40 enhancer. It is known that the SV40 and the β-globin CACCC sequences are able to bind Sp1 (11, 31). Thus, it is very likely that the CACCC boxes found in the tyrosine aminotransferase gene and the tryptophan oxygenase gene are recognized also by members of the Sp factor

family. The CACCC motifs present in the tyrosine aminotransferase gene and in the tryptophan oxygenase gene are in close proximity to glucocorticoid-responsive elements. The glucocorticoid-responsive element and the CACCC box of the tyrosine aminotransferase enhancer act synergistically. Consequently, the glucocorticoid induction of the tryptophan oxygenase gene is abolished when the CACCC box is deleted (6). For these systems, it remains to be clarified which member of the Sp transcription family binding to the CACCC box mediates the synergistic effect with the glucocorticoid receptor.

Immediately upstream of the functional uteroglobin CACCC box lies an estrogen-responsive element (3). Our gene transfer experiments demonstrated that the replacement of the CACCC box does not influence the inducibility of the uteroglobin promoter by estrogens, indicating that no synergism occurs between the estrogen-responsive element and the CACCC box. Thus, synergism of a CACCC box and an adjacent hormone-responsive element appears to be not a general feature of this combination of promoter elements.

Element II of the Uteroglobin Promoter Contains an Unusual Sp Binding Site—A second Sp binding site in the uteroglobin promoter has been detected in element II around −64 relative to the transcriptional start site. Compared with the CACCC box binding site or a classical GC box, the affinity of the Sp recognition sequence in element II is weaker. Nevertheless, this site appears to be more important for the uteroglobin promoter strength than the CACCC box-containing binding site. Very likely, its location adjacent to the TATA box is optimal for the activation properties of Sp1.

Our methylation protection experiments with recombinant Sp3 and Sp1 (data not shown) revealed protection of guanines on both strands of element II. This finding is consistent with the observation that Sp1 does not bind efficiently to single-stranded DNA (32). Contacts with guanine residues on both strands have been described also for the Sp1 binding sites present in the HTLV-III retrovirus (32) and in a parvovirus promoter (33). These binding sites also differ from the classical GC boxes found for instance in the SV40 promoter region (34). With respect to the flexibility for deviations from the consensus Sp binding site (35), the previous work of Thiesen and Bach (36) is illuminating. Using a target detection assay, they determined putative DNA binding sites for Sp1 and rescued 11 strong binding sites. One of the selected binding sites (S16: GGGGCAGGGC) differs only in one position compared with the core sequence of element II (AGGGCAGGGC). Thus, our results further demonstrate the high variability of the recognition sequence for the members of the Sp transcription factor family. The variability of Sp binding sites may explain why promoters that do not contain GC boxes are stimulated by Sp1 (37). Indeed, as far as we know, there exists no strong promoter whose activity is independent of Sp1 in transfection experiments.

Functional Relevance of the Sp Binding Sites for the Expression of the Uteroglobin Gene—Expression of the uteroglobin gene is restricted to epithelial tissues of various organs including the endometrium, the oviduct, the male genital tract, and the lung (Ref. 38, and references therein). Consistently, the activity of the uteroglobin promoter appears to be stronger in cell lines derived from endometrium and lung as compared with fibroblast cell lines following DNA-mediated transfection (2). Both Sp1 and Sp3 are present in all the cell lines that we have tested and in all the tissues examined *in situ*.[2] Moreover, the relative amounts of the two proteins vary only moderately between various cell lines (15). Thus, the Sp binding sites of the uteroglobin promoter appear to be of a general rather than of a cell type-specific nature. So far, we have been unable to delineate clearly the elements of the uteroglobin gene that mediate its cell type specificity. It appears that the combinatorial action of various transcription factors rather than a single transcription factor determines the cell type-specific expression of the uteroglobin gene in the uterus. Lung Clara cell expression has been analyzed for the rat homologue of rabbit uteroglobin. Different members of the HNF-3 family of transcription factors seem to contribute, at least in part, to its cell type-specific expression in lung (39, 40).

The control of uteroglobin gene expression is even more complex. The level of expression in endometrium and lung is regulated by various steroid hormones (41). The enhancer region, which is responsible for the induction of transcription of the gene in the endometrium by progesterone, is located 2.4 kilobases upstream of the transcriptional start site (42). So far, we do not know how this enhancer region communicates with the promoter and whether the described Sp binding sites are directly involved in this interaction. However, Sp1 seems to be active in mammalian cells only in response to a remote enhancer (43). Thus, it is possible that element II and/or VI of the uteroglobin promoter are directly involved in promoter/enhancer interactions. Further investigations will be necessary to unravel the functional interplay between the uteroglobin enhancer and promoter and to determine the specific roles of Sp1 and Sp3 in this interaction.

Acknowledgments—We thank W. Lorenz for technical assistance. Dr. R. Tjian kindly provided us with Sp1 cDNA clones. We gratefully acknowledge Drs. A. Baniahmad, M. Kalff-Suske, and J. Klug for critically reading the manuscript.

[2] J. Dennig, G. Hagen, M. Beato, and G. Suske, unpublished data.

