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TNFα and GM-CSF-induced activation of the CAEV promoter is independent of AP-1

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Abstract

Caprine arthritis encephalitis virus transcription is under the control of the viral promoter within the long terminal repeat. Previous studies with the closely related maedi visna lentivirus have indicated that viral transcription is dependent upon the AP-1 transcription factor. Other studies have indicated a potential role for the cytokines TNFα and GM-CSF in CAEV pathogenesis by increasing viral loads in infected tissues. The hypotheses that AP-1 transcription factors are necessary for transcriptional activation of the CAEV promoter and that CAEV transcriptional activation results from treatment with the cytokines GM-CSF and TNFα were tested with a stably transduced U937 cell line. Here, we found that TNFα and GM-CSF activated CAEV transcription in U937 cells. However, this activation effect was not blocked by SP600125, an inhibitor of Jun N-terminal kinase. SP600125 effectively prevented Jun phosphorylation in cells subsequently treated with cytokines. The cytokines TNFα and GM-CSF therefore activate CAEV transcription, and this effect occurs independently of AP-1. A set of progressive deletion mutants was utilized to show that TNFα-induced expression depends on an element or elements within the U3 70-bp repeat.

Keywords: CAEV; TNFα; GM-CSF; AP-1; Transcription

Introduction

Caprine arthritis encephalitis virus (CAEV) and maedi visna virus (MVV) are closely related lentiviruses that infect goats and sheep, respectively. These two small ruminant lentiviruses (SRLV) share many key features including similarities in genome structure, disease pathogenesis and host cell tropism. CAEV is a monocyte–macrophage tropic lentivirus that causes a nonsuppurative inflammatory disease complex in naturally and experimentally infected goats (Cheevers and McGuire, 1988; Crawford et al., 1980; Cush and Lipsky, 1991; von Bodungen et al., 1998; Zvaifler and Firestein, 1994). Virus replication within the monocyte–macrophage results in lesions characterized by mononuclear cell infiltration of the synovial joints (caprine arthritis, CA), central nervous system, lungs and mammary gland (Kennedy-Stoskopf et al., 1987). CA, the most clinically relevant consequence of CAEV infection, is a chronic progressive synovitis resulting in accumulation of synovial fluid, periarticular swelling, soft tissue mineralization and erosion of articular surfaces (Wilkerson et al., 1995).

The precise mechanism(s) whereby CAEV infection results in tissue pathology remains controversial. However, several in vivo and in vitro experiments indicate that the cytokines tumor necrosis factor-alpha (TNFα) and granulocyte–macrophage colony stimulating factor (GM-CSF) play a role in CA lesion development. In rheumatoid arthritis, a related chronic inflammatory disease of humans, TNFα is considered a key regulatory cytokine (Lechner et al., 1996), and both TNFα and GM-CSF mRNA are elevated within the joint synovium (Firestein et al., 1990). CAEV-infected goats have elevated serum TNFα levels compared to uninfected control goats (Mdurvwa et al., 1994). Macrophages producing abundant TNFα mRNA have been demonstrated in synovial tissue of goats with CA lesions and correlate with lesion severity (Lechner et al., 1996, 1997b). TNFα mRNA has been detected

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in the infected synovium of CA lesions as early as 6 days after infection (Lechner et al., 1997b). Macrophages infected with CAEV in vitro and stimulated with LPS express more GM-CSF mRNA than mock infected cells (Lechner et al., 1997a). In MVV infected sheep, GM-CSF mRNA expression in alveolar macrophages correlates with the presence of pulmonary lesions (Woodall et al., 1997; Zhang et al., 2002). These observations, coupled with the observation that CAE viral loads correlate with lesion severity (Cheevers et al., 1991, 1988), suggest that the cytokines TNFα and GM-CSF may augment CA lesion severity by increasing viral loads within the infected tissues.

AP-1 is a collective term referring to dimeric transcription activators composed of homodimers or heterodimers of Jun, Fos or closely related factors (Krauss, 2003). AP-1 has been proposed as a key regulator of both MVV (Gabuzda et al., 1989; Hess et al., 1989) and CAEV transcription (Kalinski et al., 1994). The SRLV promoters are located within the U3 region of the viral long terminal repeat (LTR). Within the U3 region, the MVV promoter has a consensus AP-1 site ($TGA^{G}/_{C}TCA$) proximal to the TATA box which appears to be critical for efficient transcription (Gdovin and Clements, 1992; Hess et al., 1989; Neuveut et al., 1993) and is required for phorbol ester-inducible gene expression (Gabuzda et al., 1989). In addition to the consensus site, multiple degenerate sites are present within the MVV promoter. It has been proposed that the degenerate AP-1 sites discriminate between AP-1 complexes of different compositions (Sutton et al., 1997). In contrast to MVV, the CAEV CO (CAEV Cork isolate) promoter has a near consensus AP-1 sequence (TGAGACA) near the 5′ end of the U3 region and four degenerate copies of the AP-1 consensus sequence of unknown relevance. AP-1 binding activity has been found to be decreased in CAEV-infected macrophages leading to the speculation that in cells replicating CAEV, free AP-1 becomes sequestered by viral AP-1-like binding sites resulting in reduced levels of nuclear AP-1 (Lechner et al., 1997a).

The cytokines TNFα and GM-CSF activate AP-1-mediated transcription through mechanisms involving mitogen-activated protein (MAP) kinase signaling cascades (Karin et al., 1997; Shaulian and Karin, 2002). Agents known to trigger AP-1 activity are also potent inducers of monocyte differentiation and macrophage activation (Sutton et al., 1997). CAEV viral antigen expression is repressed in monocytes (Gendelman et al., 1986; Hess et al., 1986), while differentiation of monocytes into macrophages results in viral RNA expression (Gabuzda et al., 1989; Shih et al., 1992) indicating that cellular factors such as AP-1 are involved in the regulation of viral gene expression (Neuveut et al., 1993). These observations suggest that TNFα and GM-CSF-induced activation of AP-1 factors may be transcriptionally linked to both macrophage differentiation and CAEV transcription. Clarifying this connection may be important, since activation of CAEV transcription augments CA lesion severity by increasing viral loads within the infected tissue. Here, we tested two hypotheses: (i) whether CAEV transcriptional activation results from exposure to the cytokines GM-CSF or TNFα, and (ii) whether AP-1 factors are necessary for transcriptional activation of the CAEV promoter.

Results

TNFα and GM-CSF activate CAEV expression in $U937_{CAEV}$ cells

To test the hypothesis that TNFα and GM-CSF activate the CAEV promoter, $U937_{CAEV}$ and GSM_{CAEV} cells were exposed to various concentrations of recombinant human cytokines for varying lengths of time. Exposure to a phorbol ester (PMA) was used as a positive control. Phorbol esters have been used in many studies as activators of SRLV promoters (Gabuzda et al., 1989; Shih et al., 1992; Tong-Starksen et al., 1996). $U937_{CAEV}$ cells were exposed to PMA for varying lengths of time, and the Gag mRNA copy number was determined by real-time RT-PCR. $U937_{CAEV}$ cells exposed to 10 μM PMA for 0 to 48 h had peak Gag mRNA expression at 24 h, approximately 10-fold activation over the background expression at time zero ($P < 0.05$, Fig. 1). This experiment was independently repeated three times with similar results. The data presented in Fig. 1 indicate that exposure of $U937_{CAEV}$ cells to PMA results in statistically significant increases in Gag mRNA expression.

