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A role of the TATA box and the general co-activator hTAF$_{II}$130/135 in promoter-specific *trans*-activation by simian virus 40 small t antigen

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The small t antigen (st-ag) of simian virus 40 can exert pleiotropic effects on biological processes such as DNA replication, cell cycle progression and gene expression. One possible mode of achieving these effects is through stimulation of NFκB-responsive genes encoding growth factors, cytokines, transcription factors and cell cycle regulatory proteins. Indeed, a previous study has shown that st-ag enhanced NFκB-mediated transcription. This study demonstrates that promoters possessing a consensus TATA box (i.e. TATAAAAG) in the context of either NFκB- or Sp1-binding sites are *trans*-activated by st-ag. Overexpressing the general transcription factor hTAF$_{II}$130/135, but not hTAF$_{II}$28 or hTAF$_{II}$80, stimulated the activity of promoters in a consensus TATA box-dependent mode. Converting the consensus TATA motif into a non-consensus TATA box strongly impaired activation by st-ag and hTAF$_{II}$130/135. Conversely, mutating a non-consensus TATA motif into the consensus TATA box rendered the mutated promoter inducible by st-ag and hTAF$_{II}$130/135. Mutation of the TATA box had no effect on TNFα- or RelA/p65-mediated induction of NFκB-responsive promoters, indicating a specific st-ag effect on hTAF$_{II}$130/135. St-ag stimulated the intrinsic transcriptional activity of hTAF$_{II}$130/135. Substitutions in the conserved HPDKGG motif in the N-terminal region or a mutation that impaired the interaction with protein phosphatase 2A abrogated the ability of st-ag to activate hTAF$_{II}$130/135-mediated transcription. These results indicate that *trans*-activation of promoters by st-ag may depend on a consensus TATA motif and suggest that such promoters recruit the general transcription factor hTAF$_{II}$130/135.

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INTRODUCTION

The early region of simian virus 40 (SV40) encodes the two regulatory proteins large T antigen (LT-ag) and small T antigen (st-ag). While much research has been focused on LT-ag, less attention has been paid to st-ag because results derived from studies in cultured cells or transgenic mice demonstrated that this protein is dispensable for lytic infection and transformation (reviewed by Cole, 1996; Arrington & Butel, 2001; Rundell & Parakati, 2001). However, several studies have shown that st-ag augments viral and cellular DNA replication and promotes cell cycle progression in CV-1 cells and human fibroblasts (Shenk *et al.*, 1976; Cicala *et al.*, 1993; Sontag *et al.*, 1993; Howe *et al.*, 1998; Porrás *et al.*, 1999; Whalen *et al.*, 1999). Efficient transformation of growth-arrested cells and enhancement of the transforming activity of limiting concentrations of LT-ag in cell cultures depend on st-ag. Moreover, st-ag may protect against LT-ag-induced apoptosis and a role for st-ag in tumourigenesis *in vivo* has been demonstrated (Sleigh *et al.*, 1978; Martin *et al.*, 1979; Bikel *et al.*, 1987; Choi *et al.*, 1988; Carbone *et al.*, 1989; Cicala *et al.*, 1993; Howe *et al.*, 1998; Kolzau *et al.*, 1999; Hahn *et al.*, 2002). These observations suggest an important helper function for st-ag in LT-ag-mediated processes.

Numerous proteins assemble on the promoter/enhancer region of genes to accomplish transcription of genes. These proteins include the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and RNA polymerase II. TFIID comprises the TATA box-binding protein (TBP) and about 12 TBP-associated factors (TAF$_{II}$s). Although referred to as general transcription factors, distinct TAF$_{II}$ proteins seem to be recruited selectively by specific promoters and are, therefore, essential for the transcription of a subset of genes (Albright & Tjian, 2000; Tsukihashi *et al.*, 2000; Li *et al.*, 2002; and references therein). In addition to these general transcription factors, sequence-specific DNA-binding transcription factors, co-activators and chromatin-remodelling proteins are required to ac2tivate transcription (reviewed by

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Näär *et al.*, 2001). St-ag has been shown to influence the expression of several viral and cellular genes (reviewed by Moens *et al.*, 1997). The exact mechanism by which st-ag exerts its *trans*-activation function on gene expression remains elusive, but may involve the activation of signalling pathways, probably through association with and inhibition of the serine/threonine protein phosphatase 2A (PP2A). This inhibition of PP2A by st-ag may subsequently regulate the phosphorylation pattern/activity of sequence-specific DNA-binding transcription factors. Indeed, inhibition of PP2A by st-ag led to the activation of several signalling pathways mediated by mitogen-activated protein kinases, calmodulin-dependent protein kinase IV, phosphatidylinositol 3-kinase/PKCζ and Akt/PKB (Sontag *et al.*, 1993, 1997; Frost *et al.*, 1994; Watanabe *et al.*, 1996; Howe *et al.*, 1998; Westphal *et al.*, 1998; Garcia *et al.*, 2000; Yuan *et al.*, 2002). Wild-type, but not mutant, st-ag proteins deficient in PP2A binding can influence the activity of the transcription factors cyclic AMP response element-binding protein (CREB), AP-1, NFκB, p53, Sp1, STAT3 and HOX11 (reviewed by Janssens & Goris, 2001; Lacroix *et al.*, 2002). Moreover, st-ag can regulate both serum- and Sp1-responsive promoters in a PP2A-dependent fashion (Frost *et al.*, 1994; Garcia *et al.*, 2000). Whether st-ag can affect the activity of general transcription factors has not been investigated but several studies have demonstrated a TATA-dependent mechanism for transcriptional activation of promoters by other viral proteins. Adenovirus E1A protein exhibits an absolute requirement for a TATA motif, while the varicella-zoster virus immediately-early regulatory protein IE62 *trans*-activates promoters containing a much broader pattern of TATA sequences. Viral transcriptional activators such as Zta (Epstein–Barr virus) and ICP4 (herpes simplex virus) induce transcription by enhancing or stabilizing TBP binding to the TATA box, again illustrating the importance of the TATA box for virus-induced activation of promoter activity (Perera, 2000; and references therein). The hepatitis B virus pX regulatory protein can increase levels of TBP, which serves to enhance both RNA polymerase I and III promoter activities, while the effects on RNA polymerase II promoters are different. TATA-lacking promoters are generally unaffected by increased levels of TBP, while overexpression of TBP stimulates TATA-containing promoters (Johnson *et al.*, 2000).

