Sp1-Targeted Inhibition of Gene Transcription by WP631 in Transfected Lymphocytes†

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ABSTRACT: The binding of Sp1 transcription factor to DNA is considered a potential target for small ligands designed to interfere with gene transcription. We attempted to distinguish the direct inhibition of the Sp1-binding to DNA in vivo (cell culture) from more indirect effects due to the network of pathways that modulate cell cycle progression, which may decrease transcription without direct interference with Sp1−DNA interactions. We tested whether the Sp3 protein, whose putative binding sequence overlaps the Sp1 site, can inhibit Sp1-activated transcription and interfere with drug−DNA interactions. A well-characterized model system consisting of a wtGLUT1 (wild-type glucose transporter 1) gene promoter, or a mutated mut2GLUT1 promoter, linked to a CAT (chloramphenicol acetyltransferase) reporter gene, was used to analyze the effects of overexpressed Sp1 and Sp3 transcription factors in transiently transfected Jurkat T lymphocytes. Bisanthracycline WP631, a potent inhibitor of Sp1-activated transcription in vitro, was assayed for its ability to specifically inhibit transcription in transfected Jurkat T lymphocytes. The mut2GLUT1 promoter was used to further discriminate between the WP631 interference with Sp1−DNA complexes and Sp3-induced inhibition, since the Sp3-binding site is canceled in this promoter and replaced by a high-affinity binding site for WP631.

Sp1 is a ubiquitous transcription factor that recognizes GC-rich DNA sequences, which are present in many promoters and have been shown to play a direct role in the regulation of transcription (*1*−*6*). Regulation of Sp1-dependent transcription may be affected by changes in Sp1 abundance, while protein phosphorylation has been implicated in changes in both DNA-binding and transcriptional activation (*6*). Sp1 can interact with various proteins involved in cell cycle regulation (*7*−*9*), and it is a key factor in the TGF-β-induced transcriptional stimulation of the proximal promoter of endoglin mediated by Smad proteins (*10*). Sp1 expression also increases during events associated with cell transformation (*11*).

DNA intercalators and some minor-groove binders can inhibit transcription by impeding the binding of transcription factors to certain promoters (*12*−*15*). In general, it is reasoned that drugs that merely prevent protein binding to promoter regions by steric blockage or distortion of DNA might not suffice to render a selective toxic targeting of tumor cells (*16*, *17*). However, the presence of CG-rich tracts specifically recognized by Sp1 in several promoters suggests that DNA−Sp1 complexes are potential specific targets for some small drugs, which may impair Sp1-activated gene transcription, and thus control gene expression in vivo. Recent results obtained with bisanthracycline WP631 have revealed its high preference for targeting of Sp1-binding at nanomolar concentrations (*15*, *18*, *19*).

WP631 bisintercalates into DNA with a binding constant close to that of various transcription factors (*20*). These characteristics make WP631 more effective against some tumor cell lines, including a multidrug-resistant one (*21*, *22*). There are grounds for considering that WP631 is a potent inhibitor of transcription through direct competition with the Sp1 transcription factor (*10*, *15*, *19*, *22*). Results obtained with WP631 using cells in culture suggest that transcription events can be inhibited by WP631 with high specificity. WP631 and mitoxantrone inhibited collagen COL1A1 gene expression in fibroblasts, but WP631 showed a higher potency (*19*). The crucial role of Sp1 in the regulation of COL1A1 has prompted researchers to use WP631, at low nanomolar concentrations, to control the excess fibrotic process in fibroblast diseases (*19*).

Sp1 is the prototype member of a family of related transcription factors consisting of Sp1, Sp2, Sp3, and Sp4 (*6*). All four proteins exhibit very similar structural features. Molecular, genetic, and biochemical analyses have demonstrated that Sp2, Sp3, and Sp4 are not simply functional equivalents of Sp1. Both Sp1 and Sp3 are ubiquitously expressed in human cells, competing for common target sequences (*23*). Sp1 is considered a transcriptional activator, while Sp3 contains a transcription repressive domain and can behave as either a transcriptional activator or repressor (*4*, *6*). Moreover, the behavior of the endogenous Sp1 and

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Sp3 proteins depends on the specific ratio between these proteins (*24*). An intriguing aspect of the inhibition of Sp1-activated transcription in vivo is whether molecules such as WP631 can act synergistically with Sp3 as gene inhibitors or interfere with the physiological equilibrium between the Sp1-family proteins, since all of them recognize very similar DNA sequences.

The effects of WP631 in vivo are not easy to dissect, since the direct effects due to inhibition of the Sp1-binding to DNA should be distinguished from more indirect effects due to the network of pathways related to the control of progression through the cell cycle (*8*, *9*), which may decrease transcription levels without a direct drug interference with Sp1−DNA interactions. For example, in Northern blot assays, it was observed that WP631 inhibits the transcription of *c-myc*, which contains a consensus Sp1 binding site, but also that of *p53*, which lacks putative Sp1 binding sites (*18*, *25*).

To analyze the effects of WP631 on the Sp1-activated transcription in both the presence and the absence of the Sp3 protein, we have transfected Jurkat T lymphocytes with DNA plasmids bearing a chloramphenicol acetyltransferase (CAT)[1] reporter gene, whose expression can be easily followed experimentally under the control of a promoter of the GLUT1 (glucose transporter1) gene promoter (*26*). GLUT1 is highly expressed in proliferating cells, and its 99 bp region upstream of the initiation site suffices to drive transcription (*26*−*28*). Sp1 strongly transactivates the GLUT1 promoter in cells. The Sp3 gene encodes three distinct transcription factors: a full-length Sp3 protein that can activate gene transcription and two internally initiated Sp3 isoforms described as inhibitors of gene transcription (*29*). However, this observation is not universal and might depend on the cellular context (*6*, *29*). Sp3 full-length protein and Sp3 internally translated isoforms reduced the transcriptional activity of the GLUT1 promoter in several nonmuscle and muscle cells, which contributes to the decreased expression of GLUT1 during myogenesis (*26*). Both the wild-type wtGLUT1 promoter and the mut2GLUT1 promoter, mutated at the Sp3 binding site, were cotransfected with Sp1- or Sp3-expression plasmids to analyze all combinations between both protein factors, the presence of adequate targets, and the effects of bisanthracycline WP631 on transcription in Jurkat T lymphocytes.

