Effects of *ras* transformation on the induction of the IL-1 receptor related T1 gene in response to mitogens, anisomycin, IL-1 and TNF α

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The *T1* gene gives rise to two transcripts encoding a 62 kDa membrane-bound and a 37 kDa secreted protein with similarity to the type I IL-1 receptor. It is weakly expressed in proliferating but not in resting fibroblasts and is strongly induced during the entry of quiescent cells into the cell cycle. Here we show that the *T1* gene is also transcriptionally activated in response to the treatment of fibroblasts with cycloheximide and anisomycin. These protein synthesis inhibitors are known to stimulate the JNK and p38/RK signal transduction pathways. We provide evidence that anisomycin triggers *T1* gene induction through the stimulation of the p38/RK MAP kinase. This observation is in line with our finding that physiological activators of the p38/RK pathway, the proinflammatory cytokines IL-1 and TNF α , stimulate *T1* gene expression efficiently. Growth factor mediated *T1* gene induction is a delayed early event, requiring ongoing protein synthesis. In contrast, anisomycin induces *T1* gene expression at concentrations which block translation completely. Thus, transcriptional induction of the *T1* gene via the p38/RK pathway is an immediate early event not requiring *de novo* protein synthesis. The *T1* gene is strongly induced by various mitogens in quiescent NIH3T3 fibroblasts but not in *ras* transformed NIH3T3 cells. In contrast, all of the three tested agent which activate the p38/RK pathway, IL-1, TNF α , and anisomycin led to strong *T1* gene expression in normal and *ras* transformed NIH3T3 cells alike. Thus, the *T1* gene can be induced through the activation of at least two MAP kinase pathways: signaling through the ERK pathway can occur in normal but not in *ras* transformed NIH3T3 cells, whereas the signaling through the p38/RK pathway is not affected by *ras* transformation.

Keywords: anisomycin; IL-1R family; IL-1; TNF α ; MAP kinases; SB203580; signal transduction pathway; *ras* oncogene

Introduction

T1, also known as ST2 and DER4, was identified as a gene activated by growth factors and the induced expression of the Ha-*ras* or v-*mos* oncogenes in murine fibroblasts (Klemenz *et al.*, 1989; Tominaga, 1989; Werenskiold *et al.*, 1989; Lanahan *et al.*, 1992). Later,

the human and rat homolog have been isolated (Tominaga *et al.*, 1992; Bergers *et al.*, 1994).

T1 is transcribed into two mRNA species of 2.7 kb and 5 kb which arise from alternative 3' processing (Yanagisawa *et al.*, 1993). The 5 kb *T1* transcript is found in mast cells (Gächter *et al.*, 1996), in subpopulations of cells in hematopoietic organs (embryonic liver, spleen, bone marrow) (Rößler *et al.*, 1995) and the lung (Bergers *et al.*, 1994) throughout ontogenesis. It encodes a 567 amino acid transmembrane protein (T1M) (Yanagisawa *et al.*, 1993) which belongs to the interleukin 1 receptor (IL-1R) family (Yanagisawa *et al.*, 1993; Mitcham *et al.*, 1996; Parnet *et al.*, 1996). The amino acid sequences of the ectodomain of T1 and the type I IL-1R are 28% identical and the intracellular portions share 40% identity. The genes encoding the two IL-1 receptors and T1 are tightly linked on mouse chromosome 1 (Tominaga *et al.*, 1991) and human chromosome 2 (band 2q 12–13) (Sims *et al.*, 1995). In addition, the intron/exon structures of the type I IL-1R and the *T1* gene are conserved, suggesting a common ancestor (Sims *et al.*, 1995). T1M does not bind any of the IL-1R ligands IL-1 α , IL-1 β , or IL-1ra (Rößler *et al.*, 1993; Danescu and Werenskiold, 1995; Kumar *et al.*, 1995; Gayle *et al.*, 1996). However, there is indirect evidence that it stimulates similar or identical signal transduction cascades as the IL-1R. Both, the type I IL-1R and a chimeric protein consisting of the extracellular domain of the IL-1R and the intracellular domain of T1M are able to activate NF κ B, the MAP kinase p38/RK, and transcription from the *IL-8* promoter upon IL-1 α or IL-1 β stimulation (Kumar *et al.*, 1995; Reikerstorfer *et al.*, 1995; Mitcham *et al.*, 1996).

Recently, a 227 amino acid membrane bound putative T1 ligand was cloned from human and mouse (Gayle *et al.*, 1996). Two additional soluble putative T1 ligands were partly purified and characterized as proteins of 32 kDa and 18 kDa (Kumar *et al.*, 1995). While the membrane bound putative ligand was unable to activate NF κ B and transcription from the *IL-8* promoter upon binding to T1M (Gayle *et al.*, 1996), protein fractions containing the 32 kDa and 18 kDa putative soluble ligands were able to activate the p38/RK MAP kinase via T1M (Kumar *et al.*, 1995).

The 2.7 kb *T1* transcript was found in fibroblasts, the embryonic skin, in the retina and bones (Rößler *et al.*, 1995), in developing mammary glands 3–4 weeks after birth as well as in experimental Ha-*ras* induced mammary tumors (Rößler *et al.*, 1993). It encodes a 327 amino acid protein (T1S) which is N-glycosylated and secreted (Klemenz *et al.*, 1989; Tominaga, 1989; Werenskiold, 1992; Takagi *et al.*, 1993; Kalousek *et al.*, 1994). Apart from nine amino acids at its carboxy

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terminus, T1S is identical to the extracellular domain of T1M (Yanagisawa *et al.*, 1993). Soluble forms of type II IL-1R (Symons *et al.*, 1995) as well as the related vaccinia virus B15R protein (Alcami and Smith, 1992; Spriggs *et al.*, 1992) have previously been described and postulated to function as type I IL-1R antagonists.