Previous studies in NIH 3T3 and CV-1 cells have shown that st-ag can stimulate NFκB- and Sp1-dependent transcription and that this induction of transcription was mediated by PKCζ and its upstream regulator phosphatidylinositol 3-kinase (Sontag *et al.*, 1997; Garcia *et al.*, 2000). Because NFκB and Sp1 interact physically with PP2A and st-ag inhibits PP2A, it is generally assumed that a major mechanism by which st-ag induces NFκB- and Sp1-dependent transcription relies on prevention of PP2A-mediated dephosphorylation of these transcription factors (Yamashita *et al.*, 1999; Yang *et al.*, 2001; Lacroix *et al.*, 2002). Here, we report that *trans*-activation of NFκB- and Sp1-responsive promoters by st-ag depends on a consensus TATAAAAG TATA motif. Such st-ag responder promoters were also stimulated by the general transcription factor $hTAF_{II}130/135$. Wild-type st-ag, but not a mutant unable to bind PP2A, or a mutant in the conserved hexapeptide HPDKGG, increased the intrinsic transcriptional activity of $hTAF_{II}130/135$. These data suggest that $hTAF_{II}130/135$ is a promoter-specific transcription factor whose activity may be regulated by PP2A-dependent and -independent mechanisms. Transcriptional activation of specific cellular genes by st-ag through preventing PP2A-mediated dephosphorylation of $hTAF_{II}130/135$ may, therefore, facilitate virus replication or transformation.

METHODS

Cell culture. NIH 3T3 cells (ATCC CRL 1658) were maintained as described before (Seternes *et al.*, 1999).

Materials. Recombinant murine TNFα was purchased from Alexis Biochemicals. Sonicated salmon sperm and calf thymus DNA were from Amersham Pharmacia. Newborn calf serum was from BioWhittaker. Oligonucleotides were purchased from Eurogentec or Invitrogen.

Plasmids. The NFκB-responsive luciferase reporter plasmids and their corresponding control plasmids κBconA-LUC and conA-LUC, TI-3x κB-LUC and TI-LUC, 2x κB-LUC and 2x κB-m1LUC, ENH-TK-LUC and mENH-TK-LUC were kindly provided by E. Sontag (Sontag *et al.*, 1997), X.-F. Wang (Li *et al.*, 1998), C. Scheidereit (Hirano *et al.*, 1998) and F. Bachelerie (Bachelerie *et al.*, 1991), respectively. The plasmids pTAL-LUC and pNFκB-LUC(C) were purchased from Clontech, while the plasmid pNFκB-LUC(S) was obtained from Stratagene. Expression plasmid for the dominant–negative mutant of IκB (pCMV-I$\kappa B\alpha_{S32/36A}$) has been described previously and was kindly made available by M. Körner (Ferreira *et al.*, 1998). The GAL4 fusion plasmids pGAL4-p65 and pGAL4-p65(416-550) were kindly provided by B. R. Cullen (Blair *et al.*, 1994). The RelA/p65 expression plasmid was obtained from J. A. Didonato (Zhang *et al.*, 1994), while the plasmid pG5E1bLuc was a kind gift from R. Davis (Seth *et al.*, 1991). The plasmid pCMV5 and the st-ag expression plasmid pCMV5st (wild-type st-ag) were a generous gift from E. Sontag (Sontag *et al.*, 1993). The pMIEP-LUC plasmid, containing the major immediate-early promoter of human cytomegalovirus, and the double mutant pMIEPdm-LUC with non-functional NFκB motifs have been described previously (Moens *et al.*, 2001). Plasmid pNFκB-fosTATA-LUC was constructed by annealing the double-stranded oligonucleotide 5′-AGCTCGGG-AATTTCCGGGATTTCCGGGAATTTCTCATTCATAAAACGCTTG-TTATAAAAGCAGTGGCTGCGGCGCCTCGTACTCCAAC-3′ (only one strand is given) into the *Bam*HI/*Hin*dIII sites of pO-LUC. The resulting plasmid contains three copies of the consensus NFκB-binding site linked to the TATA box region of the c-*fos* promoter. The plasmid was sequenced to verify the insertion. Plasmid $pCMVhTAF_{II}130/135$ was kindly provided by N. Tanese (Saluja *et al.*, 1998). An expression plasmid for GAL4–$hTAF_{II}130/135$ fusion protein was prepared by amplifying the $hTAF_{II}130/135$ cDNA sequences using the $pCMVhTAF_{II}130/135$ plasmid as template and the primers 5′-CAACAGCGAGGTCGACGAGAAAGTGGG-3′ and 5′-GAGCAGCAGTGAAAAGCTTGTCTCACG-3′. The PCR product was digested with *Sal*I/*Bam*HI (restriction sites are underlined) and cloned in the corresponding sites of the GAL4 DNA-binding domain expression plasmid pM (Clontech) to generate pGAL4-$hTAF_{II}130/135$. The plasmid was sequenced to ensure ligation of the

$hTAF_{II}130/135$ cDNA sequences in the correct reading frame. Expression vectors for $hTAF_{II}28$ and $hTAF_{II}80$ were a generous gift from R. G. Roeder (Guermah *et al.*, 2001).

Site-directed mutagenesis. Site-directed mutagenesis was performed with the QuickChange Site-Directed Mutagenesis kit from Stratagene, according to the instructions of the manufacturer. The TATA box motif in κBconA-LUC (see Fig. 3A) was replaced by the TATA element of pNFκB-LUC(C) (Fig. 3A) using complementary oligonucleotides (5′-GGGCTGCTCCTC**TATATTTT**GGGAAGAAAG-3′, only one primer is shown; the TATA box is shown in bold). The TATA elements of pTAL-LUC and pNFκB-LUC were converted into the TATA element of the κBconA promoter using a complementary primer set (5′-GACATGCAAA**TATAAAA**GTTCCGGGGACAC-3′, only one primer is given; the converted TATA motif is shown in bold). The st-ag mutants P43L/K45N and P101A were obtained by site-directed mutagenesis applying the primers of the complementary set (5′-GAGTTTCATCTAGATAACGGAGGAGATG-3′ and 5′-GC-AAACAATGCGCAGAGTGTGCAAAGATGTCTGC-3′, respectively, only one primer of the complementary set is shown). All mutations were verified by cycle sequencing using the Big Dye Sequencing kit (Perkin Elmer). Sequencing reactions were analysed on an ABI377 Prism Sequencer (Perkin Elmer).

