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Dose-dependent dual effect of HTLV-1 Tax oncoprotein on p53-dependent nucleotide excision repair in human T-cells

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In this study we investigated the effect of Tax on nucleotide excision repair (NER) in human T-cell lines by using the host cell repair analysis of UVC-irradiated reporter plasmid. This analysis revealed a p53-dpendent NER activity in wild type (w.t.) p53-containing T-cells and p53-independent NER in w.t. p53-lacking T-cells. Notably, in the w.t. p53-containing cells Tax exerted a dose-dependent dual effect on NER. While low Tax doses markedly stimulated this repair, high Tax doses strongly reduced it. Further experiments demonstrated that the low Tax doses enhanced, in these cells, the level and the transcriptional function of their w.t. p53 protein. On the other hand, although the high Tax doses further increased the level of p53, they functionally inactivated its accumulating molecules. Both of these Tax effects on p53 proved to be mediated by Tax-induced NF-κB-related mechanisms. Together, these data suggest that by NF-κB activation Tax elevates the level of the cellular w.t. p53. However, while at low Tax doses the elevating w.t. p53 molecules are functionally active and capable of stimulating NER, intensifying further the NF-κB activation by the high Tax doses concomitantly evokes certain mechanism(s) which functionally inactivates the accumulating p53 protein. In contrast to this dual effect on the p53-dependent NER, Tax displayed only an inhibitory effect on the p53-independent NER by its high doses, whereas its low doses had no effect on this repair. The mechanisms of the NF-κB-associated effects on the level and function of the cellular w.t.p53 and of the p53-independent NER noted in our experimental systems are further investigated in our laboratory.

Key words: HTLV-1; Tax; nucleotide excision repair (NER); host cell reactivation (HCR); p53

The stability of the cellular genome is maintained by the cell cycle coordinated centrosome duplication[1] and the mitotic spindle checkpoint,[2,3] which together secure accurate chromosomal segregation during cell replication and by several modes of DNA-repair[4,5] that protect the cells from various types of DNA damaging insults. Disruption of any of these guarding mechanisms leads to genetic instability which is linked to increased cancer incidence.[6–8]

p53 is a tumor suppressor playing a key role in mediating cell response to various stress conditions by modulating regulatory factors involved in cell cycle arrest, senescence, apoptosis, DNA repair and angiogenesis.[9] The qualitative and quantitative activity of p53 depends on its amount and posttranslational modifications induced by a variety of stress-induced signaling pathways and on its somatic mutational status.[9] Certain p53 mutants acquire modified target specificity for their transcription-activating function,[10] whereas some others loose their transcriptional activity but can act as transdominant inhibitors of the wild type (w.t.) p53 activity.[11]

In DNA damage-induced stress, the extent of the damage determine whether p53 will facilitate DNA repair or switch to induce apoptosis.[9,12,13] There are indications that p53 is directly involved in DNA repair by either physical interaction with, or modulation of the synthesis of DNA repair-associated factors.[14,15] In addition, p53 recruits histone acetylases and deacetylases for chromatin remodeling at the damage sites, which is essential for efficient detection of the lesions.[16]

Human T-cell leukemia virus type-1 (HTLV-1) is etiologically implicated with adult T-cell leukemia (ATL), tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) and certain other clinical disorders,[17,18] none of which efficiently responds to presently known therapeutic approaches.[19,20] Although the mechanism of HTLV-1 pathogenicity is not fully understood yet, its transactivator Tax protein is widely regarded as a key element in this mechanism. Tax is a potent oncoprotein capable of transforming cultured rodent cells and primary human T-lymphocytes[21] and of inducing tumors in transgenic animals.[22] This oncogenic potential is accounted for by Tax ability to modulate the synthesis or activity of many regulatory proteins which control a variety of fundamental processes in cells, like gene expression, cell cycle, apoptosis and genetic stability [for more details see recent reviews in Refs. 21,23–25].

Of note, in this context, is the interference of Tax with the regulation of the centrosome duplication[26,27] and the mitotic spindle checkpoint,[28,29] which leads to aneuploidy in HTLV-1 transformed[30] and ATL[28] cells and with the various modes DNA-damage repair[31] that leads to high mutation rate in HTLV-1 infected cells.[32] Together, these effects of Tax hamper the genetic stability of virus-harboring T-cells in HTLV-1-infected individuals.[31,33,34] Indeed, like most other tumors,[35] ATL cells display a wide range of chromosomal abnormalities which proved to be conferred by HTLV-1.[31,33,36,37]

Particular interest has been focused on Tax interference with nucleotide excision repair (NER).[31] This repair removes bulky DNA lesions, such as UV-induced cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) and adducts formed by certain carcinogens and other DNA-interacting agents. Since these lesions are the most common forms of DNA damage, NER is regarded as the most versatile pathway involved in maintaining the genome stability and defects in this repair have been implicated with increased cancer incidence.[38] Therefore, although the mechanism of Tax interference with NER is controversial,[39–43] this interference is likely one of the major functions accounting for its oncogenic potential.[31]

It should be noted that, despite the ability of HTLV-1 to infect a variety of human and nonhuman cell types, both *in vitro* and *in vivo*,[44] mature $CD4^+$ T-cells are the main targets of the HTLV-1 leukemogenic activity leading to ATL in human infections.[23,44–48] However, the various mechanisms which have been

Abbreviations: 6-4PP, pyrimidine (6-4) pyrimidone photoproducts; ATL, adult T-cell leukemia; CPD, cyclobutane pyrimidine dimers; HCR, host cell reactivation; HTLV-1, human T-cell leukemia virus type-1; MSV, murine sarcoma virus; NER, nucleotide excision repair; p53(mut), mutant p53; SE, standard error; siRNA, small interfering RNA; TSP/HAM, tropical spastic paraparesis/HTLV-1 associated myelopathy; w.t., wild type.

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proposed so far for NER inhibition by Tax[39,41–43] are mostly based on experiments performed with cell types other than mature human T-cells and, therefore, it is difficult to assess their relevance to the actual ATL-related events occurring in HTLV-1 infected people. Thus, in the present study, we chose to investigate the mechanism of Tax effect on NER in various human T-cell lines, which some of them were noted in our earlier study to resemble human primary T-lymphocytes with respect to regulation of HTLV-1 gene expression.[49] In the present study we found, indeed, that Tax effect on NER in these particular cells was largely different from those previously reported by others.[39,41–43] In contrast to all other reports that have pointed out that Tax can only inhibit NER, we show here, for the first time, that in human T-cell lines, which like primary T-lymphocytes, contain functional w.t. p53, Tax displays a dose-dependent dual effect on NER. At low level Tax rather enhances NER, whereas only at an exceedingly high level Tax inhibits this repair. Furthermore, we demonstrate that the stimulatory effect of Tax on NER in such cells is exerted by enhancing p53 level and its transcriptional function, whereas the inhibitory effect of the high Tax doses results from its functional inactivation of the accumulating p53 protein. We also obtained indications that both of these Tax effects on p53 are mediated by certain, apparently different, Tax-induced NF-κB-associated mechanisms.

Material and methods

Cells

In this study we used the Jurkat, Molt4, H-9 and SupT1 human T-cell lines which were obtained from Irvin S. Y. Chen (Department of Medicine, UCLA School of Medicine, Los Angeles, CA). The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics.

