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Sp1-p53 Heterocomplex Mediates Activation of HTLV-I Long Terminal Repeat by 12-*O*-Tetradecanoylphorbol-13-acetate That Is Antagonized by Protein Kinase C

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We have previously demonstrated that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activates human T-cell leukemia virus type-I long terminal repeat (LTR) in Jurkat cells by a protein kinase C (PKC)-independent mechanism involving a posttranslational activation of Sp1 binding to an Sp1 site located within the Ets responsive region-1 (ERR-1). By employing the PKC inhibitor, bisindolylmaleimide I and cotransfecting the reporter LTR construct with a vector expressing PKC-α, we demonstrated, in the present study, that this effect of TPA was not only independent of, but actually antagonized by, PKC. Electrophoretic mobility shift assays together with antibody-mediated supershift and immuno-coprecipitation analyses, revealed that the posttranslational activation of Sp1 was exerted by inducing the formation of Sp1-p53 heterocomplex capable of binding to the Sp1 site in ERR-1. Furthermore, we demonstrated that Jurkat cells contain both wild-type (w.t.) and mutant forms of p53 and we detected both of them in this complex at variable combinations; some molecules of the complex contained either the w.t. or the mutant p53 separately, whereas others contained the two of them together. Finally, we showed that the Sp1-p53 complexes could bind also to an Sp1 site present in the promoter of another gene such as the cyclin-dependent kinase inhibitor $p21^{WAF-1}$, but not to consensus recognition sequences of the w.t. p53. Therefore, we speculate that there might be several other PKC-independent biological effects of TPA which result from interaction of such Sp1-p53 complexes with Sp1 recognition sites residing in the promoters of a wide variety of cellular and viral genes.

INTRODUCTION

Human T-cell leukemia virus type-I (HTLV-I) has been implicated with the etiology of adult T-cell leukemia (ATL), tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM), and certain other clinical disorders (Uchiyama, 1997). However, following infection, this virus enters into a latent state, rendering the infected individuals seropositive asymptomatic carriers, and only a small percentage of these carriers may, eventually, develop one of the HTLV-I-related diseases 20–40 years later (Aboud *et al.,* 1993; Cann and Chen, 1997). Although the pathogenic mechanism of this virus is not yet completely clear, the viral transactivator *Tax* protein is widely regarded as a key element in this mechanism, due to its multiple effects on a variety of cellular transcription (Armstrong *et al.,* 1993; Li and Gaynor, 1999; Li *et al.*, 1999; Mesnar and Devaux, 1999) and cell-cycle controlling (Harhaj *et al.*, 1999; Mesnar and Devaux, 1999; Ressler *et al.,* 1997; Tsukahara *et al.*, 1999) factors and because of its interference with DNA repair (Cereseto *et al.,* 1996; Jeang *et al.,* 1990; Kao and Marriott, 1999; Philpott and Buehring, 1999; Ressler *et al.*, 1997; Uittenbogaard *et al.*, 1994). With these pleiotropic effects *Tax* may transform HTLV-I-infected cells (Grassmann *et al.*, 1989; Tanaka *et al.*, 1990) and set them into uncontrolled replication (Grassmann *et al.*, 1992; Harhaj *et al.*, 1999; Mesnar and Devaux, 1999; Robek and Ratner, 1999) and genetic instability (Maruyama *et al.*, 1992, 1996; Miyake *et al.*, 1999). However, during the latent period, viral gene expression in the carriers' infected cells is extremely low (Brauweiler *et al.*, 1995; Kinoshita *et al.*, 1989) and consequently the level of *Tax* protein is, plausibly, below the pathogenically effective threshold. Therefore, it is conceivable that generating an HTLV-I-related disease would require an activation of the latent virus to elevate the level of this protein. Thus, investigation of exogenous or intrinsic factors capable of initiating *Tax*-independent HTLV-I expression is of particular importance for a better understanding of the first step of activation of the dormant virus and its transition from the asymptomatic latency to its pathologically active phase.

Phorbol and diterpen esters are examples of exoge-

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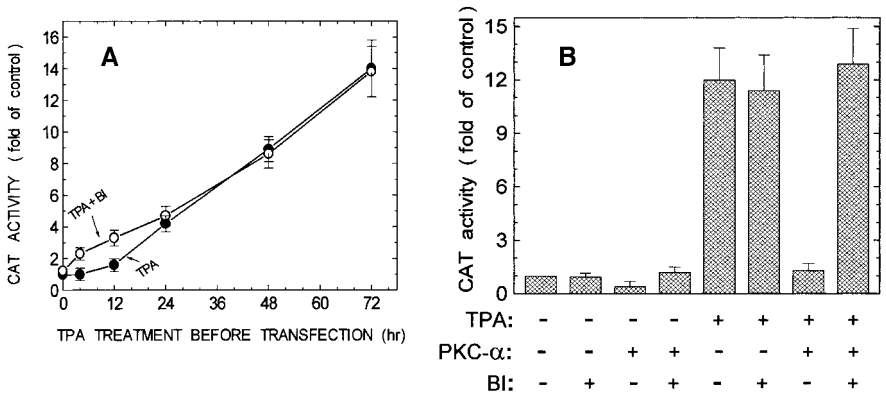

FIG. 1. Effect of TPA, BI, and PKCα on HTLV-I LTR-CAT expression. (A) Jurkat cells, pretreated with TPA alone (●) or with TPA + BI (○) for the indicated times, were transfected with HTLV-I LTR-CAT construct. (B) The HTLV-I LTR-CAT construct was transfected alone or together with the PKC-α expressing vector into the Jurkat cells under the specified conditions. Where indicated, TPA treatment was for 72 h before transfection and BI was added 1 h before TPA. CAT activity was measured 48 h after transfection, normalized to the protein concentration of each cell extract, and presented as fold of the activity detected in the untreated control cells. The illustrated data represent the average of three repeated experiments ± standard deviation.

nous agents that have been shown to activate the expression of HTLV-I and reporter genes driven by its long terminal repeat (LTR) in the absence of *Tax* protein (Aboud *et al.*, 1993; Carter and Blakeslee, 1989; Fujii *et al.*, 1987; Mor-Vaknin *et al.*, 1997; Nakao *et al.*, 1984; Radonovich and Jean, 1989; Torgeman *et al.*, 1999). These compounds are plant products (Matsuda *et al.,* 1986; Nakao *et al.*, 1984; Touraine *et al.*, 1977) potentially accessible to human contact or consumption. Therefore, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the most intensively studied representative of these compounds, may serve as a model for investigating possible mechanisms of activation of the latent HTLV-I.