Transfections and luciferase activity. For transient transfections, 2×10^5 NIH 3T3 cells were seeded per 35 mm culture dish and transfected 24 h later, as described previously (Seternes *et al.*, 1999). All transfections were performed with cell passages between 130 and 144. Cells were serum-starved (0·2 %) for 18 h before being harvested. The amount of total DNA in each transfection mixture was kept constant (5 μg per well) by adding appropriate control vector DNA and salmon sperm or calf thymus DNA (Amersham Pharmacia). All plasmids were purified with the Qiagen Plasmid Purification kit and 1 μg plasmid was used normally per well. Different plasmid DNA preparations were tested in the transfection studies. Each experiment was performed with three independent parallels and was repeated at least three times to ensure reproducibility of results. Luciferase activity was determined in 20 μl lysate using the Luciferase Assay System kit (Perkin Elmer) and a Luminoscan RT (Labsystems). Co-transfections with a β-galactosidase reporter plasmid were avoided because expression of st-ag influenced β-galactosidase values. Moreover, it is our experience that correction for protein concentrations in each cell lysate had a negligible effect on results (P. A. Olsen, unpublished results).

Western blotting. Western blotting was performed as described previously (Seternes *et al.*, 1999). GAL4 antibodies were from Santa Cruz Biotechnology (cat #sc-577). Densitometry quantification of the hybridization signals was performed with a LumiImager F1 using LumiAnalyst software (Boehringer Mannheim).

Statistics. Statistical significance of results was determined by Student's *t*-test with $P < 0{\cdot}01$.

RESULTS

St-ag selectively activates NFκB-responsive promoters

A previous study had shown that st-ag activated NFκB-dependent transcription in both NIH 3T3 and CV-1 cells (Sontag *et al.*, 1997). However, we were not able to repeat these observations using two commercially available NFκB reporter gene constructs (pNFκB-LUC from Clontech and Stratagene). This prompted us to test whether st-ag could stimulate transcription of other NFκB reporter plasmids, including that originally used by E. Sontag and colleagues. Seven different reporter plasmids containing different minimal promoters fused to NFκB-binding motifs were co-transfected with an expression plasmid for st-ag in NIH 3T3 cells. The corresponding promoters lacking the NFκB motifs were used as controls. None of the promoters lacking NFκB-binding motifs were *trans*-activated by st-ag (results not shown), while only one promoter with NFκB-binding sites (κBconA-LUC, i.e. the one used by Sontag and colleagues) was induced when st-ag was co-expressed (Fig. 1a). On average, a 4·8-fold increase in luciferase activity was measured in the presence of st-ag. Maximal induction was obtained with the reporter plasmid and the st-ag expression plasmid at a ratio of 1 : 1 (results not shown). The failure of most NFκB-responsive promoters to be *trans*-activated by st-ag was not due to impaired NFκB motifs, as these promoters were inducible by co-expression of p65/RelA (Fig. 1b) or after stimulation of transfected cells with TNFα (Fig. 1c). Corresponding promoters lacking NFκB motifs were not induced by p65/RelA and TNFα (data not shown). A synergistic increase in κBconA promoter activity was measured when st-ag and RelA/p65 were co-expressed (Fig. 1d). This suggests that st-ag may enhance further the transcriptional potentials of RelA/p65 or that st-ag *trans*-activates the promoter of the κBconA-LUC reporter plasmid through an alternative mechanism.

Trans-activation of a promoter containing NFκB-binding motifs by st-ag is partially mediated by NFκB

Studies by different groups have shown that both st-ag- and TNFα-mediated activation of PKCζ resulted in IκBα degradation, subsequent nuclear translocation of NFκB and stimulation of expression of NFκB-responsive genes (Folgueira *et al.*, 1996; Sontag *et al.*, 1997). As we observed a promoter-selective activation of NFκB-responsive promoters by st-ag, we wanted to define whether this activation required an alternative mechanism. Therefore, the dominant–negative $I\kappa B\alpha_{S32/36A}$ mutant, which is unable to be phosphorylated and proteolytically degraded, was overexpressed (Ferreira *et al.*, 1998). This mutant will retain NFκB in the cytoplasm even in the presence of st-ag. Co-expression of $I\kappa B\alpha_{S32/36A}$ completely abolished RelA/p65-induced luciferase activity from the κBconA-LUC reporter plasmid, while st-ag-enhanced NFκB-mediated transcription was reduced by 40–50 % (Fig. 2a). The observation that $I\kappa B\alpha_{S32/36A}$ could not completely block *trans*-activation of the NFκB-responsive promoter by st-ag may suggest an additional mechanism by which st-ag regulates the activity of NFκB-directed transcription. It could be imagined that, in addition to promoting nuclear translocation, as shown by Sontag *et al.* (1997), st-ag stimulated the intrinsic transcriptional potentials of RelA/p65, such as has been demonstrated for other transcription factors (reviewed by Janssens & Goris, 2001). To test this, we examined RelA/p65-mediated transcription in the presence and