Plasmids and transient transfection

The plasmids MSV-β-gal (expressing β-gal through MSV LTR) and NF-κB-Luc (expressing luciferase through NF-κB responsive promoter) were purchased from Promega Corporation, Madison, WI and from Invitrogen, UK respectively. The plasmid LTR-Luc (expressing luciferase through HTLV-1 LTR) as well as the plasmids expressing the w.t. Tax and its mutants TaxM22 and TaxM47[50] through CMV promoter, were provided by Francoise Bex (Laboratore de Microbiologie, Universite Libre de Bruxelles, B-1070, Brussels, Belgium). The plasmids pG13-Luc (expressing luciferase through a promoter containing 13 copies of the consensus binding sequence of w.t. p53) and p53-Luc (expressing luciferase through the promoter of the human p53 encoding gene) were obtained from Moshe Oren (Weizmann Institute of Science, Rehovot, Israel). The plasmids pc53-SN3 (expressing w.t. p53 through CMV promoter), pRc/CMV-p53 (residues 22 and 23), expressing the mutant p53 carrying point mutations at positions 22 and 23, which abrogate its transcriptional function [designated in this study as p53(mut)], and p21WAF-1-Luc [expressing luciferase through the p21WAF-1 promoter[51]] were provided by Dimitris. Kardassis (University of Crete Medical School, Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology of Hellas, Herakolion, Greece). A set of plasmids expressing different clones of p53-specific siRNAs (MISSION TRC shRNA Target Set) was purchased from Sigma-Aldrich Corporation. From this set, we chose to use only clones 2 and 4 which were the most effective in our experimental system. The pRL-renilla plasmid (expressing renilla through enhancer-less promoter-pRL-null) was purchased from Promega Corporation (Madison, WI). The plasmids were transfected in the indicated combinations by electroporation as previously described.[52] Each transfection included the pRL-renilla plasmid (0.1 μg) as an internal control for variation in the transfection efficiency. The total DNA was completed to 40 μg in all samples with an empty plasmid. The enzymatic activity of the β-gal reporter was assayed 48 hr after transfection, whereas that of the luciferase reporter was analyzed 24 hr post transfection. These activities were normalized to that of renilla and presented as fold or as percent of the relevant control of each experiment.

Host cell reactivation assay

The pMSV-β-gal reporter plasmid was damaged *in vitro* by exposure to UVC light (254 nm, 1.347 J/m^2/sec, 400 J/m^2) and transfected (10 μg) into the indicated T-cells. The expression of β-gal was normalized to that of the pRL-renilla. The normalized expression of nonirradiated pMSV-β-gal plasmid in the absence of any effector was taken as a control for 100% expression of this reporter. Each transfection was performed in triplicates and their average ±SE was plotted as % of the control.

Western blot analysis and antibodies

Whole cell lysates were prepared as previously described.[49] The protein concentration of the lysates was determined by Bio-Rad reagent (Bio-Rad, Richmond, CA) and equal amounts of protein of the lysates were subjected to Western blot analysis with the indicated antibodies as previously detailed.[49] Sample loading was assessed by stripping the filters from the first detecting antibodies and exposing them to anti actin antibody. Monoclonal antibody recognizing a C-terminal epitope of Tax protein (amino acids KHFRETEV), was prepared from the culture medium of the respective clone of hybridoma cells provided by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Cat. No. 168b17-46-70). Monoclonal Antibodies against p53 (DO1) and actin were purchased from Calbiochem and from ICN respectively.

Results

Tax effect on NER in human T-cells

The effect of Tax on NER was analyzed by the HCR assay in 4 different human T-cell lines, Jurkat, Molt4, H9 and SupT1. We used in this assay the MSV-β-gal reporter which does not respond to Tax transcriptional trans-activatory effect. This reporter was irradiated by a UVC dose (254 nm, 1.347 J/m^2/sec, 400 J/m^2) that was predetermined to enable the cells to recover about 50% of the full expression displayed by the nonirradiated MSV-β-gal reporter. This recovery was regarded as a basal NER activity in this experimental setting.

To characterize the effect of Tax on NER in these cells, the irradiated reporter was transfected either alone or together with increasing doses of Tax-expressing plasmid. Western blot analysis of whole cell extracts, confirmed that this plasmid efficiently expressed Tax in all 4 cell types at a dose dependent manner (Fig. 1*a* illustrates the results of Jurkat and H9 cells). We also verified that the expressed Tax was functional in these cells by demonstrating its dose dependent activation of NF-κB- and CREB- dependent reporters (Figs. 1*b* and 1*c* present the results with Jurkat and H9 cells).

The left panel of Figure 1*d* illustrates the results of the HCR assay in Jurkat and Molt4 cells, which demonstrate that, in both of these cells, Tax displayed a dose-dependent dual effect on the repair of the irradiated reporter. At low doses (0.3–0.9 μg plasmid) Tax rather stimulated this repair, peaking at almost 2-fold of the basal repair (from 50 to 95% of the nonirradiated reporter expression), whereas at higher doses Tax progressively reduced the repair down to nearly 1/3 of the basal repair (from 50 to 19% of the nonirradiated reporter expression). As expected, Tax had no effect on the expression of the nonirradiated reporter, thus excluding the possibility that the stimulation of the irradiated reporter by the low Tax doses resulted from a trivial transcriptional stimulation of some undamaged copies of this plasmid. Furthermore, the nonirradiated reporter was unaffected by the high Tax doses too, thus ruling out also the possibility that the inhibitory effect of these high Tax doses on the irradiated reporter resulted from a nonspecific cytotoxic effect. Similar results were obtained also with Molt4 cells (data not shown). In contrast; however, in H9 and