TPA is a potent protein kinase C (PKC) activator (Jaken, 1990). There are at least 12 PKC isoenzymes, which regulate a wide variety of biochemical pathways in cells (Coussens *et al.*, 1986; Mellor and Parker, 1998) and therefore most of the biological effects of TPA have been attributed to PKC activation (Jaken, 1990). Yet. we have previously shown that, although prolonged exposure of Jurkat cells to TPA severely depletes them from the TPA-responsive PKC isoenzymes, the expression of a reporter HTLV-I LTR construct is highly stimulated when transfected into such PKC-depleted cells (Mor-Vaknin *et al.*, 1997). We have also noted that addition of PKC inhibitors prior to TPA does not abrogate this stimulation (Mor-Vaknin *et al.*, 1997; Torgeman *et al.*, 1999). This excludes the possibility that the delayed effect of TPA is mediated by a long lasting factor that has been activated by PKC prior to its depletion. Subsequent experiments have indicated that this effect is exerted through a post-translational activation of the Sp1 transcription factor binding to an Sp1 recognition site located within the Ets responsive region 1 (ERR-1) of the viral LTR (Torgeman *et al.*, 1999).

The present study provides evidence that the effect of TPA on HTLV-I LTR expression, as well as the activation of Sp1, are not only independent of PKC, but are actually antagonized by its activity. Furthermore, this study demonstrates that the posttranslational activation of Sp1 is exerted by inducing the formation of Sp1-p53 heterocomplex. Notably, Jurkat and certain other T-cell lines contain both wild-type (w.t.) and mutant forms of p53 protein (Cheng and Haas, 1990; Gualberto and Baldwin, 1995; Gualberto *et al.,* 1995) and our present experiments indicate that both forms participate in the formation of this heterocomplex.

RESULTS

PKC antagonizes the TPA stimulatory effect on HTLV-I LTR expression and on the nuclear protein binding to the Sp1 site in ERR-1

We have previously shown that TPA enhances HTLV-I LTR expression in Jurkat cells by a PKC-independent mechanism (Mor-Vaknin *et al.,* 1997; Torgeman *et al.*, 1999). To further characterize this effect, it was subjected to a more detailed time-course analysis in the presence and absence of the PKC inhibitor bisindolylmaleimide (BI) (Toullec *et al.,* 1991), which proved, in our previous study (Torgeman *et al.*, 1999), to be highly efficient as an *in situ* PKC inhibitor. Figure 1A shows no or very little stimulation of HTLV-I LTR-CAT expression in Jurkat cells transfected with this construct after 4 or 12 h of TPA treatment, i.e., when active PKC was declining but still present in the cells (see Mor-Vaknin *et al.,* 1997, for detailed kinetics of the TPA-induced PKC downregulation in these cells). Notably, LTR expression at these time points was significantly enhanced when BI was added 1 h before TPA. However, when this construct was transfected after longer TPA pretreatment (i.e., when the cells were already depleted of PKC), its expression progressively increased as the duration of the TPA pretreatment increased. Moreover, no significant effect of BI was de-

tected after such extended TPA pretreatment. It is, thus, evident that the LTR stimulation by TPA is more efficient when PKC activity is either diminished by a specific inhibitor or depleted by a prolonged exposure to this phorbol ester. This notion, that active PKC antagonizes this stimulatory effect of TPA, was further tested by cotransfecting the LTR-CAT construct with a vector expressing constitutively active PKC-α (pZLPKC-α), which is one of the major PKC isoenzyme in Jurkat cells (Mor-Vaknin *et al.,* 1997). Figure 1B shows that this exogenous PKC markedly reduced the basal LTR expression in TPA-untreated control cells and completely abolished its stimulation by a prolonged TPA pretreatment. It is also shown that both of these inhibitory effects were diminished by the inhibitor BI, thus assuring that they were induced by the exogenous PKC.

Our recent study (Torgeman *et al.,* 1999) has also shown that nuclear proteins of Jurkat cells form three distinct complexes with an oligonucleotide carrying the ERR-1 sequence (see Fig. 2A for a schematic presentation of ERR-1) that we have designated as **I, II,** and **III** (see Figs. 2B and 2C). Further experiments have shown that complex **I** is preferentially stimulated by prolonged TPA treatment, whereas complex **II** is stimulated to a much lower extent and complex **III** is unaffected. Competitive electrophoretic mobility shift assay has revealed that complex **I** is formed on the Sp1 site and complex **II** on the pets site present within ERR-1, and subsequent deletion functional analysis of ERR-1 have demonstrated that the Sp1 site (i.e., complex **I**) is the element mediating the LTR stimulation in the PKC-depleted cells (Torgeman *et al.,* 1999). The significance of the moderate complex II elevation has not been clarified yet. In view of these data and the PKC antagonism against LTR stimulation by the prolonged TPA treatment illustrated in Fig. 1, it was important to assess the role of PKC in complex **I** formation. For this purpose we compared, in the next experiment, the effect of brief TPA treatments (4 and 12 h) on this complex formation to that of prolonged treatments (24 and 48 h). Figure 2B indicates that while the prolonged treatments profoundly stimulated complex **I** formation, the brief treatments elicited negligible effect, unless they were done in the presence of the PKC inhibitor, BI. This finding strongly suggests that PKC activity antagonizes the stimulatory effect of TPA on HTLV-I LTR expression by interfering with complex **I** formation.

