

# Transcriptional Regulation of ULBP1, a Human Ligand of the NKG2D Receptor\*

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Tumor cells expressing ligands of the NKG2D receptor stimulate anti-tumor immunity mediated by natural killer and T cells. In humans, NKG2D ligands (NKG2DL) are encoded by MIC and ULBP proteins. NKG2DL exhibit highly restricted expression in healthy tissues but are widely expressed in tumors. However, regulation of each NKG2DL differs substantially in different cancer cells. In this study, we characterized the mechanisms that regulate the expression of ULBP1. We show that the transcription of ULBP1 strictly depends on the binding of Sp1 and Sp3 to a CRE(1) site located in the ULBP1 minimal promoter. The mutation or deletion of this Sp1/Sp3 binding site abolished the transcription of ULBP1. It also diminished the transactivation of ULBP1 promoter by Sp3 overexpression, but not by Sp1, indicating that Sp3 is the main transcription factor that regulates ULBP1 through the CRE(1) site. Experiments in SL2 cells showed that the ULBP1 promoter was inactive in the absence of the Sp proteins and indicate that Sp3 is the essential activator of ULBP1 transcription, because the overexpression of Sp3 up-regulated its promoter activity >500-fold. Additionally, we demonstrated that AP-2 $\alpha$  repressed the expression of ULBP1 in HeLa cells by interfering with the binding of Sp3 and Sp1 to the ULBP1 promoter. These data indicate that Sp1, Sp3, and AP-2 $\alpha$  may play an important role in the immunosurveillance against cancer. Finally, the definition of ULBP1 regulation may have implications for development of new therapeutic strategies against cancer cells.

NKG2D is an activating receptor expressed by NK<sup>3</sup> cells, NK T cells, CD8  $\alpha\beta$ T cells, and  $\gamma\delta$ T cells (1, 2). Whereas most NK receptors bind to ubiquitously expressed major histocompati-

bility complex class I molecules, a peculiarity of NKG2D consists in the multitude of ligands that are induced subsequently to harmful events such as genotoxic stress or infection (3–7). NKG2D ligands (NKG2DL) are also frequently expressed on cancer cells but absent in benign tissues. Tumor cells expressing NKG2DL become susceptible to NK cell killing even if the tumor cells have normal HLA class I expression (1, 3). NKG2DL expression in mice is induced by carcinogens and genotoxic stress, and tumor cells expressing these proteins were readily eliminated by NK and CD8 T cells *in vivo* (6, 8, 9). Similarly, mice lacking  $\gamma\delta$ T cells are highly susceptible to epithelial tumors, and these cells could kill skin carcinoma cells by a NKG2D-dependent mechanism *in vitro* (10). In accordance with the role of NKG2DL in the anti-tumor immunity, cancer cells develop several means to avoid the immune system controlling the expression of NKG2D receptors on immune cells or their ligands on tumor cells (11–14). These experiments strongly support a role of the NKG2D/NKG2DL system in tumor rejection and surveillance and render this system as an interesting target for immunotherapy (15–20).

In humans, the ligands of NKG2D are MICA, MICB (21, 22), and UL16-binding proteins 1–5 (ULBP1–5) (5, 23–25). MIC and ULBP molecules exhibit highly restricted expression in healthy tissues but are widely expressed on epithelial tumors and hematological malignancies *in vivo* (15, 17, 19, 22, 26). These proteins are up-regulated in nontumor cell lines by genotoxic stress (6). Thereby, DNA-damage response, which arrests the cell cycle and enhances DNA repair functions, or triggers apoptosis, also alerts the immune system in response to potentially dangerous cells through the activation of NKG2D. It is still a matter of controversy as to why so many different ligands exist for the same receptor, because the stimulatory activity of the immune cells of different NKG2DLs are very similar. In the context of tumor transformation or infection, the multitude of ligands could be explained as a strategy to avoid the evasion of the immune system. Several studies indicate that the regulation of individual NKG2DL expression differs substantially in a particular tumor or infected cells (15, 17, 19), suggesting that the multitude of NKG2DLs do not simply reflect the redundant expression of molecules with the same function. Concordantly, despite the strong conservation of the coding sequence among all ULBPs, the sequence of the 5'-flanking regions of these genes has little homology, and this suggests that ULBPs could be specifically regulated in response to different stimuli or

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<sup>3</sup> The abbreviations used are: NK, natural killer; ULBP1, UL16-binding protein 1; NKG2DL, NKG2D ligand; MIC, major histocompatibility complex class I-related chain; HEK 293, human embryonic kidney 293; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; ChIP, chromatin immunoprecipitation; RACE, rapid amplification of cDNA ends; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CREB, cAMP-response element-binding protein.

pathologies. Nevertheless, very little is known about the mechanisms of NKG2DL induction and expression.

Here, we have analyzed for the first time the mechanism that controls ULBP1 expression. We show that ULBP1 transcription strictly depends on the CRE(1) site located at  $-115$ , because the mutation or deletion of this site practically abolished the transcription of ULBP1. We also demonstrate that the Sp1 and Sp3 transcription factors are bound to the CRE(1) sequence, and the expression of ULBP1 depends on the activity of these transcription factors. Nevertheless, chromatin immunoprecipitation analysis (ChIP) demonstrated that Sp1 and Sp3 were the main transcription factors bound to this promoter *in vivo*. Mutation of the CRE(1) site significantly diminished the transactivation of ULBP1 promoter by Sp3 expression, but not the Sp1, indicating that Sp3 is the main transcription factor that regulates ULBP1 through the CRE(1) site. Furthermore, cotransfection experiments into *Drosophila* SL2 cells lacking Sp factors demonstrated that ULBP1 promoter was practically inactive in the absence of the Sp proteins and showed that the long isoform of Sp3 was a 90-fold stronger activator of ULBP1 transcription than Sp1 was. This indicates that Sp3 plays an essential role in the expression of ULBP1 suggesting that the regulation of the expression of Sp3 isoforms or its activity may play a key role in the induction of ULBP1 expression that is observed in cancer cells. We also show that AP-2 $\alpha$  may mediate transcriptional repression of the ULBP1 gene through competition with the transcriptional activator Sp1 and Sp3 for binding to the ULBP1 minimal promoter. AP-2 $\alpha$  is a transcription factor that acts as a tumor suppressor that favors epithelial differentiation and induces cell cycle arrest (27–29). Loss of AP-2 $\alpha$  expression has been associated with progression of melanoma, breast, and colon cancer (30–35). Thus, our results indicate that Sp1, Sp3, and AP-2 $\alpha$  may play an important role in the immunosurveillance against cancer cells regulating the expression of ULBP1.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—HeLa (human cervix carcinoma) cells were obtained from the American Type Culture Collection (Manassas, VA), and human embryonic kidney 293 (HEK 293) were obtained from Dr. P. Sanchez Lazo (Universidad de Oviedo, Spain). Cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and gentamicin in a humidified 95% air, 5% CO<sub>2</sub> incubator. SL2 cells were a gift from Dr. P. Dominguez (Universidad de Oviedo) and were grown in Schneider's *Drosophila* medium (Invitrogen) with 10% fetal calf serum, 2 mM L-glutamine, and gentamicin at 28 °C without CO<sub>2</sub>.

**Mapping of the Transcription Start Sites of ULBP1 Gene**—The transcription start sites of the ULBP1 gene in HeLa and HEK 293 cells were determined by 5'-rapid amplification of cDNA ends (5'-RACE) as previously described (36). Briefly, total RNA was isolated from cell cultures by TRIzol reagent (Invitrogen). A synthetic oligonucleotide (GSP1) complementary to mRNA, spanning bases  $+334$  to  $+314$  (5'-CAC GTC TCT TAG TGT TTC AG-3'), was used to generate 5'-end ULBP1 dA-tailed. This cDNA was subjected to two rounds of

PCR. The first PCR was conducted with a specific primer (GSP2) spanning bases  $+266$  to  $+242$  (5'-C AAA GGC TTT GGC GTT GTG GTT AA-3') and an oligonucleotide containing deoxythymidines at the 3'-end, Sp6-(dT)<sub>18</sub> (5'-ATT TAG GTG ACA CTA TAG AAT ACG TCG ACA TCG ATG G(dT)<sub>18</sub>-3'). The second one was performed with a nested specific primer (GSP3) spanning bases  $+28$  to  $+10$  (5'-CCG TGG ACA GGA GGC GCT-3') and the Sp6 primer lacking the dT tail. All PCR products were 5'-end phosphorylated using T4 polynucleotide kinase (Amersham Biosciences) and blunt ended with T4 DNA polymerase (Promega, Madison, WI) and then cloned in the EcoRV site of the Bluescript II KS plasmid (Stratagene, La Jolla, CA).