Serum responsive genes have been divided into 'immediate early' and 'delayed early' genes based on the kinetics of their induction and the dependence on ongoing protein synthesis, the former being independent and the latter dependent on translation (Lau and Nathans, 1991). *T1* mRNA accumulates in response to serum with a kinetics typical for delayed early gene transcripts peaking around 6–10 h after serum stimulation (Tominaga, 1989; Werenskiold *et al.*, 1989). Delayed early genes are thought to be the targets of immediate early transcription factors. Indeed, we and others have demonstrated that the *T1* gene and its rat homologue *fit-1* are induced in response to the expression of the immediate early serum response protein c-Fos and FosB (Bergers *et al.*, 1994; Kalousek *et al.*, 1994). Thus, it can be assumed that *T1* gene induction in response to the mitogenic stimulation of quiescent cells is the consequence of c-fos gene activation. Similarly, the onset of *ras* oncogene expression, as it is artificially achieved in conditional expression systems, is followed by the transient accumulation of c-Fos which in turn triggers the activation of the *T1* gene. However, sustained expression of oncogenic *ras* results in the down-regulation of the c-fos and consequently of the *T1* gene (submitted for publication). This repression occurs in spite of permanent activation of the MAP kinases ERK-1 and ERK-2 and is the consequence of elevated AP-1 activity (unpublished observation).

The classification of the *T1* gene as an immediate- or delayed early serum responsive gene based on its serum inducibility in the presence of the protein synthesis inhibitor cycloheximide has been controversial. Three groups have described *T1* and *fit-1* as delayed early genes based on the observation that the protein synthesis inhibitor cycloheximide blocked serum inducibility in Swiss 3T3 (Kalousek *et al.*, 1994), Balb/c 3T3 (Lanahan *et al.*, 1992) and rat-1A cells (Bergers *et al.*, 1994). In contrast, Yanagisawa *et al.*, 1992 classified *T1* as an immediate early gene since cycloheximide in their experiments did not abolish the serum induction of *T1* in Balb/c 3T3 cells.

In this study we demonstrate that the *T1* gene is efficiently induced by the translation inhibitors cycloheximide and anisomycin in NIH3T3 but not in Swiss 3T3 cells. The substance SB203580 (Cuenda *et al.*, 1995), an inhibitor of the MAP kinases p38/RK and certain isoforms of JNK (Whitmarsh *et al.*, 1997) blocked anisomycin mediated *T1* gene expression completely. This finding and the observation that u.v. does not result in *T1* gene induction suggest that p38/RK is involved in the transmission of the anisomycin triggered signal to activate the *T1* gene.

The MAP kinase p38/RK (also known as CSBP and Mpk2) (Freshney *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994) is activated in mammalian cells in response to IL-1, TNF α , and lipopolysaccharide and by stress signals (Han *et al.*, 1994; Rouse *et al.*, 1994; Cuenda *et al.*, 1995; Raingeaud *et al.*, 1995; Beyaert *et al.*,

1996). Its activator is the dual specificity kinase MKK6 (Raingeaud *et al.*, 1996) which phosphorylates p38/RK at both threonine and tyrosine residues (Raingeaud *et al.*, 1995). Once activated, it phosphorylates and thereby activates MAPKAP kinase-2 (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Cuenda *et al.*, 1995; Beyaert *et al.*, 1996) and MAPKAP kinase-3 (Ludwig *et al.*, 1996; McLaughlin *et al.*, 1996) which in turn catalyze the phosphorylation of the small heat shock protein Hsp25 (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Cuenda *et al.*, 1995; Beyaert *et al.*, 1996; Ludwig *et al.*, 1996; McLaughlin *et al.*, 1996). p38/RK has been shown to phosphorylate the transcription factors ATF-2 (Dérjard *et al.*, 1995; Raingeaud *et al.*, 1995, 1996), Elk-1 (Cano *et al.*, 1995; Zinck *et al.*, 1995; Raingeaud *et al.*, 1996), and Max (Zervos *et al.*, 1995) *in vitro*, however the *in vivo* activation of these transcription factors by p38/RK has not yet been demonstrated. Here we show that anisomycin treatment resulted in the transcriptional activation of the *T1* gene. This response was observed using anisomycin concentrations which do not block translation as well as high concentrations which inhibit protein synthesis completely. Hence, the *T1* gene responds in an immediate early mode to the p38/RK signal transduction cascade stimulated by anisomycin while previous studies have shown that mitogenic stimuli, signaling through the MAP kinases of the ERK family, stimulate the *T1* gene in a delayed early manner. We further demonstrate that two physiological activators of the p38/RK pathway, IL-1 and TNF α also efficiently trigger *T1* gene induction.

Serum and growth factor triggered *T1* gene induction is abrogated in *ras* transformed NIH3T3 cells. However, anisomycin, IL-1, and TNF α mediated *T1* gene induction is not affected by the expression of the *ras* oncogene. p38/RK is not phosphorylated in unstimulated *ras* transformed NIH3T3 cells while the MAP kinases ERK-1 and ERK-2 are permanently in the phosphorylated, activated state. Thus, while the expression of a *ras* oncogene leads to the sustained activation of the ERK pathway and presumably through some feedback mechanism to *T1* gene down-regulation, it does not affect the p38/RK pathway and does not interfere with anisomycin, IL-1, and TNF α mediated *T1* gene induction.

Results

Two different pathways are used for the induction of T1 by serum and cycloheximide/anisomycin

We have previously reported that serum growth factors fail to induce the *T1* gene in Swiss 3T3 fibroblasts if protein synthesis was blocked by the treatment of the cells with cycloheximide (Kalousek *et al.*, 1994). However, we later observed that cycloheximide did not prevent *T1* mRNA accumulation in NIH3T3 cells, another murine fibroblastic cell line (Figure 1a). On first view this is in contradiction to our previous claim that *T1* is a delayed early serum responsive gene. However, cycloheximide is known to have pleiotropic effects; besides binding to ribosomes and thereby blocking translation it can also activate the JNK pathway (Kyriakis *et al.*, 1994). Other protein synthesis

Figure 1 Accumulation of *T1* mRNA in response to serum and protein synthesis inhibitors. (a) Serum starved NIH3T3 cells were stimulated with 10% FCS in the presence or absence of 50 μ g/ml cycloheximide. Total RNA was prepared before (0) and after stimulation for the time periods (in hours) as indicated below the lanes. (b) Serum starved NIH3T3 and Swiss 3T3 cells were stimulated with 10% FCS in the presence or absence of 10 μ g/ml puromycin or 10 μ g/ml emetine for 6 h. (c) Proliferating and serum starved NIH3T3, NRF-2, Swiss 3T3, and Balb/c 3T3 cells were treated with protein synthesis inhibitors (cycloheximide, 50 μ g/ml; anisomycin, 1 μ g/ml) or 10% FCS, respectively. RNA was collected after the time periods (h) indicated above the lanes and 5 μ g of total RNA was subjected to Northern blot analysis using *T1* cDNA as the probe

inhibitors such as puromycin and emetine do not share this property with cycloheximide (Cano *et al.*, 1994; Kyriakis *et al.*, 1994) and were used to test whether serum growth factor mediated *T1* gene expression in NIH3T3 cells is also dependent on ongoing protein synthesis. As can be seen in Figure 1b, serum stimulation in the presence of either puromycin or emetine did not result in the accumulation of *T1* mRNA indicating that *T1* is indeed a delayed early serum responsive gene.