EXPERIMENTAL PROCEDURES

WP631. Bisanthracycline WP631 was synthesized as previously described (*21*). A 500 μM WP631 stock solution was prepared with sterile 150 mM NaCl and diluted to the desired concentration using RPMI-1640 medium (Gibco) just before use.

Cell Culture Conditions. Jurkat T lymphocytes were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (Gibco), and 2 mM Hepes (pH 7.4), at 37 °C in a humidified atmosphere with 5% CO_2.

[1] Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; MOPS 3-(*N*-Morpholino)propanesulfonic acid, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PMSF, phenylmethyl-sulphonyl fluoride; PBS, phosphate-buffered saline.

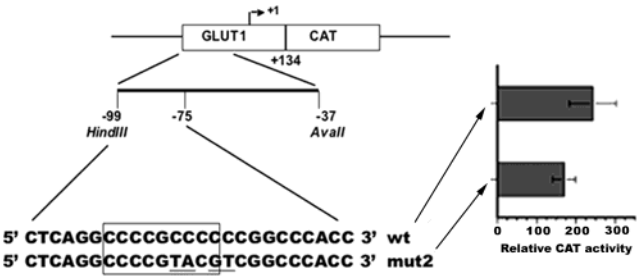

FIGURE 1: Map of the proximal region of the GLUT1 promoter linked to a CAT reporter gene. The sequences of the −99 to −75 fragments of wtGLUT1 and mut2GLUT promoters (upper strands) are shown. The −93 to −85 region contains the CG box that binds Sp1 and Sp3. The mut2GLUT1 promoter contains a high-affinity WP631 binding site, CGTACG (*33*). Plasmids containing these promoters were used for transient transfection of Jurkat T lymphocytes. The figure also shows the expression of GLUT1 promoters transfected in Jurkat T lymphocytes. Data are expressed as relative CAT activity/β-galactosidase activity after 24 h incubation. Results are the means ± SD from four transfection experiments.

Plasmids. The expression plasmids pCMV-Sp1 and pCMV-Sp3 were kindly provided by Dr. R. Tjian (University of California at Berkeley) and Dr. J. M. Horowitz (Duke University Medical Center), respectively. Wild-type (wt) and mutant (mut2) pGLUT1-CAT reporter plasmids were a gift from Dr. A. Zorzano (University of Barcelona). These CAT reporter vectors contain the −99/+134 region of the rat GLUT1 genomic sequence cloned into the pCAT-basic vector (Promega) (*26*, *30*). Partial sequences of the promoters relevant to the present study are presented in Figure 1. A pCMV-β-galactosidase expression plasmid was used as a control of efficiency in the transfection protocol and pCMV-Script vector (Stratagene) as a carrier.

Cell Growth and Cell-Cycle Analyses. The capacity of WP631 to interfere with the growth of Jurkat cells was determined using the MTT assay in the presence of various concentrations of WP631 at 37 °C for 24 h, as described elsewhere (*31*). Absorbance was determined at 570 nm using a SPECTRAmax-250 microplate reader (Molecular Devices). Viable cell number was determined on the basis of the exclusion of Trypan Blue dye (Fluka) using a hemocytometer.

After treatment with WP631 for various periods, the cells were harvested and stained with propidium iodide (Sigma) as described elsewhere (*32*). Nuclei were analyzed with a Coulter Epics-XL flow cytometer at the Serveis Cientifico-Tecnics of the University of Barcelona. Cell percentages at each phase of the cell cycle were estimated from their DNA content histograms after drug treatment.

Transfections, Cotransfections, and Reporter (*CAT ELISA*) *Assays*. Jurkat T lymphocytes were transfected with 2.5 μg of either the wild-type or the mut2 pGLUT1-CAT reporter plasmids and cotransfected with 5 μg of pCMV-Sp1 or pCMV-Sp3 expression vectors and 5 μg of pCMV-β-galactosidase expression plasmid to normalize for the efficiency of transfection. DNA total concentration was ajusted to 20 μg by the addition of parental "empty vector" pCMV-script (Stratagene) as a carrier. Cells subcultured at a density of 1×10^6 cells/mL were transfected by electroporation using a Gene Pulser II (Bio-Rad) and maintained in 24-well plates at 37 °C for 48 h, in a humidified atmosphere with 5% CO_2.

Transfected cells were incubated, when required, with 60 nM WP631 (its IC_{75} concentration in Jurkat T cells, determined after 72 h of continuous treatment (*31*)) for 24 h. Experiments were repeated 3−5 times.

Transfected cells (WP631-treated or untreated cells) were washed with ice-cold PBS and collected by centrifugation. The pellets were resuspended in 1 mL of a lysis buffer consisting of 10 mM MOPS (pH 6.5), 10 mM NaCl, 1mM EGTA, 1% Triton X-100, and 0.1 mM PMSF and incubated for 30 min at room temperature. Cell extracts were centrifuged at 15 000*g* (4 °C) for 10 min, and the supernatants were stored at −80 °C.

