486–489 *Nucleic Acids Research, 1998, Vol. 26, No. 2* *© 1998 Oxford University Press*

Activation of stress-activated MAP protein kinases up-regulates expression of transgenes driven by the cytomegalovirus immediate/early promoter

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Received September 25, 1997; Revised and Accepted November 20, 1997

ABSTRACT

The immediate/early promoter/enhancer of cytomegalovirus (CMV promoter) is one of the most commonly used promoters for expression of transgenes in eukaryotic cells. In practice, the CMV promoter is often thought of as a constitutively active unregulated promoter. However, we have observed that transcription from the CMV promoter can be up-regulated by a variety of environmental stresses. Many forms of cellular stress stimulate MAP kinase signalling pathways, resulting in activation of stress-activated protein kinases [SAPKs, also called Jun N-terminal kinases (JNKs)] and p38 kinases. We have found that the same conditions that lead to activation of SAPK/JNKs and p38 kinases can also dramatically increase expression from the CMV promoter. Inhibitors of p38 kinases abolished basal transcription from the CMV promoter and completely blocked stress-induced up-regulation of the CMV promoter. Overexpression of a dominant negative JNK kinase had no effect on basal transcription, but significantly reduced up-regulation caused by stress. These results have grave implications for use of the CMV promoter. If the CMV promoter can be up-regulated by cellular stresses, inadvertent activation of the stress kinase pathways may complicate, if not invalidate, the interpretation of a wide range of experiments.

INTRODUCTION

It has recently been discovered that a variety of environmental insults, such as UV irradation, osmotic shock and oxidative stress, mediate some of their effects on the cell through activation of MAP kinase signalling pathways (1,2). These kinase cascades ultimately activate the c-Jun N-terminal kinases (JNKs), also called stress-activated protein kinases (SAPKs, here referred to as SAPK/JNKs), a family of kinases which increase the activity of transcription factors such as c-Jun and ATF-2 (3,4). A parallel pathway which responds to many of the same stimuli results in activation of the p38 MAP kinases (5), a family of kinases that increase the activity of MAPKAP kinase 2 (6), ATF-2 (7) and Max (8).

During the course of our work we observed that many of the same stresses that activate the SAPK/JNK pathway could also dramatically increase expression of transgenes regulated by the immediate/early cytomegalovirus promoter/enhancer complex (CMV promoter). This is one of the most commonly used promoters in eukaryotic expression vectors. Its popularity derives from its ability to drive high levels of expression in nearly all mammalian cells. If the CMV promoter can be up-regulated by stress, its use may complicate a wide range of experiments, particularly those involving components of the stress-activated kinase pathways.

MATERIALS AND METHODS

Cell culture

All cell lines were originally from ATCC. Cell lines were maintained in DMEM supplemented with 10% fetal calf serum. Cells were transfected with Lipofectamine (Gibco-BRL, Burlington, Ontario) following the manufacturer's instructions. Equal amounts of expression vector were added in each transfection; when necessary an empty CMV expression vector (pcDNA-3; Invitrogen, San Diego, CA) was added to equalize the total amount of plasmid DNA. Under these conditions ~80% of NIH 3T3 cells are transfected.

β-Galactosidase activity assays

Forty-eight hours after transfection cells were lysed in PBS, 0.5% Nonidet P-40. Insoluble debris was pelleted and the supernatant diluted 10-fold with Z buffer [140 mM sodium phosphate, pH 7.4, 1 mM magnesium sulfate, 140 mM potassium chloride, 0.27% β-mercaptoethanol, 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG)]. After incubation at 37°C for 30 min, the absorbance at 420 nm was measured with an LKB Ultrospec 4050

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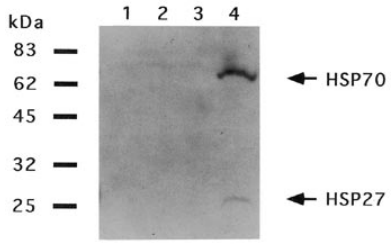

Figure 1. NIH 3T3 cells exposed to arsenite have elevated levels of heat shock/stress proteins. Western blots were probed with an antibody that recognizes only the inducible form of HSP70 (K20; Santa Cruz Biotechnology) and an antibody against HSP27 (M20; Santa Cruz Biotechnology). Lane 1, untreated control; lane 2, 1 μM arsenite; lane 3, 10 μM arsenite; lane 4, 50 μM arsenite. Cells were treated with sodium arsenite for 6 h before harvesting and blotting as described in Materials and Methods.