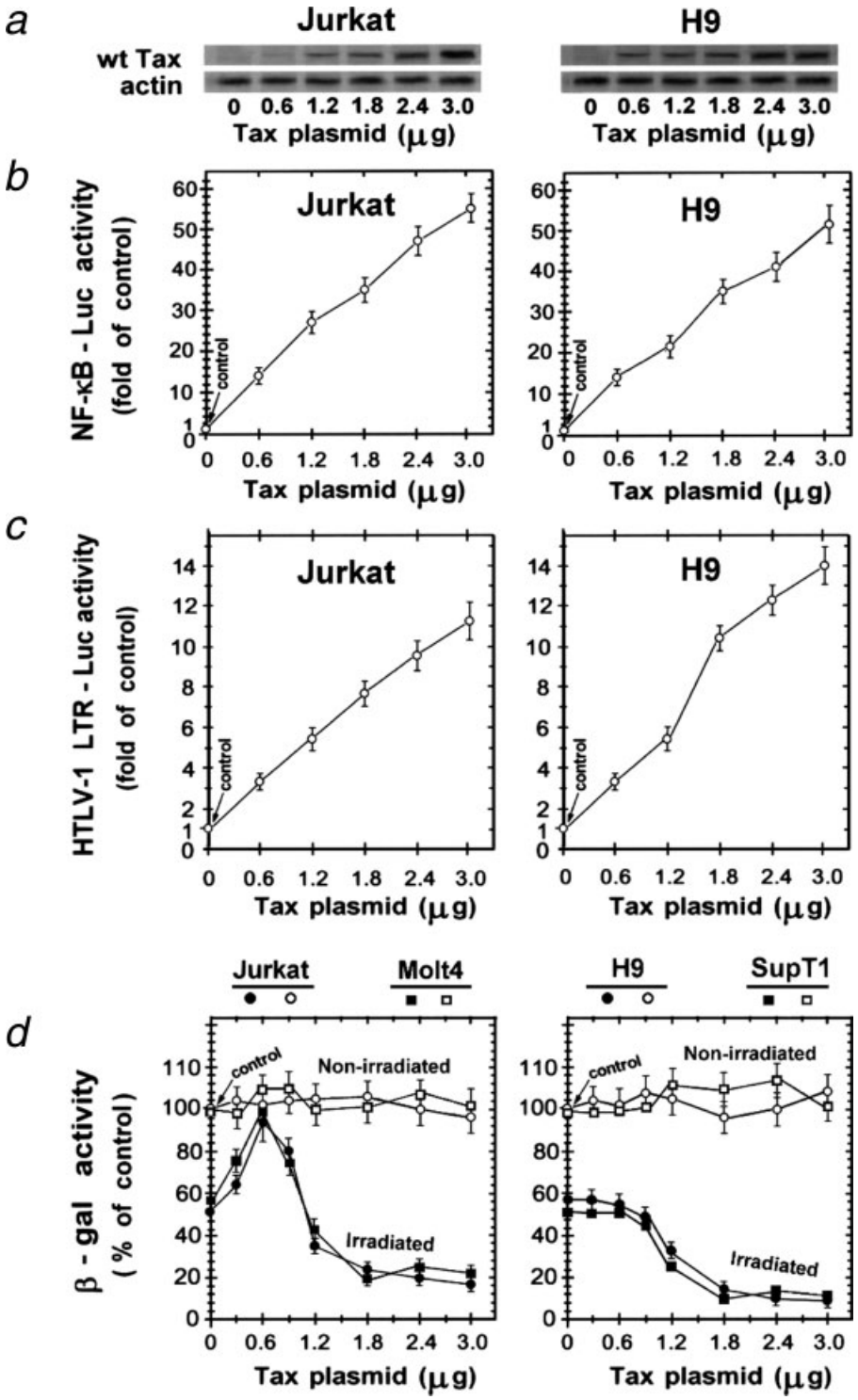

FIGURE 1 – Comparison of Tax effect on NER in Jurkat and H9 human T-cell lines. (*a*) The efficiency of Tax expression was first compared in these two cell types by transfecting them with increasing doses of the Tax plasmid and subjecting equal protein amounts of their whole cell extracts to Western blot analysis with anti-Tax monoclonal antibody at 24 hr post infection. Equal loading of the various aliquots was assessed by stripping the filters from the first detecting antibodies and re-exposing them to anti-actin antibody. (*b*) The NF-κB-activating capacity of Tax was assessed in these cells by transfecting them with 10 μg NF-κB-Luc reporter alone (control) or together with increasing doses Tax plasmid. Each transfection included 0.1 μg pRL-renilla plasmid which served for assessing the relative transfection efficiency. The enzymatic activities were measured in the cell lysates at 24 hr post-transfection and the luciferase activity was normalized to that of renilla. (*c*) The CREB-activating capacity of Tax was assessed in these cells as described in B, except that the HTLV-1 LTR-Luc reporter replaced the NF-κB-Luc reporter. (*d*) Tax effect on NER in these cells was examined by the HCR assay in which UVC-irradiated MSV-β-gal reporter (10 μg) was transfected together with the indicated dose of the Tax plasmid. In parallel, the effect of these Tax doses on the nonirradiated reporter was also examined. The normalized β-gal activities were normalized to that of renilla as described in *b* and *c* and plotted as % of the normalized activity obtained in the control cells that were transfected with the nonirradiated reporter alone. The results in *b*, *c*, and *d* are the average of triplicates ± SE.

SupT1 cells (Fig. 1*d*, right panel) Tax exerted only inhibitory effect by its high doses, but no NER stimulation by its low doses could be detected in these cells. The expression of the nonirradiated reporter in H9 and SupT1 cells (Fig. 1*d*, right panel) was comparable to its expression in Jurkat Molt4 cells (Fig. 1*d*, left panel) and was unaffected by Tax.

p53 status in the employed T-cell lines

Earlier reports have described various functions of p53 in NER.[14–16,53–57] It was, therefore, of interest to look for a possible variation in the functional status of p53 in the T-cell lines employed in this study that might explain the different patterns of Tax effects on NER in these cells. Although several laboratories claim that Jurkat cells are p53 null, we have previously shown that the Jurkat cells used in our experiments, express comparable levels of both w.t. and mutant p53 proteins.[51,58] This finding is compatible with the sequence data reported by Cheng and Hass,[59] showing that Jurkat cells are heterozygous for p53, carrying a point mutation in one of their p53 alleles. Molt4 cells have been shown to contain only w.t. p53.[60,61] In contrast, immunoprecipitation (our unpublished data) and immunostaining[62] analyses with monoclonal antibodies recognizing only w.t. p53 (pAb 1620) or only mutant p53 (pAb 240), have revealed that H9 cells express only mutated p53 protein, and SupT1 cells have been reported to carry multiple mutations on the DNA-binding domain of p53 which hamper its transcriptional transactivation function.[63]

To validate that these differences are reflected by correspondingly different p53 functional status we compared the ability of these cells to support the expression of the w.t. p53-responding pG13-Luc reporter. This was done by transfecting the reporter, either alone or together with the plasmid expressing the negative dominant p53 mutant [p53(mut)] described in Material and Methods section. We found that p53(mut) displayed a dose-dependent inhibitory effect on pG13-Luc expression in Jurkat (Fig. 2*a*) and Molt4 (not shown) cells. This observation proved that both of these cell lines contained a functionally active endogenous w.t. p53 capable of inducing an appreciable basal expression of this reporter which could be blocked by a molar excess of an ectopic inhibitory p53 mutant. In contrast, extremely low pG13-Luc expression was apparent in H9 (Fig. 2*a*) and SupT1 (not shown) cells regardless of the presence or absence of the ectopic p53(mut). This finding confirmed that H9 and SupT1 cells did not contain transcriptionally functional w.t. p53 protein.

The negative dominance capacity of p53(mut) was further substantiated by showing that its molar excess could also inhibit the transcriptional activity of ectopically expressed w.t. p53 in both Jurkat and H9 cells (Fig. 2*b*). Figure 2*c* shows a comparable dose-dependent expression of the transfected p53(mut) and w.t. p53 plasmids in both cells. It should be noted that although in transient transfections, plasmids are, usually, taken-up by only a certain fraction of the cell population, it is evident from these data that the amount of the endogenous p53 protein in these cells is low enough to enable detection of the dose-dependent increase of the ectopically expressed p53 proteins.

An additional evidence for the functional activity of the endogenous w.t. p53 protein emerged from our finding in Jurkat cells that the plasmids expressing clones 2 and 4 of the p53-specific siRNAs, strongly repressed the basal pG13-Luc expression (Fig. 2*d*). The specificity of these siRNAs was confirmed by showing that they efficiently suppressed also the activation of pG13-Luc by the ectopic w.t. p53 (Fig. 2*e*), while they had no effect on the activation of HTLV-1 LTR-Luc by Tax (Fig. 2*f*). Clone 4 (Fig. 2*g*) and clone 2 (not shown) siRNAs also markedly reduced the level of the endogenous as well as of the ectopically expressed p53 proteins. However, clone 1 and clone 3 which were also included in the MISSION TRC shRNA Target Set, had no or very little inhibitory effect on the level or function of the endogenous and ectopic p53 (not shown), thus further proving the specificity of clone 2 and 4.

Involvement of the endogenous w.t. p53 in the basal NER activity of Jurkat cells

As shown in Figure 1, both Jurkat and H9 cells could repair, under our experimental conditions, about 50% of the damaged reporter. In the next experiment we found that, this basal repair was efficiently reduced in Jurkat cells by both p53(mut) (Fig. 3*a*) and p53-siRNAs (Fig. 3*b*) at a dose-dependent manner. Neither of these inhibitors had any effect on the expression of the none-irradiated reporter, thus excluding the possibility that the declining expression of the irradiated reporter might result from a general cytotoxic effect of these inhibitors. In contrast, neither p53(mut) (Fig. 3*a*) nor p53-siRNAs (Fig. 3*b*) had any effect on the basal expression of the irradiated reporter in H9 cells. These data suggest that while the endogenous w.t. p53 of Jurkat cells plays an essential role in their basal NER activity, the basal NER of H9 cells is p53-independent.