**Generation of ULBP1 Promoter Reporter, Mutant, and Deletion Constructs**—A 900-bp ULBP1 promoter fragment was amplified by PCR from human genomic DNA using the following primers: ULBP1s, 5'-TAA GCC CTC CCT TGC GGC C-3', and the reverse *ULBP1as*, 5'-CCG TGG ACA GGA GGC GCT-3'. The fragment obtained was ligated into the SmaI site of the pGL2 Basic vector (Promega). Different deletions were produced by PCR using this construct as a DNA template with the following forward primers:  $-603$ , 5'-CAC AAC TGA TCA CAT CAT GAT TGA TCA C-3';  $-288$ , 5'-GGG GTA ATG ATA AAT GCT GTG TTG AA-3';  $-200$ , 5'-CGC ACG CCG CCA CCA A-3';  $-137$ , 5'-GTC AGA TGA CGA GCC CGG G-3';  $-75$ , 5'-CCC GCC CAG TGT ATC CCT G-3'; and  $-42$ , 5'-GGG CTG GGC AGC TTT ATA AAC AG-3'. The PCR products were subcloned into the SmaI site of pGL2 Basic vector. The integrity of constructs was confirmed by DNA sequencing. Mutant promoter constructs were generated using the QuikChange kit (Stratagene) with the following primers (only forward primers are shown; mutated oligonucleotides were underlined): mTATA, 5'-CCG GGC TGG GCA GCT TCG CGA ACA GCC GTG GTG TG-3'; mGC(1), 5'-ATC CCT GCG CGC GGC TTTG CCG GGC TGG GCA GC-3'; mGC(2), 5'-GCC TAG GAT CCC GAA CAG TGT ATC CCT GCG CGC-3'; mNF- $\kappa$ B, 5'-GGC GTG ACG GGG TGG AGC ATA AAC AAA AAA GTG CAT GCC-3'; mGC(3), 5'-GCC CGG GGC GTG ACG GTT TGG AGC ATC CCC-3'; mCRE(1), 5'-GAG CCC GGG GCG GCT GTG GGT GGA GCA TCC C-3'; mGC(4)/AP-2, 5'-GAT GAC GAG CCC GGT TCG TGA CGG GGT GGA GC-3'; and mCRE(2), 5'-GCC AGG CCG CTG TCA GGC TGT GAG CCC GGG GCG TGA CGG-3'. All these constructs were verified for their nucleotide sequence and reading frame by DNA sequence analysis.

**Expression and Dominant Negative Plasmids**—The constructs for mammalian expression pN3-Sp1, pN3-Sp3FL (a cytomegalovirus-driven expression vector for full-length Sp3 with both upstream AUGs) and pN3-Sp4 (37) and the *Drosophila* expression vectors pPac, pPacSp1, pPacSp3 (which expresses a short isoform of Sp3), pPacUSp3 (expression vector for long Sp3 isoform), and pPacSp3FL (expression vector for full-length Sp3, which is equivalent to the mammalian vector pN3-Sp3FL) (38, 39) were kindly provided by Dr. G. Suske (Philips-Universität Marburg, Germany). Human AP-2 $\alpha$  and murine AP-2 $\beta$  in pCMX-PL1 vector were generously provided by Dr. R. Buettner (40). pCDNA3.1/AP-2 $\gamma$  was a gift from Dr. R. J. Weigel (41). The AP-2B vector pSG5/AP-2 and the expres-

sion vectors pCDNA6/AP-2 $\alpha$ -(1–165) and -(165–437) (42) were kindly provided by Dr. P. Kannan (Metro Health Medical Center, Case Western Reserve University). The reporter plasmid pGL3-waf1-LUC was described before (43).

**Transient Transfections and Luciferase Reporter Assays**—HeLa and HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's recommendations. Briefly,  $5 \times 10^4$  cells were transfected in 24-well plates with 300 ng of each reporter construct and 12 ng of pRL-null vector (Promega) as an internal control plasmid. Cells were harvested at 24 h and assayed for luciferase activity using the Dual Luciferase Reporter System (Promega). For co-transfection assays with dominant negative or expression vectors, pRSV  $\beta$ -galactosidase plasmid was included and luciferase measures were normalized with  $\beta$ -galactosidase activity. In some experiments, 200 nM mithramycin A (Sigma) was added 4 h after DNA transfections, and luciferase activity was assayed 24 h later. *Drosophila* SL2 cells ( $5 \times 10^5$  cells) were co-transfected in 24-well plates using Lipofectamine 2000 with 500 ng of reporter plasmid plus 300 ng of each expression vector. Cells were harvested 48 h post-transfection, and luciferase activities were normalized against the total protein concentration as measured using the BCA Protein Assay kit (Pierce).

**Real-time PCR and Flow Cytometry**—Real-time PCR was performed using SYBR Green from reverse-transcribed cDNA samples for relative quantification of mRNA levels for the genes of interest. The mRNA level of the GAPDH gene was determined in each assay to normalize the expression of genes of interest. The primers used were as follows: ULBP1 sense, 5'-ATC AGC GCC TCC TGT CCA C-3'; ULBP1 antisense, 5'-AAA GAC AGT GTG TGT CGA CCC AT-3'; MICA sense, 5'-AAT GGA ACC TAC CAG ACC TGG G-3'; MICA antisense, 5'-ACA TGG AAT GTC TGC CAA TGA CT-3'; GAPDH sense, 5'-CGG AGT CAA CGG ATT TGG TC-3'; and GAPDH antisense, 5'-AAT CAT ATT GGA ACA TGT AAA CCA TGT AGT-3'. Cell surface expression of ULBP1 and MICA/B were detected by flow cytometry using a BD Biosciences FACSCalibur (Sunnyvale, CA). Detached cells were incubated with a monoclonal antibody anti-ULBP1 (4G7) or a monoclonal anti-MICA/B (6G6), which were kindly provided by Dr. Thomas Spies (Fred Hutchinson Cancer Research Centre, Seattle, WA), for 30 min on ice; followed by incubation with a phycoerythrin-labeled goat anti-mouse IgG1 secondary monoclonal antibody (BD Pharmingen, San Diego, CA) for another 30 min on ice.

To investigate the effect of Sp1, Sp3, and AP-2 $\alpha$  on endogenous ULBP1 expression, HeLa and HEK 293 cells were grown in 60-mm dishes and co-transfected with 2  $\mu$ g of pN3, pN3-Sp1, pN3-Sp3FL, pCMX, or pCMX-AP-2 $\alpha$  expression vectors and with 0.5  $\mu$ g of pCS2-GFP plasmid (44). After 48 h, ULBP1 surface expression was analyzed in GFP-positive cells using a monoclonal antibody anti-ULBP1 (4G7) as described above.

**Electrophoretic Mobility Shift Assay**—90% confluent HeLa and HEK 293 cells were used for preparing nuclear extracts as previously described (45). The protein concentration of the extracts was determined by using BCA assay (Pierce). A wild-type double-stranded oligonucleotide corresponding to positions -128/-99 of ULBP1 promoter (5'-GAG CCC GGG GCG

TGA CGG GGT GGA GCA TC-3') was 5'-end-labeled using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP (3000Ci/mmol, Nucliber, Spain). The DNA binding reactions were performed in a volume of 20  $\mu$ l containing 5  $\mu$ g of nuclear protein together with the  $^{32}$ P-end-labeled wild-type probe and 2  $\mu$ g of poly(dI-dC) (Amersham Biosciences) in binding buffer (10 mM HEPES-KOH, pH 7.9; 5% (v/v) glycerol; 50 mM KCl; 5 mM MgCl<sub>2</sub>; 0.5 mM EDTA; 0.1% Nonidet P-40; and 1 mM dithiothreitol) on ice for 35 min. The binding reactions were electrophoresed on 6% native acrylamide gels in Tris-Borate-EDTA buffer. For competition experiments, a 100 molar excess of unlabeled probes was incubated with the extracts at 25 °C for 15 min before adding the radioactive probe. The oligonucleotides used in competition assays were as follows: Sp1 consensus, 5'-ATT CGA TCG GGG CGG GGC GAG C-3'; CREB consensus, 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3'; AP-2 consensus, 5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3'. Mutant ULBP1 promoter probes included the same punctual mutations described above. In supershift assays, nuclear extracts and 2  $\mu$ g of commercial antibody were preincubated for 60 min on ice prior to the addition of the remaining components of the binding reaction. Rabbit polyclonal antibodies anti-Sp3 (sc-644X), anti-AP-2 $\alpha$  (sc-184X), and mouse monoclonal antibody anti-Sp1 (sc-420X) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-CREB (ab503, ABCAM, United Kingdom) was a generous gift from Dr. JM Freije (Universidad de Oviedo).