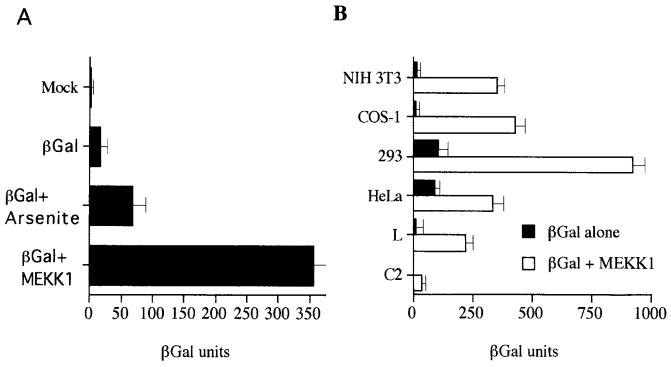

Figure 2. Activation of stress-activated MAP kinases up-regulates expression from the CMV promoter. (**A**) NIH 3T3 cells were transfected with pCMV-*lacZ* as described in Materials and Methods. Forty hours after transfection some of the cells were treated with 50 μM sodium arsenite for 6 h. (**B**) The indicated cell lines were transfected with pCMV-*lacZ* or co-transfected with pCMV-*lacZ* and pCMV-*MEKK(Act)*. Forty-six hours after transfection the cells were harvested and assayed for β-galactosidase activity as described in Materials and Methods. Each experiment was done three to five times, in duplicate, and the average was taken.

spectrophotometer. Protein concentration of the extracts was measured with a bicichoninic acid–copper assay (Sigma, St Louis, MO). β-Galactosidase activity is defined as $(OD_{420\ nm} \times 1000) \div$ (time of incubation)(mg extract). All assays were done in duplicate.

Immunoblots

Cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Insoluble debris was pelleted and the protein concentration of the supernatant determined using a bichichoninic acid–copper assay (BioRad, Mississauga, Ontario). Samples of 20 μg were electrophoresed by standard SDS–PAGE and transferred to Immobilon-P membranes (Millipore). Blots were probed with the indicated antibodies using standard techniques and developed with ECL chemiluminescence reagents (Amersham).

Northern blots

Forty-eight hours after transfection, RNA was extracted from the cells with Trizol (Gibco-BRL), following the manufacturer's instructions. The RNA was quantified by measuring optical density at 260 nm and the values were confirmed by ethidium staining after gel electrophoresis. Five micrograms of RNA were separated on a MOPS/formaldehyde–agarose gel and transferred to Zetabind membrane (BioRad) by capillary blotting. The blot was probed with full-length *lacZ* cDNA using standard techniques.

Immunoprecipitation kinase assay of JNK1

Activation of JNK1 was assayed as described previously (9). Briefly, following cell lysis and centrifugation to remove cell debris the enzyme was immunoprecipitated using anti-JNK1 polyclonal antibody (C-17; Santa Cruz Biotechnology, Santa Cruz, CA) and GST–cJun (Santa Cruz Biotechnology) was used as substrate to determine the activity.

Plasmids and reagents

Unless otherwise stated, chemicals were purchased from Sigma and cell culture reagents were from Gibco-BRL. The p38 inhibitor SB203580 was from Calbiochem. The pCMV-*lacZ* plasmid was pUT535 (Cayla, Toulouse, France). Plasmid p*JNKK*(–) encodes a JNK kinase (also called SEK1) mutated at the sites of MEKK1 phosphorylation, such that activated MEKK1 can bind to the sites but not phosphorylate them, thus preventing some of the activated MEKK1 from activating endogenous JNKK. Plasmid pCMV-*MEKK(Act)* expresses a constitutively active truncated form of MEKK1 and was a gift from M.Karin.