These findings suggest that cycloheximide treatment is sufficient to promote the accumulation of *T1* mRNA. Indeed, the addition of cycloheximide to NIH3T3 cells resulted in the synthesis of *T1* mRNA (Figure 1c). However, no *T1* mRNA accumulated under the same conditions in Swiss 3T3 cells which is in accordance with our previous finding (Kalousek *et al.*, 1994). Anisomycin is another protein synthesis inhibitor which shares with cycloheximide the property of stimulating signal transduction pathways thereby triggering the expression of susceptible genes (Almendral *et al.*, 1988; Quantin and Breathnach, 1988; Mahadevan and Edwards, 1991; Liang *et al.*, 1996). Anisomycin treatment resulted in the accumulation of even higher levels of *T1* mRNA in all fibroblastic cell lines tested. In contrast to serum growth factors which

induced the *T1* gene with similar efficiency in the different, non-transformed fibroblastic cell lines tested (Figure 1c), anisomycin treatment led to very high, intermediate and low levels of *T1* mRNA accumulation in NIH3T3, Balb/c 3T3 and Swiss 3T3 cells, respectively.

We have previously observed that serum-mediated *T1* gene induction is attenuated in NIH3T3 cell lines which express the Ha-ras oncogene such as the NFR-2 cell line used in this study (Figure 1c). In contrast, we found that cycloheximide and anisomycin treatment led to similar levels of *T1* mRNA accumulation in normal and ras-transformed NIH3T3 cells (Figure 1c). This is a further indication that growth factors and cycloheximide/anisomycin operate through different signal transduction pathways.

Anisomycin inducibility is not a general phenomenon among delayed early serum responsive genes

We tested whether anisomycin treatment affects two other well studied delayed early serum responsive genes. For this experiment we used Balb/c 3T3 cells because the delayed early serum responsive genes encoding stromelysin and interstitial collagenase are well induced by EGF, serum, and TPA in these but not in NIH3T3 cells. Balb/c 3T3 cells were rendered quiescent by serum deprivation and subsequently stimulated with EGF, serum, TPA and anisomycin. Serum- and TPA stimulation led to the well documented induction of the three tested delayed early serum responsive genes (reviewed in Aoyama and Klemenz, 1993). In contrast, anisomycin treatment resulted only in *T1* mRNA accumulation but did not stimulate stromelysin and collagenase gene transcription (Figure 2). This shows that the observed pattern of gene induction by mitogens and anisomycin is overlapping but not congruent.

Figure 2 Anisomycin does not induce the delayed early serum responsive genes encoding stromelysin and interstitial collagenase. Serum starved Balb/c 3T3 cells were treated with 35 ng/ml epidermal growth factor (EGF), 10% FCS, 200 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA), or 1 μ g/ml anisomycin (aniso) for 6 h. Three identical Northern blots were prepared and hybridized with radioactive labeled probes recognizing stromelysin, collagenase and *T1* mRNA

T1 is transcriptionally induced by anisomycin

Different mechanisms can be envisaged for anisomycin mediated *T1* mRNA accumulation. Anisomycin affects cellular physiology in two ways. By virtue of binding to ribosomes it can block translation and by an unknown mechanism it can stimulate MAP kinase pathways. The two modes of operation exhibit different dose dependencies and can thus be distinguished. While protein synthesis is only inhibited at anisomycin concentrations above 40 ng/ml (Figure 3b) it turns on signal transduction pathways at lower concentrations (Mahadevan and Edwards, 1991). As can be seen in Figure 3a, anisomycin led to the significant accumulation of the 2.7 kb *T1* mRNA at concentrations which are insufficient to block protein synthesis and the maximal level of *T1* mRNA was reached at a concentration of 100 ng/ml which still allowed translation to proceed at ca. 40% of its efficiency in the absence of the drug.

Another mRNA which we fortuitously found to accumulate in response to anisomycin treatment of NIH3T3 cells encodes mouse tissue factor (Figure 3c). However, this response is only observed at anisomycin concentrations which are sufficiently high to block protein synthesis completely, i.e. approximately 30 times higher than the concentrations needed to achieve accumulation of *T1* mRNA. We assume that the accumulation of tissue factor mRNA is the result of mRNA stabilization possibly due to the lack of the synthesis of a labile RNase.

This result suggests that transcriptional activation of the *T1* gene is the cause of or contributes to anisomycin-mediated *T1* mRNA accumulation. In order to test whether anisomycin does indeed trigger *T1* gene transcription we performed nuclear run on assays. Nuclei were isolated from NIH3T3 cells which were serum starved for 1 day and exposed to serum or anisomycin for 2 and 3.5 h, respectively. Radioactively labeled run on RNA was prepared and hybridized to Southern blots containing genomic and complementary *T1* DNA (Figure 4). All DNA fragments containing transcribed portions of the *T1* gene hybridized to run-on RNAs from anisomycin and serum stimulated but not from unstimulated cells. One genomic DNA fragment contains repetitive DNA. It scores positive in this hybridization experiment with all three different run-on probes and serves as an internal control for equal quality of the nuclear run-on transcripts and the DNA transfer. We conclude from this experiment that anisomycin treatment leads to *T1* gene expression and that the extent of transcription in response to serum and anisomycin is similar.

Using run on transcription assays we could show that anisomycin was also able to induce *T1* transcription in the *ras* transformed cell line NFR-2 (data not shown).