Total protein in cell lysates was quantified by the Bradford assay (Bio-Rad). CAT activities of about 50 μg of cell extracts were determined with the CAT ELISA kit (Roche Diagnostics). Absorbance was measured at 405 nm, using 492 nm as a reference wavelength, in a Spectramax-250 microplate reader (Molecular Devices). β-Galactosidase activities were measured using the β-Gal Reporter Gene Assay (Roche Diagnostics) and a Sirius luminometer (Berthold Detection Systems).

Western Blot Analysis. Jurkat cells transfected with either the wild-type or mut2 GLUT1 promoters and the Sp1 and Sp3 expression vectors were used to obtain cell extracts, as described above. Equal amounts of protein (50 μg) were separated by SDS-polyacrylamide on 10% gels and transfected to Optitran BA-S85 membranes (Schleicher and Schuell). The blots were incubated with 5 μg mL^{-1} rabbit polyclonal anti-Sp1 or anti-Sp3 antibodies (both from Santa Cruz Biotechnology). The membrane was washed and incubated with a secondary peroxidase-conjugated anti-(rabbit IgG) antibody, and the bound antibody was detected by chemiluminiscence using luminol (Sigma). Signal intensities were quantified in a Molecular Dynamics computing densitometer and used to determine the relative Sp1 and Sp3 contents in the different transfection experiments.

Electrophoretic Mobility Shift Assays. EMSA assays were performed in a buffer of 10 mM Tris·HCl (pH 7.4) containing 50 mM KCl, 1 mM $MgCl_2$, 5 μM $ZnCl_2$, 2 mM EDTA, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 0.1% Nonidet NP-40, and 5% glycerol. A typical reaction contained about 20 ng of pure Sp1 protein (Promega) and 1500−3000 cpm (about 2 nmol in bp) of a radioactively end-labeled *Hind*III-*AvaII* 62-mer fragment from either promoter (Figure 1), in the presence of 0.5 μg of poly[d(I−C)] (Roche Diagnostics). In reactions containing WP631, the Sp1 protein and the drug were added at the same time to mimic the conditions of the transcription experiments. Following 20 min of incubation, the samples were analyzed on 4.5% nondenaturing polyacrylamide gels containing 45 mM Tris·borate, 1 mM EDTA, and 0.1% Nonidet NP-40 (pH 8.3). After running at 12 V/cm, the gels were dried under vacuum and subjected to autoradiography. Quantitative analysis was performed with a Molecular Dynamics computing densitometer.

RESULTS

We were interested in analyzing changes in gene transcription under strict conditions of cell growth in the presence of WP631. Jurkat T lymphocytes were chosen because with this cell line we had performed previous experiments on the pharmacological effects of WP631, including the effects of the drug on the transcription of genes involved in the control of cell cycle (*18*). Three types of experiments were performed to analyze whether bisanthracycline WP631 inhibits the Sp1-activated transcription of GLUT1 promoters in Jurkat T cells. First, WP631 was added to test the inhibition of a CAT gene reporter under the control of the wild-type wtGLUT1 promoter or the mutated mut2GLUT1 promoter, which lacks canonical Sp3 binding sites (Figure 1) (*26*), in transiently transfected cells, in experiments driven by the intracellular (endogenous) Sp1 and Sp3 and the basal transcription machinery. Second, WP631 was used to inhibit transiently transfected cells with either the wtGLUT1 or mut2GLUT1 promoters, in cells also cotransfected with an Sp1- or an Sp3-expresion plasmids, or both, to obtain various Sp1/Sp3 ratios. Third, EMSA analysis was used to assess the differences in Sp1-binding affinity for the two GLUT1 promoters in vitro and to elucidate how the Sp1 binding to the promoter regions was affected at the molecular level by the presence of increasing concentrations of WP631.

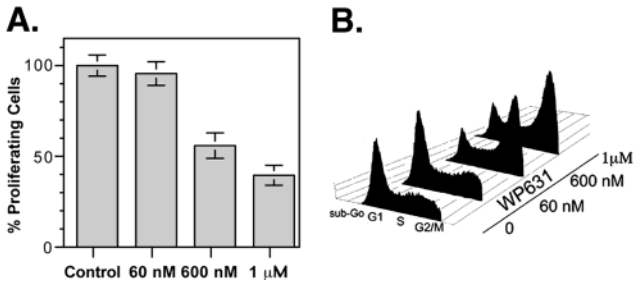

FIGURE 2: Cell proliferation and cell cycle distribution of Jurkat cells treated with various concentrations of bisanthracycline WP631. (A) Percentage of proliferating cells measured as cells able to metabolize MTT after treatment with 60 nM, 600 nM, or 1 μM for 24 h (means ± SD, six experiments) compared to an untreated control. (B) Flow-cytometry analysis of Jurkat T cells incubated with 60 nM, 600 nM, or 1 μM for 24 h.

Cell Growth in the Presence of WP631. Proliferation of Jurkat T lymphocytes treated with three concentrations of WP631 (60 nM, 600 nM, and 1 μM) for 24 h is presented in Figure 2A. WP631 at a concentration of 60nM (which corresponds to its IC_{75}, as determined after 72 h of treatment (*31*)) did not alter the percentage of proliferating cells compared to the untreated control. Only 55% cells treated with 600 nM were proliferating, and 40% were proliferating in the presence of 1 μM WP631. However, the MTT assay fails to distinguish between growth arrest and a reduction in cell number due to cell death. Therefore, the number of viable cells was also determined as the capacity of these cells to exclude the Trypan Blue dye in the experimental conditions shown in Figure 2. Trypan Blue assays indicated that about 5% (600 nM) and 13% (1 μM) cells died during the 24 h treatment. Since the number of proliferating cells decreased after 24 h of treatment with 600 nM or 1 μM while most of these cells remained viable, we also analyzed the cell-cycle distribution to identify the phase of the cell cycle at which proliferation stopped. Figure 2B displays the cell cycle distribution of Jurkat cells treated with various concentrations of WP631 for 24 h. Cells accumulated, in a dose-dependent manner, in G2 after 24 h of treatment (Figure 2B). Cells in G2 were committed to die after longer incubation time (data not shown) in agreement with our previous observations (*31*). Therefore, experiments on gene transcription under strict