At 4 h, $U937_{CAEV}$ cells exposed to the human cytokines TNFα (10 ng/ml) or GM-CSF (50 ng/ml) had approximately 3.2-fold increased CAEV Gag mRNA expression ($P < 0.05$) over control samples at time zero (Figs. 2a and c). $U937_{CAEV}$ cells exposed to 0–10 ng TNFα/ml or 0–50 ng GM-CSF/ml for 2 h had a dose-response activation of CAEV Gag mRNA expression with a maximal activation at 10 ng TNF α/ml or 5 ng GM-CSF/ml, respectively ($P < 0.05$; Figs. 2b and d). These experiments were repeated a minimum of three times for each cytokine with similar results. Exposure of $U937_{CAEV}$ cells to both GM-CSF and TNFα for 4 h did not result in additional promoter activation (i.e., a synergistic effect was not detected, data not shown). Goat-derived GSM_{CAEV} cells exposed to the human cytokines TNFα (10 ng/ml) or GM-CSF (50 ng/ml) for 4 h had a statistically significant 1.9- or 1.4-fold increase in CAEV Gag expression, respectively (Fig. 3). This experiment was performed two times with similar results. In all of the

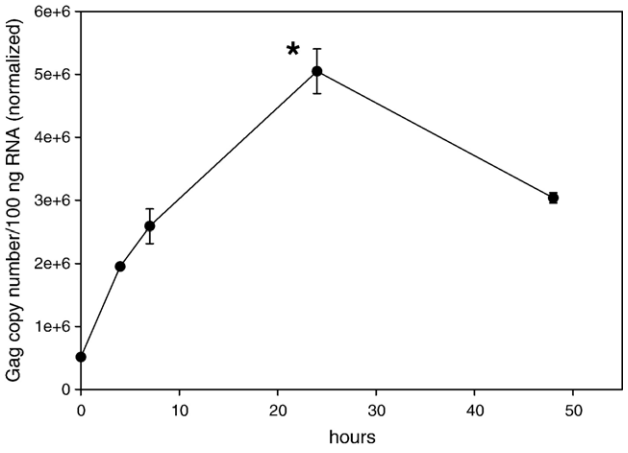

Fig. 1. PMA activates CAEV transcription in U937 cells. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) time course analysis for Gag mRNA of $U937_{CAEV}$ cells exposed to 10 μM PMA at varying time points.

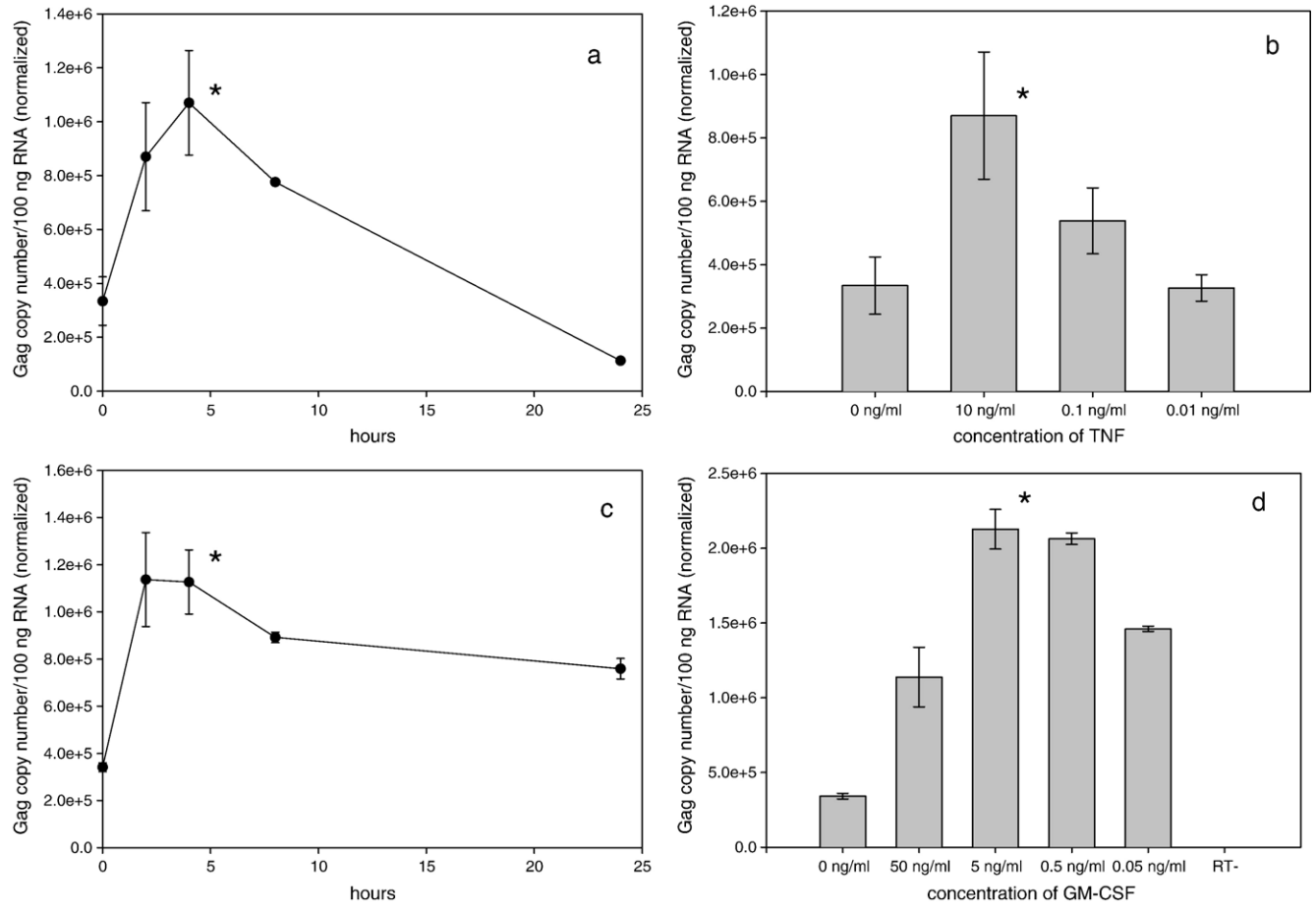

Fig. 2. TNFα and GM-CSF activate CAEV transcription in U937 cells. (a) Real-time RT-PCR time course analysis for Gag mRNA of $U937_{CAEV}$ cells exposed to an initial concentration of 10 ng TNFα/ml media. (b) Real-time RT-PCR dose-response analysis for Gag mRNA of $U937_{CAEV}$ cells exposed to 0–10 ng TNFα/ml for 2 h. (c) Real-time RT-PCR time course analysis for Gag mRNA of $U937_{CAEV}$ cells exposed to an initial concentration of 50 ng GM-CSF/ml. (d) Real-time RT-PCR dose-response analysis for Gag mRNA of $U937_{CAEV}$ cells exposed to 0–50 ng GM-CSF/ml for 2 h. The RT− lane corresponds to untreated control sample lacking reverse transcriptase.

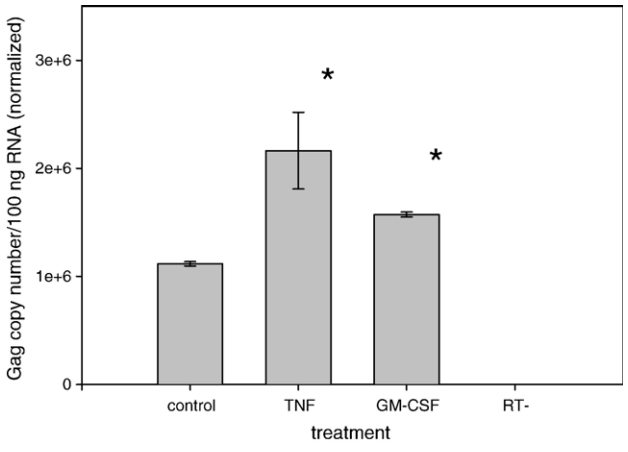

Fig. 3. TNFα and GM-CSF activate CAEV transcription in GSM cells. Real-time RT-PCR analysis for Gag mRNA of GSM_{CAEV} cells exposed to media alone (control), an initial concentration of 10 ng TNFα/ml media or an initial concentration of 50 ng GM-CSF/ml for 4 h. The RT− lane corresponds to untreated control sample lacking reverse transcriptase.

described experiments, samples without reverse transcriptase (RT−) did not yield Gag amplification products indicating that the DNase treatment step was effective.

Tumor necrosis factor alpha and GM-CSF therefore activate the CAEV promoter, both in human-derived U937 cells and in goat-derived GSM cells. In regard to the expression kinetics, $U937_{CAEV}$ cells treated with TNFα or GM-CSF had statistically significant increases in CAEV Gag mRNA expression with a maximal effect at 4 h. TNFα treatment produced a transient increase in Gag mRNA expression which decayed to background levels by 24 h. In contrast, cells treated with GM-CSF had a persistent increase in Gag expression at 24 h.

The inhibitor SP600125 does not reverse PMA, TNFα and GM-CSF induced promoter activation

We tested the hypothesis that PMA and cytokine-induced CAEV promoter activation is mediated through AP-1 factors by SP600125-induced inhibition of Jun N-terminal kinase activity (JNK). Activation of JNK results in phosphorylation of Jun at Ser 63 and Ser 73 (Krauss, 2003). Phosphorylation of Jun

enhances its transcriptional activity (Shaulian and Karin, 2002; Smeal et al., 1994) and increases the protein's stability (Karin et al., 1997; Kracht and Saklatvala, 2002). Previous experiments with SP600125 have demonstrated a greater than 300-fold specificity of JNK inhibition relative to other MAP kinases (Bennett et al., 2001).