A T1 reporter gene construct can be induced by anisomycin

Based on the finding that anisomycin stimulation leads to the transcriptional activation of the *T1* gene we investigated whether a *T1* reporter gene construct can be stimulated similarly. A plasmid (pFH6) was constructed which contains a CAT gene flanked by

7.4 kb and 5 kb of the *T1* gene 5' and 3' flanking regions, respectively. It includes the first and part of the second as well as part of the eighth exon and was stably introduced into NIH3T3 cells (Figure 5). Since

Figure 3 *T1* is induced at concentrations of anisomycin which are subinhibitory for translation. Proliferating NFR-2 cells were treated with different concentrations of anisomycin (ng/ml) as indicated below the lanes. (a) Total RNA was collected 8 h after the addition of anisomycin and analysed by Northern blot with a *T1*-specific probe. The ethidium bromide stained gel is shown as a control for equal loading of RNA. The same result was obtained with non transformed NIH3T3 cells except that the amount of accumulated 5 kb *T1* mRNA was always lower than in anisomycin treated NFR-2 cell. (b) Anisomycin was added to proliferating NFR-2 cells 30 min prior to the metabolic labeling of proteins with ³⁵S-methionine for 1 h. TCA precipitable radioactivity was measured and the values corrected for total protein content. The value obtained for the cell culture which received no anisomycin was set to 100%. (c) NIH3T3 cells were treated with anisomycin at the concentrations indicated above the lanes and RNA was isolated 6 h later. 5 µg of total RNA was subjected to Northern blot analysis using a mouse tissue factor and a *T1* cDNA probe

Figure 4 *T1* is transcriptionally induced by anisomycin. Nuclei were prepared from serum starved NIH3T3 cells before (lanes 1, 5) or 2 and 3.5 h after stimulation with 10% FCS (S, lanes 2, 6) or 1 μ g/ml anisomycin (A, lanes 3, 7), respectively. Nuclear run on experiments were performed and the labeled run-on products hybridized to a Southern blot of restriction enzyme digested plasmid DNA. Left panel: *T1* cDNA; *EcoRI* digested pT1.10, giving rise to a 1 kb and a 1.6 kb *T1* cDNA fragment (arrowheads) and a 3.4 kb vector fragment. Right panel: *T1* genomic DNA; pT1.28, restricted with *EcoRI* and *PstI* giving rise to a 1.2 kb DNA fragment from the transcribed portion of the gene (arrowhead). A genomic DNA fragment between 3.3 kb and 6.4 kb upstream of the transcription start site containing repetitive, transcribed sequences is indicated (rep. DNA). The ethidium bromide stained gels are shown in lanes 4 and 8

anisomycin blocks translation the cell clones could not be scored for CAT activity in response to anisomycin stimulation. Therefore we prepared total RNA of two transfected cell clones which were rendered quiescent by serum starvation and were either stimulated with serum or anisomycin for 4 and 8 h. The RNA was subjected to Northern blot analysis using a CAT specific DNA probe (Figure 5). The same Northern blot was reprobed with a *T1* cDNA probe in order to monitor the induction of the endogenous *T1* gene. We found very efficient expression of the transfected gene in response to serum and anisomycin stimulation. The amount of CAT mRNA which accumulated in anisomycin treated cells was higher than in mitogenically stimulated cells. The ratio of accumulated CAT mRNA under the two conditions is similar to that of the endogenous *T1* gene.

The inhibitor of the MAP kinase p38/RK and some JNK isoforms, SB203580, interferes with anisomycin-mediated T1 gene induction

The p38/RK MAP kinase was recently shown to be essential for anisomycin induced glucose uptake into KB cells (Gould *et al.*, 1995). This observation motivated us to test whether the p38/RK pathway is also involved in anisomycin-mediated *T1* gene induc-

Figure 5 A *T1*-CAT reporter gene is inducible by serum and anisomycin. The construct pFH6, shown in the lower part of the figure, was stably transfected into NIH3T3 cells. Two of the transfected cells clones, P1.3 and PA3.4, were rendered quiescent by serum starvation for 24 h. Fresh medium containing 10% FCS or anisomycin at a final concentration of 100 ng/ml was added and RNA collected after 4 and 8 h. The RNA was subjected to Northern blot analysis using in succession a *CAT* and a *T1* specific probe to detect the exogenous and endogenous *T1* transcripts, respectively. In the lower part of the figure the genomic organization of the *T1* gene (upper part) and the pFH6 construct (lower part) are shown. pFH6 contains 7.4 kb and 5 kb of the 5' and 3' regions of the *T1* gene, respectively, including exon 1 and part of the exons 2 and 8. The identification and characterization of the enhancer element has been described earlier (Trüb *et al.*, 1994). Relevant cloning sites, the translational initiation and stop codons and the transcriptional start site which is used in fibroblasts are indicated. Filled and striped boxes represent non coding and coding regions of the *T1* transcript, respectively

tion. To test this possibility we used the recently described inhibitor of p38/RK, SB203580 (Cuenda *et al.*, 1995). Anisomycin-mediated *T1* mRNA accumulation was blocked by SB203580 in a dose dependent manner and to completion at a concentration of 100 μ M (Figure 6). Run on transcription assays showed that the inhibitor SB203580 blocked anisomycin triggered *T1* gene induction (data not shown). Initially SB203580 was described as a specific inhibitor of p38/RK. However, it was later reported that this substance can also inhibit most of the JNK isoforms at high, and the two isoforms JNK β 1 and JNK β 2 even at low concentrations (Whitmarsh *et al.*, 1997). If anisomycin treatment led to *T1* gene induction via the stimulation of these JNK isoforms we expect to observe *T1* mRNA accumulation in response to u.v. irradiation. To test this possibility we subjected a NIH3T3 derived cell line which harbours a u.v. responsive TRE-CAT reporter gene to u.v. treatment. RNA was collected at different times following the irradiation and subjected to Northern blot analysis (Figure 7a). No increase in accumulated *T1* mRNA was observed at all time points examined and in response to three different doses of irradiation. As a control for the u.v. response we monitored the activation of the CAT reporter gene. CAT activity was induced following u.v. irradiation in a dose and time dependent manner (Figure 7b). u.v. treatment, in contrast to anisomycin treatment resulted in only very weak phosphorylation of p38/RK in the studied cell line as revealed from Western blot analysis using an antibody which selectively recognizes phosphorylated p38/RK (data not shown). These results suggest that anisomycin mediated *T1* gene expression is not the consequence of JNK but rather of p38/RK activation.