Dose-dependent dual effect of ectopic w.t. p53 on NER

Earlier studies have noted that NER suppression by Tax can be rescued by a functional p53 signal.[40] Therefore, to assess further the importance of w.t. p53 in NER in our cell sytem, the irradiated and nonirradiated reporters were cotransfected with increasing doses of the w.t. p53-expressing plasmid. The results illustrated in Figure 3*c* demonstrate that the ectopic w.t. p53 exerted in Jurkat cells a dose-dependent biphasic effect on the irradiated reporter. At low doses (up to 25 ng) p53 enhanced the repair, whereas at higher doses the reporter activity was progressively reduced. The low p53 doses had no effect on the nonirradiated reporter, thus excluding the possibility that their enhancing effect on the irradiated reporter resulted from a trivial transcriptional activation of some undamaged copies of this reporter rather than from enhancing its repair. However, unlike the high Tax doses, which had no effect on the nonirradiated reporter (see Fig. 1*d*), the high doses of p53 were found to reduce also the expression of the nonirradiated reporter. FACS analysis demonstrated that these high p53 doses progressively increased the percentage of apoptotic cells up to 35% (data not shown). In contrast, no such apoptotic effect was displayed by the high Tax doses (data not shown). It is, therefore, reasonable to postulate that while the reduction of the irradiated MSV-β-gal reporter by the high Tax doses reflected a true NER inhibition, the declining expression of the irradiated and the nonirradiated reporter, conferred by the high p53 doses, reflected a dose-related transition of p53 function from DNA repair to apoptosis induction.

Notably, higher ectopic w.t. p53 doses were needed for inducing both phases of its effects on NER in the presence of 10 μg p53(mut) (Fig. 3*c*) or 30 μg clone 4 (Fig. 3*c*) or clone 2 (not shown) p53-siRNAs. These higher doses were, most likely, required for compensating the reduction of the w.t. p53 transcriptional activity by p53(mut) and the inhibition of its protein synthesis by the p53-siRNAs.

Interestingly, Figure 3*d* shows that the ectopic w.t. p53 displayed a similar biphasic effect on the irradiated reporter and a similar reduction of the expression of the nonirradiated reporter by its high doses in H9 cells as well. Furthermore, these p53 effects were, similarly, influenced in these cells by p53-siRNA (Fig. 3*d*). However, while p53(mut) and p53-siRNA markedly reduced the basal NER activity in Jurkat cells (Fig. 3*c*), p53-siRNA had no effect on the basal NER activity of H9 cells (Fig. 3*d*). These findings indicate that although the basal NER activity of H9 cells is p53-independent, these w.t. p53-deficient cells still contain the other functional factors required for p53-dependent NER which can be revived by introducing ectopic w.t. p53.

Involvement of w.t. p53 in the dual Tax effect on NER

The above data on p53 effect in NER, together with reported studies on Tax-mediated p53 functional inactivation[64,65] and on the ability of ectopically expressed w.t. p53 to block NER inhibition by Tax,[40] led us to explore whether the basal endogenous w.t.

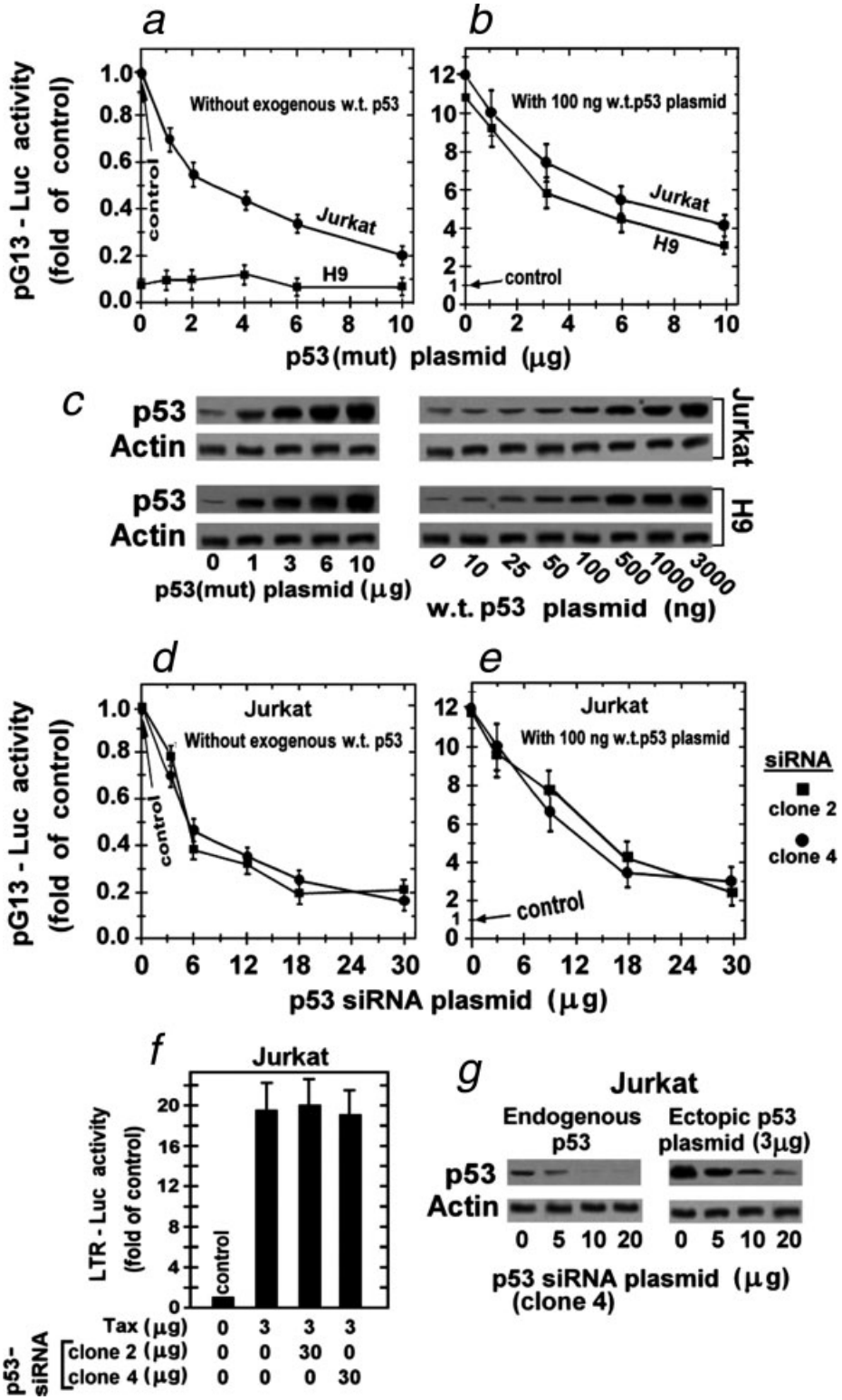

FIGURE 2 – Effect of p53(mut) and p53-specific siRNAs on the transcriptional function of the endogenous and ectopic w.t. p53. The effect of p53(mut) on the transcriptional function of the endogenous and ectopic w.t. p53 was determined by transfecting Jurkat and H9 cells with the pG13 reporter alone or together with the indicated doses of the p53(mut) plasmid in the (*a*) absence or (*b*) presence of 100 ng ectopic w.t. p53 plasmid. The expression efficiency of the p53(mut) (*c*, left panels) and the ectopic w.t. p53 (*c*, right panels) plasmids in these cells was determined by Western blot analysis of whole cell extracts of the transfected cells with p53-specific monoclonal DO1 antibody. Equal loading of the various samples was assessed as in Figure 1*a*. The effect of p53-specific siRNAs on the transcriptional function of the endogenous (*d*) and ectopic (*e*) w.t. p53 was examined as described in *a* and *b* respectively, except that the p53-specifc siRNAs clone 2 (square symbols) and clone 4 (circle symbols) replaced p53(mut). To asses the specificity of these siRNAs to p53 we examined their effect in Jurkat cells on activation of a nonrelevant gene like the activation of HTLV-1 LTR-Luc by Tax which depends on CREB function (*f*). The effect of the p53-siRNA (clone 4) on the endogenous p53 basal level (*g*, left panel) and on the ectopic p53 level (*g*, right panel) was analyzed in Jurkat cells as in *c*. The reporter activity in all cases was normalized according to renilla as describe in Figure 1 and the results present the average of triplicates ±SE.