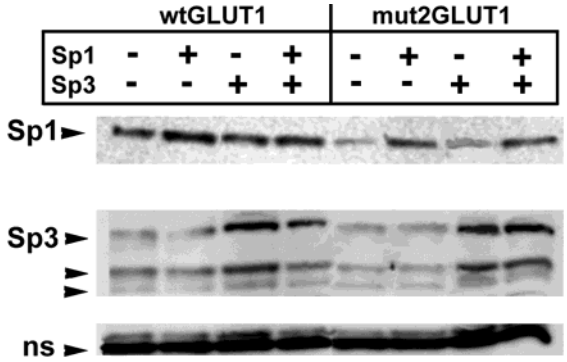

FIGURE 3: Changes in the Sp1 and Sp3 protein levels in Jurkat T cells transfected with the wtGLUT1 and mtGLUT1 promoters. A representative Western blot showing the effect of cotransfecting cells with Sp1 and/or Sp3 expression plasmids. Quantification of the corrected band intensities was used to determine the relative Sp1 and Sp3 contents under the different experimental conditions. pCMV-β-galactosidase expression plasmid was used to normalize for the efficiency of the different transfection experiments. Three isoforms of Sp3 are shown together with a nonspecific 45 kDa band that cross-reacts with the Sp3 antibody, which was used as a normalization control.

conditions of cell growth, in which intracellular Sp1 and Sp3 are directly involved, require the use of a low-nanomolar range concentration of WP631 for 24 h. Concentrations much lower than 60 nM (e.g., 6 nM) were also used in gene expression experiments (see below).

Analysis of the Expression of GLUT1 Promoters after Transient Expression of Sp1 and Sp3 and in the Presence of WP631. Experiments to asses the effects of drug treatment on cells transfected with either wild-type or mutant GLUT1 promoters, in the presence of cotransfected Sp1- and Sp3-expresion plasmids, or the empty expression vector (pCMV-script) as a control of endogenous Sp1 and Sp3 activities, were also optimized to measure the expression of the proteins in proliferating cells. We hypothesized that because the gene expression experiments were based on the quantification of the CAT protein, see Experimental Procedures, the experimental data should be obtained when the transiently expressed plasmids expressed protein levels high enough to transactivate the GLUT1 promoter. Western blot analysis established that changes in Sp1 and Sp3 levels could be observed 24 h after transfection (Figure 3). The experiments presented in Figures 2 and 3 indicated that experiments aimed to analyze the effects of WP631 on the wild-type GLUT1 and mut2GLUT1 promoters in vivo (cell culture) should be carried out 24 h after cotransfection with the plasmids, and in the presence of low (e. g. 60 nM) WP631 concentrations. In these experimental conditions, we deemed that 'sufficient' gene expression of the transformed plasmids, and cell proliferation, occurred.

Transient transfection of pCMV-Sp1 and pCMV-Sp3 separately, or in cotransfection experiments, led to changes in the protein intracellular concentrations and the Sp1/Sp3 ratios (Figure 3). The presence of transfected Sp1-expression plasmid caused a 12% increase in Sp1 protein content in cells (Figure 3), while transfection with the Sp3-expression plasmid caused a 40% increase in the Sp3 content. It is conceivable that this apparently modest increase might represent a major change of the relative levels of both proteins inside the cells transformed with the GLUT1 promoters, since not all cells were cotransfected. Therefore, cotransfection with an Sp3-expression plasmid should produce a large reduction of the Sp1/Sp3 ratio, which might alter the activity of either wtGLUT1 and mut2GLUT1 promoters, as described below.

WP631 Inhibits the Expression of GLUT1 Promoters Driven by the Endogenous Transcription Machinery. Results plotted in Figure 4 (bars wt and mt, and their equivalent bars in Figure 1) would indicate that the transient expression of the construct containing wtGLUT1 was more activated by the endogenous cell transcription machinery. The presence of 60 nM WP631 in the cell culture media sufficed to inhibit, by about 30%, the CAT reporter activity linked to either promoter under the control of the endogenous Sp1 and Sp3 and the basal transcription machinery of lymphocytes (cf. bars wt and mt versus wt + W and mt + W in Figure 4). WP631 and endogenous Sp1 and Sp3 protein factors might compete for DNA binding, with almost identical WP631 efficiency, to attenuate the transcription of both promoters. A lower WP631 concentration, 6 mM, was also assayed, but it did not alter the GLUT1-driven transcription significantly (not shown).

In the Presence of Cotransfected Sp1 and Sp3, the Transcription of wtGLUT1 and mut2GLUT1 Promoters Was Differently Inhibited by WP631. We compared the relative CAT activities in cells transfected with GLUT1 promoters in the presence/absence of cotransfected Sp1 and/or Sp3 and WP631 (Figure 4, bars a−l).

Overexpressed Sp1 activated gene transcription similarly in experiments using both promoters (Figure 4, bars a and c), which was inhibited by bisanthracycline WP631, yet with distinct effectiveness: 30% inhibition versus 100% inhibition (Figure 4, bars b and d). The effect of WP631 was stronger on the mut2GLUT1 promoter, which contains a high-affinity CGTACG binding-site for WP631 (Figure 1) (33). For the sake of comparison, Figure 4 displays, side by side, the experimental conditions with and without WP631. WP631 and overexpressed Sp3 did not suffice to completely inhibit the transcription of the wtGLUT1 promoter in vivo in the presence of overexpressed Sp1 (Figure 4, bar j), but they did when the mutant promoter was used (Figure 4, bar l). In all cases analyzed, the presence of WP631 significantly inhibited gene transcription (Student's t-test, $p < 0.05$; see legend to Figure 4), except when the wtGLUT1 promoter was cotransfected with both expression plasmids. In this case, the drug did not significantly alter the transcription levels (Figure 4, bars i and j), which is further analyzed in the Discussion section. Taken together, these results are in agreement with the view that the Sp1/Sp3 ratio determines the transcription levels of GLUT1 promoters in proliferating cells (*26*−*28*) and the three distinct gene isoforms encoded by the Sp3 expression plasmid ((*29*) and Figure 3) act as repressors on GLUT1 promoters (*26*).