Anisomycin leads to p38/RK activation

To further substantiate the finding that anisomycin triggers p38/RK activation we investigated whether p38/RK becomes phosphorylated in response to

Figure 6 The p38/RK inhibitor SB203580 blocked *T1* gene induction by anisomycin. Growth arrested NIH3T3 cells were pretreated with the indicated concentrations of SB203580 for 15 min prior to the addition of 1 μ g/ml anisomycin. 6 h later total RNA was prepared and subjected to Northern blot analysis with a *T1*-specific probe (top). The same blot was reprobed with a *GAPDH*-specific probe to control for equal loading and the integrity of the RNA (bottom)

Figure 7 u.v. irradiation did not induce *T1* mRNA accumulation. CT-7 cells (a NIH3T3 derived cell line which is stably transfected with the u.v.-inducible reporter construct 5 $\times$ TRE-TATA-CAT) were irradiated with u.v. doses of 0.7, 1.4, 2.1, and 2.8 J/m². After irradiation, the cells were allowed to recover for the time periods indicated. In parallel, NIH3T3 cells were treated with 1 μ g/ml anisomycin for 6 h. (a) Total RNA was harvested and subjected to Northern blot analysis using a *T1* specific probe. (b) Protein extracts were prepared and CAT activity measured in lysates normalized for protein content

anisomycin treatment. NIH3T3 cells were serum starved for 24 h and exposed to either emetin, cycloheximide, anisomycin or serum. Only cycloheximide and anisomycin led to p38/RK phosphorylation as revealed by Western blot analysis using an antibody which selectively recognizes the phosphorylated form of p38/RK (Figure 8a). A parallel Western blot was reacted with an anti p38/RK antibody which does not discriminate between the phosphorylated and the unphosphorylated form to demonstrate that equal amounts of p38/RK are present in all extracts (Figure 8b).

In several *ras* transformed fibroblasts the MAP kinases ERK-1 and ERK-2 were found to be permanently phosphorylated (Leevers and Marshall, 1992; Minden *et al.*, 1994). We investigated whether the Ras oncoprotein affects p38/RK similarly. The basal level of phosphorylated p38/RK in *ras* transformed NFR-2 cells was low, comparable to that in normal NIH3T3 cells. Anisomycin treatment resulted in the rapid and strong phosphorylation of p38/RK (Figure 8a,b). Thus, *ras* transformation does not influence basal and anisomycin mediated p38/RK phosphorylation.

One of the targets of p38/RK is MAP kinase activated protein kinase-2 (MAPKAPK-2) which upon activation phosphorylates the small heat shock protein Hsp25 (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Cuenda *et al.*, 1995). Hence, anisomycin treatment should lead to Hsp25 phosphorylation. There are two major phosphorylation sites on murine Hsp25. Unphosphorylated, mono- and diphosphorylated Hsp25 have distinct migration properties on 2 D gels and give rise to the spots a, b, and c, respectively as indicated in Figure 8c. Fibroblasts are devoid of Hsp25 under normal conditions (Aoyama *et al.*, 1993; Klemenz *et*

Figure 8 Anisomycin treatment led to the phosphorylation of the MAP kinase p38/RK. (a) Serum starved NIH3T3 and NFR-2 cells were incubated with 10 μ g/ml emetine (Eme), 50 μ g/ml cycloheximide (CHX), 1 μ g/ml anisomycin (Ani) or 10% FCS (Se) for 15 min. Total cellular proteins were separated on a 12.5% SDS polyacrylamide gel and subjected to Western blot analysis with an antibody directed selectively against p38/RK which is phosphorylated at Y182. (b) An identical Western blot was developed with a p38/RK specific antibody which does not discriminate between the phosphorylated and the unphosphorylated form of p38/RK. (c) C2C12 myoblasts were either incubated at 44°C for 20 min (heat shock), treated with 1 μ g/ml anisomycin for 15, 60 and 90 min or left untreated. Cellular proteins were subjected to 2-dimensional Western blot analysis with an antibody directed against Hsp25. The unphosphorylated and the mono- and diphosphorylated forms of Hsp25 are marked a, b, and c, respectively

al., 1993). Several stress conditions such as hyperthermia result in both, Hsp25 synthesis and phosphorylation which make it difficult to test anisomycin-mediated Hsp25 phosphorylation in these cells. We therefore chose to use the myoblast cell line C2C12 which contains high amounts of Hsp25 under normal conditions. Anisomycin treatment led to the rapid and transient phosphorylation of Hsp25 as revealed from the substantial increase in the abundance of spot c in Figure 8c. The extent of phosphorylation is similar to that seen after a heat shock (Crête and Landry,

1990). This is consistent with the notion that anisomycin leads to the activation of the p38/RK-MAPKAPK-2-Hsp25 cascade.

T1 gene expression is induced in response to IL-1 and TNF α

Two well studied physiological activators of the p38/RK signalling cascade are the proinflammatory cytokines IL-1 and TNF α (Freshney et al., 1994; Geng et al., 1996; Raingeaud et al., 1995; Modur et al., 1996; Nahas et al., 1996; Beyaert et al., 1996). Since fibroblasts express receptors for both ligands we expected to observe T1 gene induction in IL-1 and TNF α stimulated NIH3T3 cells. Indeed, IL-1 and TNF α treatment led to T1 mRNA accumulation in NIH3T3 cells with similar kinetics as was observed following serum stimulation (Figure 9). Likewise, T1 mRNA accumulated in response to IL-1 and TNF α in the ras transformed NFR-2 cell line. These cells do not express the T1 gene in response to the stimulation with serum (Figures 1c, 9a), TPA, PDGF, FGF, and LPA (Kessler et al., submitted). Thus, those agents which stimulate T1 gene expression via the activation of a stress activated MAP kinase pathway (IL-1, TNF α , anisomycin) can stimulate T1 gene expression irrespective of ras transformation, whereas activators of the ERK pathway (TPA, serum, LPA, PDGF, and FGF) can stimulate T1 gene expression only in the non-transformed cells.