An additional indication for this antagonism emerged from the experiment presented in Fig. 2C, which demonstrated that 72-h treatment of the cells with the calcineurin inhibitor, cyclosporin A (CSA), markedly stimulated complex **I** formation, but when added together with TPA, their combined effect was considerably lower than that of each of them alone. Calcineurin dephosphorylates serine/threonine targets of various serine/threonine protein kinases, including PKC (Scott, 1997). Therefore, these results suggest that preventing dephosphorylation of certain serine/threonine targets of a non-PKC protein kinases stimulates complex **I** formation, whereas preserving the phosphorylation of certain serine/threonine targets of PKC rather inhibits this complex formation. Moreover, it appears from these results that the negative effect of the PKC-mediated phosphorylation is dominant over the stimulatory phosphorylation by the non-PKC protein kinases. Consistent with this interpretation, Fig. 2C shows that when the PKC-mediated phosphorylation was blocked by BI, the joint effect of CSA and TPA became cumulative.

A similar TPA-induced PKC-independent stimulation of HTLV-I LTR expression (Fig. 3A) and complex **I** formation (Fig. 3B) was also observed in two other T-cell lines, SupT1 and CEM×174, indicating that this phenomenon was not unique to Jurkat cells.

Complex I is formed by Sp1 and p53 proteins

We have previously shown that complex **I** formation is stimulated by TPA through a posttranslational activation of Sp1 binding to the Sp1 site in ERR1 (Torgeman *et al.,* 1999). Other laboratories have demonstrated that under certain conditions some missense mutants of p53 protein associate with the Sp1 protein to form a complex that activates the expression of HIV-1 LTR and certain other promoters by binding to their Sp1 recognition sites (Borellini and Glazer, 1993; Gualberto and Baldwin, 1995; Ohlsson *et al.,* 1998; Sawaya *et al.,* 1998). There are also indications for a similar association between Sp1 and w.t. p53 protein (Gualberto and Baldwin, 1995; Gualberto *et al.,* 1995; Sawaya *et al.,* 1998). Therefore, we investigated whether p53 was involved in the TPA-induced posttranslational Sp1 activation. This was done by analyzing the effect of anti-Sp1 and anti-p53 (pAb421) antibodies on complex **I** electrophoretic mobility. Figure 4A shows that each of these antibodies supershifted the migration of this complex, while completely eliminating the original band **I** (lanes 3 and 4, respectively). This finding indicates that band **I** represents a single complex, containing both the Sp1 and the p53 proteins together, rather than a mixture of two distinct but similarly migrating complexes, one that contains only Sp1 and the other only p53. This conclusion was further supported by the observation that a combination of these two antibodies supershifted complex **I** significantly further as a single complex (band **Ic** in lane 5) rather than separating it into the two differently migrating supershifted complexes seen in lane 3 and 4. The specificity of this supershift was confirmed by the failure of two irrelevant antibodies, anti-albumin (lane 2) and anti-c-Jun (lane 6), to affect complex **I** migration.

Complex I contains both wild-type and mutant forms of p53 protein

Jurkat cells, as well as certain other T-cell lines, have been reported to contain both w.t. and mutant forms of