**Chromatin Immunoprecipitation**—ChIP was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) following the manufacturer's recommendations. Briefly, HeLa and HEK 293 cells were treated with 1% formaldehyde, pelleted, and resuspended in SDS lysis buffer. Chromatin was sonicated to sizes between 200 and 500 bp using a Branson Sonifier 450. Diluted chromatin was precleared with 80  $\mu$ l of salmon sperm DNA/protein A-agarose-50% slurry (Upstate Biotechnology) and then incubated overnight at 4 °C with 5  $\mu$ g of anti-Sp1, -Sp3, -AP-2 $\alpha$  antibodies or normal rabbit IgG (sc-2027, Santa Cruz Biotechnology). 20  $\mu$ l of NaCl 5 M was added, the immune complexes were eluted, and cross-links were reversed by overnight incubation at 65 °C. DNA samples were purified by phenol/chloroform extraction and ethanol precipitation. A 95-bp fragment of the ULBP1 proximal promoter spanning the region from -137 to -43 was amplified by PCR using the following primers: 5'-GTC AGA TGA CGA GCC CGG G-3' (forward) and 5'-GGC CCG CCG CGC GCA-3' (reverse). The samples were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

**Western Blotting**—To obtain total protein extracts, HeLa and HEK 293 cells were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 1 mM dithiothreitol, complete inhibitor mixture (Roche Applied Science) and phosphatase inhibitor cocktails I and II (Sigma). Soluble proteins were then separated by centrifugation at 15,000  $\times$  g for 5 min at 4 °C. Protein concentration was determined using BCA assay (Pierce). Nuclear extracts from HeLa and HEK 293 cells were obtained as described above. Following heat denaturation, samples containing 15  $\mu$ g of protein were loaded on 10% SDS-PAGE gels. Proteins were then transferred to nitrocellulose



## Transcriptional Regulation of ULBP1

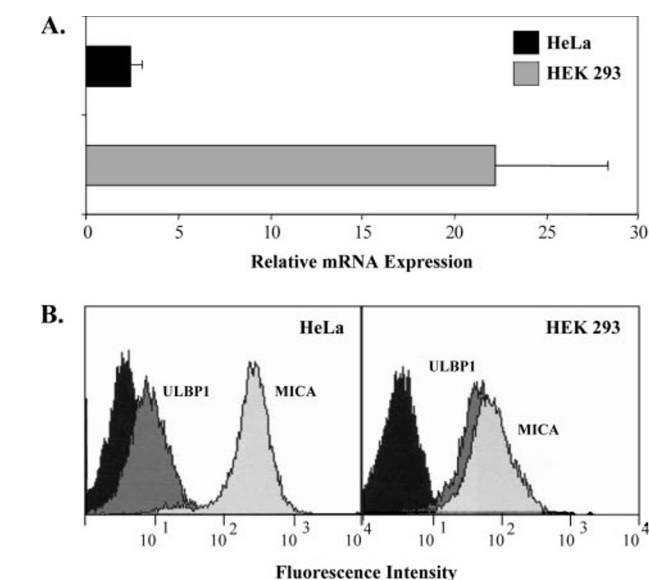
membranes and stained with Ponceau to verify that similar amounts of protein had been loaded. Blots were blocked with 3% nonfat dry milk and incubated overnight at 4 °C with a dilution 1/600 of the rabbit polyclonal antibodies anti-Sp3 (sc-644X) and anti-AP-2α (sc-184X) and of the mouse monoclonal antibody anti-Sp1 (sc-420X). Finally, blots were incubated with 1/7500 donkey anti-rabbit-horseradish peroxidase

or goat-anti-mouse-horseradish peroxidase (Amersham Biosciences) in 1.5% nonfat dry milk washed and developed with West Pico Chemiluminescent substrate (Pierce).

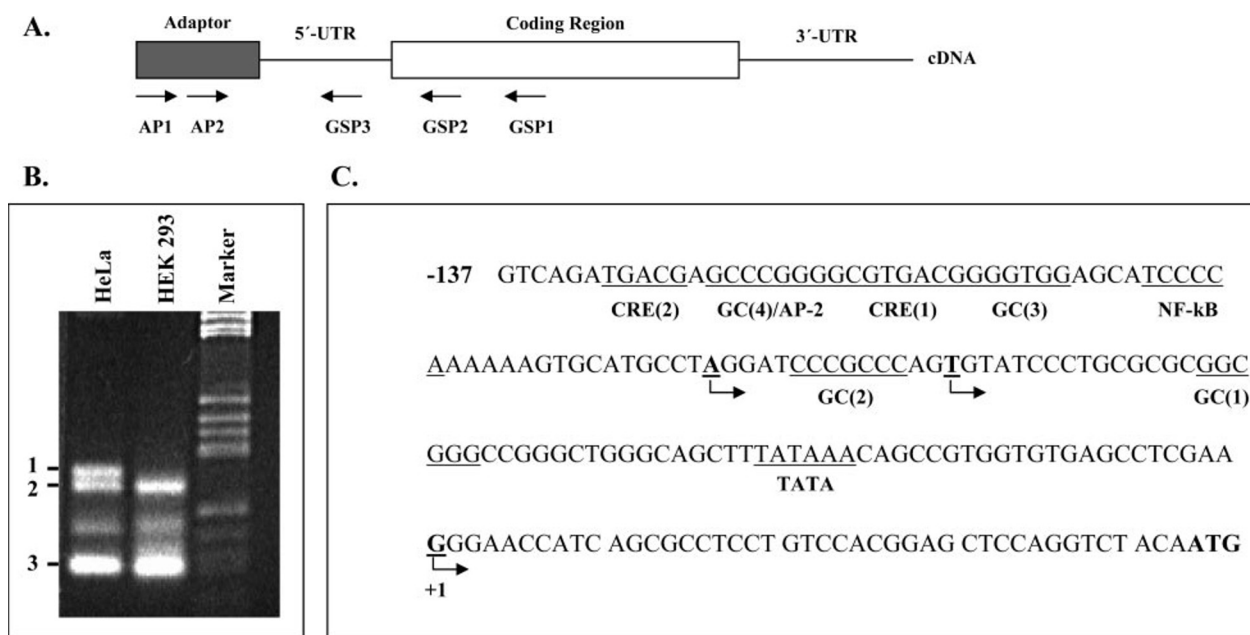
## RESULTS

**Expression of ULBP1 and Characterization of Transcription Start Sites**—The expression of ULBP1 was analyzed in HeLa and HEK 293 cells at RNA and protein level. The ULBP1 mRNA was quantified by real-time PCR and normalized using GAPDH level within each cell line. HEK 293 showed 9.3-fold higher expression of ULBP1 mRNA than HeLa (Fig. 1A). Concomitantly, HEK 293 cells expressed significant amounts of ULBP1 surface protein; however, only a scarce expression of ULBP1 was detected on HeLa cells (Fig. 1B).

To determine the transcription initiation site(s) of the ULBP1 gene, 5'-RACE was performed using total RNA from HeLa and HEK 293 cells (Fig. 2A). Three and two ULBP1-specific transcription start sites were identified in HeLa and HEK 293, respectively (Fig. 2B). Sequencing of an additional PCR product that appeared between bands 2 and 3 indicated that it did not correspond to a ULBP1 transcription start site (data not shown). The sequence of the PCR products 2 and 3 revealed that both cell lines shared two common transcription start sites located 43 bp and 108 bp upstream of the ATG codon. HeLa presented an additional transcription start site located 132 bp relative to ATG (Fig. 2B, band 1). Fragment 3, shared by HEK 293 and HeLa, showed the highest intensity of all three bands and is located 28 bp downstream of a canonical TATA box. This site corresponds with the previously determined 5'-end of the ULBP1 mRNA (5) suggesting that this is the main transcription start site of the ULBP1 gene (Fig. 2C).



**FIGURE 1. Analysis of expression of ULBP1.** A, expression of ULBP1 mRNA in HeLa and HEK 293 cells was analyzed by real-time PCR. The level of ULBP1 mRNA was normalized using the GAPDH level within each cell line. The results represent the mean  $\pm$  S.E. of three independent experiments. B, protein expression was analyzed by immunofluorescence and FACS analysis using 4G7 (anti-ULBP1) and 6G6 (anti-MICA/B) monoclonal antibodies. Isotype controls are represented in black, ULBP1 in dark gray, and MICA in light gray. The histogram shows one representative experiment.