Sp1/Sp3 Protein Ratio Determines the wtGLUT1 Promoter Activity in Transfected Lymphocytes and Modulates the Effects of WP631. The control of transcription by the Sp1/Sp3 ratio was corroborated using the wtGLUT1 promoter, whose activity was inhibited by Sp3 in the absence of cotransfected Sp1 (Figure 4, bar e), which correlates with the changes in protein levels detected by Western blot (Figure 3). In addition, the presence of Sp3 protein and WP631 abrogated the transcription activity of the wtGLUT1 promoter (Figure 4, bar f). Cotransfection with both protein-expression

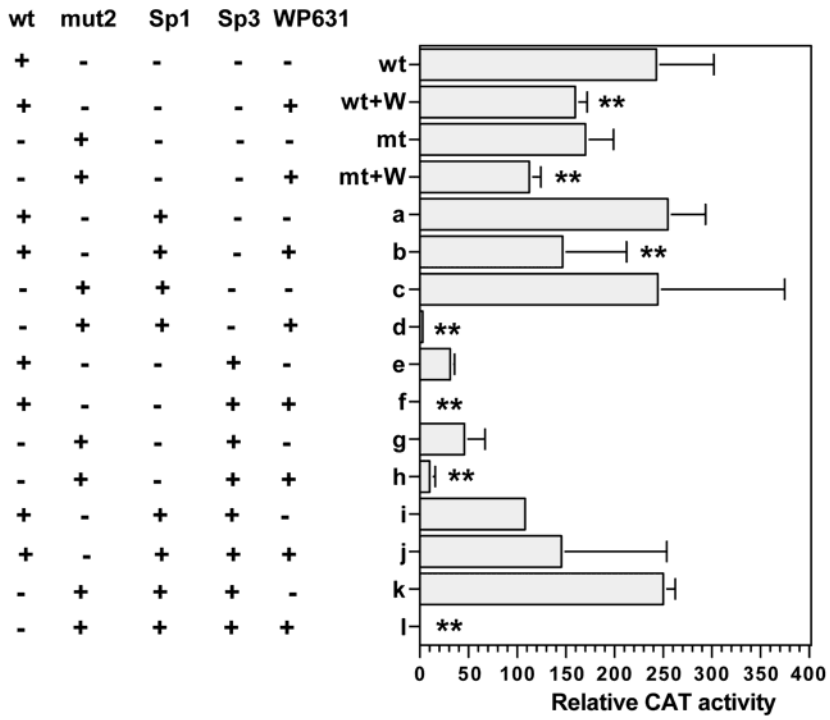

FIGURE 4: Inhibition of transcriptional activity by WP631. The figure shows the effects of proteins Sp1 and Sp3 and bisanthracycline WP631 on the transcriptional activity of transfected wtGLUT1 and mut2GLUT1 promoters in Jurkat T lymphocytes. Transcriptional activity was measured by the CAT reporter assay and expressed as relative CAT activity/β-galactosidase activity (means $\pm$ SD) from three to four experiments. Bars labeled as wt and mt correspond to the CAT activities measured in Jurkat cells that were cotransfected with wtGLUT1 and mut2GLUT1 promoters (see legend to Figure 1 for further details), while bars a, c, e, g, i, and k are the expression levels in the presence of cotransfected protein expression plasmids. Differences in the relative CAT activity due to the presence of bisanthracycline WP631 that were statistically significant (**$p < 0.05$) are indicated.

plasmids almost halved the CAT reporter activity driven by the wtGLUT1 promoter (cf. bars a and i in Figure 4), suggesting that overexpressed Sp3 partially inhibited Sp1 binding to the wtGLUT1 promoter. WP631 did not act efficiently when the two proteins were overexpressed (Figure 4, bar j), in contrast to what was observed in the experiments performed without cotransfection with protein expression plasmids (cf. bars wt + W and j in Figure 4). In any case, the results obtained with the wtGLUT1 promoter do not allow us to differentiate clearly the inhibition by Sp3 from that caused by the drug.

Using mut2GLUT1 Promoter Provides Direct Dissection of WP631 Effects on Sp1-Activated Gene Expression in Vivo. The capacity of WP631 to inhibit DNA−Sp1 interactions in vivo was further supported by experiments using the mut2GLUT1 promoter, whose transcription was completely inhibited by WP631 (Figure 4, bars h and l). We would like to highlight that the Sp3-binding site is canceled in this promoter (*26*, *30*), while EMSA experiments showed, see below, that Sp1 can bind to this promoter yet with a lower affinity than to the wild-type promoter (Figure 5A), in agreement with the observed Sp1-activated transcription of the mut2GLUT1 promoter (Figure 4) (*26*).