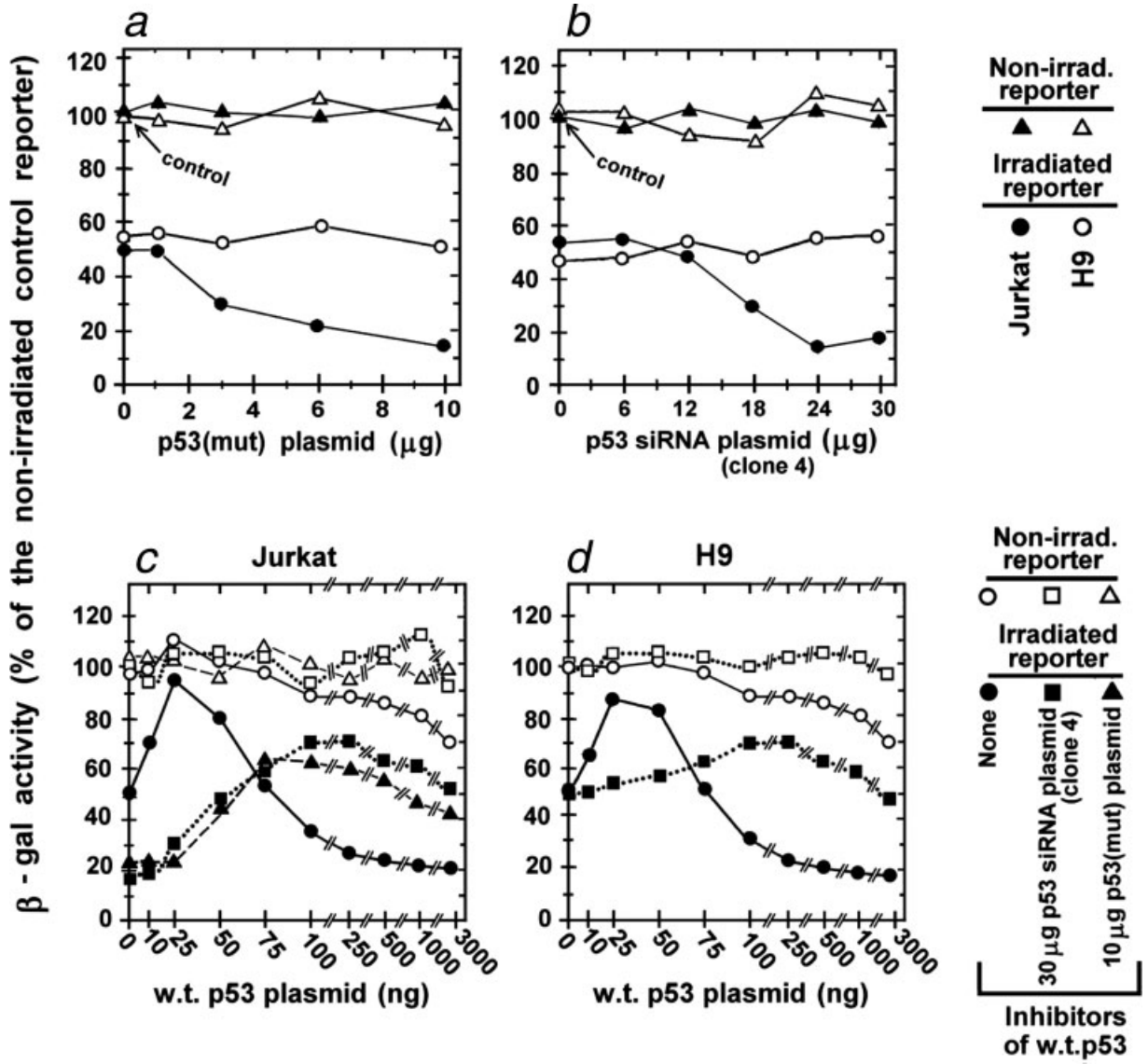

Figure 3 – Effect of p53(mut) and p53-siRNA on NER in the absence and presence of increasing ectopic w.t. p53 doses. The effect of increasing doses of p53(mut) (*a*) and p53-siRNA clone 4 (*b*) on the expression of the irradiated and nonirradiated MSV-β-gal reporter was examined in Jurkat and H9 cells by the HCR assay as described in Figure 1*d*. In addition, the effect of the highly inhibitory doses of p53(mut) (10 μg) or p53-siRNA (30 μg) on the irradiated and nonirradiated reporter was similarly analyzed in Jurkat (*c*) and H9 (*d*) cells in the absence and presence of increasing amounts of ectopic w.t. p53.

p53 activity of Jurkat cells was involved in the dual Tax effect on NER depicted in Figure 1*d*. For this purpose we reexamined the effect of Tax on NER in the presence of the high doses of p53(mut) (10 μg) and p53-siRNAs (30 μg) which displayed in the previous experiment (Figs. 3*a* and 3*b*) the maximal inhibition of the basal NER activity of these cells. Figure 4*a* shows that both of these p53 inhibitors completely abolished the NER-stimulating effect of the low Tax doses, but did not affect the maximal NER-inhibition by the high Tax doses. For comparison, we carried out a similar experiment with H9 cells and, as expected, no effect of these p53 inhibitors on either the basal NER activity or on Tax effect on NER was detected (Fig. 4*b*).

Tax effect on the endogenous w.t. p53 level and transcriptional function in Jurkat cells

Although the results shown in Figure 4*a* seem to corroborate with an involvement of the endogenous w.t. p53 of Jurkat cells in the dual Tax effect on NER, they still cannot properly explain the nature of Tax and p53 interplay in this repair. A clue on this issue may be gained from our finding depicted in Figures 3*c* and 3*d* that increasing the cellular level w.t. p53 by ectopic w.t. p53, up to a certain point, profoundly elevated NER activity. This finding raised the possibility that the NER-stimulating effect of the low Tax doses in Jurkat and Molt4 cells resulted from Tax-induced elevation of their endogenous w.t. p53 to a NER-stimulating level. This possibility was tested by Western blot analysis which revealed that Tax increased the level of p53 in these cells (Fig. 5*a* illustrates representative results obtained with Jurkat cells).

Studies from other laboratories have shown that p53 protein accumulates in HTLV-1-infected and Tax-expressing cells because of its stabilization rather than due to enhancement of its *de novo* synthesis, and that this accumulating protein is functionally inactive.[64,66] In contrast, our next experiment revealed that Tax stimulated the promoter of the human p53-encoding gene in a dose-dependent manner (Fig. 5*b*), suggesting that the p53 eleva-

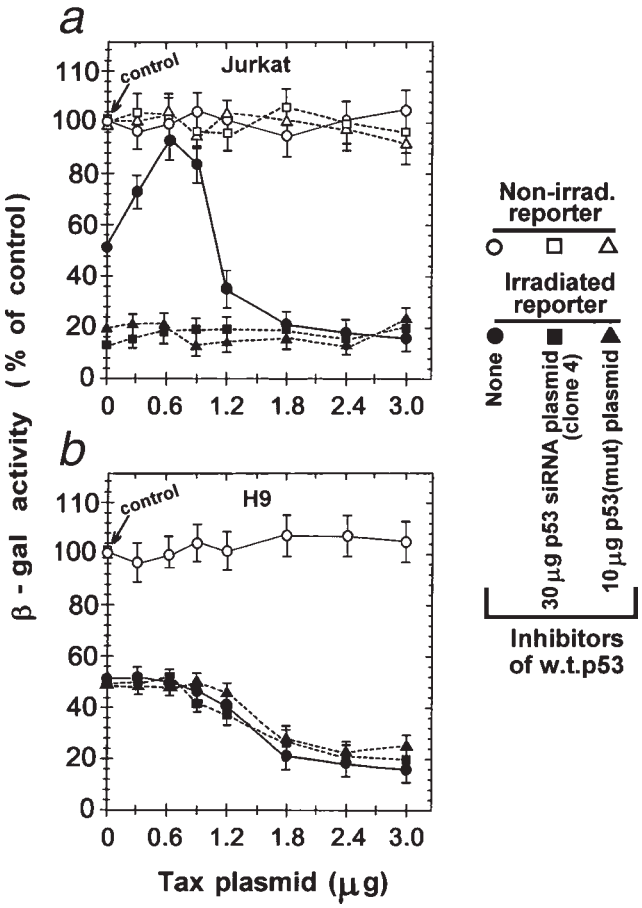

FIGURE 4 – Effect of Tax on the basal NER activity in the absence and presence of p53-siRNA and p53(mut). The effect of increasing amounts of Tax plasmid on the irradiated and nonirradiated MSV-β-gal reporter was examined (*a*) Jurkat and (*b*) H9 cells in the absence or presence 30 μg p53-siRNA clone 4 or 10 μg p53(mut) plasmids as described in Fig. 1*d*.