$U937_{CAEV}$ cells treated with 40 μM SP600125 for 2 h prior to exposure to 10 μM PMA for 24 h demonstrated no decrease in Gag mRNA copy number relative to PMA-treated cells alone (Fig. 4a). Exposure of $U937_{CAEV}$ cells to 40 μM SP600125 for 2 h prior to a 4-h exposure to 10 ng TNFα/ml or 50 ng GM-CSF/ml (Figs. 4b and c, respectively) did not block cytokine-induced U3 promoter activation.

Surprisingly, a statistically significant increase in Gag mRNA expression was identified in cells treated with both cytokine and SP600125 (*) when compared to cells treated with cytokine alone. These experiments were repeated four or more times with each agent with similar results. $U937_{CAEV}$ cells exposed to 30 or 40 μM SP600125 for 6 h had a statistically significant increase in expression of Gag mRNA (*) relative to untreated control cells (Fig. 4d).

$U937_{CAEV}$ cells treated with SP600125 and PMA had approximately the same Gag mRNA expression relative to PMA-treated cells alone (10-fold over the untreated control cells, Fig. 4a). Cells treated with both a cytokine and SP600125 had 2- to 4-fold more Gag expression relative to cytokine-treated cells alone (Figs. 4b and c). CAEV promoter activation may be maximized (saturated) in PMA-treated cells, preventing further activation by SP600125 treatment. In cytokine-treated cells, promoter activation does not appear to be saturated. This observation suggested that PMA and cytokines may activate the CAEV promoter by different molecular mechanisms, which is consistent with the different time course kinetics of activation (Figs. 1 and 2).

To confirm the effectiveness of JNK inhibition by SP600125 in $U937_{CAEV}$ cells, a Western blot was performed with lysates from cytokine- or PMA-treated cells. $U937_{CAEV}$ cells were treated with or without 40 μM SP600125 for 2 h then exposed to either TNFα (10 ng/ml), GM-CSF (50 ng/ml) or 10 μM PMA and incubated an additional 4 h prior to the lysate harvest. Equivalent amounts of protein were loaded per lane onto two gels run in parallel. The subsequent blots

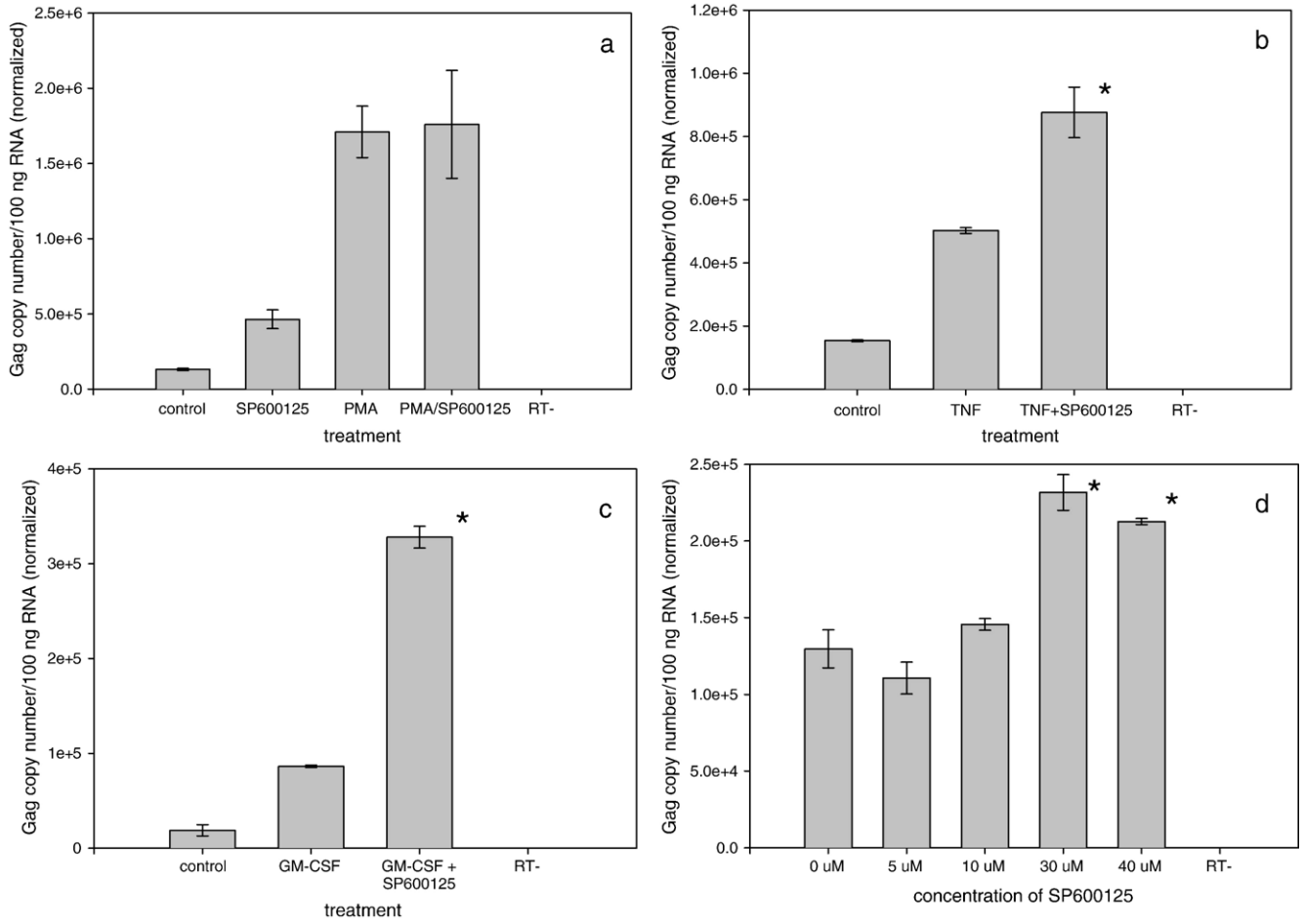

Fig. 4. The JNK inhibitor SP600125 does not inhibit PMA and cytokine-induced CAEV promoter activation but activates the CAEV promoter. (a) Real-time RT-PCR analysis for Gag mRNA of $U937_{CAEV}$ cells treated with 40 μM SP600125 2 h prior to a 24-h exposure to 10 μM PMA. (b and c) Real-time RT-PCR analysis for Gag mRNA of $U937_{CAEV}$ cells treated with 40 μM SP600125 for 2 h prior to exposure to either 10 ng TNFα/ml (b) or 50 ng of GM-CSF/ml (c) for 4 h. The treated cells were harvested after a total incubation time of 6 h. (d) Real-time RT-PCR analysis for Gag mRNA of $U937_{CAEV}$ cells exposed to 0–40 μM SP600125 for 6 h. RT− lanes correspond to untreated control sample lacking reverse transcriptase.

were incubated with either a polyclonal antibody against phosphorylated c-Jun (Ser 73) or a monoclonal antibody against c-Jun. Bands corresponding to phosphorylated c-Jun were present in the TNFα, GM-CSF and PMA-treated samples but were either absent or greatly attenuated in samples previously treated with SP600125 (Fig. 5a). The phosphorylated c-Jun antibody also recognizes phosphorylation of JunD (ser 100); bands corresponding to phosphorylated JunD were not detected for any treatment. The control blot treated with the c-Jun monoclonal antibody (Fig. 5b) confirms that a similar amount of c-Jun was expressed for each treatment condition. Cells treated with cytokine or PMA alone have an additional band (between 40 and 50 kDa) corresponding to the phosphorylated form of c-Jun; this band is absent in the SP600125-treated lanes. This experiment was repeated twice with similar results. A dose-response experiment was performed with SP600125 in order to titrate the effect of the inhibitor (Fig. 5c). U937$_{CAEV}$ cells were treated with 0–40 μM SP600125, incubated for 2 h and exposed to 0 or 10 ng TNFα/ml for 4 h prior to lysate harvest. The Western blot was performed as described previously with anti-phosphorylated c-Jun (Ser 73) as the primary antibody. An inhibitory dose-response effect was evident with increasing concentrations of SP600125. In previously published experiments, 10 μM SP600125 effectively inhibits 90 to 100% of Jun N-terminal kinase activity (Bennett et al., 2001). To demonstrate that SP600125 treatment decreases AP-1-mediated transcription, we utilized the AP-1-dependent gene metalloproteinase (Wu et al., 2001). Treatment of U937$_{CAEV}$ cells with 40 μM SP60015 for 19 h blocked mRNA expression of metalloproteinase 2.3-fold (Fig. 5d). This experiment was repeated three times with similar results.