In the presence of overexpressed Sp1, WP631 reduced the Sp1-activated transcription levels in the mut2GLUT1 promoter by more than about 100% (Figure 4, bar d). This suggests that the presence of a specific drug-binding site accounted for the differences observed. We found the same degree of inhibition by WP631 in lymphocytes cotransfected with Sp1 plus Sp3, in agreement with the fact that Sp3-binding site is canceled in this promoter (*26*) (cf. bars d and l versus bars c and k in Figure 4). Unexpectedly, cotransfection of Jurkat T cells that contained the transfected mt2GLUT1 promoter with Sp3-expression plasmid resulted in a lower activity compared to the same promoter driven by the endogenous proteins (cf. bar g versus bar mt in Figure 4). This behavior may be accounted for by postulating that the cotransfected "extra" Sp3 (Figure 3) inhibited transcription by squelching intracellular Sp1; thus, the effective concentration of Sp1 that can bind to the mut2GLUT1 promoter was reduced. When the two transcription factors were cotrasfected, the mut2GLUT1 promoter activity was higher than in the presence of overexpressed Sp3 (cf. bars k and g in Figure 4). In fact, the CAT activity measured after cotransfection was higher (Figure 4, bar k) than in the mutant promoter driven by the endogenous proteins (Figure 4, bar mt), a result that emphasizes the importance of the relative Sp1 and Sp3 levels (*26*) in the behavior of the GLUT promoters.

In contrast to the transcription driven only by the endogenous cell factors, WP631 exerted a stronger effect on the mut2GLUT1promoter, regardless of the presence of cotransfected Sp1, Sp3, or Sp1 plus Sp3 (Figure 4, bars d, h, and l). Our data support that WP631 was the unique active inhibitor of the mutated promoter and that it directly inhibited the Sp1−DNA interaction in vivo.

Sp1−Promoter Complexes Are Distinctly Susceptible to WP631 in Vitro. Figure 5 shows band shift (EMSA) analyses of the Sp1 binding to 62-mer fragments of the wtGLUT1 and mut2GLUT1 promoters (Figure 1), which contain consensus protein-binding sites. Cancellation of the Sp3 site in mut2GLUT1 did not destroy the overlapping Sp1-binding

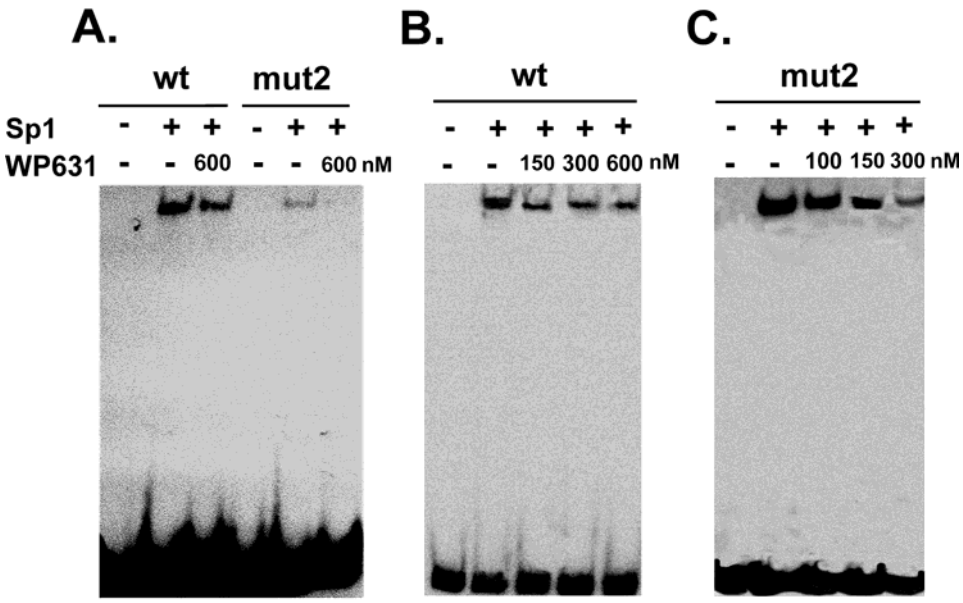

FIGURE 5: Effect of WP631 on the binding of the Sp1 protein factor to the 62-mer HindIII−AvaII fragment of wtGLUT1 and mut2GLUT1 promoters. (A) EMSA experiment performed in parallel for both promoters under strictly the same conditions from sample preparation and manipulation to gel electrophoresis in the same polyacrylamide gel to show that Sp1 binds better to the wild-type promoter and that the WP631 concentrations required to inhibit the DNA−Sp1 complex differ. (B and C) representative EMSA experiments showing the effect of increasing concentrations of WP631 on the stability of Sp1−DNA complexes using the 62-mer fragment of the wtGLUT1 or mut2GLUT1 promoters, respectively. In the wild-type GLUT 1 promoter, 150, 300, and 600 nM WP631 produced 26, 35, and 39% inhibition of the Sp1−DNA complex, respectively, while in the mut2GLUT1 promoter, 100, 150, and 300 nM WP631 produced 23, 32, and 47% inhibition, respectively.

site but altered the putative Sp1-binding sequence (*26*), yet Sp1 transactivated the mutated promoter (Figure 4). Experiments performed in parallel using both promoter fragments demonstrated that the Sp1 binding to the wild-type promoter was about 6-fold higher than to the mutant promoter in the presence of a large excess of competing DNA (poly[d(I−C)]) (Figure 5A).

Figures 5B and 5C show representative examples of experiments carried out in duplicate, in which WP631 inhibits the formation of Sp1−DNA complexes. When a fragment of the wild-type promoter was used, 600 nM WP631 inhibited the formation of Sp1−DNA complex by about 40%. The same WP631 concentration inhibited completely the Sp1−DNA interaction when the mutant promoter was used (Figures 5A and 5B). These results suggest that the protein binds tighter to the wtGLUT1 promoter. In the EMSA experiments, 300 nM WP631 achieved 35% inhibition of the Sp1−DNA band for the wild-type promoter, while approximately the same inhibition required only 150 nM WP631 in the mut2GLUT promoter, in which 300 nM drug produced a 47% inhibition (Figures 5B and 5C). These results were consistent with previous studies on Sp1 binding to each promoter, which revealed stronger Sp1-binding to wtGLUT1 (*26*), and they are also in agreement with the distinct susceptibility of the two promoters to transcription inhibition by WP631. Nevertheless, the drug concentrations required to disrupt the complex in vitro (Figure 5) and to inhibit the CAT reporter activity (Figure 4) were not quantitatively comparable because of the differences in experimental conditions.