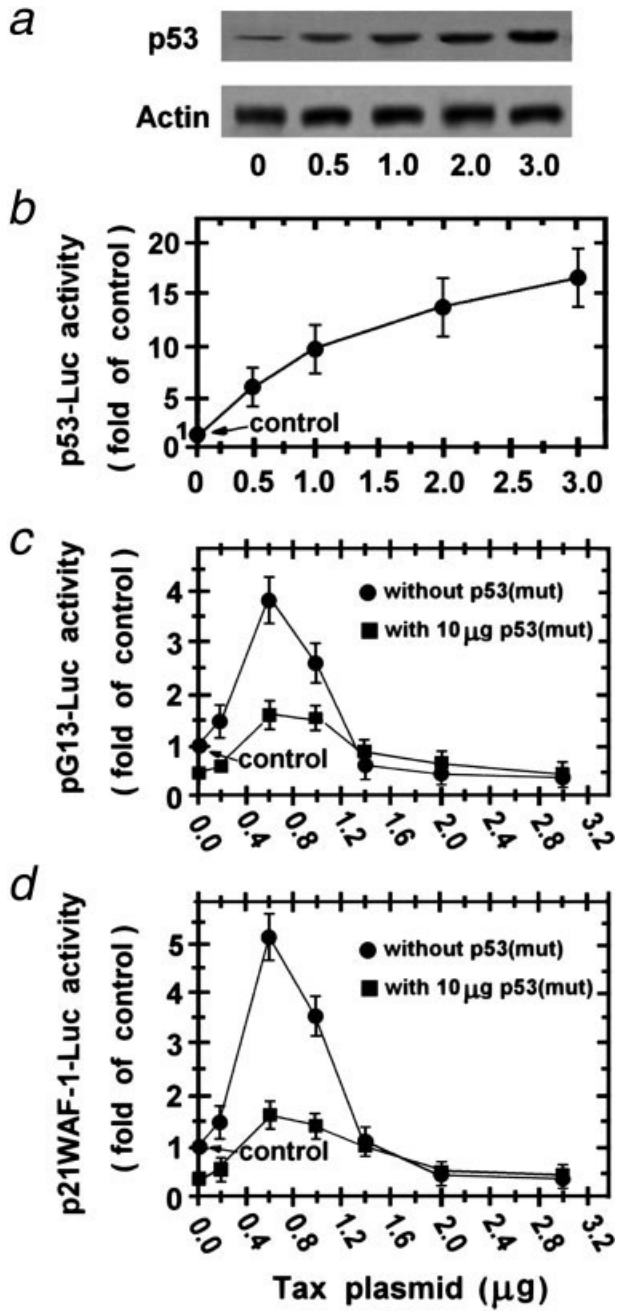

FIGURE 5 – Effect of Tax on the level, gene expression and transcriptional function of the endogenous p53. The effect of increasing Tax doses on the endogenous p53 level was estimated in Jurkat cells by Western blot analysis (*a*) as describe in Figure 2*c*, whereas the effect of these Tax doses on the expression of the human p53-encoding gene was determined by measuring their activation of Luc reporter driven by the promoter of this gene (*b*). The effect of these Tax doses on the transcriptional function of the endogenous p53 was determined by analyzing their activation of pG13-Luc (*c*) and p21WAF-1-Luc (*d*) expression in the absence and presence of 10 μg p53(mut) as described in Figure 2.

tion depicted in Figure 5*a* resulted, at least partly, from stimulation of its gene expression. Moreover, we also proved that low Tax doses rather enhanced the transcriptional function of the elevated endogenous w.t. p53 by showing that these low Tax doses stimulated the expression of pG13-Luc (Fig. 5*c*) and p21WAF-1-Luc (Fig. 5*d*) and that this stimulation was abrogated by p53(mut) (Figs. 5*c* and 5*d*). However, high Tax doses rather reduced the expression of these reporters in a dose-dependent manner (Figs. 5*c* and 5*d*), although these high doses further increased the level of the p53 protein (Fig. 5*a*) and its gene expression (Fig. 5*b*). These findings suggest that at low doses Tax enhances the level of the functionally active w.t. p53 molecules, which, in turn, enhance NER. However, while at high doses, Tax increases further the accumulation of p53 molecules, it concomitantly induces a mechanism that inactivates their function and therefore, NER drops down below its basal level. This model is consistent also with our finding depicted in Figure 4*a*, that in Jurkat cells both p53-siRNAs and p53(mut) diminished the NER-stimulation by the low Tax doses, but they had no influence on the NER-inhibition by its high doses which already inactivated the w.t. p53 by themselves.

NF-κB involvement in Tax effect on p53 function

There is a substantial controversy over the mechanism of Tax effect on w.t. p53 activity (reviewed by Tabakin-Fix *et al.*[64]). While a number of studies suggest that Tax inactivates p53 through NF-κB-associated pathways,[67–69] others indicate that Tax affects p53 function through CREB/ATF-associated pathways.[70,71] To clarify this point in our experimental system we examined the effect of TaxM47 and TaxM22[50] on p53 level and its transcriptional function in Jurkat cells. Figures 6*a* and 6*b* confirm that TaxM47 can activate NF-κB- but not CREB/ATF-dependent gene expression, whereas TaxM22 can activate CREB/