The results indicated that 40 μM SP600125 effectively inhibited JNK activity, as measured by blockade of c-Jun phosphorylation and inhibition of mRNA transcription of the AP-1 dependent gene metalloproteinase. 40 μM SP600125 effectively inhibited phosphorylation of c-Jun in cells subsequently treated with cytokines or PMA. Collectively, these results indicated that phosphorylated Jun protein (and therefore, AP-1 transcription factor) is not required for activation of the CAEV promoter by PMA, GM-CSF or TNFα. Activation of the CAEV promoter therefore occurs independently of AP-1.

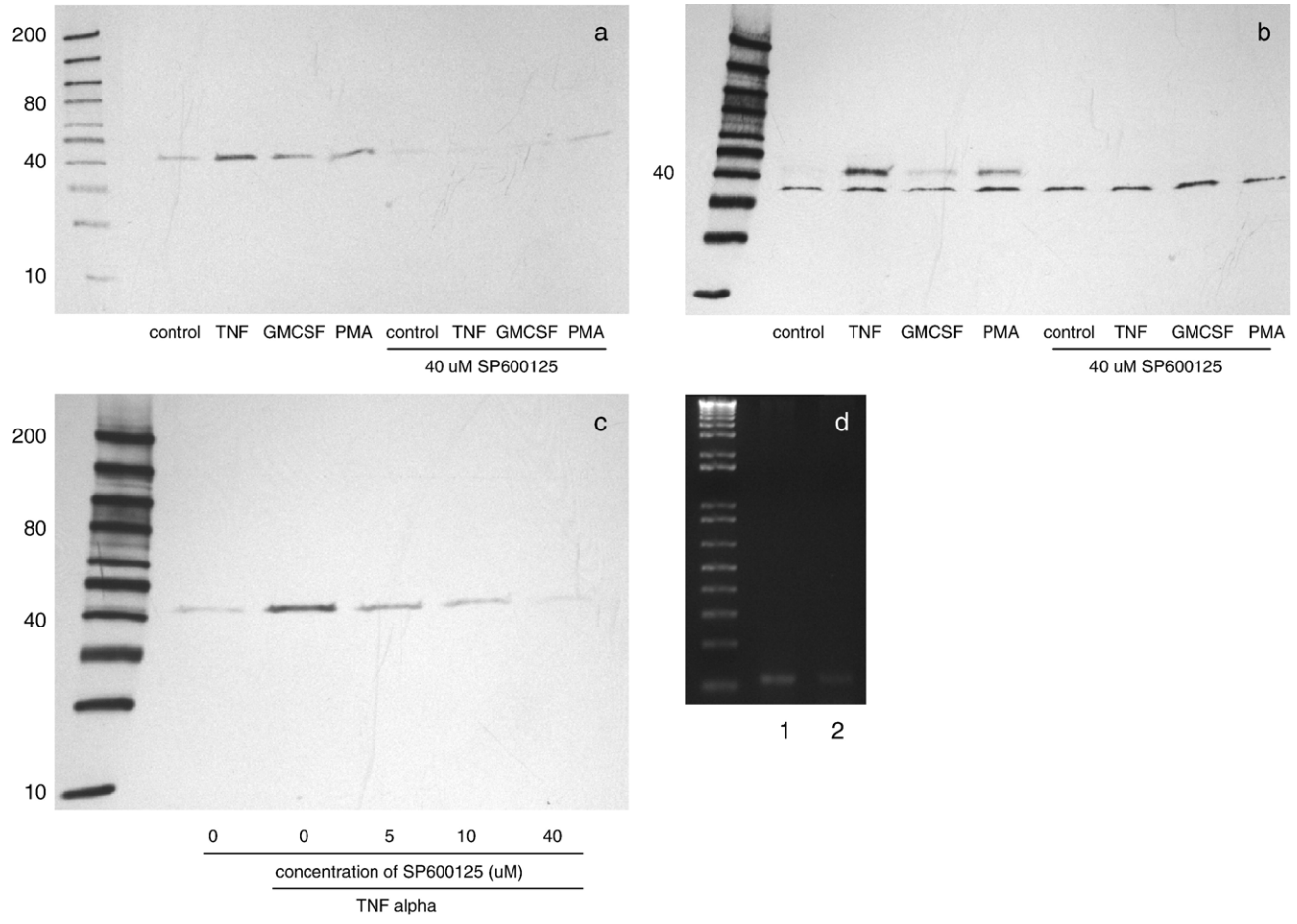

Fig. 5. The JNK inhibitor SP600125 blocks cytokine and PMA-induced phosphorylation of Jun and inhibits metalloproteinase transcription. (a and b) Western blot analyses of U937$_{CAEV}$ cell lysates (50 μg protein/lane) with or without exposure to 40 μM SP600125 for 2 h prior to incubation with 10 ng TNFα/ml, 50 ng GM-CSF/ml, 10 μM PMA or no treatment (control) for 4 h. The primary antibodies were anti-phosphorylated Jun-73 (a) or c Jun (b). (c) Western blot analysis of U937$_{CAEV}$ cell lysates (50 μg protein/lane) exposed to 0, 5, 10 or 40 μM SP600125 for 2 h prior to exposure to 10 ng TNFα/ml or no treatment for 4 h. 50 μg of total protein was loaded per lane. (d) Human matrix metalloproteinase 12 RT-PCR analysis of RNA extracted from U937$_{CAEV}$ cells exposed to control media (lane 1) or 40 μM SP600125 (lane 2) for 19 h. This experiment was repeated three times with similar results.