RESULTS

Transcription from the CMV promoter can be up-regulated

While performing routine transfection experiments with expression vectors containing the CMV promoter, we observed that certain stressors would dramatically increase expression from these constructs. To study this phenomenon further we employed a plasmid in which expression of the *Escherichia coli* gene *lacZ*, which encodes β-galactosidase, is regulated by the CMV promoter. This system allowed us to readily quantify the amount of CMV-driven expression obtained.

NIH 3T3 cells were transfected with the CMV-*lacZ* plasmid as described in Materials and Methods. Forty hours after transfection the cells were treated with 50 μM sodium arsenite. Six hours were left between initiation of the treatment and harvesting to allow time for transcription and translation. The concentration of arsenite used was chosen to be sublethal but extremely stressful. Under these conditions of exposure to arsenite induction of a stress response was also demonstrated by increased levels of two heat shock/stress proteins, HSP27 and HSP70 (Fig. 1). As shown in Figure 2A, treatment of transfected NIH 3T3 cells with sodium arsenite, a reagent which activates SAPK/JNK (10), increased β-galactosidase activity 3-fold. Other stressors, such as osmotic shock, hydrogen peroxide and heat shock, were also able to increase β-galactosidase activity (data not shown).

To confirm that the observed CMV up-regulation was a direct consequence of stress kinase activation, rather than some side-effect of drug treatment, we employed a plasmid that expresses a cDNA encoding an active MEKK1, one of the initial kinases in the MAP kinase cascade. Low levels of active MEKK1 will preferentially activate SAPK/JNKs, but high levels of expression, such as are found here, will also activate the p38 kinases (2,11). Co-transfection of NIH 3T3 cells with pCMV-*lacZ*

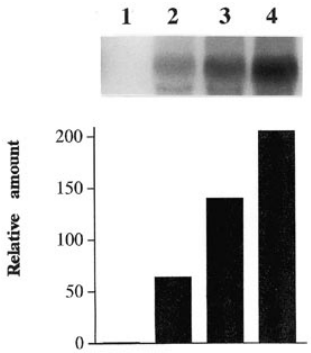

Figure 3. Stress-induced up-regulation of the CMV promoter occurs at the level of transcription. NIH 3T3 cells were transfected with pCMV-*lacZ* or co-transfected with pCMV-*lacZ* and pCMV-*MEKK(Act)*. Some cells were treated with 50 μM sodium arsenite 40 h after transfection for 6 h. The cells were harvested 46 h after transfection. mRNA was extracted, separated by gel electrophoresis and blotted as described in Materials and Methods. The relative amount of β-galactosidase mRNA was measured by phosphorimaging. Lane 1, mock transfection; lane 2, transfected only with pCMV-*lacZ*; lane 3, transfected only with pCMV-*lacZ* and treated with sodium arsenite; lane 4, transfected with pCMV-*lacZ* and pCMV-*MEKK(Act)*. The experiment was repeated several times and a representative result is shown.

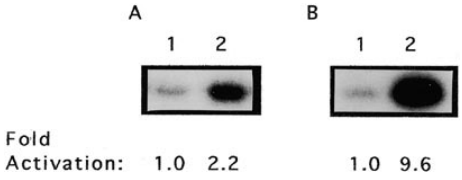

Figure 4. JNK1 is activated by the same conditions that up-regulate CMV expression. NIH 3T3 cells were transfected with pCMV-*MEKK(Act)* (**A**, lane 2), mock transfected (**A**, lane 1), untreated (**B**, lane 1) or treated with sodium arsenite as described in the legend to Figure 1 (**B**, lane 2). The cells were harvested and assayed for JNK1 activity as described in Materials and Methods. The relative JNK1 activity was measured by phosphorimaging and is indicated under each lane. The experiment was repeated several times and a representative result is shown.

and pCMV-*MEKK(Act)* resulted in dramatically increased β-galactosidase activity (Fig. 2A). To see if this effect was cell type specific a number of different cell lines were tested. Each cell line was co-transfected with pCMV-*lacZ* and pCMV-*MEKK(Act)*. Forty-eight hours later, β-galactosidase levels were measured. For all cell types tested co-expression of activated MEKK1 caused a significant increase in β-galactosidase levels (Fig. 2B). The inductive effect was not limited to β-galactosidase, as other proteins expressed from the CMV promoter were also found to be up-regulated by stressors (data not shown).