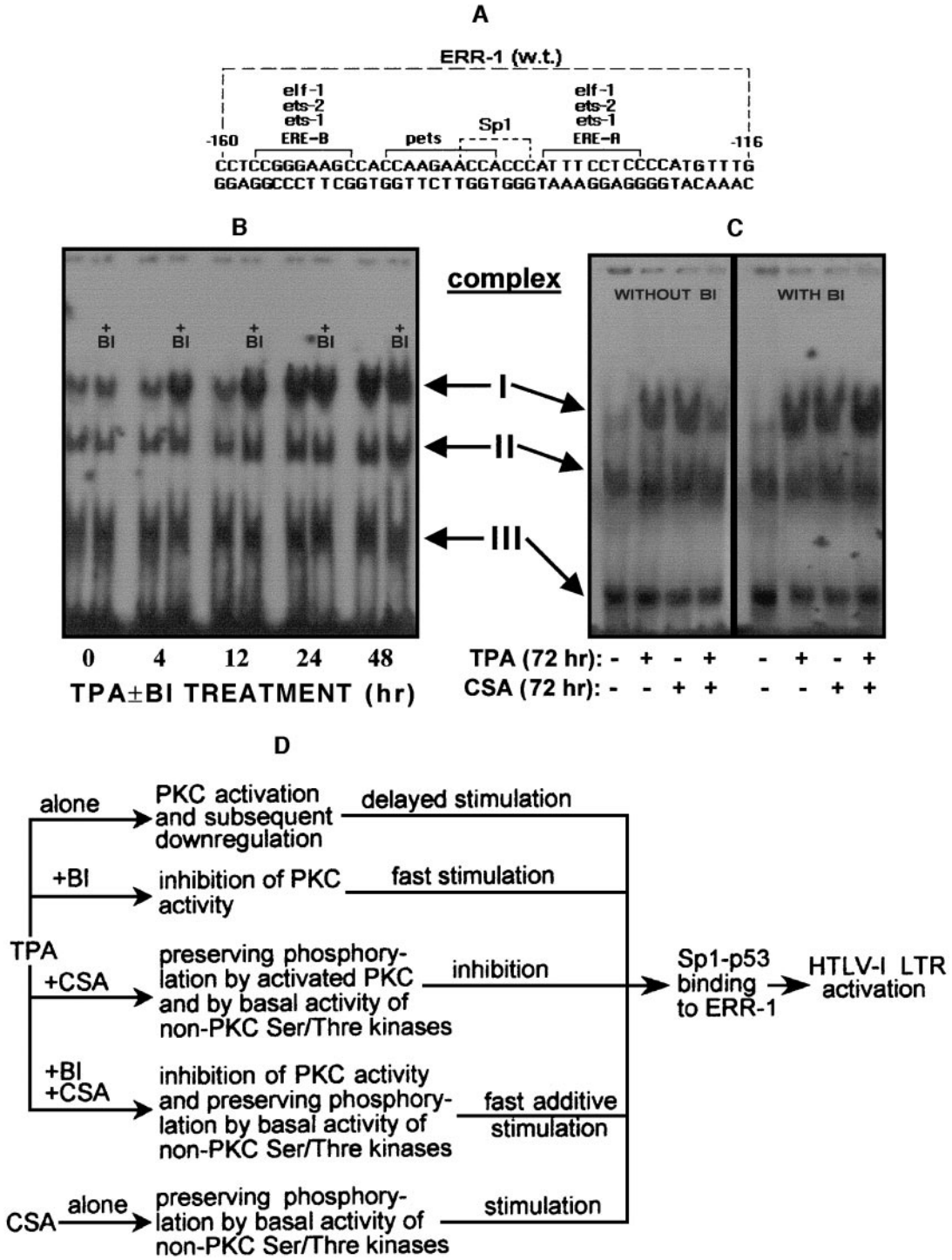

FIG. 2. Effect of TPA, CSA, and BI on the binding of nuclear proteins of Jurkat cells to ERR-1. (A) Schematic presentation of ERR-1. The nucleotide sequence of ERR-1 and the location of the binding sites of various transcription factors known to interact with ERR-1 are shown. The numbers represent the position of the nucleotides in the viral LTR relatively to the transcription starting point. (B) Nuclear extracts, prepared from cells treated with TPA alone or with TPA + BI for the indicated times, were analyzed for protein binding to P^{32}-labeled ERR-1oligonucleotide. (C) Cells were treated with TPA or TPA + CSA in the absence (left) or presence (right) of BI for 72 h (BI was added 1 h before the other agents), and their nuclear extracts were tested for protein binding ^{32}P-labeled ERR-1 oligonucleotide. (D) The data in B and C are schematically summarized.

p53 protein (Cheng and Haas, 1990; Gualberto and Baldwin, 1995; Gualberto *et al.,* 1995). The presence of both p53 forms in our Jurkat cells was confirmed by reacting their nuclear extract with either pAb1620 or pAb240 antibodies (which exclusively recognize only w.t. and only mutant p53, respectively) and by subjecting the immunoprecipitates to Western blot analysis with rabbit polyclonal anti p53 antibodies. Notably, a comparable level of

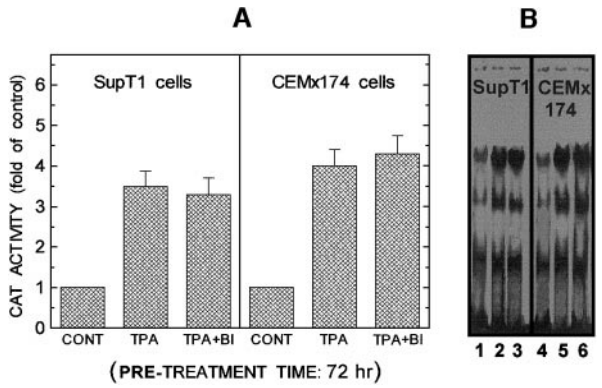

FIG. 3. Effect of TPA and BI on HTLV-I LTR-CAT expression and on nuclear protein binding to ERR-1 in SupT1 and CEM×174 cells. (A) SupT1 (left) and CEM×174 (right) cells pretreated with TPA alone or with TPA + BI for the indicate times were transfected with HTLV-I LTR-CAT construct and assayed for CAT activity 48 h after transfection as in Fig. 1A. (B) SupT1 (left) and CEM×174 (right) cells were treated for 72 h with TPA alone (lanes 2 and 5) or with TPA + BI (lanes 3 and 6). Untreated cells (lanes 1 and 4) served as control. Nuclear extracts prepared from these cells were tested for protein binding to ^{32}P-labeled ERR-1 oligonucleotide.

both p53 forms was detected in the nuclear extract of TPA-treated and untreated cells (Fig. 4C). Since both p53 forms can potentially associate with Sp1 (Gualberto and Baldwin, 1995; Gualberto *et al.,* 1995; Sawaya *et al.,* 1998), it was of interest to find out which of them participated in the TPA-induced complex **I** formation. For this purpose, we performed supershift analysis of complex **I** with pAb1620 and pAb240 antibodies. Figure 4B illustrates that each of these two antibodies only partially supershifted complex **I** (lanes 3 and 4). Furthermore, in addition to the remaining band **I,** each of these antibodies formed two new bands, **IA** and **IB**. This suggests that both forms of p53 participate in forming a mixture of Sp1-p53 complexes with variable w.t./mutant p53 combinations. Some of these complexes seem to contain Sp1 with only w.t. p53 molecules and therefore are maximally supershifted by pAb1620 antibodies (band **IB** in lane 4), but are not supershifted at all by pAb240 antibodies (band **I** in lane 3). Others contain Sp1 with only mutant p53 molecules and therefore are maximally supershifted by the pAb240 antibodies (band **IB** in lane 3), but not at all by the pAb1620 antibodies (band **I** in lane 4). The remaining complexes contain Sp1 associated with both w.t. and mutant p53 molecules together. Such complexes can bind fewer molecules of each of these antibodies and are therefore intermediately supershifted by both pAb240 (band **IA** in lanes 3) and pAb1620 (band **IA** in lanes 4). As predicted, all these heterocomplexes were maximally supershifted by pAb421 antibodies, which recognize both w.t. and mutant p53 (band **IB** in lane 2).

TPA induces the Sp1-p53 association independently of their binding to DNA

Next, we elucidated whether Sp1 and p53 proteins were interdependent on each other for their binding to the same Sp1 site. For this purpose, aliquots of the nuclear extract of Jurkat cells, treated with TPA for 72 h, were incubated with the pAb421 anti-p53 or with the anti-Sp1 antibodies and after removal of the immunoprecipitates the supernatants were tested for binding to ERR-1 oligonucleotide. If Sp1 and p53 could bind the Sp1 site independently of each other, each of these supernatants would be expected to form an electrophoretically faster complex in place of the original complex **I**. However, Fig. 5A excludes this possibility by showing that both supernatants lost, indeed, their ability to form the original complex **I,** but did not form any new complex instead (see lane 3 for the Sp1-free supernatant and lane 4 for the p53-free supernatant). Both supernatants retained their ability to form the other two complexes, thus verifying the specificity of the immunoprecipitation. This specificity was further confirmed by the failure of the two irrelevant antibodies, the anti-albumin (lane 2) and the anti-c-Jun (lane 5) to affect the formation of any of the three complexes.