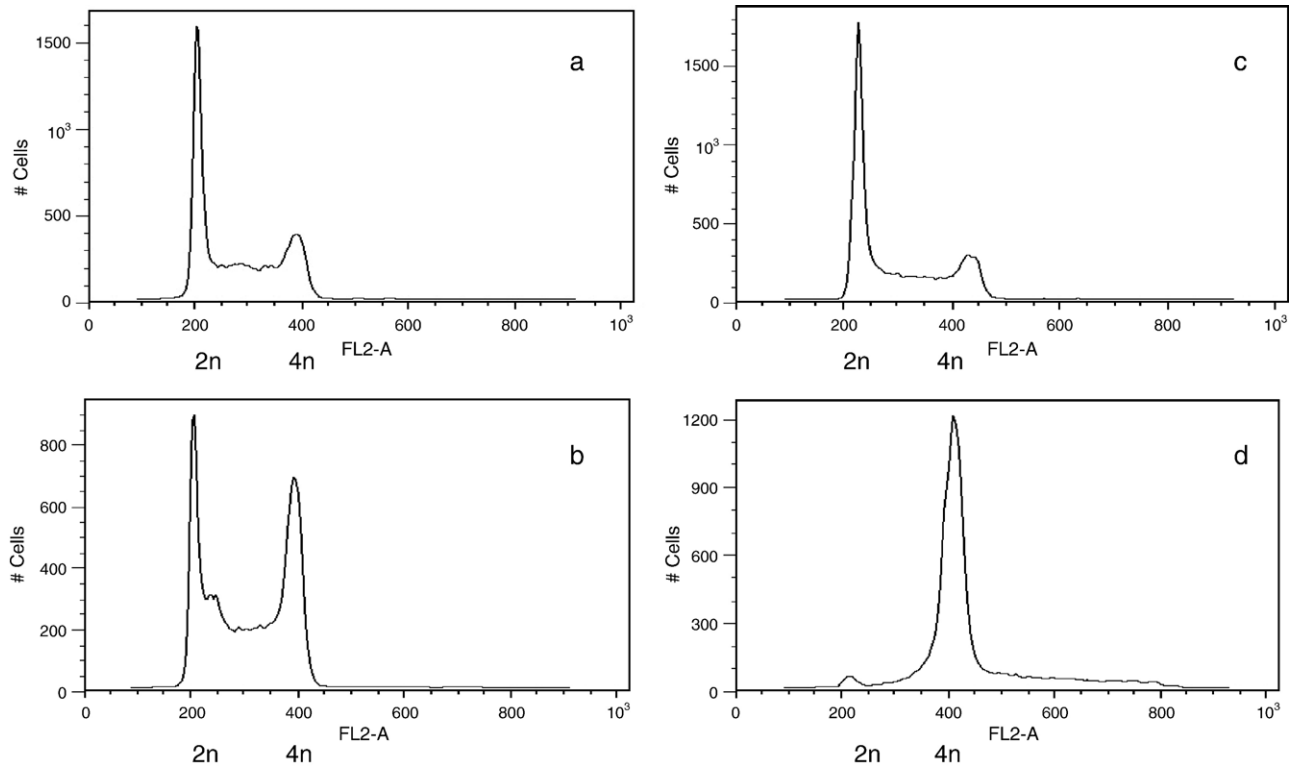

Fig. 6. Flow cytometry analysis of U937$_{CAEV}$ cells treated with SP600125. U937$_{CAEV}$ cells were incubated with or without 40 μM SP600125 for 5 or 24 h: (a) 5 h, no treatment; (b) 5 h with SP600125; (c) 24 h, no treatment; (d) 24 h with SP600125. The cells were fixed, stained with propidium iodide and analyzed for DNA content via FlowJo software.

SP600125 blocks U937 cells in the G2 phase of the cell cycle

In order to explain the anomalous SP600125-induced activation of the CAEV promoter identified in Fig. 4, we determined the DNA content of U937$_{CAEV}$ cells treated with or without SP600125. In HIV-infected cells, G2 arrest has been shown to be associated with an enhancement of transcriptional activity (Bouzar et al., 2003; Goh et al., 1998; Mueller and

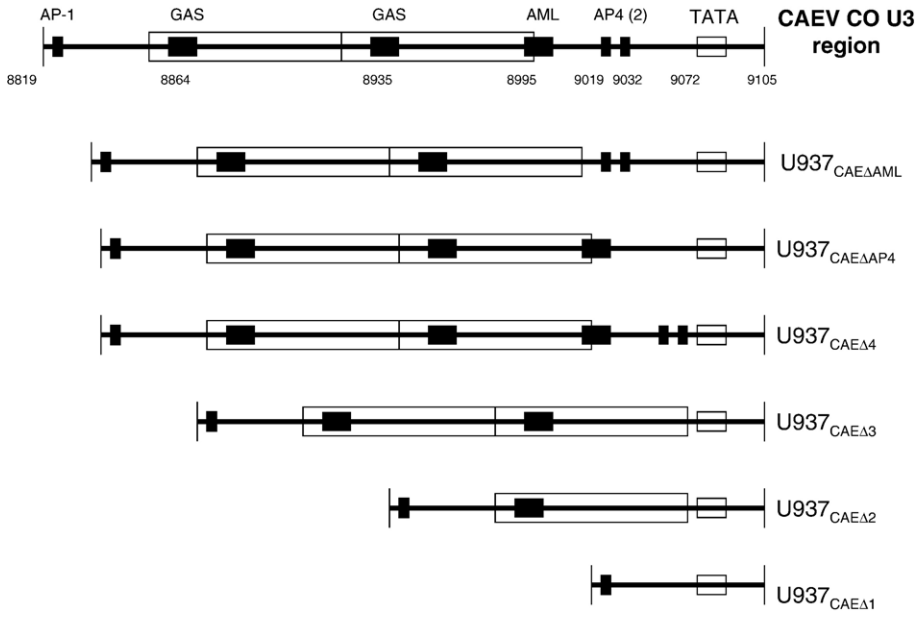

Fig. 7. Maps of the CAEV-CO U3 region and derived deletion mutants. The transcription factor binding motifs are represented as labeled black boxes, and the two 70-bp repeats are denoted by large open boxes. Portions of the U3 region deleted in each mutant are as follows: nucleotides 8856–9069, 8923–9069, 8994–9069, 9037–9069, 9007–9037 and 8995–9014, for U937$_{CAEΔ1}$, U937$_{CAEΔ2}$, U937$_{CAEΔ3}$, U937$_{CAEΔ4}$, U937$_{CAEΔAP4}$ and U937$_{CAEΔAML}$, respectively.

Lang, 2002). We hypothesized that the increased transcriptional activity of the SP600125-treated cells was the result of G2/M block. Cells blocked in the G2 phase of the cell cycle accumulate a 4n complement of genomic DNA, which can be quantified by flow cytometry.

$U937_{CAEV}$ cells were incubated with or without 40 μM SP600125 for 0 to 24 h. Both treated and untreated cells were fixed and stained at 0, 2, 5, 7 and 24 h, as described in the Materials and methods. $U937_{CAEV}$ cells exposed to SP600125 for 5 and 24 h (Figs. 6b and d) had an increase in the 4n DNA content relative to untreated cells (Figs. 6a and c). The G2/G1 ratio increases from 0.55 at time zero to 0.82, 1.6, 2.5 and 9.7 at 2, 5, 7 and 24 h, respectively. This experiment was performed twice with similar results. Cells treated with 40 μM SP600125 for 2 h and subsequently exposed to cytokines or PMA for 4 h had the same G2/G1 ratio as cells exposed to SP600125 alone for 6 h (data not shown).

The results show that the JNK inhibitor SP600125 effectively blocks $U937_{CAEV}$ cells in the G2 phase of the cell cycle, resulting in an increase in the 4n DNA content of the treated cells.

The U3 70-bp repeat is required for TNFα-induced promoter activation

Since activation of the CAEV promoter occurs independently of AP-1, a set of six U3 deletion mutants were constructed (Fig. 7) in order to define the locus of TNFα-induced transcriptional activation. For each mutant, the U3 region was sequenced and determined to match the predicted sequence. Stably integrated, U937-based cell lines were generated from each CAEV-mutant construct as described in the Materials and methods. A PCR reaction was performed utilizing genomic DNA from each cell line with the Rev_{for} and