DISCUSSION

Previous studies have shown that WP631 is a potent inhibitor of transcription, as this bisanthracycline inhibits Sp1-activated transcription at low nanomolar range concentrations both in vitro and in vivo (cell culture) (*15*, *18*, *19*, *22*). However, certain drugs can lose "potency" as assay conditions become more complex (*13*, *34*). Therefore, we aimed to dissect the inhibiting effects produced by the drug from those arising from the presence of some transcription factors binding to overlapping sequences. We have shown that the conditions used in our experiments (60 nM drug, CAT determination performed 24 h after transfections) were essential to ensure that cells proliferated in the presence of the drug (Figure 2) and that the cotransfected expression plasmids were functional. These observations are consistent with the view that transcriptional activity depends on a given Sp1/Sp3 ratio, which can be modified by transient expression of plasmids (Figures 3 and 4), and it is subjected to changes in cell cycle and cellular conditions (*6*). They are also consistent with the view that cells with enhanced growth are more susceptible to changes in gene expression produced by WP631 than barely proliferating cells (*35*).

We sought to gain new insights into the WP631 effects on transcription in vivo by testing whether the Sp3 protein, whose putative binding sequence overlaps the Sp1 site, inhibits Sp1-activated transcription and interferes in drug−DNA interactions. We took advantage of the mut2GLUT1 promoter that lacks the Sp3-binding site (*26*), while encompassing a high-affinity binding site for WP631 (CGATCG) elucidated by NMR and X-ray studies (*33*, *36*), to dissect the transcription attenuation produced by the drug (Figure 4).

Sp3 has been described as a bifunctional transcription regulator because it can repress Sp1-mediated transcription by competing with Sp1 for their overlapping binding sites or behave as an activator of gene transcription (*4*, *24*, *37*). The Sp3/Sp1 ratio is known, for example, to modulate the

transcription of the GLUT1 promoter during myogenesis (*26*, *27*). In this paper, we have differentiated some specific effects of Sp1, Sp3, and WP631 on the transcription of the GLUT1 promoter (Figure 4). High levels of endogenous Sp1 have been described in Jurkat T cells (*38*), which may limit the response to additional (transiently expressed) Sp1, yet about a 12% increment in the Sp1 protein levels can be detected after cotransfection experiments (Figure 3). Thereby, the similar levels of CAT reporter activity observed under the control of the wtGLUT1 promoter (Figure 1, and Figure 4, bars wt and a). However, coexpression of Sp1 and Sp3 altered the situation in transiently transfected Jurkat T cells. The presence of overexpressed Sp3 (Figure 3) reduced significantly the Sp1/Sp3 ratio and inhibited wtGLUT1 promoter activity even in the presence of additional Sp1 (Figure 4, bars a and i), which resembles the regulation of the GLUT1 promoter in some cells (*26*). These results are in agreement with the view that the Sp1/Sp3 ratio, which changed depending on the cotransfected plasmids (Figure 3), determines the transcription levels of GLUT1 promoters in proliferating cells (*26*−*28*) and in accordance with the view that the three distinct gene products encoded by the Sp3 expression plasmid (*29*) act as repressors in GLUT1 promoters (*26*). Inside cells, the binding of one or another of these protein factors to the overlapping sites may depend not only on their relative concentrations but also on the intrinsic affinities of Sp1 and Sp3 for their putative sites. The finding of gene repression after the cotransfection with wtGLUT1 promoter is consistent with previous studies showing that Sp3 efficiently competes for the binding to the wtGLUT1 promoter (*26*, *28*). Sp3 partially inhibited the activation by Sp1 in this promoter (Figure 4).

WP631 shows a clear preference for interfering with Sp1−DNA complexes (*10*, *15*, *18*, *19*, *39*). Our results support the hypothesis that it is an extremely potent inhibitor of Sp1 binding in vivo (*19*, *22*) and also indicate that drug effects depend on two key elements: the relative levels of Sp3 protein and the exact composition of the Sp1 binding site, since the drug effects on activated transcription differed in wtGLUT1 and mut2GLUT1 promoters (Figure 4). WP631 binds better to the mutant promoter (Figure 5), which encloses a high-affinity binding site (Figure 1). This is consistent with the fact that higher concentrations of the bisanthracycline were required to disrupt the tighter binding of Sp1 to the wtGLUT1 promoter in vitro (Figure 5). Previous experiments, using an unrelated promoter, showed the strong capacity of WP631 to inhibit Sp1−DNA complexes, even in the absence of a canonical 6 bp binding site for WP631 (*15*), thus suggesting that WP631 binding is facilitated by Sp1-induced bending (*40*).