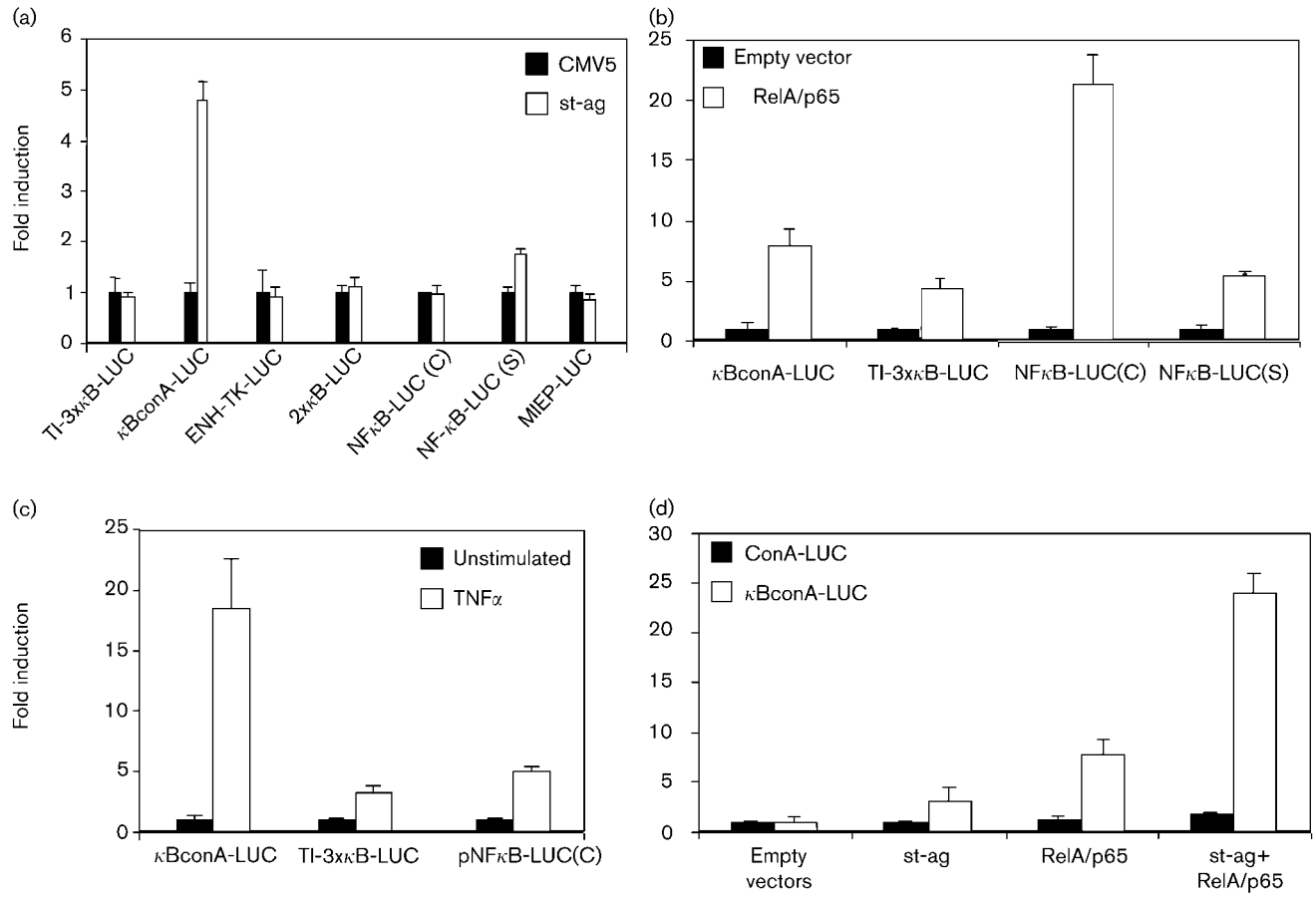

Fig. 1. St-ag selectively activates NFκB-responsive promoters. (a) NIH 3T3 cells were transfected with 1 μg luciferase reporter plasmid and 1 μg of the empty expression vector pCMV5 or 1 μg of a plasmid expressing st-ag. After transfection, cells were grown for 18 h in 0·2 % serum before being harvested. Luciferase activity in each cell extract was determined. Results are shown as fold induction, with luciferase activity of the reporter plasmid in the absence of st-ag arbitrarily set as 1·0. Results represent a typical experiment and data are presented as the average of three independent parallels (±SD). (b) Co-expression of RelA/p65-induced NFκB-mediated transcription. As in (a) but cells were co-transfected with either 1 μg empty pRcCMV vector or RelA/p65 expression vector. (c) TNFα stimulates the transcriptional activity of NFκB-responsive promoters. At 18 h after transfection, cells were treated for 5 h with 30 ng TNFα ml^{-1} before being harvested. Luciferase activity in cell extracts of unstimulated cells was set arbitrarily as 1·0. (d) Simultaneous expression of st-ag and RelA/p65 results in synergistic *trans*-activation of the κBconA promoter.

absence of st-ag by using GAL4 fusion proteins. Cells were co-transfected with the empty expression vector for st-ag (CMV5) or the expression vector for st-ag and with a plasmid encoding GAL4–p65 (full-length) or GAL4–p65(416-550) fusion proteins. The latter encompasses the transactivation domain of RelA/p65. St-ag failed to increase transcription mediated by the GAL4–p65 or GAL4–p65(416-550) fusion proteins, indicating that st-ag does not stimulate the intrinsic transcriptional potentials of NFκB (Fig. 2b). Together, these results may suggest that supplementary events or factors, in addition to NFκB, are involved in mediating *trans*-activation of NFκB-responsive promoters by st-ag.

The TATA box motif is crucial for st-ag-stimulated NFκB-mediated transcription

Comparing the promoter sequences of the seven reporter plasmids that were tested, we noticed a difference in the TATA box motif (Fig. 3a). Only the st-ag-inducible κBconA promoter possessed a TATA motif with perfect identity to the consensus TATA motif, while the other six promoters, which were not *trans*-activated by st-ag, possessed TATA sequences that diverged from this consensus sequence (Périer *et al.*, 2000). To elucidate the importance of the TATA motif in st-ag-stimulated NFκB-mediated transcription, promoter mutation experiments were performed. The TATA motif of the non-st-ag-responsive

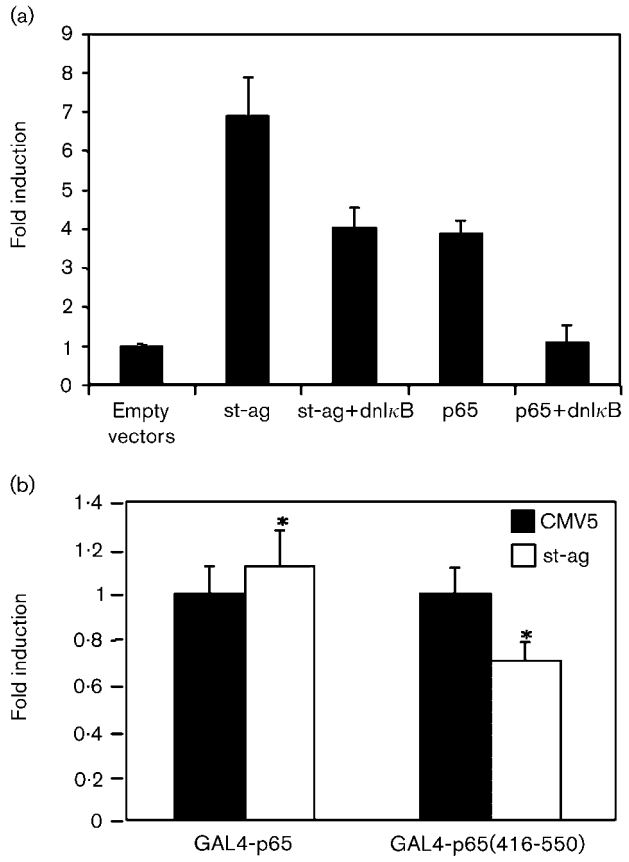

Fig. 2. The dominant–negative IκBα mutant ($I\kappa B\alpha_{S32/36A}$) partially abrogates st-ag-induced NFκB-mediated transcription. (a) Cells were transfected with the κBconA-LUC reporter plasmid (1 μg) in the presence of st-ag or/and $I\kappa B\alpha_{S32/36A}$ (dnIκB) expression plasmid (1 μg). As a control, cells were co-transfected with the RelA/p65 expression plasmid (1 μg) and/or $I\kappa B\alpha_{S32/36A}$. (b) St-ag fails to stimulate the transcriptional activity of GAL4–RelA/p65. Cells were transfected with 1 μg expression plasmids for the GAL4–p65 fusion proteins. The plasmid pGAL4-p65 encodes full-length RelA/p65, while the plasmid pGAL4-p65(416-550) encompasses the transactivator domain of RelA/p65 spanning aa 416–550. GAL4–p65- and GAL4–p65(416-550)-mediated transcription of the luciferase gene was measured in the presence of empty expression vector for st-ag (CMV5) or st-ag. Luciferase activity measured in the absence of st-ag was set arbitrarily as 1·0. Results represent the average of three independent parallels (±SD). Asterisks indicate that the observed differences were not significant ($P<0{\cdot}01$).