**FIGURE 2. RACE 5' analysis of the transcription start site of ULBP1 gene.** A, RACE 5' was conducted using two adapter primers (AP1 and AP2) and three specific ULBP1 primers (GSP1, -2, and -3). The GSP1 primer was used to generate the 5'-end ULBP1 dA-tailed cDNA. This cDNA was subjected to two rounds of PCR using the GSP2 and the AP1 primers, and GSP3 and AP2 primers, respectively. B, analysis on agarose gel of the nested PCR products obtained from HeLa and HEK 293 mRNA. C, nucleotide sequence of the 5'-untranslated region and proximal promoter of ULBP1 gene. The arrows indicate the transcription start sites. The main transcription start site is numbered +1. The potential binding sites for transcription factors are underlined.

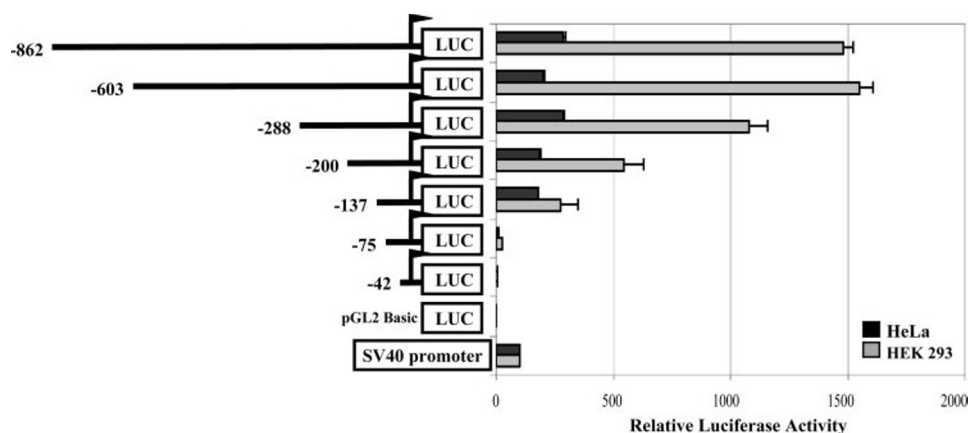


FIGURE 3. Identification of the minimal promoter region of ULBP1 using progressive deletions of the 5'-region in luciferase reporter constructs. The schematic presentation of the luciferase reporter constructs is shown. These plasmids were transiently transfected in HeLa and HEK 293 cells, and luciferase activity was assayed after 24 h. To correct the differences in transfection efficiencies, the *Renilla* luciferase plasmid pRL-null was included in each transfection. The activity of the luciferase reporter construct under the control of the SV40 promoter (pGL2-Control) was set as 100. The results represent the mean  $\pm$  S.E. of three independent experiments made in triplicate.

**Identification of DNA Elements Involved in Transcriptional Regulation of ULBP1**—Approximately 1 kb of the ULBP1 5'-flanking region (−862/+28) was cloned into a luciferase reporter vector, and it was transiently transfected in HeLa and HEK 293 cells. The transfection of this construct revealed that this sequence had promoter activity in both cell lines, but HEK 293 cells had a 4.2-fold higher ULBP1 promoter activity than HeLa cells (Fig. 3). To localize the promoter region responsible for ULBP1 transcriptional activity, a series of six truncated promoter fragments were generated by PCR, and these were cloned into a promoterless luciferase reporter vector. The deletion of the ULBP1 5'-flanking region progressively decreased the luciferase activity, nevertheless the construct −137/+28 still retained significant promoter activity in both cell lines (Fig. 3). The further deletion of the region that encompasses the sequence −137 to −75 practically abolished the transcriptional activity of the ULBP1 promoter suggesting that this region plays a key role in the basal expression of the ULBP1 gene. By sequence analysis we established that the difference in ULBP1 transcription between HeLa and HEK 293 is not due to any difference in the genomic sequence of the ULBP1 promoter region amplified from both cell lines. However, we have detected in HEK 293 cells the existence of a possible single nucleotide polymorphism (G/C) located at −15 bp relative to the ULBP1 main transcription start site, which is out of the core region of the minimal promoter of ULBP1 (data not shown).

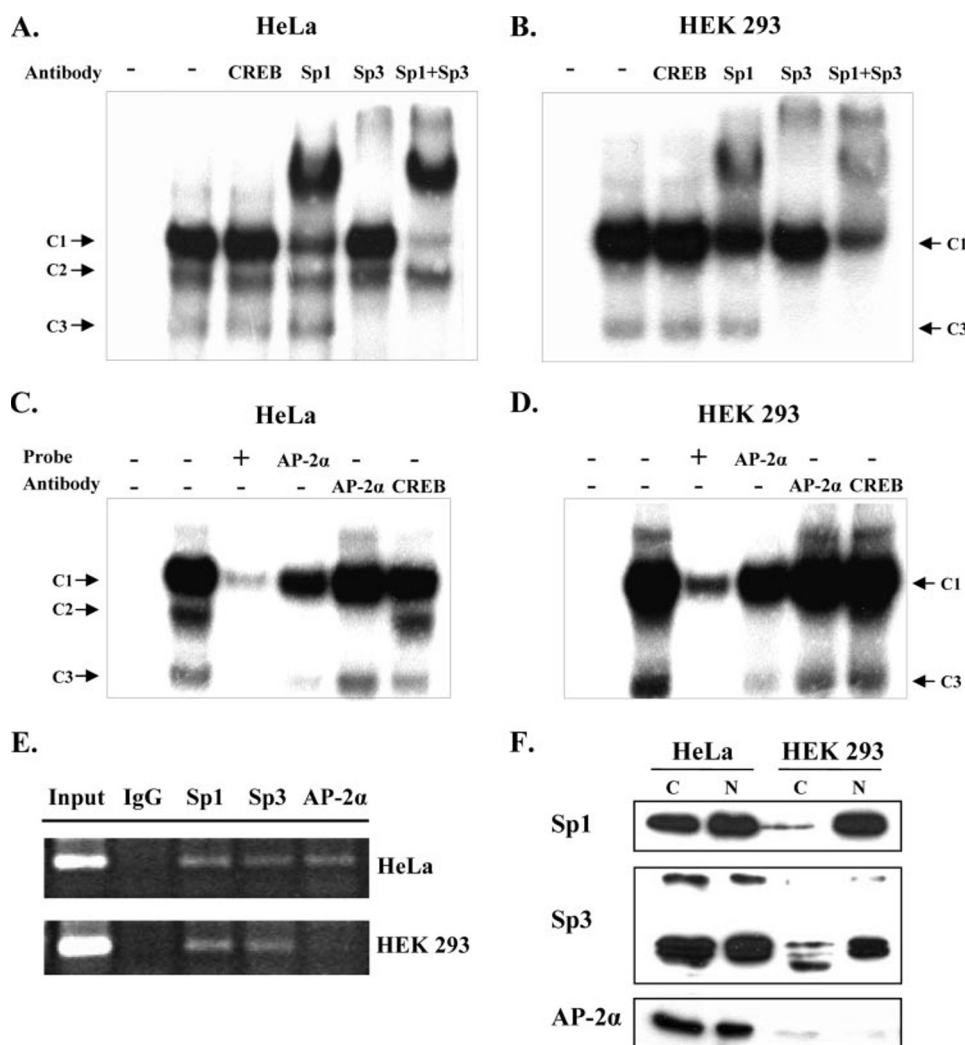
The analysis of the minimal promoter (−137/+28) for consensus motifs for transcription factors revealed a canonical TATA box located at −28, three GC boxes, one overlapping sequence GC(4)/AP-2, two CRE-like sequences (CRE(1) and CRE(2)), and one site for NF- $\kappa$ B (Fig. 2C). The functional significance of these elements was studied by mutation of their sequences in the −862/+28 construct (Fig. 4A). Mutation of the TATA box decreased the activity of the ULBP1 promoter in HeLa and HEK 293 by 19 and 80%, respectively (Fig. 4B). Remarkably, the most significant results were obtained with the mutation of the sequences located in the region −137/−75. The mutation of the CRE(1) at −116 practically abolished

the ULBP1-mediated transcription activity in both cell lines (Fig. 4B). The sequences juxtaposed to this CRE(1) also showed functional activity. Thus, the mutation of the GC(3) box, located just downstream, reduced the transcriptional activity by 25 and 55% in HeLa and HEK 293, respectively; and the mutation of GC(4)/AP-2, which is located just upstream of the CRE(1) site, resulted in a 65% increase of luciferase activity in HeLa cells, but it had little effect on HEK 293 cells. This indicates that the GC(4)/AP-2 site is a negative regulator of ULBP1 transcription in HeLa. Finally, the mutation of the CRE(2) site also showed an important reduction of the ULBP1-mediated transcription in HeLa and HEK 293 cells (Fig. 4B).