To determine whether the association between Sp1 and p53 requires their binding to the target DNA, the immunoprecipitates obtained in the experiment depicted in Fig. 5A were subjected to Western blot analysis. As shown in Fig. 5B, anti Sp1 antibodies readily detected the 97-kDa Sp1 protein in the immunoprecipitate pulled down by pAb421 antibodies from the nuclear extract of

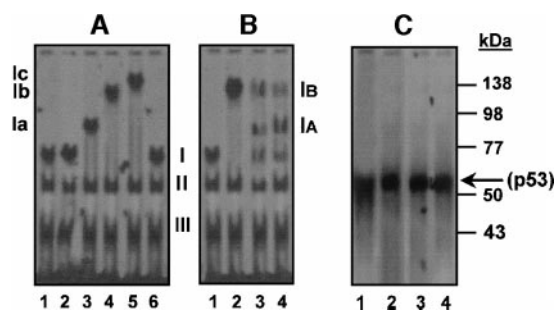

FIG. 4. Supershift analysis of complex I and detection of w.t. and mutant p53 in nuclear extract of Jurkat cells. The nuclear extract of cells treated with TPA for 72 h was reacted with ^{32}P-labeled ERR-1 oligonucleotide in the presence of the following antibodies: (A) none (lane 1), anti-albumin (lane 2), anti-Sp1 (lane 3), pAb421 which recognizes both w.t. and mutant p53 (lane 4), anti-Sp1 + pAb421 (lane 5), and anti-c-Jun (lane 6); (B) none (lane 1), pAb421 anti-p53 (lane 2), pAb240 which recognizes only mutant p53 (lane 3), and pAb1620 which recognizes only w.t. p53 (lane 4). The resulting complexes were analyzed for their gel electrophoretic mobility. (C) Nuclear extracts of the TPA-treated (lane 1 and 2) and untreated (lane 3 and 4) cells were reacted with pAb1620 (lane 1 and 3) or pAb240 (lane 2 and 4) antibodies and the immunoprecipitates were subjected to Western blot analysis with rabbit anti-p53 polyclonal antibodies and peroxidase-conjugated mouse antibodies against rabbit IgG.

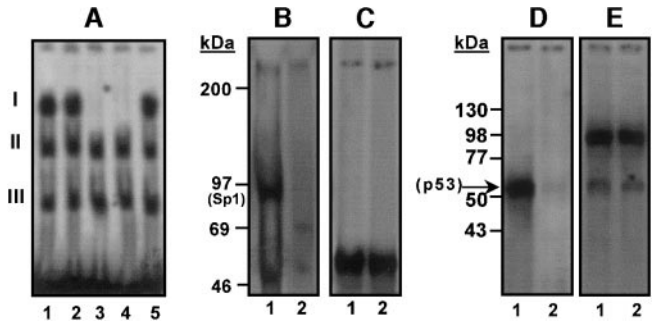

FIG. 5. Characterization of complex I formation by immuno-coprecipitation analysis. (A) Aliquots of the nuclear extract of Jurkat cell, treated with TPA for 72 h, were reacted with the following antibodies: none (lane 1), anti-albumin (lane 2), anti-Sp1 (lane 3), pAb421 anti-p53 (lane 4), and anti-c-Jun (lane 5). The resulting immunoprecipitates were removed by protein A-bound Sepharose CL-4B beads and the supernatants were tested for binding to ^{32}P-labeled ERR-1 oligonucleotide. (B) The immunoprecipitates pulled down by the pA421 anti p53 antibodies from nuclear extracts of TPA-treated (72 h, lane 1) and untreated (lane 2) Jurkat cells were subjected to Western blot analysis with mouse anti-Sp1 antibodies and peroxidase-conjugated rabbit anti-mouse IgG antibodies. (C) The filter of B was stripped and reacted with rabbit anti-p53 antibodies and peroxidase-conjugated mouse anti-rabbit IgG antibodies. (D) The immunoprecipitates, pulled down by the mouse anti-Sp1 antibodies from nuclear extracts of the above TPA-treated (lane 1) and untreated (lane 2) cells, were subjected to Western blot analysis with rabbit polyclonal anti-p53 antibodies and peroxidase-conjugated mouse antibodies against rabbit IgG. (E) The filter of D was stripped and reacted with mouse anti-Sp1 antibodies and peroxidase-conjugated rabbit anti-mouse IgG antibodies. Size markers are shown at the left side of B and D and they apply also for C and E, respectively.

the TPA-treated cells (lane 1), whereas this protein was below detectable level in the immunoprecipitate prepared from TPA-untreated control cells (lane 2). Stripping the blot from the anti Sp1 antibodies and reacting it with rabbit anti p53 polyclonal antibodies revealed comparable p53-specific bands in both lanes (Fig. 5C). A reciprocal Western blot analysis of the immunoprecipitates pulled down by the mouse anti-Sp1 antibodies is illustrated in Fig. 5D. In this analysis p53 protein was readily detected by rabbit anti-p53 antibodies in the immunoprecipitate of the TPA-treated cells (lane 1) but not of the untreated control cells (lane 2). Reacting this blot with anti Sp1 antibodies, after stripping it from the anti-p53 antibodies, revealed comparable Sp1 specific bands in lanes 1 and 2 (Fig. 5E). Notably, semiquantitative Western analysis of the whole nuclear extracts confirmed that exposure to TPA had no effect on the nuclear level of either Sp1 or p53 proteins (Fig. 6). This finding, together with the coprecipitation results shown in Figs. 5B and 5D, strongly suggests that TPA induces the Sp1/p53 association by a posttranslational event, independently of their binding to DNA targets.