To confirm that up-regulation was at the level of transcription NIH 3T3 cells were transfected with pCMV-*lacZ* and treated with sodium arsenite or co-transfected with pCMV-*MEKK(Act)*. Levels of β-galactosidase mRNA were examined by Northern blotting. As can be seen in Figure 3, both sodium arsenite treatment and co-expression of activated MEKK1 up-regulated the amount of mRNA driven by the CMV promoter.

Both SAPK/JNKs and p38 kinases are involved in regulating transcription from the CMV promoter

As mentioned briefly, the stressors that induce CMV up-regulation are known to activate the SAPK/JNK pathways. To confirm that SAPK/JNK activation correlates with CMV up-regulation we assayed SAPK/JNK activity directly. As shown in Figure 4, treating NIH 3T3 cells with either arsenite or transfection with pCMV-*MEKK(Act)* strongly increases the kinase activity of JNK1. Although MEKK1 does not activate JNK1 as strongly as does arsenite, MEKK1 up-regulates expression from the CMV promoter to a much greater extent than does arsenite (Fig. 1A). However, this apparent contradiction can easily be explained if we consider that cells transfected with pCMV-*MEKK(Act)* are stimulated for 46 h, allowing a steady accumulation of products expressed from the CMV promoter, while arsenite treatment only allows 6 h worth of products to accumulate.

To further investigate the role of SAPK/JNK in CMV up-regulation we employed a dominant negative JNK kinase. The plasmid p*JNKK*(–) expresses a JNKK (also called SEK1) mutated such that it partially blocks activation of SAPK/JNKs. When pCMV-*lacZ* and p*JNKK*(–) were co-expressed together in NIH 3T3 cells basal levels of β-galactosidase were unaltered. However, when cells co-transfected with pCMV-*lacZ* and p*JNKK*(–) were treated with sodium arsenite the presence of JNKK(–) reduced up-regulation of β-galactosidase activity by half (Fig. 5). Since p38 kinases and SAPK/JNKs are both activated by the same or similar stresses (2), we also investigated the role of p38 kinases in CMV stress-induced up-regulation. To study their function we used a specific inhibitor that blocks activity of the p38 kinases (12). We found that if p38 inhibitor was added at the time of transfection almost no product was expressed from the CMV promoter (Fig. 5), indicating that basal levels of p38 kinase activity are essential for basal CMVpromoter activity. If, however, p38 inhibitor was added 40 h after transfection, at the same time sodium arsenite was added, the presence of p38 inhibitor completely blocked stress-induced up-regulation of the CMV promoter (Fig. 5).

DISCUSSION

Activation of the stress-activated MAP kinase cascades has many effects on the cell that appear to be mediated primarily through regulation of transcription. Activated SAPK/JNK is known to phosphorylate and thereby activate key transcriptional factors such as c-Jun and ATF-2 (3,4) and the p38 kinases also regulate ATF-2 (7). The immediate/early CMV promoter/enhancer complex contains AP-1 and ATF binding sites (13). It is, therefore, not particularly surprising that activity of the CMV promoter should be altered in a stressed cell. The stress-induced activation of the CMV promoter that we have observed may simply be a side-effect caused by a general up-regulation of transcription, but it is also possible that cytomegalovirus could have evolved to take advantage of the stress response. Like many other viruses, cytomegalovirus evades the immune system by remaining quiescent within the cell until conditions become appropriate for viral replication (14). Stimulation of cytomegalovirus replication appears to be primarily mediated by inducing transcription from the cytomegalovirus immediate/early promoter (15) and many of the conditions known to stimulate CMV replication, such as oxidative stress, immunosuppressive drugs and irradiation, are also conditions that can activate SAPK/JNKs and p38 kinases (1–4,13,16). These conditions are also likely to result in immunosuppression of the host, allowing cytomegalovirus to replicate only under conditions which it is able to survive. Thus