**Interaction of the ULBP1 Proximal Promoter with Transcription Factors Sp1, Sp3, and AP-2 $\alpha$** —The mutational analysis showed that several potential binding sites for transcription factors are functional in the minimal promoter of ULBP1. In this study, we focused on the GC(4)/AP2, CRE(1), and GC(3) sequences, which as we have shown, played a crucial role in the expression of ULBP1. EMSA was performed to determine the transcription factors that bind to these sequences. A radiolabeled double-stranded oligonucleotide encompassing the potential GC(4)/AP-2, CRE(1), and GC(3) sites (Fig. 5A) was incubated with nuclear extracts from HeLa cells, which resulted in the formation of three complexes (called C1, C2, and C3) (Fig. 5B, lane 2), whereas only two complexes (C1 and C3) were observed using nuclear extracts from HEK 293 cells (Fig. 5C, lane 2). These complexes were specific, because they competed with a 100-fold molar excess of the unlabeled probe (Fig. 5, B and C, lane 3). To characterize the *cis*-acting elements of ULBP1 promoter that interacted with these three complexes, competition experiments with mutated double-stranded oligonucleotides were performed. The unlabeled probe with a mutated GC(3) box competed with complexes C2 and C3, and it only partially competed with C1, indicating that C1 was partially bound to the GC(3) box (Fig. 5, B and C, lane 4). The mutant CRE(1) oligonucleotide only abolished complex C2 in HeLa, and it had no effect on HEK 293, indicating that the complexes C1 and C3 were bound to this sequence in both cell lines (Fig. 5, B and C, lane 5). The mutant GC(4)/AP-2 oligonucleotide was unable to compete with any complex, indicating that the three complexes were bound to this sequence (Fig. 5, B and C, lane 6). Together, these results indicate that complexes C1 and C3 were originated by interaction with CRE(1) and GC(4)/AP-2 sites in HeLa and HEK 293, and the binding of C1 was also extended to the GC(3) box. The complex C2 resulted from interaction with the GC(4)/AP-2 site exclusively in HeLa cells. To further characterize the transcription factors that composed these complexes, competition experiments using unlabeled double-stranded oligonucleotides encoding consen-







**FIGURE 6. Identification of proteins in complexes by supershift assays.** Antibodies for CREB, Sp1, and/or Sp3 were preincubated with HeLa (A) and HEK 293 (B) nuclear extracts before the addition of the probe. In C and D, HeLa and HEK 293 nuclear extracts were incubated with unlabeled probe (lane 3), AP-2 consensus probe (lane 4), and antibodies for AP-2α (lane 5) or CREB (lane 6). Lane 1 shows the free probe, and lane 2 shows the binding between the probe and the nuclear extracts. E, *in vivo* binding of Sp1, Sp3, and AP-2α to ULBP1 minimal promoter was examined using ChIP analysis. Control (lane 2), Sp1 (lane 3), Sp3 (lane 4), and AP-2α (lane 5) were immunoprecipitated from the sonicated lysates of HeLa and HEK 293 cells using normal rabbit IgG or antibodies against human Sp1, Sp3, and AP-2α proteins. After reversing the cross-linking, DNA was precipitated, and PCR was performed using primers to amplify the ULBP1 core promoter DNA. The PCR products were then run on 2% agarose gels and visualized by ethidium bromide staining. Lane 1 contains input DNAs used as controls. These data are representative from two independent experiments with similar results. F, Western blotting of the expression of Sp1, Sp3, and AP-2α using cytoplasmic and nuclear extracts from HeLa and HEK 293 cells. The proteins transferred to nitrocellulose membranes were stained with Ponceau to verify that similar amounts of protein had been loaded in each lane.

agreement with the fact that Sp1 and Sp3 are ubiquitous transcription factors, Sp1 and the short and long isoforms of Sp3 were expressed in both cell lines, but AP-2α was only significantly expressed in the cytoplasm and nucleus of HeLa cells. Remarkably, HEK 293 cells only showed a scarce expression of AP-2α, indicating that the lack of AP-2α binding to the ULBP1 minimal promoter in HEK 293 cells is due to the loss of the expression of AP-2α in this cell line.

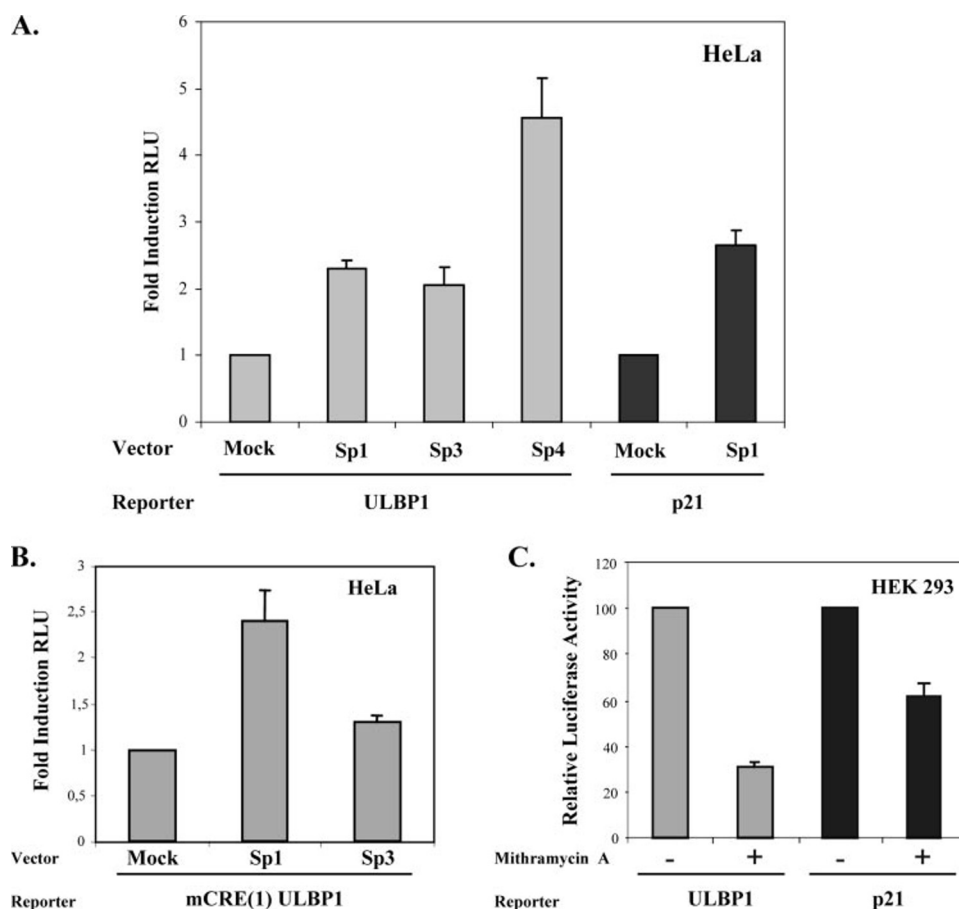
**Sp3 and Sp1 Potently Transactivate the ULBP1 Promoter—**The role of Sp1 and Sp3 in the regulation of ULBP1 promoter was tested by the overexpression of these transcription factors in HeLa cells. The cotransfection of Sp1 and the full-length Sp3 expression vectors with the ULBP1 reporter construct –862/

+28 increased ULBP1-mediated promoter activity 2.2- and 2.1-fold, respectively (Fig. 7A). The cotransfection of Sp4, which has similar transcriptional properties to Sp1, was also able to significantly transactivate the activity of the ULBP1 promoter. Co-transfection of Sp1 expression vector and p21 promoter construct, which has been shown to be regulated by Sp1 (46), resulted in an induction similar to ULBP1 promoter (Fig. 7A). To further investigate the role of Sp transcription factors in controlling the expression of the ULBP1 promoter, we cotransfected the Sp expression vectors with the ULBP1 promoter constructs –862/+28 with a mutation in the CRE(1) site (which is the binding site for Sp1 and Sp3) (Fig. 7B). Despite the fact that the mutation of CRE(1) reduced dramatically the activity of ULBP1 promoter, the Sp1 expression vector activated the CRE(1) mutant in a similar extent to the wild-type promoter (2.3-fold). Nevertheless, the mutation of CRE(1) abolished the transactivation capacity of the Sp3 expression vector, indicating that Sp3 is the main transcription factor that positively regulates the expression of ULBP1 through the CRE(1) site.