Sp1-p53 complex binds to consensus recognition sites of Sp1 but not of w.t. p53

Since some of the Sp1-p53 complexes formed in the TPA-treated cells contain w.t. p53 protein, it was important to verify whether these complexes also bind to p53 consensus recognition sites and activate promoters containing such sites. In the first experiment, the pG13-CAT construct (which contains 13 copies of p53 consensus recognition sequences) was transfected into Jurkat cells pretreated for 72 h with TPA and to untreated control cells. Figure 7A shows that the basal expression of this construct in the untreated cells was extremely low. This low expression can be attributed to the mutant p53 present in these cells, since it is well known that mutant p53 negatively transdominates the transcriptional transactivation function of the w.t. p53 (Kerns *et al.,* 1992). Furthermore, the expression of this construct was not affected at all by the TPA pretreatment (Fig. 7A). The trivial possibility that the employed pG13-CAT construct might be defective was ruled out by showing that its expression was markedly stimulated when cotransfected with the w.t. p53-expressing pc53-SN3 vector (Fig. 7A). Therefore, these data strongly suggest that the TPA-induced Sp1-p53 complex does not activate gene expression through w.t. p53 consensus recognition sites. This was further supported by demonstrating that an oligonucleotide, carrying the w.t. p53 consensus recognition sequence (p53, r.s.), could not compete out complex I formation on ERR-1 probe (Fig. 7B, lane 2), neither could it form any complex with nuclear proteins of TPA-treated or untreated cells (Fig. 7B, lanes 5 and 6). This oligonucleotide proved, however, to be highly functional in forming a specific complex with a purified w.t. p53 protein (Fig. 7B, lane 7), thus ruling out the possibility that it might have been defective. These data are consistent with other reports showing that under certain conditions, w.t. and mutant p53 proteins associate with certain other transcription factors and influence their specific activity rather than conferring a p53 DNA-binding specificity on the resulting complexes (Agarwal *et al.,* 1998).

In contrast; Fig. 7B shows that excess of an oligonucleotide with the Sp1 site of the p21^{WAF-1} promoter (Sp1/

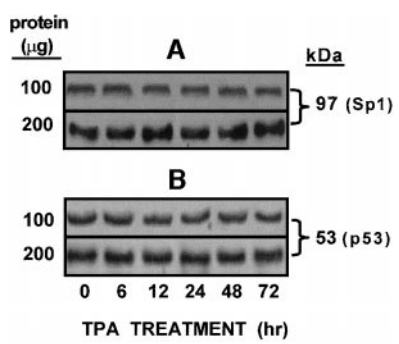

FIG. 6. Semiquantitative Western blot analysis of TPA effect on the nuclear level of Sp1 and p53 proteins. The indicated quantities (100 and 200 μg protein) of nuclear extracts prepared from Jurkat cells after treatment with TPA for the indicated times were subjected to Western blot analysis with (A) anti-Sp1 or (B) pAb421 anti-p53 antibodies. Size markers and purified Sp1 and p53 proteins (not shown) were run in parallel in the corresponding gels.

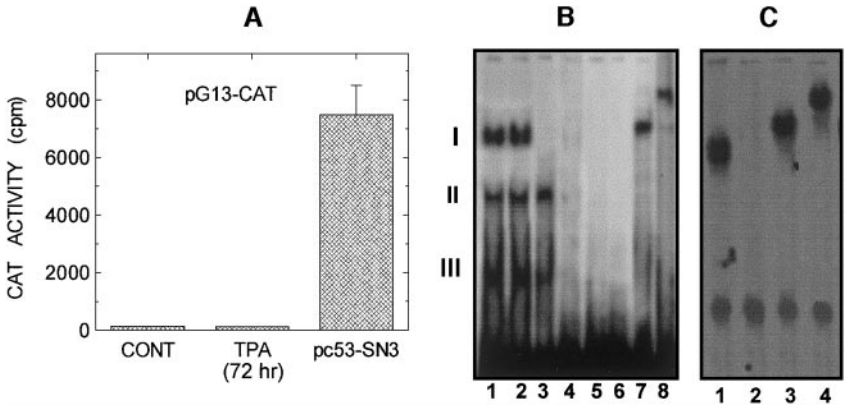

FIG. 7. Effect of TPA on the expression of w.t. p53 responsive promoter and examination of Sp1-p53 binding to w.t. p53 and Sp1 consensus recognition sites. (A) The pG13-CAT construct was transfected to Jurkat cells pretreated for 72 h with TPA and to untreated control cells. To verify that pG13 promoter is functional and can be activated in these cells, the pG13-CAT construct was cotransfected with the w.t. p53-expressing pc53-SN3 vector. (B) The nuclear extract of the TPA-treated cells was reacted with ^{32}P-labeled ERR1 oligonucleotide without competitor oligonucleotides (lane 1) or with 50-fold molar excess of unlabeled p53, r.s. (lane 2), Sp1, WAF-1 (lane 3), and ERR-1 (lane 4) oligonucleotides. Lanes 5 and 6 show the results of reacting ^{32}P-labeled p53, r.s. oligonucleotide with the nuclear extracts of the TPA-treated and untreated cells, respectively. Lane 7 shows the complex formed by purified w.t. p53 protein on p53, r.s. oligonucleotide, whereas lane 8 shows a supershift of this complex by pAb421 anti p53 antibodies. (C) The nuclear extract of the TPA-treated cells was reacted with ^{32}P-labeled Sp1, WAF-1 oligonucleotide alone (lane 1) or in the presence of 50-fold molar excess of unlabeled ERR1 oligonucleotide (lane 2), anti-Sp1 antibodies (lane 3), and pA421 anti-p53 antibodies (lane 4). The resulting complexes were subjected to gel electrophoretic mobility analysis.

WAF-1) specifically blocked only complex I formation on the ERR-1 probe (lane 3), whereas excess of the ERR-1 oligonucleotide eliminated all the three bands formed on this probe (lane 4). In addition, Fig. 7C shows that the Sp1/WAF-1 oligonucleotide formed two complexes with nuclear proteins of TPA treated cells (lane 1) and that only the upper band was competed out by excess of the ERR-1 oligonucleotide (lane 2). Moreover, this complex was supershifted by anti Sp1 (lane 3) as well as by the pAb421 anti p53 antibodies (lane 4). Together these data suggest that the Sp1-p53 complex can bind to Sp1 consensus recognition sites of various genes.

DISCUSSION

The present study demonstrated that the stimulatory effect of TPA on HTLV-I LTR expression and complex I formation on ERR-1 probe was not only independent of PKC activity but was actually antagonized by this activity. In addition, by employing the calcineurin inhibitor, CSA, in various combinations with TPA and BI, we provided evidence to suggest that certain non-PKC serine/threonine protein kinase(s) stimulate complex I formation and that this stimulation was also antagonized by PKC. Of note in this context is our previously reported observation that the basal activity of protein kinase A (PKA), present in cells in the absence of cAMP-stimulating signals, is essential for the basal expression of HTLV-I LTR (Turgeman and Aboud, 1998). It is tempting to speculate that the stimulation of LTR expression, noted when the cells were exposed to CSA alone, might result from preserving the phosphorylation mediated by this basal PKA activity. We are currently investigating whether the effective phosphorylated serine/threonine residues reside in the proteins comprising complex I or in a certain other factor that indirectly affects this complex formation.