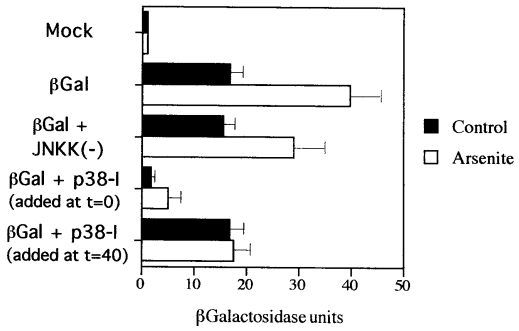

Figure 5. MAP kinases regulate expression from the CMV promoter. NIH 3T3 cells were mock transfected (Mock), transfected with pCMV-*lacZ* alone (βGal), co-transfected with pCMV-*lacZ* and p*JNKK*(–) [βGal + JNKK(–)] or transfected with pCMV-*lacZ* and treated with 0.5 μM SB203580 (βGal + p38-I). Forty hours after transfection some of the cells were treated with arsenite as described in the legend to Figure 1. Some cells were treated with SB203580 for the entire 46 h (added at t = 0), while others were only treated with SB203580 40 h after transfection for 6 h (added at t = 40). All cells were harvested 46 h after transfection and assayed for β-galactosidase activity as described in Materials and Methods. Each experiment was done three times in duplicate and the average was taken.

viral promoters may have been selected to be able to maximize transcription under stressed conditions.

In addition to variation in transcriptional activity of the CMV promoter according to cell type and developmental age (17), two previous reports have suggested that the CMV promoter can be up-regulated under specific conditions. In one the major immediate/early enhancer of cytomegalovirus was found to be up-regulated by cAMP in lymphoid cell lines (18). This up-regulation was mediated through a CRE element contained within the enhancer. In the other report NF-κB was shown to play a central role in activation of the CMV promoter by cytomegalovirus gene products (13). Sambucetti *et al*. were also able to artificially activate transcription from the CMV promoter by stimulating the cells with phorbol esters (13).

Our results are the first to show that stress-activated MAP kinases are involved in regulating transcription from the CMV promoter. We find that without basal p38 kinase activity the CMV promoter is transcriptionally silent (Fig. 4). This may be mediated by lower ATF-2 activity, as p38 kinases have been shown to directly stimulate ATF-2 activity (7), or it may be mediated indirectly through loss of NF-κB function. Wesselborg *et al*. were able to demonstrate that p38 kinases are necessary for NF-κB-dependent gene expression (19). p38 kinase inhibitors were also able to completely block stress-induced up-regulation of the CMV promoter (Fig. 4). It is unclear whether this blockage is simply due to complete shut-off of the CMV promoter in the absence of p38 kinases or whether p38 kinases are also essential for stress-induction of the CMV promoter.

It is unlikely that NF-κB activity is involved in stress induction of the CMV promoter. Although some stresses are able to activate NF-κB, sodium arsenite does not (19). Thus the observed up-regulation of the CMV promoter is probably mediated by increased activity of c-Jun and ATF-2.

The pertinence of the data presented in this paper stems from the widespread use of the CMV promoter in expression vectors. Experimental protocols that might activate the stress response could be complicated to interprete due to up-regulation of the CMV promoter. For example, the pCMV-*MEKK(Act)* plasmid used in our experiments sets up a self-stimulating loop, expressing increasing amounts of active MEKK1 until the cell dies. Experiments which use inhibitors of p38 kinases would be devastated by simultaneous use of the CMV promoter. On the other hand, protocols that only require high levels of expression could exploit stress activation of the CMV promoter to generate even more transgenic protein and perhaps p38 kinase inhibitors could be used to switch the CMV promoter on and off.

ACKNOWLEDGEMENTS

This work was supported by grants from the ALS Society of Canada, the Muscular Dystrophy Association of Canada and the Medical Research Council of Canada. H.D.D. is an MNI Killam Scholar. We thank M.Karin for his kind gifts of expression vectors.

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