The transfection of Sp1, Sp3, and Sp4 in HEK 293 cells also transactivated the activity of the ULBP1 promoter (2-, 2.1-, and 0.97-fold, respectively) (data not shown). To confirm the role of Sp transcription factors on ULBP1 expression in HEK 293, which expresses significant amounts of ULBP1, we used mithramycin A, which is an inhibitor of Sp transcription factor binding (47).

Treatment of HEK 293 cells with 200 nM mithramycin A for 24 h reduced the ULBP1 and p21-mediated promoter activities by 70 and 28%, respectively (48) (Fig. 7C).

**The Long Isoform of Sp3 Is a Potent Activator of the ULBP1 Promoter in *Drosophila* SL2 Cells—**To determine the role of Sp1 and Sp3 on the transcriptional activity of the ULBP1 promoter, transient expression experiments were performed in *Drosophila* SL2 cells, which lack Sp proteins (49). ULBP1 promoter was practically inactive in this cell line indicating that Sp1 and Sp3 are essential for its promoter activity. The cotransfection of the ULBP1 reporter with the pPacSp1 plasmid approximately increased 6-fold the ULBP1 transcriptional activity relative to the empty vector (pPac) (Fig. 8). The overex-



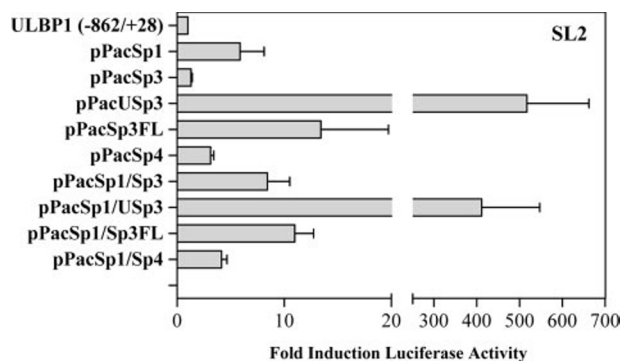
**FIGURE 7. The transcription factors Sp1 and Sp3 and Sp4 activate ULBP1 promoter activity.** A, the ULBP1 promoter construct (−862/+28) (200 ng) was cotransfected with 400 ng of the expression plasmids for Sp1, Sp3, and Sp4 in HeLa cells. The cotransfection of p21 promoter reporter and the Sp1 expression vector was used as a control in these experiments. B, the mutant CRE(1) construct was cotransfected with the expression plasmids for Sp1 and Sp3. To correct the differences in transfection efficiencies, the pRSV-β galactosidase plasmid was included in each transfection. The -fold induction represents the -fold increase in luciferase activity relative to that obtained from cotransfection of backbone of the expression vector. Data are the mean ± S.E. from three independent experiments with triplicates for each experiment. C, HEK 293 cells were transfected with 300 ng of the ULBP1 promoter construct (−862/+28) or p21 promoter construct and 30 ng of the pRSV-β galactosidase plasmid and treated with 200 nM of mithramycin A or Me<sub>2</sub>SO. Cells were harvested after 24 h, and luciferase and galactosidase activity was measured. Relative luciferase activity is expressed as arbitrary units that represent the mean ± S.E. of three independent experiments with triplicates for each experiment. The luciferase activity was normalized by β-galactosidase activity as described under "Experimental Procedures."

pression of Sp4 also led to an increase of 3-fold of ULBP1 promoter activity. The overexpression of different isoforms of Sp3 had a dual effect. The short isoform (pPacSp3) had practically no effect on ULBP1 promoter activity (1.3-fold induction); however, the long isoform of Sp3 (pPacUSp3) led to a 517-fold increase of ULBP1 promoter activity. Transfection of the full-length Sp3 (pPac-Sp3FL), which expresses the short and long isoforms of Sp3 and is equivalent to the mammalian pN3-Sp3FL expression vector previously used in HeLa and HEK 293 cells, only increased the promoter activity of ULBP1 13-fold (Fig. 8). A combination of Sp1 and Sp3 or Sp4 expression vectors showed little change in activity, suggesting that Sp1 and Sp3 do not interact synergistically on the ULBP1 promoter. Collectively, these data indicate that transcription factor Sp3 plays an essential role in the transcription of the ULBP1 gene, because it was a 90-fold stronger activator of ULBP1 promoter activity than Sp1 was.

**AP-2α Repressed ULBP1 Promoter Activity in HeLa Cells**—The role of AP-2 in the transcription of ULBP1 was analyzed by the expression of AP-2α, -β, and -γ transcription factors, which share a common DNA binding domain. In HEK 293, the expression vectors for these transcription factors were unable to significantly modify the transcriptional activity of the ULBP1 promoter (data not shown). Similarly, the AP-2α expression vector was also unable to regulate the expression of the p21 promoter luciferase construct in these cells (data not shown). The lack of response of the p21 promoter that has been shown to be regulated by AP-2α in other cell lines (27) suggests that AP-2α activity is impaired in HEK 293 cells. In HeLa cells, the expression vectors for AP-2α, -β, and -γ repressed the ULBP1 promoter by 32, 56, and 40%, respectively (Fig. 9A). The opposite effect was obtained with the cotransfection of AP-2α with the p21 promoter construct, which resulted in a 2.5-fold increase of luciferase activity (data not shown) (27). These results are in agreement with the fact that the mutation of this AP-2 site increased the transcription activity of the ULBP1 promoter in HeLa cells, but had no effect on HEK 293 cells, and all data together indicate that the AP-2α transcription factor is a repressor of ULBP1 promoter activity in HeLa cells.

The mechanism of AP-2α repression of ULBP1 transcription was further analyzed in HeLa cells. First, the role of the AP-2 binding site (GC(4)/AP-2) in response to Sp expression vectors was studied. The mutation of the AP-2 site significantly increased the transactivation capacity of the Sp expression vectors compared with the wild-type promoter. The overexpression of Sp1, Sp3, and Sp4 increased the activity of the ULBP1 promoter 6.9-, 4.8-, and 6-fold (Fig. 9B). Concordantly, the expression vector AP-2α-(165–437), which only expresses the DNA binding domain of AP-2α, repressed the ULBP1-mediated luciferase activity (Fig. 9C). These observations indicate that AP-2α represses ULBP1 transcription, at least in part, by preventing the binding or activity of Sp1 and Sp3 transcription factors to the CRE(1) element, which overlaps with the AP-2α binding site (Fig. 2C). To further analyze the mechanism of repression of ULBP1 transcription by AP-2α, two expression vectors that lack the DNA binding domain, AP-2B and AP-2α-(1–165), were cotransfected with the ULBP1-luciferase promoter construct. AP-2B is a naturally occurring splice variant of





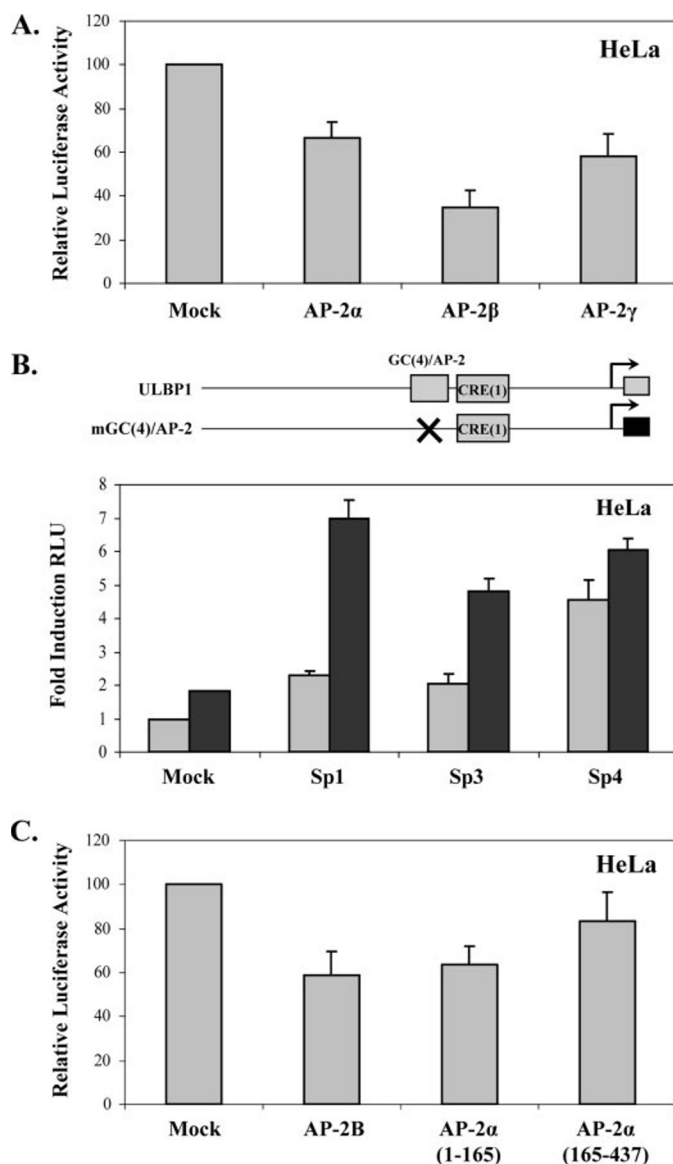
**FIGURE 8. The long isoform of Sp3 is a potent activator of ULBP1 transcription in SL2 cells.** The ULBP1 promoter construct (–862/+28) was cotransfected with the *Drosophila* expression plasmids for Sp1 (pPacSp1), for the short isoform (pPacSp3), the long isoform (pPacUSp3), and the full-length of Sp3 (pPacSp3FL) and Sp4 (pPacSp4) in *Drosophila* SL2 cells. The amount of plasmid DNA was kept constant in all experiments using the pPac vector. Data are expressed as -fold induction of normalized luciferase activity relative to that obtained following co-transfection of the ULBP1 promoter vector with empty pPac. The data are the mean  $\pm$  S.E. of luciferase activity from three independent experiments with triplicates for each experiment.