Supershift analysis with anti-Sp1 antibodies and the pAb421 anti-p53 antibodies indicated that complex I contains both Sp1 and p53 proteins. Jurkat and certain other T-cell lines are heterozygous for p53 and produce both w.t. and mutant forms of p53 protein (Cheng and Haas, 1990; Gualberto and Baldwin, 1995; Gualberto *et al.,* 1995). Further analysis of complex I, with antibodies specifically recognizing only mutant (pAb240) or only w.t. (pAb1620) p53, revealed that both p53 forms were present in this complex at variable combinations. Interestingly, the effect of TPA differs in this respect from those of GM-CSF and TNF, which have been shown by others to stimulate a selective association of Sp1 only with mutant p53 (Borellini and Glazer, 1993; Gualberto and Baldwin, 1995). However, *in vitro* experiments have shown that association between Sp1 and w.t. p53 is also possible (Kerns *et al.,* 1992; Sawaya *et al.,* 1998).

Immuno-coprecipitation analysis revealed that the TPA-induced Sp1-p53 complex formation occurs independently of the binding of these proteins to the target DNA sequences. In addition, no significant TPA-induced elevation could be detected in the level of either Sp1 or any of the p53 forms, indicating that TPA stimulated the formation of their heterocomplexes by a posttranslational event. This finding is consistent with numerous studies showing that the activity of Sp1 and p53 is regulated by posttranslational events, such as phosphorylation (Anderson, 1993; Baudier *et al.,* 1992; Chernov *et al.,* 1998; Esposito *et al.,* 1997; Legget *et al.,* 1995; Milne *et*

al., 1995; Zhang and Kim, 1997), dephosphorylation (Yan *et al.,* 1997; Zhang and Kim, 1997), and protein–protein interactions (Borellini and Glazer, 1993; Chen *et al.,* 1994; Farmer *et al.,* 1996; Gu *et al.,* 1997; Gualberto and Baldwin, 1995; Hansen *et al.,* 1996; Lu and Levin, 1995; Mundt *et al.,* 1997; Ohlsson *et al.,* 1998; Thut *et al.,* 1995, 1997).

Gel-shift and competition analyses demonstrated that the Sp1-p53 complex can bind to Sp1 sites but not to consensus recognition sequences of the w.t. p53. Since promoters of many cellular genes contain Sp1 binding sites (Azizkhan *et al.,* 1993), it is possible that at least some of the cellular responses to TPA and other agents capable of inducing Sp1-p53 complex formation (Borellini and Glazer, 1993; Gualberto and Baldwin, 1995; Ohlsson *et al.,* 1998; Sawaya *et al.,* 1998) are mediated by this complex. In addition, this complex may explain cases where genes lacking p53 binding sites have been found to be activated by w.t. or mutant forms of p53 (Borellini and Glazer, 1993; Gualberto and Baldwin, 1995; Ohlsson *et al.,* 1998). Also notable is our finding that Sp1-p53 complex binds to the Sp1 site of the $p21^{WAF-1}$ promoter. This gene is an immediate downstream target of the w.t. p53 and its transactivation by this tumor suppressor leads to cell-cycle arrest which frequently precedes the onset of apoptosis (El-Diery *et al.,* 1994; Levin, 1997). However; $p21^{WAF-1}$-mediated cell-cycle arrest has been reported to occur also independently of w.t. p53 (Zeng and El-Diery, 1996). It is quite possible that in some of these cases $p21^{WAF-1}$ activation is mediated by Sp1-p53 complex. Indeed in a parallel study (in preparation) we have found that exposure of Jurkat cells to TPA induces a temporary elevation of $p21^{WAF-1}$ protein, leading to a temporary cell-cycle arrest which is followed by apoptosis. Because of the presence of the mutant p53 in these cells, which negatively transdominates the w.t. p53 (Kerns *et al.,* 1992), it very likely that this $p21^{WAF-1}$ elevation was exerted though the binding of the Sp1-p53 complex rather than through w.t. p53. In addition, we have noted that a genotoxic agent, like cisplatin, also induces apoptosis and HTLV-I LTR expression in these cells through a similar Sp1-p53 complex (unpublished data). This may suggest that environmental or/and intrinsic DNA damaging agents and other factors capable of inducing cell-cycle arrest and apoptosis might be involved in activating the latent virus in HTLV-I carriers. It is of interest to mention in this context certain earlier reports demonstrating that the viral *Tax* protein inhibits cell-cycle arrest and apoptosis, whereas other studies have indicated that *Tax* rather enhances the effect of cell-cycle arresting and apoptosis inducing agents. However, the potential of *Tax* to protect from cell-cycle arrest and apoptosis appears to be dominant in HTLV-I-transformed T-cells (Mesnar and Devaux, 1999). It is, therefore, reasonable to postulate that after activation of the latent virus in HTLV-I carriers the emerging *Tax* protein acts to rescue the host cells from the cell-cycle arrest and the apoptotic death induced by the virus activating agents. In this manner *Tax* may enable the subsequent stepwise progression of these cells toward the HTLV-I-related adult T-cell leukemia.

Finally, the ability of TPA to exert its stimulatory effect on the LTR expression in the absence of *Tax* further supports the view that this effect may serve as a model for the mechanism of the latent virus activation in HTLV-I carriers, since this activation is supposed to take place in cells containing extremely low *Tax* level. Although we have found *Tax* to synergistically enhance the expression of HTLV-I LTR in cells subjected to prolonged TPA pretreatment (unpublished data), it is rather unlikely that *Tax* acts to enhance complex I formation on ERR-I, since *Tax* has been shown in two of our previous studies (Mor-Vaknin *et al.,* 1997; Torgeman *et al.,* 1999) to elicit comparable stimulatory effects on w.t. LTR and ERR-1 lacking mutant LTR.