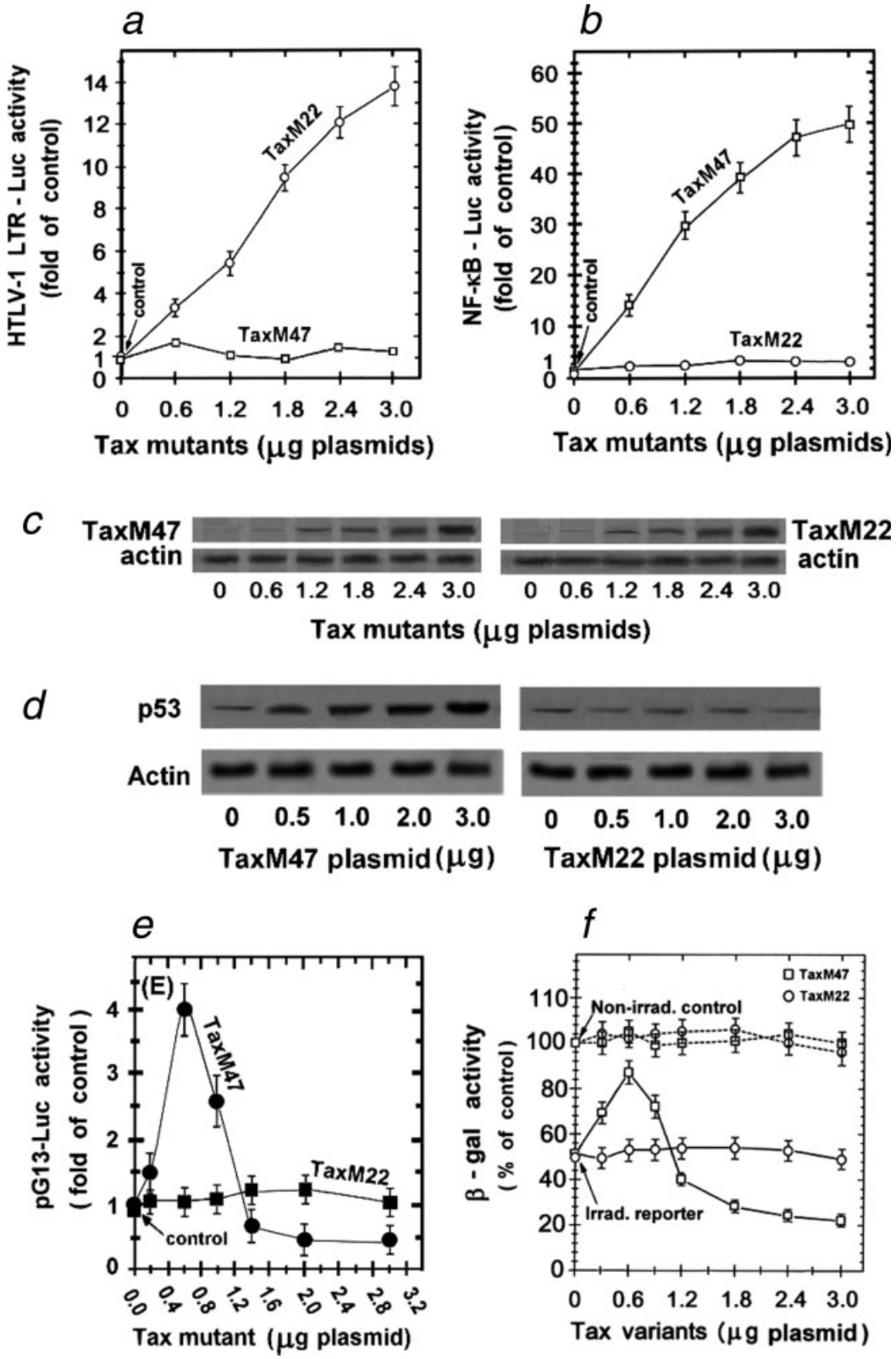

Figure 6 – Role of NF-κB in Tax effect on w.t. p53 expression and on NER in Jurkat cells. The CREB- and NF-κB-activating ability of TaxM47 and TaxM22 was first assessed by checking their dose-dependent activation of the HTLV-1 LTR-Luc (*a*) and NF-κB-Luc (*b*) reporters. The expression of TaxM47 and TaxM22 in these cells was assessed by transfecting them with increasing doses of their encoding plasmids and measuring the level of their protein in the whole cell extract prepared at 24 hr after transfection by Western blot analysis with the anti Tax monoclonal antibody (*c*) as described in Figure 1*a*. The dose-dependent effect of TaxM47 and TaxM22 on the level on the endogenous p53 was determined by subjecting the whole cell extracts of the transfected cells to Western blot analysis with the DO1 anti p53 antibody at 24 hr post-infection (*d*). The dose- dependent effect of TaxM47 and TaxM22 on the transcriptional function of the endogenous w.t. p53 was determined by measuring their ability to activate the pG13-Luc reporter (*e*). The dose-dependent effect of TaxM47 and TaxM22 on NER was examined as described in Figure 1*d*.

ATF- but not NF-κB- dependent gene expression. Figure 6*c* shows that both mutants were expressed by their encoding plasmids at the same efficiency and that this expression was comparable to that of the w.t. Tax (compare with the left panel of Fig. 1*a*). Interestingly, TaxM47 enhanced the level of the endogenous p53 (Fig. 6*d*, left panel) and activated the human p53 promoter (not shown) at the same dose-dependent efficiency as the w.t. Tax, whereas TaxM22 had no effect on either the cellular p53 level (Fig. 6*d*, right panel) or on the expression of the p53 promoter (not shown). It is, thus, evident that the stimulatory effects of Tax on p53 gene expression and its protein accumulation were exerted by an NF-κB-related mechanism. This notion is compatible with the observation of Wu and Lozano that the NF-κB p65(RelA) factor stimulates the expression of the p53-encoding gene.[72] Furthermore, Figure 6*e* shows that, like the w.t. Tax (Fig. 5*a*), TaxM47 displayed a dose-dependent dual effect on the endogenous w.t. p53 transcriptional function measured by the pG13-Luc activation, whereas TaxM22 had no effect on this reporter. These findings indicate that NF-κB-related factors were involved not only in the stimulatory but also in the inhibitory effect of Tax on p53 transcriptional function.

NF-κB involvement in the dual Tax effect on the p53-dependent NER

The earlier evidence for p53 involvement in the dual Tax effect on NER and the indications that Tax affects p53 function through NF-κB activation led to us to hypothesize that NF-κB activation might be an upstream event in a multi-factorial mechanism of the dual Tax effect on the p53-depended NER. This hypothesis was supported by our findings that while TaxM47 displayed in Jurkat cells the same dual effect on NER (Fig. 6*f*) as the w.t. Tax (Fig. 1*d*), TaxM22 had no effect on this repair (Fig. 6*f*).

Of note is that our finding that Tax22 does not affect NER in Jurkat cells is contrasting the observation of Marriot's group that this Tax mutant effectively inhibits NER in CREF cells.[39] By testing a panel of Tax variants, these authors have noted that the ability of w.t. Tax, TaxM22 and certain other Tax mutants to inhibit NER is linked to their ability to activate the expression of the proliferating cell nuclear antigen (PCNA). Furthermore, in contrast to their observation in another study,[73] we were unable to show activation of the human PCNA promoter by w.t. Tax or by its mutants TaxM22 and TaxM47 in our experimental system (not shown).

Effect of high Tax dose on the NER activity of ectopic w.t. p53

After showing the biphasic effect of p53 on NER (Figs. 3*c* and 3*d*) and demonstrating the functional p53 inactivation by high Tax doses (Figs. 5*c* and 5*d*), it was of interest to explore the effect of high Tax dose on p53 function in NER. For this purpose Jurkat cells were cotransfected with the irradiated MSV-β-gal reporter and increasing doses of w.t. p53 plasmid alone, or together with 3 μg plasmid expressing the w.t. Tax or its TaxM22 and TaxM47 mutants. We found that in the presence of w.t. Tax (Fig. 7*a*), and TaxM47 (Fig. 7*b*), substantially higher doses of p53 were required for inducing its stimulatory effect on NER as well as for exerting its inhibitory effect on the irradiated and nonirradiated reporter, which, as noted above, reflected its apoptotic function. No such effects were displayed by TaxM22 (Fig. 7*c*). These data indicate that Tax impairs both the NER activity of p53 and its apoptotic function and that both of these Tax effects are exerted through an NF-κB-associated pathway.