AP-2 $\alpha$ , which lacks the DNA binding domain, and AP-2 $\alpha$ -(1–165) is an artificial construct in which the DNA binding domain has been deleted. Both expression vectors also significantly repressed the ULBP1-mediated luciferase activity (Fig. 9C). These data suggest that AP-2 $\alpha$  may repress the ULBP1 promoter activity in HeLa cells by both mechanisms, dependent and independent of DNA binding.

**Physiological Roles of Sp1, Sp3, and AP-2 $\alpha$  on ULBP1 Expression**—To determine the effect of Sp1, Sp3, and AP-2 $\alpha$  on endogenous ULBP1 expression, HeLa and HEK 293 cells were cotransfected with the expression vectors for these transcription factors in combination with a GFP plasmid, and ULBP1 protein levels were analyzed on the surface of GFP-positive cells by flow cytometry. Sp1 and Sp3 transfection increased ULBP1 expression in HeLa (3.32- and 2.27-fold, respectively) and HEK 293 cells (3.15- and 1.8-fold, respectively). However, the overexpression of AP-2 $\alpha$  reduced ULBP1 protein levels by 18% in HeLa cells, whereas this transcription factor was unable to regulate the expression of ULBP1 in HEK 293 cells (Fig. 10). These results correlate with the data previously obtained with the reporter assays, suggesting the importance of Sp1, Sp3, and AP-2 $\alpha$  in the regulation of ULBP1 expression.

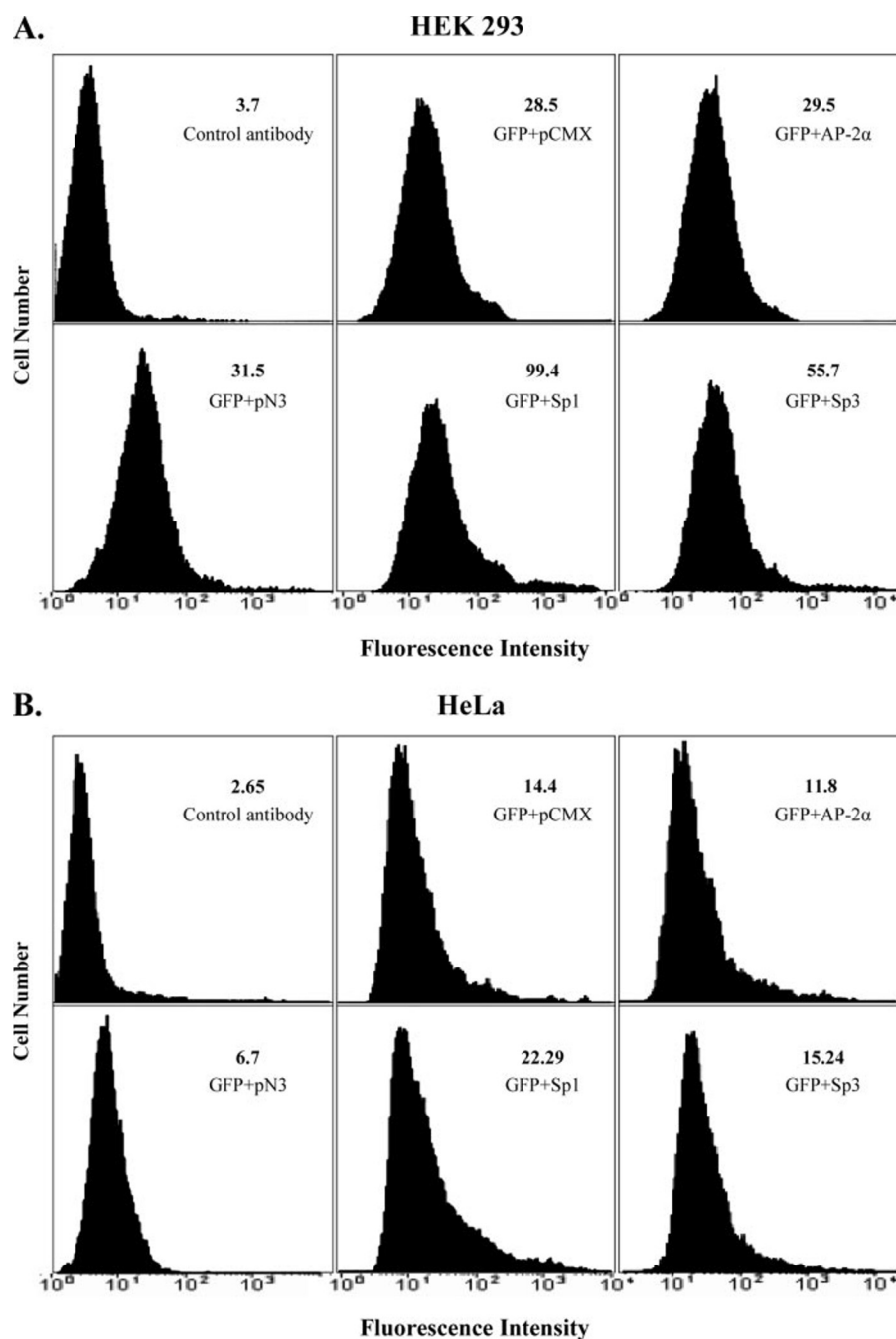
## DISCUSSION

It is well established that transformation can induce NKG2DL expression, and cancer cells expressing NKG2DL become susceptible to NK cell killing *in vitro* and *in vivo* (1–3, 8, 22). These strongly support a role of the NKG2D/NKG2DL system in tumor rejection and surveillance, rendering this system as an interesting target for immunotherapy (15–20). However, the mechanisms involved in NKG2DL induction are largely unknown. Several studies have shown that the expression of individual NKG2DL expression differs substantially in tumor cells, suggesting that individual NKG2DL expression may be induced in response to different stimuli or pathologies. To understand the mechanisms that induce the expression of NKG2DL in cancer cells, we have functionally characterized for the first time the regulation of ULBP1 transcription. Our data



**FIGURE 9. The transcription factors AP-2 $\alpha$ , - $\beta$ , and - $\gamma$  repressed ULBP1 promoter activity in HeLa cells.** A, the ULBP1 promoter construct (–862/+28) (200 ng) was cotransfected with 400 ng of the expression vectors for AP-2 $\alpha$ , - $\beta$ , and - $\gamma$  in HeLa cells. B, the ULBP1 promoter construct with the mutation of the GC(4)/AP-2 site was cotransfected with the expression plasmids for Sp1, Sp3, and Sp4 in HeLa cells. C, two AP-2 $\alpha$  expression vectors, which lack the DNA binding domain, AP-2B and AP-2 $\alpha$ -(1–165) and the expression vector AP-2 $\alpha$ -(165–437), which only expresses the DNA binding domain of AP-2 $\alpha$ , were cotransfected with the ULBP1 promoter construct. In all the experiments, the pRSV- $\beta$  galactosidase plasmid was included to correct the differences in transfection efficiencies. Data are the mean  $\pm$  S.E. of luciferase activity from three independent experiments with triplicates for each experiment.