promoter in pNFκB-LUC(C) was replaced with the κBconA TATA region and vice versa: the κBconA TATA sequence was converted into the TATA motif present in pNFκB-LUC(C). These mutations reduced the basal activity of κBconA-LUC by almost 50 %, while the mutated NFκB-LUC plasmid had a slightly increased basal activity in the absence of st-ag compared to the non-mutated promoter (data not shown). The mutated κBconA promoter (κBmutconA) was still induced by the st-ag expression plasmid; however, an almost 50 % reduction was measured compared to the wild-type promoter. Interestingly, pNFκBmut-LUC with consensus TATA box showed a 4-fold increase in *trans*-activation by st-ag as compared to the non-mutated pNFκB-LUC (Fig. 3b). Neither RelA/p65- nor TNFα-induced transcription was affected by mutations in the TATA motif (Fig. 3b). These results clearly indicate the importance of the TATA box motif for st-ag-mediated activation of a promoter. To test experimentally the importance of the TATA motif in the induction of NFκB-responsive promoters by st-ag further, we investigated whether a chimeric promoter consisting of the c-*fos* TATA motif (a consensus TATA motif; Fig. 3a) and three copies of the NFκB-binding site could be *trans*-activated by st-ag. As depicted in Fig. 3(c), this promoter was also induced by st-ag. These results underscore further the importance of the TATA motif in mediating st-ag-induced *trans*-activation of NFκB-responsive promoters.

A Sp-1-responsive promoter with the consensus TATA motif but not with a non-consensus TATA box is also *trans*-activated by st-ag

The minimal chicken conalbumin (conA) promoter lacking the NFκB-binding motifs was not stimulated by st-ag (Fig. 1d). This suggested to us that the TATA box per se is not sufficient to induce activation by st-ag but, in addition to an appropriate TATA motif, an upstream binding motif (e.g. NFκB) is required to mediate promoter *trans*-activation by st-ag. A recent report demonstrated that st-ag could activate Sp1-responsive promoters (Garcia *et al.*, 2000). The authors used promoter constructs containing either the adenovirus major late promoter or the human immunodeficiency virus LTR promoter/enhancer. Both promoters possess a consensus TATA motif (Fig. 3a). This observation indicates that Sp1 motifs in concert with a consensus TATA box sequence may also mediate st-ag-induced *trans*-activation. This assumption was investigated by testing whether st-ag could activate promoter activity of the plasmid pTAL-LUC. This plasmid contains a single Sp1 motif linked to the same non-consensus TATA box present in pNFκB-LUC(C) (Fig. 3a). We also converted the TATA motif in pTAL-LUC into the conA TATA box to generate the reporter plasmid pTALmTATA-LUC. St-ag did not induce transcription from the pTAL-LUC plasmid but changing the TATA box into a consensus TATA motif stimulated luciferase activity about 2-fold in the presence of st-ag (Fig. 4). These results prove that the consensus TATA box combined to specific upstream binding elements can mediate st-ag induction.

Promoters with consensus TATA motifs are activated by overexpression of $hTAF_{II}130/135$

Our results demonstrated that NFκB- or Sp1-binding motifs combined with the conA promoter could be *trans*-activated by st-ag. The transcription factors NFκB and Sp1 have been shown to interact with the general transcription factors