MATERIALS AND METHODS

Cells

The human T-cell lines Jurkat, SupT1, and CEM×174 employed in this study were kindly provided by Dr. Irvin S. Y. Chen (UCLA, Los Angeles, CA). They were grown in RPM1–1640 medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies

Mouse monoclonal antibodies against Sp1, c-Jun, and albumin, rabbit polyclonal anti-p53, rabbit anti-mouse-IgG, and mouse anti rabbit-IgG antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Mouse anti-human p53 monoclonal antibodies clones pAb421 (recognizing both w.t. and mutant forms of p53), pAb240 (recognizing only mutant forms of p53), and pAb1620 (recognizing only w.t. p53) were a generous gift from Professor Varda Rotter (Weizman Institute of Science, Rehovot, Israel).

Plasmids

The plasmids used in this study were HTLV-I LTR-CAT (Mor-Vaknin *et al.,* 1997), pG13-CAT expressing CAT through a promoter containing 13 copies of the consensus binding sequence of the w.t. p53 protein (Kerns *et al.,* 1992), pc53-SN3 expressing w.t. p53 through the hCMV promoter (Kerns *et al.,* 1992), and pZLPKC-α expressing the PKC-α isenzyme through MoMuLV LTR (Eldar *et al.,* 1990).

Treatment with TPA, bisindolylmaleimide I, and cyclosporin A

Cells were suspended in complete medium at an initial density of 3×10^5 cells/ml and TPA (50 nM, Sigma Chemicals Israel Ltd., Holon, Israel) was added at differ-

ent time intervals. Cells grown in parallel without TPA, and harvested simultaneously with the TPA-treated cells at the end of the experiment, served as a control to monitor possible effect of the culture aging. When specified, 1 μm of the PKC inhibitor bisindolylmaleimide I (Calbiochem, La Jolla, CA; Toullec *et al.,* 1991) was added 1 h before TPA to ensure its penetration into the cells before the TPA-induced PKC-activation took place. In experiments with the calcineurin inhibitor, CSA (Sigma Chemicals Israel Ltd.; Guerini, 1997), this drug (1 μg/ml) was added either alone or simultaneously with TPA.

Preparation of nuclear extracts

Cells (10^7) were washed twice with cold phosphate-buffered saline and allowed to swell on ice for 10 min in a buffer containing 10 mM Hepes–KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin; NP-40 was then added (0.5% final concentration) and the suspension was vigorously vortexed for 10 s and briefly centrifuged at 14,000*g*. The pellet was resuspended in a buffer containing 20 mM Hepes–KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. This suspension was allowed to stand on ice for 20 min and then centrifuged at 14,000*g* for 2 min. Protein concentration of the resulting supernatant was determined by the Bio-Rad reagent and this extract was stored in small aliquots at −70°C.

Electrophoretic mobility shift assay (EMSA)

The following oligonucleotides were used in this study: the complete ERR-1 sequence: 5′-CCTCCGGGAAGCCACCAAGAACCACCCATTTCCTCCCCATGTTTG-3′ (Bosselut *et al.,* 1990; Gitlin *et al.,* 1991), which was designated as ERR1; the Sp1 binding site from the $p21^{WAF-1}$ gene promoter 5′-CTGGGCCCCGGGGAGGGCGGTCCCGGGCGGCGCG-3′ (Biggs *et al.,* 1996) that we designated as Sp1/WAF-1 and the consensus w.t. p53 recognition sequence 5′-GGACATGCCCGGGCATGTC-3′ (Funk *et al.,* 1992) designated as p53/r.s.

The oligonucleotides, used as probes for DNA binding assay of nuclear proteins, were labeled by filling in with the Klenow fragment of DNA polymerase-I and [α-^{32}P]dCTP as detailed by Gitlin *et al.* (1991). The labeled probe was added (1 ng, ~5 × 10^4 cpm) to a binding reaction mixture (30 μl final volume) containing 15 μg nuclear proteins, or 300 ng recombinant w.t. p53 protein (kindly provided by Professor Moshe Oren from the Weizman Institute of Science, Rehovot, Israel), 10 mM Tris–HCl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 12.5% glycerol, 0.1% NP-40, and 3 μg poly(dI-dC). The mixture was first pre-incubated on ice for 20 min before adding the probe and further incubated at room temperature for an additional 20 min after the probe addition.

For competition analysis, 50-fold molar excess of the competing oligonucleotide over the labeled probe was added together with the probe.

For supershift analysis the indicated monoclonal antibodies (1 μg) were added together with the labeled probe. For negative controls with irrelevant antibodies we used the anti-c-Jun and anti-albumin monoclonal antibodies.

To remove the Sp1 or p53 protein from the nuclear extracts, anti-Sp1 or pAb421 monoclonal antibodies (20 μg) were added, respectively, to aliquots of the examined extract (200 μg extract protein) and the resultant immunocomplexes were precipitated by protein A–Sepharose CL-4B beads (Pharmacia Biothech). The supernatant was then examined for DNA binding as described above.

In all cases the DNA binding reaction mixtures were subjected to 5% polyacrylamide gel electrophoresis in 0.25 × TBE (1 × TBE = 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) at 110 V for 3–4 h at 4°C. The gel was dried and subsequently autoradiographed on Kodak X-O-Mat S film at −70°C with intensifying screen.

Western blot analysis of Sp1 and p53 proteins

The nuclear level of Sp1 and p53 (w.t. + mutant) was determined by a semiquantitative Western blot analysis of 100 and 200 μg of nuclear extract protein as described by Mor-Vaknin *et al.* (1997), using the anti Sp1 and the pAb421anti p53 monoclonal antibodies, respectively.

To determine the level of the w.t. versus the mutant p53 protein, aliquots of the examined nuclear extract (200 μg protein) were immunoprecipitated with pAb1620 or pAb240 anti p53 antibodies (20 μg protein), respectively, and the immunoprecipitates were collected by protein A–Sepharose beads as described above. These precipitates were dissociated from the protein A–Sepharose beads by heating at 95°C for 5 min and then subjected to Western blot analysis with the indicated antibodies. Since the heavy chain of the immunoprecipitating antibodies has the same SDS–PAGE mobility as p53, the presence of p53 in immunoprecipitates pulled down with mouse antibodies was determined by performing the Western blot analysis of these precipitates with rabbit polyclonal anti-p53 antibodies and peroxidase-conjugated mouse antibodies against rabbit IgG.

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