clearly indicate that Sp3 and Sp1 play a critical role in ULBP1 promoter activity and protein expression. These transcription factors bind to the CRE(1) site located in the minimal promoter of the ULBP1 gene *in vitro* and *in vivo* (Fig. 11), and we demonstrated that the expression of ULBP1 strictly depends on Sp1/Sp3 activity. Thus, the mutation or deletion of the Sp1/Sp3 binding site practically abolished the activity of the ULBP1 promoter in HeLa and HEK 293 cells and in several cell lines analyzed (data not shown). Furthermore, experiments in SL2 cells showed that ULBP1 promoter was practically inactive in



**FIGURE 10. Regulation of ULBP1 protein expression by Sp1, Sp3, and AP-2α.** HEK 293 (A) and HeLa (B) were transiently transfected with pN3, pN3-Sp1, pN3-Sp3FL, pCMX, or pCMX-AP-2α plasmids (2 μg) and with a GFP expression vector (0.5 μg), as indicated. After 48 h, surface expression of ULBP1 protein was analyzed in GFP-expressing cells by flow cytometry using a monoclonal antibody anti-ULBP1 (4G7) as described under "Experimental Procedures." Numbers in the upper right corner indicate the mean fluorescence intensity from ULBP1. These data are representative from three independent experiments with similar results.

absence of the Sp1 family proteins, and it also indicates that Sp3 is the essential activator of ULBP1. Sp1 and Sp3 are ubiquitously expressed transcription factors. Consistent with the high conservation of the DNA-binding zinc finger domain, both Sp1 and Sp3 bind to GC boxes with identical affinity (50–53). This suggested, at first, that both proteins share similar functional properties. However, the data obtained from the analysis of Sp1 and Sp3 knock-out mice and from functional analysis indicated that the physiological roles of these proteins appear to be dif-

ferent (52). This is due to the presence in Sp3 of an inhibitory domain, which suggests that this transcription factor may be an important target of regulatory events (39). Thus, while Sp1 usually stimulates transcription, Sp3 has a dual function as an activator or repressor whose activity is dependent upon context of DNA-binding sites in a promoter. Additionally, the transcriptional role of Sp3 is more complicated, because there are at least four isoforms derived from alternative transcription start sites. The short isoforms lack the transactivation domain, and they are inactive or weak activators (54, 55). With respect to ULBP1, transfection experiments with SL2 cells revealed that the long isoform of Sp3 was a potent regulator of ULBP1 transcription, increasing >500-fold the activity of the ULBP1 promoter, whereas the short isoform of Sp3 was practically inactive. These data together indicate that the regulation of the expression of the different isoforms of Sp3 or the relative levels of Sp1 and Sp3 may have a dramatic impact on ULBP1 expression, and, thus, in the immune response against cancer or infected cells. ULBP1 and other NKG2DLs exhibit a highly restricted pattern of expression in benign tissues, but they are frequently expressed by many epithelial tumors and some hematological malignancies (15, 17, 19, 22, 26). We observed that Sp3 plays an essential role in the expression of ULBP1 in cell lines such as HEK 293 cells, which express significant

amounts of ULBP1, suggesting that regulation of the expression of Sp3 isoforms or its activity may be involved in the induction of ULBP1 expression in cancer cells. A significant shift toward the long isoforms of Sp3 is observed in Sp1<sup>-/-</sup> cells demonstrating that the Sp3 isoform expression can change *in vivo* (52, 56). Unfortunately, it is not known under which physiological conditions the alteration of Sp3 isoform ratio may take place.

Regulation of Sp3 activity may be also achieved post-translationally. For instance, the post-translational modification of Sp3 by sumoylation within its inhibitory domain leads to its inactivation (37, 57), but acetylation stimulates Sp3 activity (58). Sp1 and Sp3 activity may also be regulated by interaction

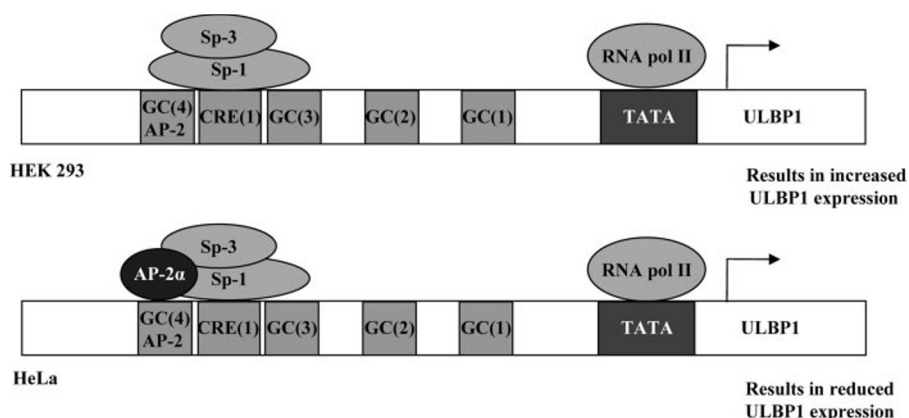


FIGURE 11. **Model of regulation of ULBP1 transcription.** Schematic representation of the ULBP1 promoter and the transcription factors involved in its regulation and shown.

with other proteins such as p300, which is a coactivator with potent histone acetylase activity that may directly acetylate Sp3 or by recruiting repressors with deacetylase activity such as histone deacetylases 1 and 2 (59). Nevertheless, little is known about the regulation of these mechanisms during the transformation process. Recent evidence exists that indicates that the DNA damage control pathway induced the expression of all NKG2DLs, including ULBP1 (6). NKG2DL up-regulation was prevented by pharmacological or genetic inhibition of ATM (ataxia telangiectasia, mutated), ATR (ATM- and Rad3-related) or Chk1, but p53 is not essential for ligand up-regulation. It has been suggested that the expression of NKG2DL in tumor cells may be dependent on ATM activity that is chronically activated in cancer cells due to genomic instability. The mechanism of induction of ULBP1 in cancer cells and the potential role of Sp3 in this process should be explored in the future.

Our findings also revealed that ULBP1 expression may be regulated by AP-2 $\alpha$ . The transcription factor AP-2 $\alpha$  is a prototype of a family of closely related and conserved DNA-binding proteins harboring helix-loop-helix dimerization motifs composed of, at least, AP-2 $\alpha$ , - $\beta$ , and - $\gamma$  (40, 60, 61). We have demonstrated that AP-2 $\alpha$  was bound to the GC(4)/AP-2 site in HeLa cells. Mutation of this sequence and the overexpression of AP-2 $\alpha$ , - $\beta$  and - $\gamma$  indicate that this element is a repressor of ULBP1 transcription in this cell line. Our results also indicate that AP-2 $\alpha$  may repress ULBP1 transcription by DNA-dependent and -independent mechanisms. Of special interest, the repressive effect of an AP-2 $\alpha$  expression vector, which only expresses the DNA binding domain and the positive effect of the mutation of the AP-2 site in the basal activity and in the Sp1- and Sp3-mediated induction of ULBP1 promoter, indicate that displacement of the Sp1/Sp3 is one mechanism of regulating ULBP1 expression. The AP-2 and Sp1/Sp3-binding sites are juxtaposed on the ULBP1 promoter (Fig. 9), and EMSA experiments revealed an overlapping between the Sp1/Sp3 and AP-2 $\alpha$  binding sites in HeLa cells. This indicates that competition of AP-2 $\alpha$  with Sp1/Sp3 for the binding to this sequence may modulate the level of ULBP1 expression. All these data together suggest that the binding to ULBP1 promoter of Sp1 and Sp3 in most of the mammalian tissues may also be modulated by the presence of other transcription factors such as AP-2 $\alpha$ , which shows a restricted expression pattern in adult

tissues, and thereby may be blocked by the regulatory pathways that activate AP-2 $\alpha$ . This transcription factor exerts a key regulatory function in vertebrate embryonic development. In adult cells, expression of AP-2 $\alpha$  favors epithelial differentiation and induces cell cycle arrest and apoptosis (27–29). All these effects together support the role of AP-2 $\alpha$  as a tumor suppressor gene. In agreement, reduction or loss of its expression or activity has been reported in many human cancers of the breast, ovary, colon, skin, and prostate (30–35). The loss of AP-2 $\alpha$

decreases the rate of apoptosis, diminishes the differentiation of epithelial cells, and increases the proliferation rate that allows the cancer cells to progress. However, our experiments have shown that AP-2 $\alpha$  down-regulates the promoter activity and protein expression of ULBP1 in HeLa cells. To conclude, our results indicate that regulation of expression of Sp1, Sp3, and AP-2 $\alpha$  may act as a mechanism that controls ULBP1 expression and, consequently, these transcription factors play an important role in the immunosurveillance against cancer cells. Consequently, the pathways involved in ULBP1 induction may be a productive target for designing new therapeutic agents that enhance immunogenicity of tumor cells.

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