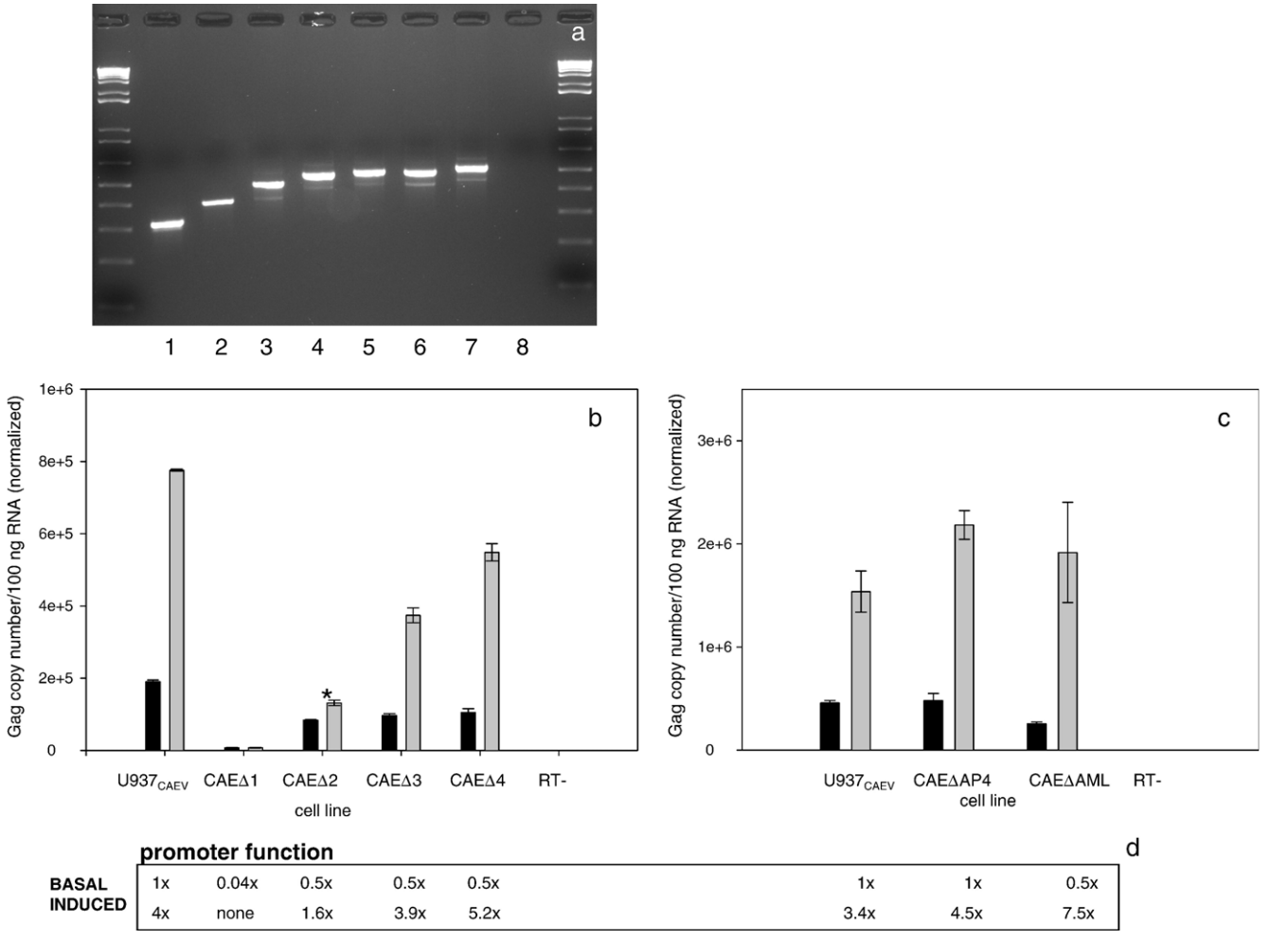

Fig. 8. The U3 70-bp repeat is necessary and sufficient for TNFα-induced promoter activation. (a) PCR products of genomic DNA isolated from $U937_{CAEΔ1}$, $U937_{CAEΔ2}$, $U937_{CAEΔ3}$, $U937_{CAEΔ4}$, $U937_{CAEΔAP4}$, $U937_{CAEΔAML}$, $U937_{CAEV}$ and parent U937 cells (lanes 1–8, respectively). PCR was performed with the Rev_{for} and R_{rev} primer set. (b) Real-time RT-PCR results for Gag mRNA of $U937_{CAEV}$, $U937_{CAEΔ1}$, $U937_{CAEΔ2}$, $U937_{CAEΔ3}$ and $U937_{CAEΔ4}$ cells with (grey bars) or without (black bars) exposure to 10 ng TNFα/ml for 4 h. The difference between TNFα exposed and unexposed Gag mRNA copy number for $U937_{CAEΔ2}$ is statistically significant (*). The RT− lane corresponds to untreated control sample lacking reverse transcriptase. (c) Real-time RT-PCR results for Gag mRNA of $U937_{CAEV}$, $U937_{CAEΔAP4}$ and $U937_{CAEΔML}$ cells with (grey bars) or without (black bars) exposure to 10 ng TNFα/ml for 4 h. The RT− lane corresponds to untreated control sample lacking reverse transcriptase. (d) Relative basal and TNFα-induced promoter function for the data pictorially presented in panels b and c. For both figures, 1× basal promoter function was defined as the Gag copy number for untreated $U937_{CAEV}$ cells.

R_{rev} primer set. The PCR products matched the predicted product size (Fig. 8a, lanes 1–6). To confirm the presence of an unmodified U3 promoter region within the $U937_{CAEV}$ cell line, genomic DNA was isolated, and a PCR reaction was performed utilizing the same primer set (Fig. 8a, lane 7). This PCR product was sequenced and determined to be identical to the wild-type CAEV-CO U3 sequence. No product was detected when the same PCR reaction was performed with genomic DNA isolated from parent U937 cells (negative control, Fig. 8a, lane 8).

The U3 deletion mutant cell lines were treated with or without TNFα (10 ng/ml) for 4 h prior to RNA harvest. The Gag mRNA copy number was determined by real-time RT-PCR. When corrected for equal numbers of integrating constructs, $U937_{CAE\Delta1}$ cells had a 26-fold attenuation of the basal Gag mRNA expression (Fig. 8b). Importantly, $U937_{CAE\Delta1}$ had no increase in Gag mRNA expression in response to TNFα treatment. However, $U937_{CAE\Delta2}$ cells, with a single copy of the U3 70-bp repeat, had a 0.5-fold basal Gag mRNA expression relative to the wild-type $U937_{CAEV}$ cells and a statistically significant 1.6-fold activation in response to TNFα exposure (Figs. 8b and d). The deletion mutants $U937_{CAE\Delta3}$ and $U937_{CAE\Delta4}$ had a similar basal expression level and a progressively stronger response to TNFα exposure (3.9- and 5.2-fold, respectively). Two other deletion mutants, $U937_{CAE\Delta AP4}$ and $U937_{CAE\Delta AML}$, had a 4.5- and 7.5-fold activation in response to TNFα, and a 1- and 0.5-fold basal expression, respectively (Figs. 8c and d). Samples without reverse transcriptase (RT-) did not yield gag amplification products. These experiments were repeated once with similar results.

In $U937_{CAE\Delta1}$ cells, the basal promoter function was severely attenuated, and the TNFα-induced promoter activation was abrogated. However, in $U937_{CAE\Delta2}$ cells, the basal and TNFα-induced promoter functions were attenuated but present. These results suggest that TNFα-induced expression is dependent upon an element or elements within the U3 70-bp repeat. In comparison to the 1.6-fold activation demonstrated with $U937_{CAE\Delta2}$ cells, the cell line $U937_{CAE\Delta3}$ has two copies of the 70-bp repeat and a 3.9-fold response to TNFα treatment. This suggests a dosage effect with the additional repeat element. These data also indicate that basal U3 promoter function is dependent on multiple loci within the U3 region including: the 70-bp repeat, AML and nucleotides 9037–9068 (the region deleted in $U937_{CAEV\Delta4}$ cells). Note that the 5′ near consensus AP-1 motif is present in all of the deletion mutants (Fig. 7) supporting the concept that AP-1 is not sufficient to confer TNFα-induced responsiveness in the CAEV promoter.

These promoter deletion results are consistent with the concept that cytokine-induced activation is not the result of random integration of the construct adjacent to a cytokine-responsive cellular promoter. In addition, these results indicate that the cytokine-induced increase in Gag mRNA is a transcriptional effect and not a result of transcript stabilization.

Discussion

Previous investigations into the transcriptional regulation of SRLV have utilized transient transfections with LTR promoter–reporter gene constructs. The in vivo biological relevance of these experiments has been limited by the episomal location of the viral promoter, the lack of viral protein expression and confounded by variation in transfection efficiency among experimental groups. For these reasons, CAEV constructs were stably integrated into the U937 cell genome. The human-derived U937 cell line was chosen as it facilitates the utilization of a greater variety of research reagents. This cell line allows the exploration of viral promoter regulation in the more biologically relevant context of an integrated provirus.

Exposure of $U937_{CAEV}$ cells to PMA, GM-CSF or TNFα resulted in 10-, 3.2- and 3.2-fold CAEV promoter activation, respectively. Goat-derived GSM_{CAEV} cells exposed to recombinant human TNFα and GM-CSF had a statistically significant but attenuated activation. Recent findings indicate that GSM cells are not of the monocyte–macrophage lineage (B. Davis, personal communication). As CAEV is a monocyte–macrophage tropic virus, it is not surprising that viral promoter activation in GSM cells is attenuated relative to the monocyte–macrophage U937 cell line. In previously published experiments, TNFα did not activate the CAEV promoter in U937 cells above baseline levels (Tong-Starksen et al., 1996). However, these experiments relied on a transient transfection system with a LTR-CAT reporter construct and were conducted with a 24-h cytokine exposure time. The data presented here clearly indicate a maximal TNFα induction effect at 4 h which degrades to background levels by 24 h. This is the first report describing TNFα and GM-CSF-induced transcriptional activation of an integrated CAEV construct. These results are consistent with the hypothesis that CAEV transcriptional activation results from exposure to the cytokines GM-CSF or TNFα. As it has previously been demonstrated that increased CAE viral loads are associated with increased lesion severity (Cheevers et al., 1991, 1988), the cytokines TNFα and GM-CSF may play a role in CA pathogenesis by activation of the CAE viral promoter leading to increased virus production.