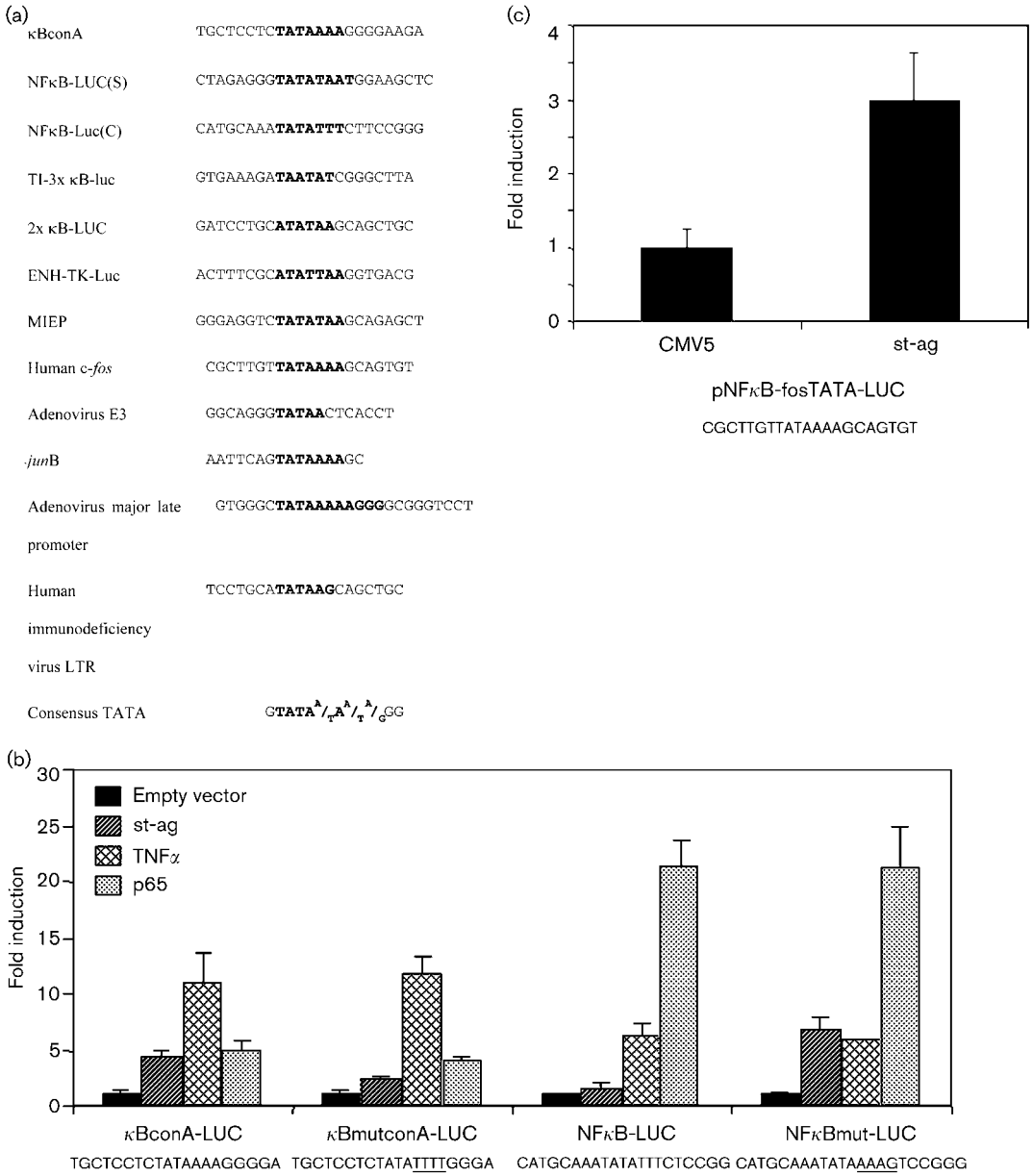

Fig. 3. The TATA box motif is crucial for st-ag-enhanced NFκB-mediated transcription but not for RelA/p65- or TNFα-induced activation of NFκB-responsive promoters. (a) Comparison of the TATA box sequences found in the different NF-κB-responsive reporter plasmids, used in this study, and in st-ag-responsive promoters. The TATA box regions of TI-3x κB-LUC, κBconA-LUC, 2x κB-LUC, ENH-TK-LUC, MIEP-LUC and pNFκB-LUC(C) were confirmed by sequencing, while the TATA sequences of NFκB-LUC(S), human c-*fos* (Van Straten *et al.*, 1983), adenovirus E3 (Cladaras & Wold, 1985), *jun*B (Apel *et al.*, 1992), the adenovirus major late promoter, the human immunodeficiency virus LTR (Olsen & Rosen, 1992) and the consensus TATA box (Patikoglou *et al.*, 1999) have been published previously. (b) *Trans*-activation of NFκB-responsive promoters by st-ag but not RelA/p65 or TNFα requires the conA TATA motif. Transfections and calculation of relative luciferase activities were as described in detail in the legend of Fig. 1. (c) The c-*fos* TATA box, when fused to NFκB-binding sites, can be induced by st-ag. The corresponding sequence of the TATA box region of each plasmid is shown. Mutations are underlined.

hTAF$_{II}$105 and hTAF$_{II}$130/135, respectively (Saluja *et al.*, 1998; Yamit-Hezi & Dikstein, 1998). hTAF$_{II}$105 shares regions of high homology with hTAF$_{II}$130/135. Interestingly, st-ag has been shown to interact *in vitro* with dTAF$_{II}$110, the *Drosophila* homologue of hTAF$_{II}$130/135 (Damania & Alwine, 1996). This prompted us to investigate whether hTAF$_{II}$130/135 is utilized by the Sp1- and NFκB-responsive promoters that are activated by st-ag. Therefore, the effect of hTAF$_{II}$130/135 protein on promoters with consensus and non-consensus TATA boxes was monitored.

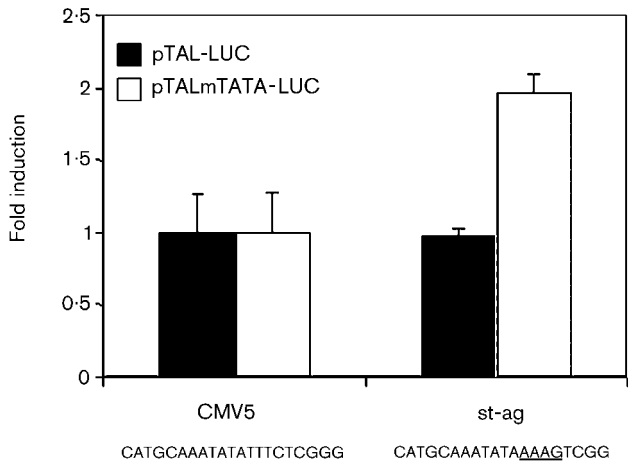

Fig. 4. A Sp-1-responsive promoter in the context of the appropriate TATA motif is *trans*-activated by st-ag. Cells were transfected with the plasmid pTAL-LUC or pTALmTATA-LUC. A single Sp1 site and a non-consensus TATA box drive the luciferase reporter gene in the former plasmid, while the TATA box is replaced by the conA TATA box in the plasmid pTALmTATA-LUC. Luciferase activity was monitored in the presence of empty vector (CMV5) or st-ag. Luciferase activities were determined as described in the legend of Fig. 1. Results of a representative experiment are shown. The corresponding sequence of the TATA box region of each plasmid is shown. Mutations are underlined.

Overexpression of $hTAF_{II}130/135$ activated the promoters containing the consensus TATA motif 2- to 5·5-fold, while mutating these promoters into the corresponding promoters with non-consensus TATA sequence reduced *trans*-activation by $hTAF_{II}130/135$ (Fig. 5a). These results favour a model in which st-ag induced activation of specific promoters that utilize the general transcription factor $hTAF_{II}130/135$. The activation of promoters with consensus TATA box by $hTAF_{II}130/135$ was specific for this general transcription factor because neither $hTAF_{II}28$ nor $hTAF_{II}80$ could induce promoters, irrespective of the sequence of the TATA box (Fig. 5b).

St-ag potentiates the intrinsic transcriptional activity of $hTAF_{II}130/135$

Next, we tested whether st-ag could affect $hTAF_{II}130/135$-mediated transcription. Co-expression of wild-type st-ag stimulated the intrinsic transcriptional potentials of $hTAF_{II}130/135$ about 4-fold (Fig. 6a). The st-ag mutant protein P101A, which fails to bind PP2A (Mungre *et al.*, 1994), did not activate transcription mediated by $hTAF_{II}130/135$. The st-ag double mutant P43L/K45N, shown previously to be unable to activate the adenovirus E2A promoter but not impaired in PP2A binding (Mungre *et al.*, 1994), did not induce $hTAF_{II}130/135$-mediated transcription. The differences in activation of $hTAF_{II}130/135$-mediated transcription by the distinct st-ag variants are not the result of unequal expression levels of the various st-ag proteins, as previous studies have shown that the mutant proteins are expressed with comparable levels and stability as wild-type st-ag (Mungre *et al.*, 1994; Porrás *et al.*, 1996; Watanabe *et al.*, 1996; Howe *et al.*, 1998). Enhanced GAL4–$hTAF_{II}130/135$-directed transcription in the presence of st-ag was not the result of increased GAL4–$hTAF_{II}130/135$ protein levels, as co-expression of st-ag hardly (1- to 1·5-fold increase in independent experiments) affected the amount of GAL4–$hTAF_{II}130/135$ in transfected cells (Fig. 6b).