Figure 9 T1 mRNA accumulates in response to IL-1 and TNF α treatment. (a) Proliferating NIH3T3 and NFR-2 cells were stimulated with 20 ng/ml IL-1 and serum starved cells with 10% FCS for the indicated times. (b) Quiescent NIH3T3 and NFR-2 cells were stimulated with 20 ng/ml TNF α for 3 h. RNA was harvested and subjected to Northern blot analysis using a T1 specific probe

p38/RK activation is insufficient to induce the T1 gene in Swiss 3T3 cells

As shown previously, treatment of Swiss 3T3 cells with cycloheximide did not result in *T1* mRNA accumulation at all concentrations tested and anisomycin had only a very weak inducing effect (Figure 1c). Since we have shown that p38/RK activation is required for anisomycin-mediated *T1* gene induction we tested whether either the amount of p38/RK or its phosphorylation state in response to anisomycin differs between NIH3T3 and Swiss 3T3 cells. As shown in Figure 10, both the total amount of p38/RK as well as the extent of its phosphorylation after anisomycin addition are comparable in the two cell lines as revealed by Western blot analysis.

Anisomycin treatment also led to the accumulation of *c-fos* mRNA in NIH3T3 cells (Figure 11a). Similar amounts of *c-fos* mRNA were found if cells were treated with low concentrations of anisomycin (30 ng/ml) which hardly inhibited translation and at a high concentration (1 µg/ml) which blocked translation efficiently (Figure 11a). Hence, we assume that *c-fos* mRNA accumulation in NIH3T3 cells is the consequence of transcriptional induction as has been shown previously in C3H 10T1/2 cells (Mahadevan and Edwards, 1991). Following anisomycin addition, Swiss 3T3 cells accumulated *c-fos* mRNA to levels which even exceeded those in NIH3T3 cells. Again, this effect was similar if cells were treated with low or high concentrations of anisomycin and is therefore probably due to transcriptional stimulation. The anisomycin mediated *c-fos* mRNA synthesis could be inhibited with SB203580, indicating that p38/RK is involved in the induction. (Figure 11a, lane 10). This experiment revealed that Swiss 3T3 cells are not refractory to anisomycin mediated gene induction and that anisomycin stimulated activation of the *c-fos* gene apparently needs an additional signal which is weak in NIH3T3 and strong in Swiss 3T3 cells.

We have shown above that *T1* mRNA accumulates to a similar extent in NIH3T3 and NFR-2 cells in response to anisomycin stimulation (Figure 1c). In contrast, anisomycin mediated *c-fos* mRNA accumulation is severely repressed in NFR-2 cells. (Figure 11b). This is reminiscent of serum stimulated *T1* gene

induction which is only observed in normal but not in *ras* transformed fibroblasts. These results indicate that anisomycin mediated induction of the *T1* and the *c-fos* genes operate through different mechanisms.

Discussion

This study was carried out to clarify an apparent contradiction in the classification of *T1* as an immediate early or delayed early mitogen responsive gene. It has led us to the finding that the *T1* gene can be induced through two different signal transduction cascades, the ERK and the p38/RK pathways. The stimulation of the ERK pathway by mitogens such as serum, LPA and PDGF leads to the phosphorylation of transcription factors which are involved in the stimulation of immediate early genes (Moolenaar, 1994; Hill and Treisman, 1995). We have previously shown that *T1* gene expression in response to serum

Figure 10 The amount of p38/RK and the extent of its phosphorylation in response to anisomycin is comparable in NIH3T3 and Swiss 3T3 cells. NIH3T3 and Swiss 3T3 cells were treated with the doses of anisomycin (ng/ml) indicated below the lanes. After 15 min, total cellular protein extracts were prepared and analysed on two identical Western blots developed with antibodies directed against either Y182 phosphorylated or total p38/RK

Figure 11 *c-fos* mRNA accumulated in response to anisomycin in both, NIH3T3 and Swiss 3T3 but not in NFR-2 cells. (a) NIH3T3 and Swiss 3T3 were incubated with anisomycin at concentrations which blocked translation only partially (30 and 100 ng/ml) or completely (1 µg/ml). A Northern blot was prepared with total RNA isolated before and 1 h after anisomycin treatment and hybridized with a *c-fos* specific probe. In a separate experiment Swiss 3T3 cells were treated with the p38/RK inhibitor SB 203580 (+) or not (-) prior to the stimulation with 1 µg/ml anisomycin for 1 h. All RNA samples except those in lanes 9 and 10 were analysed on the same Northern blot. The ethidium bromide stained gels are shown as a control for equal loading of RNA. (b) NIH3T3, NFR-2, and Swiss 3T3 cells were treated with 100 ng/ml anisomycin for the time periods indicated below the lanes. RNA was prepared and analysed on the same Northern blot which was developed with a *c-fos* specific DNA probe

Figure 12 Model for the induction of the *T1* gene in response to mitogenic stimulation and anisomycin, IL-1, and TNF α treatment in normal NIH3T3 and *ras* transformed NFR-2 cells. In normal NIH3T3 fibroblasts the *T1* gene can be induced through the stimulation of the p38/RK and the ERK pathway, the former being an immediate early and the latter a delayed early effect, as defined by the requirement for ongoing protein synthesis. In the *ras* transformed NFR-2 cells *c-fos* gene induction is blocked and consequently no *T1* gene expression in response to mitogenic stimulation is observed. However, p38/RK mediated *T1* gene activation is not affected in these cells

stimulation requires the transcription factor c-Fos (Kalousek *et al.*, 1994, Trüb *et al.*, 1994). c-Fos levels are very low in quiescent cells but increase drastically and transiently during the early phase of cell cycle entry (Lau and Nathans, 1991). If the synthesis of c-Fos is blocked, no *T1* gene induction ensues which categorizes *T1* as a delayed early gene (Figure 12). This classification holds true despite the finding that cycloheximide treatment of NIH3T3 cells does not preclude *T1* gene expression in response to mitogenic stimulation because we have shown that puromycin and emetine which inhibit translation without stimulating signal transduction cascades block serum mediated *T1* gene expression in all three fibroblastic cell lines studied. We provided evidence that the second signalling pathway which results in *T1* gene expression is the p38/RK cascade.

Pretreatment of cells with low concentrations of SB203580 causes the inhibition of p38/RK (Cuenda *et al.*, 1995) and certain JNK isoforms (Whitmarsh *et al.*, 1997) and prevented anisomycin mediated *T1* gene expression. Since u.v. irradiation did not result in *T1*

gene activation and did not cause substantial phosphorylation of p38/RK we conclude that anisomycin acts through the activation of the p38/RK pathway to stimulate *T1* gene activation. This is in accordance with the observation that anisomycin stimulated *T1* gene expression is not affected in *ras* transformed fibroblasts. *ras* oncogene expression results in the permanent activation of JNK and ERK but not p38/RK. Sustained activation of a signal transduction pathway should result either in the permanent stimulation of target gene expression or in gene silencing if a feedback inhibitory loop is involved.