As mentioned in Results, the experiments using wtGLUT1 cotransfected with Sp1 and Sp3 plasmids brought about a condition in which the presence of added WP631 in transiently transfected Jurkat T lymphocytes did not abrogate gene activity, since the drug did not compete efficiently when the proteins were in excess (Figure 4, bars i and j). WP631 may thus be incapable of increasing the inhibiting capability of Sp3 because the protein and the drug can compete for overlapping binding sites. Further discussion on the origin of the competition between both proteins and WP631 can be only tentative. Figure 6 presents a model in which DNA bending by Sp3 can be required for correct (efficient) drug

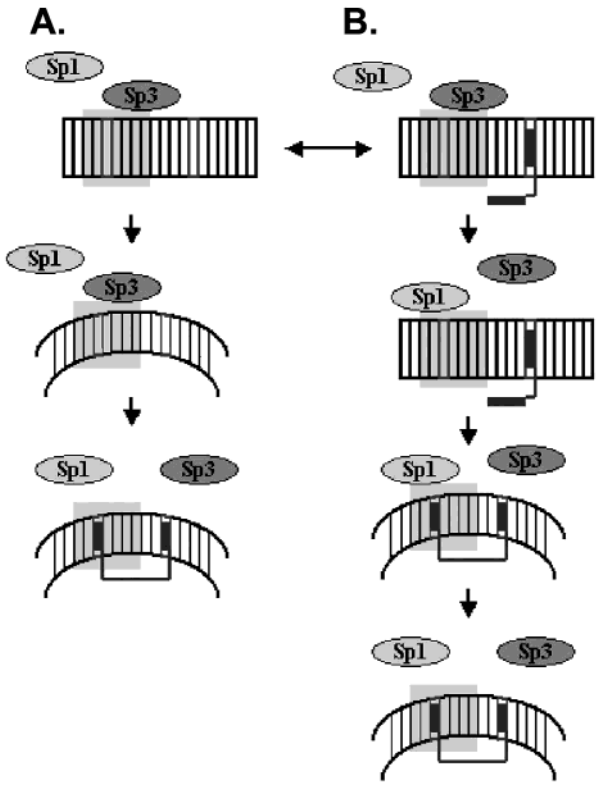

FIGURE 6: Schematic model that illustrates the opposite effects of overexpressed Sp1 and Sp3 on the wtGLUT1 promoter in the presence of WP631, which result in the partial abrogation of transcription. Panels A and B correspond to two alternative pathways that might end in the repression of the CAT reporter activity under the control of wtGLUT1 promoter and in the presence of overexpressed Sp1 and Sp3 proteins and bisanthracycline WP631. The model is based on results presented in Figure 4 (experiments represented by bars i and j should be compared with those represented by bar a). To construct this model, we have considered the bending that can be induced by Sp1 or Sp3 upon binding (*40*) and the change in DNA structure due to the DNA unwinding produced by the bisintercalated WP631, which is known to displace the binding of Sp1 to DNA (*15*) and may also displace that of Sp3. (A) Model suggests Sp3-induced bending of DNA. (B) Model considers that monointercalation of WP631, in the vicinity of the Sp3 binding site, displaced the protein, thus allowing Sp1-binding. Eventually, Sp1 is displaced by WP631, and thus repression is achieved.

binding, as suggested for a Sp1−WP631−DNA complex (*15*). The WP631 molecule is rather flexible and may monointercalate at isolated CG sites, yet with a lower binding affinity than when bisintercalating at 6 bp CG-rich tracts (*41*). We envisage a scenario, schematized in Figure 6, in which, to some extent, the presence of drug molecules, monointercalated in some neighboring CG sites, should alter protein binding after that drug intercalation unwinds the double helix, thereby changing the Sp3-binding affinity, which in turn allows the binding of overexpressed Sp1. DNA bending can be induced by Sp1 or Sp3 upon binding (*40*), and the change in DNA structure would facilitate the formation of bisintercalated WP631−DNA complex. The DNA unwinding produced by bisintercalated WP631 is known to displace the Sp1 binding to DNA (*15*). It is worth noting that WP631 completely abrogated the mut2GLUT1 activity in vivo, even though Sp3 did not bind to the mutated promoter. Simply, the protein-induced bending of DNA was not required because the promoter encompassed a specific 6 bp drug-binding site (Figure 1).

Neither of the techniques used here can establish the nature of the structural variations occurring after binding by itself, but the magnitude of Sp1-induced DNA bending is known to have key consequences on drug binding (*15*). In any case, the protein-induced curvature of a particular promoter may depend on the exact composition of the G-box and the neighboring sequences (*42*). Sp1 binds to the wtGLUT1 promoter better than to the mut2GLUT promoter (Figure 5) (*26*), while WP631 appears to better inhibit the Sp1-binding to the mut2GLUT1 promoter by two complementary, Sp3-independent mechanisms. First, WP631 binds tightly to the mut2GLUT1 promoter that contains a high-affinity CGATCG binding site. Second, it competes more efficiently with Sp1 (Figure 5), which, in turn, displays a looser binding to the mut2GLUT1 promoter.

The effects of WP631 on both transiently expressed GLUT1 promoters were considered to be independent of those on other intracellular genes, which either contain Sp1 binding sites (including an active endogenous GLUT1 promoter in lymphocytes) or can be indirectly repressed through the complex network of control pathways in the cell. Inside the cells, several genes involved in cell cycle control may be regulated by Sp1 and/or Sp3 and by some gene products that could directly interact with Sp1 (*8*, *9*). Our approach has allowed us to dissect WP631 and Sp3 effects on Sp1-transactivated transcription in vivo. The concentration of WP631 used in the experiments with Jurkat T lymphocytes (60 nM WP631) is lower than that normally used to inhibit transcription by other DNA-binding ligands (*12*, *13*, *43*), and thus it is more likely to be attained in physiological conditions (*19*, *22*, *31*). In fact, an analysis of the WP631 uptake rate revealed that even smaller concentrations of the drug may be located inside the nuclei of cells treated with this drug concentration (*31*), and thus the transcription-inhibiting effect may well be due to a rather lower intracellular concentration of WP631, within the nanomolar range.

Collectively, our results show that WP631 specifically inhibits Sp1-activated transcription and may compete with Sp3 binding to finally inhibit transcription in vivo. Hitherto, only the unrelated drug ecteinascidin 743, which does not intercalate into DNA (*16*, *44*), also recognizes Sp1−DNA at low nanomolar concentrations and inhibits transcription (*45*).

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