DISCUSSION

The *trans*-activating st-ag protein of SV40 can govern important cellular processes by controlling the activity or expression of specific cellular proteins, thereby creating an environment that allows optimal virus replication in the host cells (Hahn *et al.*, 2002). It is generally assumed that a major mechanism by which st-ag induces transcription is through inhibition of PP2A-dependent dephosphorylation of transcription factors (reviewed by Janssens & Goris, 2001). Here, we describe a novel mechanism of promoter-specific *trans*-activation by st-ag. St-ag preferentially activated promoters consisting of a consensus TATAAAAG TATA motif and binding sites for the transcription factor NFκB or Sp1. Previous studies had shown that st-ag regulates the activities of the promoter/enhancer of adenovirus E2A and E3 genes, the oncogenes c-*fos* and *jun*B, the cyclins A and D1 and the proliferating cellular nuclear antigen (PCNA) gene (reviewed by Moens *et al.*, 1997). A closer examination of the TATA boxes in the human c-*fos*, E3, *jun*B and conA promoters reveals the presence of the TATA consensus motif, supporting the importance of the sequence of the TATA motif. We found that the amplitude of induction of the NFκB-responsive promoters with consensus TATA motif (the conA and the chimeric c-*fos* promoters) by st-ag was comparable to the stimulation reported previously for the phosphoenolpyruvate carboxykinase promoter (Wheat *et al.*, 1994), the cyclin D1 promoter (Watanabe *et al.*, 1996), the cyclin A promoter (Schüchner *et al.*, 2001; Porrás *et al.*, 1996) and the c-*fos* promoter (Mullane *et al.*, 1998). The consensus TATA promoter fused to a single Sp1 site was only induced 2-fold in our study. This is in agreement with the thymidine kinase promoter. This promoter, which contains a single Sp1 motif and a single CRE motif, was induced to comparable levels (2·5-fold) by st-ag in JEG-3 cells (Watanabe *et al.*, 1996).

The fact that st-ag also affects the activities of the TATA-less cyclin A, cyclin D1 and PCNA promoters (Travali *et al.*, 1989; Herber *et al.*, 1994; reviewed by Moens *et al.*, 1997) may suggest that the flanking regions are important. This observation supports further a plausible importance of the flanking sequences. Converting the consensus TATAAAAGGG motif of the κBconA promoter into a non-consensus TATATTTTGG sequence of the NFκB-LUC plasmid did not completely abrogate *trans*-activation by

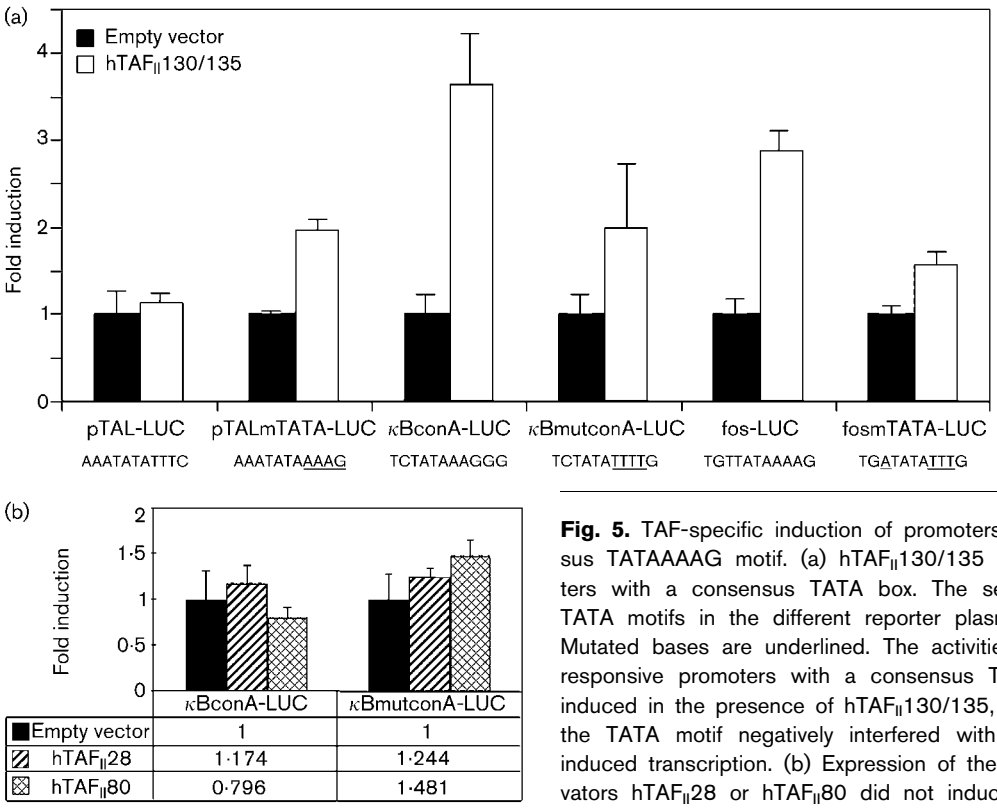

Fig. 5. TAF-specific induction of promoters with a consensus TATAAAAG motif. (a) hTAF$_{II}$130/135 activates promoters with a consensus TATA box. The sequences of the TATA motifs in the different reporter plasmids are shown. Mutated bases are underlined. The activities of the NFκB-responsive promoters with a consensus TATA motif were induced in the presence of hTAF$_{II}$130/135, while converting the TATA motif negatively interfered with hTAF$_{II}$130/135-induced transcription. (b) Expression of the general co-activators hTAF$_{II}$28 or hTAF$_{II}$80 did not induce the activity of the tested promoters. Transfections were with 1 μg of the expression and reporter plasmids each and calculations of luciferase activity were as described in the legend of Fig. 1. Relative fold induction values are shown.

st-ag or hTAF$_{II}$130/135, as this mutated promoter was still induced 2·5-fold compared to 4- to 5-fold for the unmutated promoter. On the other hand, the promoter of the NFκB-LUC plasmid was not responsive to st-ag or hTAF$_{II}$130/135. This discrepancy may be explained by assuming that sequences flanking the TATA box could be involved in recruiting hTAF$_{II}$130/135. Indeed, recent studies have demonstrated that TAF$_{II}$s are not only recruited through protein–protein interaction with TBP and each other but also that they can bind DNA directly in a sequence-specific mode, thereby contributing to promoter selectivity (Albright & Tjian, 2000; Green, 2001). Moreover, binding of hTAFII130/135 to the flanking sequences may affect the binding of TBP to the TATA box, as was shown recently (Furukawa & Tanese, 2000). Point mutations of the flanking sequences and in the consensus TATA motif may enable the identification of crucial nucleotides required to mediate *trans*-activation by st-ag.