The ability of anisomycin to stimulate the p38/RK pathway was further supported by the finding that anisomycin treatment resulted in the phosphorylation of p38/RK and Hsp25. The latter is a substrate of MAPKAPK-2, a downstream target of p38/RK (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Cuenda *et al.*, 1995). This is in concordance with recent observations of anisomycin mediated activation of p38/RK in KB (Gould *et al.*, 1995) and 293 cells (Brunet and Pouyssegur, 1996).

Anisomycin caused *T1* gene induction over a wide range of concentrations. The highest concentrations used in this study inhibited protein synthesis efficiently. The transcription factors mediating *T1* gene induction under these conditions must have been present before the onset of the stimulation with anisomycin. By this criterion *T1* has to be classified as a immediate early gene, not requiring *de novo* synthesis of the transcriptional stimulators. This is unlike the mechanism operating in response to growth factor mediated *T1* gene induction. Mitogenic stimulation leads to the synthesis of immediate early gene products which are involved in *T1* gene activation.

Anisomycin can probably cause the accumulation of specific mRNAs through different mechanisms. By virtue of its activity as a stimulator of signal transduction cascades it can cause the direct transcriptional activation of some genes such as the *T1* gene. By virtue of its ability to inhibit translation it might cause the loss of labile transcriptional repressors or RNases and thereby induce transcription in an indirect manner or cause mRNA stabilization, respectively. The accumulation of tissue factor mRNA might be the result of such a mechanism as it is only observed under conditions of complete protein synthesis inhibition. *T1* is one of the few genes for which transcriptional induction in response to the activation of the p38/RK pathway has been demonstrated. Such genes are the true targets of the p38/RK signal transduction cascade and the study of the mechanisms of their transcriptional induction should lead to the identification of the transcription factors which are the nuclear targets of this signalling pathway.

We have observed that the *c-fos* gene is induced during the onset of *ras* oncogene expression but that it is silenced upon the prolonged presence of oncogenic p21^{ras}. There is evidence that this is the consequence of sustained *fra-1* gene expression and the activation of c-Jun through its N-terminal phosphorylation by JNK. The Fra-1/c-Jun heterodimeric AP-1 complex which predominates in *ras* transformed NIH3T3 cells and which is present in high amounts throughout the cell cycle is an efficient repressor of the *c-fos* gene (manuscript submitted). The lack of *c-fos* gene

induction in *ras* transformed cells is the likely cause for the unresponsiveness of the *T1* gene to mitogen mediated activation in these cells. However, as we have shown here, *T1* gene induction occurs in response to anisomycin, IL-1, and TNF α in normal and *ras* transformed NIH3T3 cells alike (Figure 12). This is a further indication that different transcription factors mediate the activation of the *T1* gene in response to the stimulation of the p38/RK and ERK1/ERK2 pathways and in particular that c-Fos is only involved in the latter response.

The extent of anisomycin mediated gene induction varies strongly among different murine fibroblastic cell lines. While the *T1* gene is induced with high, intermediate, and low efficiency in NIH3T3, Balb/c 3T3, and Swiss 3T3 cells, respectively, the inverse is true for the *c-fos* gene. The presence of different amounts of p38/RK in these cell lines or its differential susceptibility to phosphorylation in response to anisomycin could be excluded as possible explanations. We assume that different downstream target molecules of the p38/RK pathway are involved in the activation of the *T1* and the *c-fos* gene and that these molecules are present in different amounts in the tested cell lines. Alternatively, other transcription factors which cooperate with the p38/RK target molecules in gene activation might be the limiting components and accumulate to different levels in the studied cell lines. Currently only two *in vivo* substrates of p38/RK are known, the related MAPKAP kinase-2 (Cuenda *et al.*, 1995) and MAPKAP kinase-3 (Ludwig *et al.*, 1996; McLaughlin *et al.*, 1996). Their only known substrate is the small heat shock protein Hsp25, an unlikely candidate for gene activation. The MAPKAP kinase-3 is a target of stress and mitogen stimulated MAP kinase pathways. It is interesting to note that its activation is transient in response to mitogenic stimulation and sustained in response to stress (Ludwig *et al.*, 1996). Similarly, the *T1* gene is induced for only a short time following cell cycle entry of quiescent cells, but its expression is prolonged in the presence of anisomycin. These kinetic considerations make the substrates of MAPKAP kinase-3 appear as good candidates for *T1* gene stimulators. We have shown in this report that a *T1* gene construct can be induced by anisomycin treatment in NIH3T3 cells. Promoter dissection studies should lead us to the identification of the crucial DNA sequence motives involved in anisomycin mediated *T1* gene activation. The transcription factors interacting with such sequence motives are expected to be the critical targets of the p38/RK signal transduction pathway. Thus, the *T1* gene is a very useful tool to investigate the p38/RK pathway.

It is interesting to note that the stimulation of a hybrid protein consisting of the extracellular domain from the IL-1 receptor and the intracellular domain of T1 leads to the activation of the p38/RK pathway (Kumar *et al.*, 1995). We speculate that the activation of the membrane anchored form of T1 may result in enhanced *T1* gene induction. An autoregulatory inhibition or stimulation of the *T1* response might ensue, depending of whether the synthesis of the secreted or the membranous form of T1 occurs.

The membrane anchored form of T1 is predominantly found on the surface of mast cells. These cells

store TNF α in cytoplasmatic granules. Upon antigenic stimulation they release TNF α and start to produce several cytokines including IL-1. Secreted TNF α and IL-1 might stimulate *T1* gene expression in neighbouring cells resulting in the synthesis and secretion of the soluble T1 protein. Assuming that the soluble form of T1 can bind the putative T1 ligand with similar affinity as the membranous form this could lead to the quenching of the T1 response. The recently described putative T1 ligand will be a useful tool to study this model.