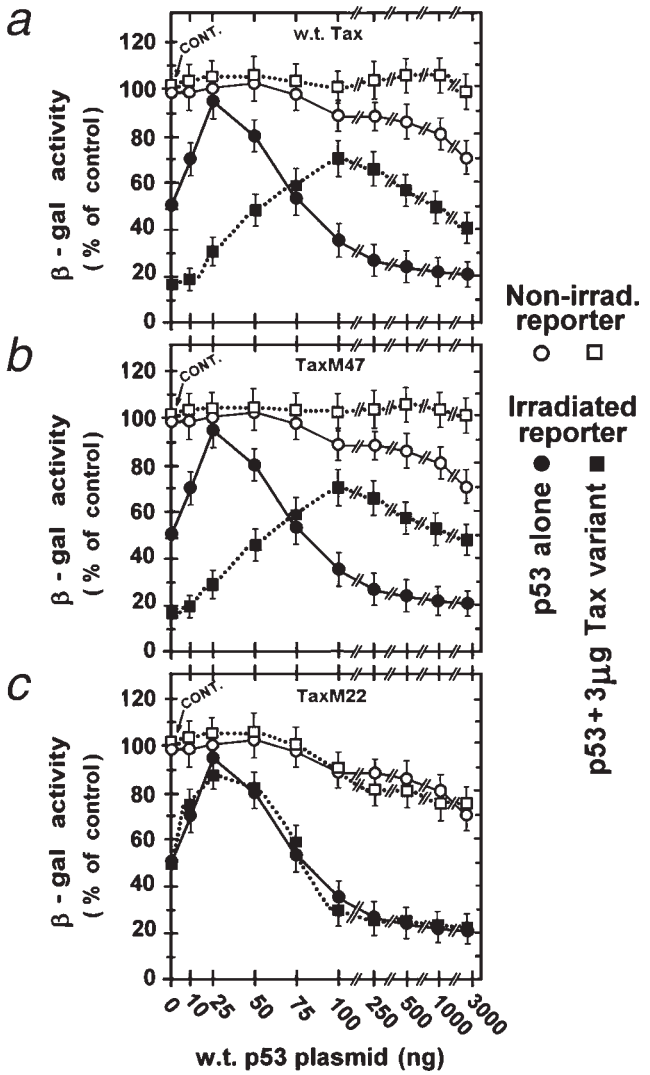

FIGURE 7 – Effect of w.t. Tax, TaxM47 and TaxM22 on the NER function of ectopically expressed p53. Jurkat cells were transfected with irradiated or nonirradiated MSV-β-gal reporter (10 μg) and the indicated doses of the w.t. p53 plasmid alone or together with 3 μg w.t. Tax (*a*), TaxM47 (*b*) or TaxM22 (*c*) plasmid. NER was analyzed as described in Figure 1*d*.

Discussion

A substantial part of Tax oncogenic potential is ascribed to its ability to destabilize the cellular genome.[26,28,31,33,34,37] In this study we were particularly interested in NER, which is regarded as one of the major pathways used by eukaryotic cells for maintaining their genomic integrity.[38] From many earlier studies performed with various cell types, Tax appears as capable of exerting only an inhibitory effect on NER. Our present experiments with different human T-cell types revealed that this is true only in T-cells lacking functional w.t. p53. However, in T-cells that contain functional w.t. p53 we found Tax to display a dose-dependent dual effect on this repair. At low doses Tax rather enhanced NER and only at high doses Tax inhibited this repair. Since the target peripheral blood T-lymphocytes of HTLV-1 in infected people contain functional w.t. p53, the dual Tax effect on the p53-dependent NER observed in our experiments plausibly reflects more faithfully its actual effect on this repair in the infected T-cells of HTLV-1 carriers.

While we are still investigating the mechanism of Tax effect on NER in the w.t. p53-lacking T-cells, the present study was rather focused on the mechanism of the biphasic Tax effect on NER in the w.t. p53-containing T-cells. We proved that the cellular w.t. p53 of these cells was involved in their basal NER activity as well as in the two phases of Tax effect on NER. We also provided evidence that low Tax doses elevated the level and the transcriptional function of the w.t. p53, which in turn was plausibly involved in

enhancing NER. On the other hand, we demonstrated that although high Tax doses further increased the level of w.t. p53, these doses concomitantly activated a mechanism that functionally inactivated the accumulating p53 protein and consequently led to NER inhibition. Notably, we found that this Tax-mediated p53-inactivation impaired also the apoptotic function of exceedingly high p53 levels.

There is a considerable controversy over the mechanism of Tax effect on o53 synthesis.[64] Several studies indicate that p53 protein accumulates in HTLV-1-infected and Tax-expressing T-cells due to its stabilization rather than because of enhancement of its *de novo* synthesis. In contrast, we found in this study that Tax stimulated the transcriptional expression of the promoter of the human p53-encoding gene. This finding indicates that at least part of the elevation of p53 protein level that we observed in the w.t. p53-containing T-cells resulted from increasing its *de novo* synthesis. Furthermore, we proved that this transcriptional activation of the p53 promoter is mediated by an NF-κB-associated pathway. This finding is compatible with the observation of Wu and Lozano who have identified an NF-κB-responsive element within the promoter of the human p53-encoding gene, through which the p65(RelA) NF-κB factor can activate its transcriptional expression in Hela cells.[72] On the other hand, it is contrasting the observation of Uittebogaard *et al*., that in CV-1 cells, Tax represses the expression of the p53 promoter through a basic helix-loop-helix (bHLH) recognition site residing in this promoter.[74] These conflicting results likely reflect a cell type-related variation.[70]

Interestingly, we found that the Tax-mediated p53-functional inactivation is also exerted by an NF-κB-related pathway. While this finding is compatible with several earlier studies,[67–69] it is contrasting others which indicate that p53-functional inactivation by Tax is not dependent on NF-κB[75] or that it is rather exerted through a CREB/ATF-associated pathway.[70,71] Furthermore, several different mechanisms have been proposed for the NF-κB-associated p53 dysfunction by Tax.[67–69,76–78] Studies aimed to define which of these mechanisms is operating in our experimental system are presently in progress in our laboratory.

Haoudi and Semmes have shown that cell cycle arrest facilitates NER activity.[41,42] However, it is rather unlikely that induction of cell cycle arrest was involved in the NER stimulation by the low Tax doses observed in our experiments with the w.t. p53-containing T-cells, since we (unpublished data) and others[42,79] have shown that Tax rather avoids cell cycle arrest. In addition, these authors have noted that forcing the cells into cell cycle arrest by serum starvation abrogated Tax-induced NER inhibition.[42] Therefore they suggest that Tax-mediated NER inhibition might be an indirect consequence of its attenuation of the cell cycle arrest rather than a direct effect on this repair. However, we could not confirm this serum starvation effect in our experimental system (data not shown).

It is important to note, that shortly after human infection, HTLV-1 enters into a latent state, in which viral gene expression is low and so is the level of Tax.[21,23,46] This low Tax level is evidently insufficient for exerting its wide range of oncogenic activities since the vast majority of the HTLV-1 carriers never develop ATL. Therefore, we believe that initiating the leukemogenic process in HTLV-1 infected individual needs activation of the dormant virus for elevating Tax to a pathogenically effective level. Our previous studies[49,58] suggest that this activation can be promoted by stress-inducing agents that are widely prevailing in the daily human surroundings.[23,46] Moreover, in view of the dose-dependent biphasic Tax effect on the p53-dependent NER, observed in this study, it would be interesting to investigate the possibility that the low Tax level existing in cells harboring the latent virus may rather protect these cells from mutagenesis by enhancing NER, and that such protection might partially account for the long HTLV-1 clinical latency. On the other hand, the leukemogenic process may plausibly start, according to this speculation, when the dormant virus is activated by external stimuli, since the elevated Tax, emerging from this activation may destabilize the host cell genome by interfering with NER and certain other guarding mechanisms of the genome integrity. We plan to test this speculation by comparing NER capacity of primary T-cells from latent HTLV-1 carriers, which contain low Tax level, to that of T-cells from TSP/HAM patients, in which HTLV-1 is highly active and its Tax level is markedly elevated.[23,46] Primary T-cells from uninfected people may serve as control for these analyses.

Of note, however, since the leukemic ATL cells contain no or very little amount of Tax,[23,46] we assume that this putative activation of the latent virus is rather transient. On the basis of numerous indications that are thoroughly discussed in our review articles,[23,46] we propose that after activation of the latent virus, the elevated level of Tax transiently enables accumulation of mutations which may include such that hamper DNA repair and the cell-cycle check-points or prevent apoptosis. These mutations can later replace the oncogenic Tax functions after re-silencing the activated virus, and promote further the progression of the leukemogenic process in the absence of Tax. In other words, this hypothetic model implies that Tax only initiates the leukemogenic process, whereas additional Tax-unrelated events are required to complete the progression of this process towards the ultimate ATL state.

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