St-ag-responsive promoters were also activated when hTAF$_{II}$130/135 was overexpressed and vice versa: st-ag non-responsive promoters were not induced by hTAF$_{II}$130/135. The exact molecular mechanism(s) underlying the activator effect of st-ag on promoters containing a consensus TATA box combined with a NFκB- or Sp1-binding motif remains unknown. St-ag may prevent PP2A-mediated dephosphorylation of hTAF$_{II}$130/135, as the non-PP2A binding P101A st-ag mutant was unable to stimulate the intrinsic transcriptional activity of hTAF$_{II}$130/135 and also failed to activate the κBconA promoter (result not shown). However, a PP2A-independent mechanism is suggested by the studies with the P43L/K45N double mutant. This mutant st-ag is still able to bind PP2A but failed to stimulate GAL4–hTAF$_{II}$130/135-mediated transcription and could not activate the κBconA promoter (result not shown). Previous studies have demonstrated that this mutant failed to *trans*-activate the adenovirus E2 and the cyclin A promoters (Mungre *et al.*, 1994; Porrás *et al.*, 1996) but induced the activity of the cyclin D1 promoter with comparable levels as wild-type st-ag (Watanabe *et al.*, 1996). The cyclin A and D1 promoters both lack a TATA box, while a non-consensus TATA motif is present in the adenovirus E2 promoter (Herber *et al.*, 1994). These findings, combined with our observations, add to the diversity of transcriptional regulation by st-ag and require future research to solve the exact mechanisms by which st-ag can *trans*-activate promoters.

It remains to be established whether the novel mechanism by which st-ag can activate gene expression contributes to a successful SV40 infection or transformation. Prevalence for this mechanism derives from the following observations:

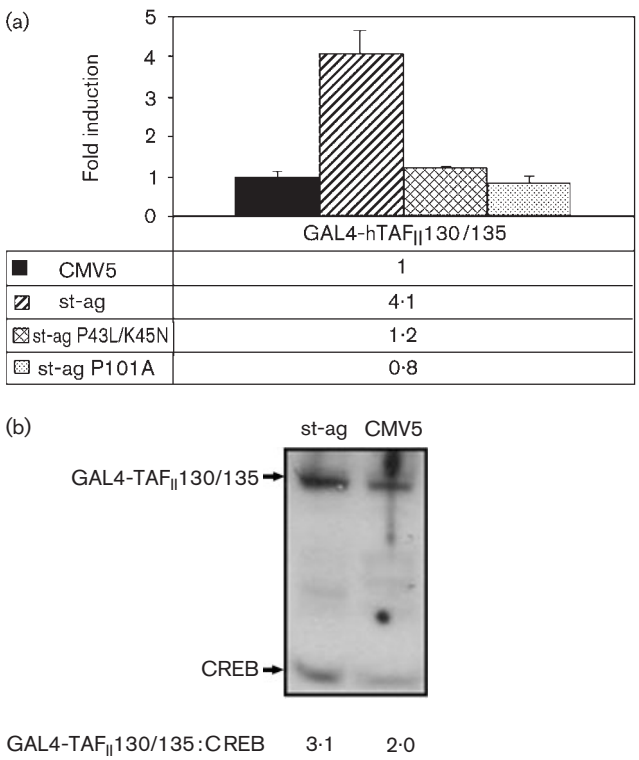

Fig. 6. Wild-type st-ag increases the intrinsic transcriptional potentials of hTAF$_{II}$130/135. (A) NIH 3T3 cells were co-transfected with 1 μg of plasmid encoding the GAL4–hTAF$_{II}$130/135 fusion protein and empty expression vector CMV5 or expression vectors for wild-type st-ag or mutants P43L/K45N or P101A. The former can still bind PP2A but cannot *trans*-activate the adenovirus E2A promoter, while the latter is impaired in PP2A binding. The result of a representative experiment is shown. Luciferase activity represents the average of three independent parallels (±SD). Luciferase activity in the absence of st-ag was set arbitrarily as 1·0. (B) Co-expression of st-ag had almost no effect on the levels of GAL4–hTAF$_{II}$130/135. Cells were co-transfected with the GAL4–hTAF$_{II}$130/135 expression vector and empty pCMV5 or st-ag-encoding vector. GAL4–hTAF$_{II}$130/135 protein levels were detected using anti-GAL4 antibodies and were correlated to endogenous levels of CREB. Numbers represent the relative ratio of the GAL4–hTAF$_{II}$130/135 : CREB hybridization signals, as determined by densitometric scanning. In an independent experiment, no differences in GAL4–hTAF$_{II}$130/135 protein levels were detected in the presence or absence of st-ag.

SV40 infection induced quiescent cells to re-enter the S phase and re-entering the cell cycle coincided with increased c-*fos* transcription. St-ag was important for enhanced expression of c-*fos* (Morike *et al.*, 1988; Glenn & Eckhart, 1990; Ogris *et al.*, 1992). Another study showed that granulocyte macrophage colony-stimulating factor promoter was induced upon polyomavirus replication in haematopoietic cells. The effect of st-ag was, however, not examined (Watanabe *et al.*, 1995). Both the c-*fos* and the granulocyte macrophage-colony stimulating factor promoter contain a consensus TATA motif and Sp1 (c-*fos*) or NFκB (granulocyte macrophage-colony stimulating factor) binding sites. Finally, this mechanism may also contribute to virus immune evasion. Epstein–Barr virus encodes a protein homologous to cellular IL-10. IL-10 is a negative regulator of IL-12, itself a cytokine that both promotes IFN-γ production and influences the development of Th1- and Th2-like cytokine-producing cells, and of TAP, a protein involved in transport and presentation of processed peptide antigens (Ploegh, 1998). The promoter of IL-10 contains a consensus TATAAAAG TATA box and a crucial Sp1 site (Tone *et al.*, 2000; Ma *et al.*, 2001). This makes this gene a putative target for *trans*-activation by st-ag and a strategy for SV40 to subvert the immune system.

In conclusion, *trans*-activation of specific promoters by st-ag may rely upon at least two different mechanisms. First, st-ag may prevent PP2A-mediated dephosphorylation of specific transcription factors like CREB, Sp1, NFκB, STAT and AP-1 and thereby stimulate the activity of promoters regulated by these transcription factors (reviewed by Janssens & Goris, 2001). In addition, st-ag may stimulate the promoter activity of promoters utilizing the general transcription factor hTAF$_{II}$130/135. The exact molecular mechanism by which st-ag induces hTAF$_{II}$130/135-responsive promoters needs to be elucidated.

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