In this study, we also tested the hypothesis that AP-1 transcription factors are necessary for transcriptional activation of the CAEV promoter. To test this hypothesis, we utilized the JNK inhibitor SP600125. Western blot assays indicated that SP600125 effectively blocked phosphorylation of c-Jun and prevented TNFα, GM-CSF and PMA-induced phosphorylation of c-Jun. In addition, SP600125 attenuated transcription of the AP-1-dependent metalloproteinase gene. Treatment with SP600125, however, failed to block PMA and cytokine-induced activation of the viral promoter indicating that AP-1 transcription factors are not required for activation of the CAEV promoter by PMA, GM-CSF or TNFα. In support of this conclusion, the $U937_{CAEV\Delta1}$ mutant cell line has a near-consensus copy of the AP-1 motif yet the basal promoter function was severely attenuated, and the TNFα-induced promoter function was abrogated. Interestingly, SP600125 was found to activate the CAEV promoter. Experiments described here indicate that SP600125 effectively blocks the U937 cells in G2 phase of the cell cycle, leading to an increased G2/G1 ratio and 4n

DNA content. G2 arrest has been shown to be associated with an enhancement of transcriptional activity in HIV-infected cells. The CAEV *tat* gene product is currently thought to be functionally analogous to the lentiviral accessory protein Vpr (Villet et al., 2003). One of the functions of Vpr is to arrest virally infected cells in G2 phase (Bartz et al., 1996; Jowett et al., 1995). SP600125 treatment may complement the tat-deleted genotype of the $U937_{CAEV}$ cells resulting in cell cycle arrest and increased transcriptional activity of the CAEV promoter.

Since AP-1 is apparently not required for TNFα or GM-CSF-induced activation of the CAEV promoter, transcription experiments were performed to localize the required promoter regions. A set of progressive U3 deletion mutants indicate that the 70-bp repeat is required for TNFα-induced promoter activation. A consensus, gamma-activated site (GAS) is present within the 70-bp repeat and has been previously shown to be responsive to the cytokine interferon gamma (IFN gamma) (Tong-Starksen et al., 1996). The CAEV GAS element resembles the well-conserved GAS sequence of cellular genes and a minimal promoter composed of the GAS element is sufficient to confer responsiveness to IFN gamma (Sepp and Tong-Starksen, 1997). IFN gamma-induced CAEV promoter activation has been shown to be mediated through the transcriptional factor STAT1 (Sepp and Tong-Starksen, 1997). TNFα (Guo et al., 1998) and GM-CSF (Jackson et al., 2004) initiate signaling cascades resulting in STAT1 activation. Interestingly, STAT1 has also been shown to play a pivotal role in the differentiation of monocytes into macrophages (Coccia et al., 1999). It is possible that TNFα and GM-CSF-induced CAEV promoter activation is mediated through STAT1 binding of the U3 GAS site. Experiments are ongoing to test this hypothesis.

Materials and methods

Plasmids

The plasmid CAEVpac11 was generated from the previously described pCAEVneo11 (Hotzel and Cheevers, 2001). This plasmid has the CAEV-CO strain (Cork and Narayan, 1980) proviral sequence with a 1330-bp deletion in *env* and an SV40-pac (pac: puromycin acetyltransferase) cassette replacing the *tat* gene. The plasmids CAEΔ1, CAEΔ2, CAEΔ3, CAEΔ4, CAEΔAP4 and CAEΔAML have deletions between nucleotides 8856–9069, 8923–9069, 8994–9069, 9037–9069, 9007–9037 and 8995–9014, respectively. For each plasmid, two unique primers were designed flanking the deleted region: 5′ $U3_{rev}$/TATA-bgl_{for}, $U3_{rev}$/TATA-bgl_{for}, $U3_{rev}$/TATA-bgl_{for}, $AP4_{rev}$/TATAbgl$_{for}$, AML_{rev}/$U3_{for}$ and $U3_{rev}$/$AP4_{for}$ for plasmids CAEΔ1, CAEΔ2, CAEΔ3, CAEΔ4, CAEΔAP4 and CAEΔAML, respectively. Each primer was designed with a unique BglII restriction site at the 5′ end. For each primer set above, the reverse primer was utilized in a PCR reaction with the Rev_{for} primer, and the forward primer was utilized with R_{rev} to generate two PCR products. The unique BglII ends allow reconstruction of the U3 region with the appropriate deletion. All cloning steps involving PCR were checked by sequencing to confirm the absence of PCR-induced sequence errors. The plasmid pMEVSV-G, expressing the vesicular stomatitis virus (VSV) G glycoprotein (utilized for packaging), was obtained from Richard Sutton. All plasmids were propagated in *Escherichia coli* JM109.

Cells and tissue culture media

The U937 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The U937 cell line is frequently used for in vitro SRLV studies (Sepp and Tong-Starksen, 1997; Shih et al., 1992; Tong-Starksen et al., 1996). U937 cells were maintained at 37 °C in 5% CO_2 in RPMI 1640 media with L-glutamine (Gibco BRL, Grand Island, NY) supplemented with 12% fetal calf serum (FCS), 100 U penicillin/l, 100 μg streptomycin/l and 2-mercaptoethanol (2 μl/500 ml media). 293T cells (utilized for generating pseudotyped virus) were grown in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island NY) supplemented with 10% FCS, 100 U penicillin/l, 100 μg streptomycin/liter and 2 mM L-glutamine. Goat synovial membrane (GSM) cells were derived and maintained as previously described (Klevjer-Anderson and Cheevers, 1981).

Derivation of cell lines

U937 cells, along with the plasmids CAEVpac11, CAEΔ1, CAEΔ2, CAEΔ3, CAEΔ4, CAEΔAP4 and CAEΔAML were utilized to generate the cell lines: $U937_{CAEV}$, $U937_{CAEΔ1}$, $U937_{CAEΔ2}$, $U937_{CAEΔ3}$, $U937_{CAEΔ4}$, $U937_{CAEΔAP4}$ and $U937_{CAEΔAML}$, respectively. Pseudotyped virus was generated in 293T cells; the cells were plated in 60-mm plates and allowed to adhere overnight. On day 2, the cells were cotransfected with 8 μg of one of the CAEV plasmids and 4 μg pMEVSV-G using a commercially available calcium-phosphate transfection kit (Profection Mammalian Transfection Systems, Promega). Culture medium was replaced 24 h after transfection (day 3). Pseudotyped virus was harvested on day 4 from media spun at 3000 rpm at 4 °C for 20 min to remove cellular debris. U937 cells were seeded in a 25-cm^2 tissue culture flask with 2 ml RPMI 10%-FCS and 2 ml of pseudotyped virus. Control U937 cells were placed in 4 ml of RPMI 10%-FCS without virus. The flasks were swirled every 10 min for 90 min, and the volume was subsequently adjusted to 8 ml RPMI 10%-FCS per flask. Two days later (day 6), the cells were placed in 75-cm^2 flasks, and the media were replaced with RPMI 10%-FCS containing 0.5 μg puromycin/ml. The media were changed daily and the cells were kept in puromycin selection for 14 days until 100% of the control cells were dead. Integration of the CAEV constructs into the U937 cell genome was confirmed by RT-PCR with total RNA utilizing Gag and U3 primers and PCR with genomic DNA and U3 primers (as described below). GSM_{CAEV} cells were derived from GSM cells and the pCAEVpac11 plasmid in the same manner as for $U937_{CAEV}$ cells.

Reagents

The recombinant human cytokines TNFα and GM-CSF (R&D Systems, Inc., Minneapolis, MN) were solubilized in PBS with 0.1% bovine serum albumin at stock concentrations of 10 μg/ml and 5 μg/ml, respectively. The cytokines were filter sterilized and split into 100 μl aliquots. Phorbol myristate acetate (PMA, Sigma Aldrich) was solubilized in DMSO at a stock concentration of 1 mg/ml (1.6 mM), filter sterilized, and split into 75 μl aliquots. SP600125 (Calbiochem, La Jolla, CA) was solubilized in DMSO at a stock concentration of 10 mM, filter sterilized and split into 75 μl aliquots. Puromycin (Sigma Aldrich) was solubilized in water at a stock concentration of 2 mg/ml, filter sterilized and divided into 200 μl aliquots. A stock solution of propidium iodide (PI) was created by dissolving 5 mg of PI in 1 ml of PBS. All of the reagents were protected from light and stored at −20 °C (PI was stored at 5 °C).