REFERENCES

1. Misseyanni, A., Klug, J., Suske, G., and Beato, M. (1991) *Nucleic Acids Res.* **19,** 2849–2859
2. Suske, G., Lorenz, W., Klug, J., Gazdar, A. F., and Beato, M. (1992) *Gene Exp.* **2,** 339–352
3. Slater, E. P., Redeuihl, G., Theis, K., Suske, G., and Beato, M. (1990) *Mol. Endocrinol.* **4,** 604–610
4. Becker, P. B., Gloss, B., Schmid, W., Strähle, U., and Schütz, G. (1986) *Nature* **324,** 686–688
5. Strähle, U., Schmid, W., and Schütz, G. (1988) *EMBO J.* **7,** 3389–3395
6. Danesch, U., Gloss, B., Schütz, G., Schüle, R., and Renkawitz, R. (1987) *EMBO J.* **6,** 625–630
7. Schüle, R., Muller, M., Otsuka-Murakami, H., and Renkawitz, R. (1988) *Nature* **332,** 87–90
8. deBoer, E., Antoniou, M., Mignotte, V., Wall, L., and Grosveld, F. (1988) *EMBO J.* **7,** 4203–4212
9. Myers, R. M., Tilly, K., and Maniatis, T. (1986) *Science* **232,** 613–618
10. Zenke, M., Grundtstrom, T., Matthes, H., Wintzereith, M., Schatz, C., Wildeman, A., and Chambon, P. (1986) *EMBO J.* **5,** 387–397
11. Xiao, J.-H., Davidson, I., Macchi, M., Rosales, R., Vigneron, R., Staub, A., and Chambon, P. (1987) *Genes & Dev.* **1,** 794–807
12. Jones, N. C., Rigby, P. W. J., and Ziff, E. B. (1988) *Genes & Dev.* **2,** 267–281
13. Klug, J., Knapp, S., Castro, I., and Beato, M. (1994) *Mol. Cell. Biol.* **14,** 6208–6218
14. Hagen, G., Müller, S., Beato, M., and Suske, G. (1992) *Nucleic Acids Res.* **20,** 5519–5525
15. Hagen, G., Müller, S., Beato, M., and Suske, G. (1994) *EMBO J.* **13,** 3843–3851
16. Kingsley, C., and Winoto, A. (1992) *Mol. Cell. Biol.* **12,** 4251–4261
17. Pascal, E., and Tjian, R. (1991) *Genes & Dev.* **5,** 1646–1656
18. Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987) *Cell* **51,** 1079–1090
19. Andrew, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 2499
20. Fried, A., and Crothers, D. M. (1981) *Nucleic Acids Res.* **9,** 6505–6525
21. Garner, M. M., and Revzin, A. (1986) *Trends Biochem. Sci.* **11,** 395–396
22. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560
23. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Cato, A. C. B., Miksicek, R., Schütz, G., Arnemann, J., and Beato, M. (1986) *EMBO J.* **5,** 2237–2240
25. Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989) *EMBO J.* **8,** 1981–1986
26. Schneider, I. (1972) *J. Embryol. Exp. Morphol.* **27,** 353–365
27. DiNocera, P. P., and Dawid, I. B. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 7095–7098
28. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051
29. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. (1983) *J. Mol. Appl. Genet.* **2,** 101–109
30. Courey, A. J., and Tjian, R. (1988) *Cell* **55,** 887–898

31. Philipsen, S., Talbot, D., Fraser, P., and Grosveld, F. (1990) *EMBO J.* **9,** 2159–2167
32. Jones, K. A., Kadonaga, J. T., Luciw, P. A., and Tjian, R. (1986) *Science* **232,** 755–759
33. Pitluk, Z. W., and Ward, D. C. (1991) *J. Virol.* **65,** 6661–6670
34. Gidoni, D., Dynan, W. S., and Tjian, R. (1984) *Nature* **312,** 409–413
35. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci.* **11,** 20–23
36. Thiesen, H. J., and Bach, C. (1990) *Nucleic Acids Res.* **18,** 3203–3209
37. Saffer, J. D., Jackson, S. P., and Thurston, S. J. (1990) *Genes & Dev.* **4,** 659–666
38. Sandmöller, A., Voss, A. K., Hahn, J., Redemann-Fibi, B., Suske, G., and Beato, M. (1991) *Mech. Dev.* **34,** 57–68
39. Stripp, B. R., Sawaya, P. L., Luse, D. S., Wikenheiser, K. A., Wert, S. E., Huffman, J. A., Lattier, D. L., Singh, G., Katyal, S. L., and Whitsett, J. A. (1992) *J. Biol. Chem.* **267,** 14703–14712
40. Sawaya, P. L., Stripp, B. R., Whitsett, J. A., and Luse, D. S. (1993) *Mol. Cell. Biol.* **13,** 3860–3871
41. Beato, M., Arnemann, J., Menne, C., Müller, H., Suske, G., and Wenz, M. (1983) in *Regulation of Gene Expression by Hormones* (McKerns, K. W., eds) pp. 151–175, Plenum Publishing Corp., New York
42. Jantzen, C., Fritton, H. P., Igo-Kemenes, T., Espel, E., Janich, S., Cato, A. C. B., Mugele, K., and Beato, M. (1987) *Nucleic Acids Res.* **15,** 4535–4552
43. Seipel, K., Georgiev, O., and Schaffner, W. (1992) *EMBO J.* **11,** 4961–4968