DNA primers and probes

Primers for RT-PCR and fluorescence-interference probes for real-time PCR were ordered from Integrated DNA technologies, Inc. (IDT, Coralville, IA). The primers were utilized in PCR reactions at a final concentration of 400 nM. Primers were designed as follows: CAEV Gag_{for} primer: 5′ ATT CTG TAA TGT TCC AGC AAC TGC 3′; CAEV Gag_{rev} primer: 5′ ATA TGC CAA CTG CCT TTC AAA GTC 3′; Rev_{for} primer: 5′ CTG ACG ATG GGA ATC TGG ATA AAT GG 3′; R_{rev} primer: 5′ AGT GGA TCC TGC GAG AGC CGC TCT G 3′; 5′ $U3_{rev}$ primer: 5′ GGG AGA TCT AGC TTG TTA TTA GTC CTC TTT AGC CC 3′; TATA-bgl_{for} primer: 5′ GGG AGA TCT GCT GTA TAT AAG GGA GAA GCT TGC TGC 3′; $AP4_{rev}$ primer: 5′ GGG AGA TCT CAG CTG ATA TGT CAG CTG TTA CAT CTG 3′; $U3_{rev}$ primer: 5′ GGG AGA TCT CAT TTT GCT TTG TCA TAG TGA ACT TGC 3′; $U3_{for}$ primer: 5′ GGG AGA TCT ATG CTT GCT CAT GCT GAC ACT GTA GC 3′; $AP4_{for}$ primer: 5′ GGG AGA TCT TGT AAC AGC TGA CAT ATC AGC TGA TGC 3′; AML_{rev} primer: 5′ GGG AGA TCT GCA CTT GCG GTT ACA TTT TGC TTT GTC 3′; β actin forward primer: 5′ CTC ACG GAG CAC GGC TAT AG 3′; β actin reverse primer: 5′ GCA GAG CTT CTC CTT GAT GTC A 3′; human matrix metalloproteinase 12 forward primer (MP_{for}): 5′ CTG GAC ACA TCT ACC CTG GAG A 3′; MP_{rev} primer: 5′ ACG GTT CAT GTC AGG TGT GTA ATT 3′; 18s $rRNA_{for}$ primer: 5′ GTA ACC CGT TGA ACC CCA TT 3′; 18s $rRNA_{rev}$ primer: 5′ CCA TCC AAT CGG TAG TAG CG 3′.

The CAEV Gag and β actin fluorescence-interference probes for real-time PCR were HPLC purified (IDT, Coralville, IA) and utilized at a final concentration of 250 nM. The Gag probe was designed as follows: 5′-/5HEX/AGC AAT GCA GCA TGG CCT CGT GTC/BHQ-1/-3′ (where BHQ_1 represents Black Hole Quencher1). The β actin probe was designed as follows: 5′-/FAM/TCA CCA CCA CTG CCG AGC GGG AA/BHQ-1/-3′. The probes were protected from light and stored at −20 °C.

RNA isolation/RT-PCR assays

Total cellular RNA was purified utilizing one of the following reagents or kits: Trizol Reagent (Invitrogen, Carlsbad, CA), RNAeasy Mini Kit (Qiagen Inc., Valencia, CA) or RiboPure Kit (Ambion, Austin, TX). The RNA was isolated according to the protocols included with the kits and, if not utilized immediately, was stored at −80 °C. DNase treatment was accomplished with TURBO DNase (Ambion) utilizing 4 units DNase per 3 μg RNA and incubating the reaction for 30 min at 37 °C. The DNase was inactivated by incubating the samples at 65 °C for 15 min. RNA was reverse transcribed into cDNA with the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Indianapolis, IN). Real-time PCR assays were performed with TaqMan Universal Master Mix (Roche) or iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad iCycler detection system. CAEV Gag or 18s rRNA primer pairs were utilized for real-time PCR reactions; each assay was performed in triplicate. A control sample was run without reverse transcriptase to control for the DNase treatment effectiveness. All experiments were controlled for variations in the reverse transcriptase step and in the RNA concentration by normalizing to either β actin or 18s rRNA PCR products run in parallel with the same cDNA. The normalization calculation was performed for each sample as follows: [sample copy number/(sample actin or 18s copy number/control actin or 18s copy number)]. When experiments were performed with multiple cell lines, an integration constant was generated by the following formula: genomic $U937_{CAEV}$ *gag* copy number/cell line of interest genomic *gag* copy number. This integration constant was then multiplied by the value generated above to arrive at the final copy number normalized by both the amount of 18s rRNA and the relative number of integrated constructs within the cellular genome.

Standard PCR was performed with Platinum Taq DNA polymerase (Invitrogen) on a GeneAmp PCR System 9600 (Perkin Elmer). PCR products from standard RT-PCR were run on agarose gels, stained with ethidium bromide and digitally photographed with a Multiimage Light Cabinet (Alpha Innotech Corporation, San Leandro, CA) utilizing Alphaimager 2200 software. PCR product densitometry was performed with the same software.

Genomic DNA isolation and PCR product sequencing

Genomic DNA was isolated from 5×10^5 cells utilizing a Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, MN). Standard PCR was performed with the Rev_{for} and R_{rev} primer set. Real-time PCR was performed with CAEV Gag primers. The U3 LTR PCR product from $U937_{CAEV}$ genomic DNA was sequenced by Amplicon Express (Pullman, WA).

Western blots and protein assays

3×10^6 $U937_{CAEV}$ cells were plated in 6-well plates, treated with or without the Jun N-terminal kinase inhibitor SP600125,

incubated for 2 h at 37 °C, and subsequently treated with cytokines or PMA. The plates were incubated for 4 additional h at 37 °C, the cells were washed once with PBS and lysed with 150 μl of lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium dodecyl sulfate, 0.5% deoxycholate). The lysates were kept on ice, sheared by pipetting through a 20-gauge needle and filtered through a syringe-tip filter. If not used immediately, the lysates were stored for up to 7 days at −20 °C. The cell lysates were normalized for protein concentration utilizing a BCA Protein Assay Kit (Pierce, Rockford, IL). The assays were run in triplicate with at least two different sample dilutions. For each assay, a nine sample standard curve was generated in duplicate from the kit reagents. The sample absorption was determined on a microplate reader (Titertek Multiskan MCC/340, EFLAB, Finland) at a wavelength of 540 nm. The lysates were mixed with loading buffer (0.03 M Tris (pH 6.8) containing 2% SDS, 10% glycerol, and 0.01% bromphenol blue in the presence of 100 mM dithiothreitol) (Ozyoruk et al., 2001), heated to 100 °C for 5 min and loaded onto a 4–20% gradient polyacrylamide gel (BioRad, Hercules, CA). The gel was run at 200 V for 30 min and transblotted onto nitrocellulose at 100 V for 60 min. Western blotting was performed utilizing either rabbit polyclonal IgG phospho-c-Jun (Ser 73) or rabbit monoclonal IgG c-Jun (60A8) primary antibodies (PhosphoPlus c-Jun Antibody Kit, Cell Signaling Technology, Beverly, MA) according to the kit protocol.

Propidium iodide staining and flow cytometry

Propidium iodide (PI) staining was accomplished using a modification of a published protocol (Villet et al., 2003). Briefly, 2–3 × 10^6 $U937_{CAEV}$ cells were plated in 3-cm diameter, 6-well plates with or without 40 μM SP600125. At the appropriate time point, the cells were pelleted, washed once with PBS, pelleted and resuspended in 1 ml PBS. 0.5 ml of 1% paraformaldehyde was added, and the cells were incubated on ice for 30 min. The cells were washed once with PBS, resuspended in 0.5 ml PBS and treated with 90 μl of RNAse A (10 mg/ml, 100 U/mg) for 30 min at room temperature. The cells were washed again, resuspended in 1 ml PBS and treated with 50 μl propidium iodide (1 mg/ml). The cells were stored in the dark at 5 °C. A FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with Cell Quest software (Becton Dickinson) was used to collect data. The data were analyzed and figures generated with FlowJo software (Tree Star, Inc., Stanford University, Palo Alto, CA).

Statistics

The data are presented as the mean of three or more values (bar) with the standard deviation displayed as error bars. A one way analysis of variance (ANOVA) or Kruskal–Wallis one way ANOVA was performed on each data set with three or more parameters. Where global differences were identified, the Student–Newman–Keuls (SNK) multiple comparison test was used for select pair wise comparisons of the mean responses between treatment groups. A P value of less than 0.05 was considered to be statistically significant. All of the graphs were generated in SigmaPlot and the statistics were performed with SigmaStat software (Systat Software Inc. Richmond, CA).

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