Materials and methods

Cell cultures

The murine fibroblastic cell lines NIH3T3, Swiss 3T3, Balb/c 3T3 (clone A31), the Ha-*ras* (EJ) transfected clone NFR-2 (Aoyama *et al.*, 1993), and the myoblast cell line C2C12, were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Inc.) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 mg/ml). Cell proliferation was arrested by reducing the FCS concentration to 0.5% for 24 h. Recombinant mouse IL-1 β and TNF α were purchased from Genzyme Diagnostics (Cambridge, MA) and Sigma, respectively, and used at 20 ng/ml.

To measure the inhibitory effect of anisomycin, NFR-2 cells were incubated with anisomycin which was dissolved in 50% ethanol or with the solvent only for 30 min. Thereafter, cells were metabolically labelled with ³⁵S-methionine for 1 h, extensively washed, lysed in a hypotonic buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM DTT, 1 mM PMSF) and proteins were precipitated with trichloroacetic acid (TCA). The protein pellet was dissolved in the sample buffer for SDS polyacrylamide gels and aliquots were spotted onto two nitro-cellulose filters. One filter was stained with amido black and protein concentrations were determined according to Schaffner and Weissman (Schaffner and Weissman, 1973). The other filter was used to measure radioactivity. The amount of incorporated radioactivity was normalised for equal amount of protein.

u.v. irradiation

For u.v. irradiation of cells the medium was removed and the cells were irradiated in a Stratalinker 1800 u.v. Crosslinker (Stratagene) with a wavelength of 254 nm. After irradiation the medium was added back and the cells were allowed to recover for the indicated time periods.

RNA preparation and Northern blots

RNA was prepared according to Chomczynski and Sacchi (1987) and Northern blots prepared with 5 μ g of total RNA per lane. The following random primed, α -³²P-dCTP labelled DNA probes were used: *T1*, two *Eco*RI-fragments (1 and 1.6 kb) from the plasmid pT1.10, spanning the entire *T1* cDNA (Klemenz *et al.*, 1989); GAPDH, a 0.8 kb *Eco*RI fragment of the chicken *glyceraldehyde phosphate dehydrogenase* cDNA; *stromelysin*, a 1.8 kb *Eco*RI/*Xho*I fragment from plasmid pM124 containing cDNA of murine *stromelysin* (kind gift of R Friis); *collagenase*, a 1.5 kb *Xba*I fragment from the plasmid pCllase 1 (Angel *et al.*, 1987) containing human interstitial *collagenase* cDNA; *v-fos*, a 1 kb *Pst*I fragment from the plasmid pfos-1 (Curran *et al.*, 1982); *mouse tissue factor*, two *Eco*RI fragments of 711 bp and 1.07 kb (kind gift of D Nathans); CAT, the 1.25 kb *Eco*RI/*Hind*III fragment of the *CAT* gene in the plasmid pBLCAT2 (Tsonis *et al.*, 1988).

Nuclear run-on transcription

Approximately 6×10^7 cells were washed twice with phosphate-buffered-saline (PBS) and scraped into PBS. The cells were pelleted and resuspended in HB buffer (15 mM HEPES, pH 7.5; 60 mM KCl; 15 mM NaCl; 2 mM EDTA; 14.4 mM β -mercaptoethanol; 0.5 mM spermidine; 0.5 mM spermine; 10.3% w/v sucrose) supplemented with 0.5% NP-40. The nuclei were pelleted at 875 RCF for 10 min, resuspended in 10 ml HB buffer, pelleted again, taken up in 15 mM HEPES (pH 7.5), 75 mM KCl, 75 mM NaCl, 0.5 mM EDTA, 1.25 mM PMSF, 0.85 mM DTT, 60% glycerol and frozen in liquid nitrogen as aliquots of 2×10^7 nuclei. All procedures were performed at 0–4°C. The nuclei were stored at –80°C. 2×10^7 nuclei were incubated at 30°C for 45 min in 90 mM HEPES (pH 8.0), 160 mM KCl, 30 mM NaCl, 15 mM DTT, 2 mM MgCl₂, 1.4 mM ATP, 1.4 mM CTP, 1.4 mM GTP, 150 μ Ci [α -³²P]UTP (3000 Ci/mmol, Amersham), 0.2 mM EDTA, 0.5 mM PMSF, 0.4 mM β -mercaptoethanol, 14.2 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 0.6 units RNase inhibitor and 25% glycerol. The RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and pelleted. The pellet was dissolved in water and purified using the RNeasy system from Qiagen. The RNA was dissolved in formamide at 65°C and hybridised to a Southern blot for 17 h at 48°C in 50% formamide, 2×SSC (0.3 M NaCl, 34.2 mM Na citrate, pH 7.0), 5×Denhardt's, 1% SDS, 10 mM Tris-HCl (pH 7.5) and 100 μ g/ml denatured carrier DNA. Southern blots were prepared with the following plasmid DNAs: pT1.10 (*T1* cDNA) cut with *Eco*RI (Klemenz et al., 1989); pT1.28 which contains a 7.6 kb *Eco*RI fragment from genomic *T1* DNA spanning the region between –6.4 kb and +1.2 kb (Trüb et al., 1994). Restriction with *Eco*RI and *Pst*I gives rise to a 1.2 kb fragment from the transcribed region and three fragments of 0.8 kb, 2.3 kb and 3.1 kb, from the untranscribed region. After restriction digestion the DNA fragments were size fractionated on a 1% agarose gel, denatured and transferred to a nylon membrane (Gene-Screen Plus).

Western blots

Cells were lysed in sample buffer for SDS polyacrylamide gels, protein concentrations determined as described by

Schaffner and Weissmann (Schaffner and Weissman, 1973) and 25 μ g protein fractionated on 12.5% SDS polyacrylamide gels. For 2-dimensional gel analysis according to O'Farrell et al. (1977) the cells were lysed in sample buffer A (O'Farrell, 1975) and 30 μ g protein was used for the analysis. The primary antibodies used were directed against p38/RK phosphorylated at tyr 182, against total p38/RK (New England Biolabs) or against mouse Hsp25 (Aoyama et al., 1993). The secondary antibody was anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Promega). Immunocomplexes were visualised enzymatically according to the manufacturer's protocol.

Plasmid construction

The plasmid pFH6 was constructed in the following way: A 5 kb *Sac*I fragment containing part of exon 8 and 3.8 kb of the *T1* gene 3' flanking region was cloned by blunt end ligation into the *Sma*I site of pBC SK[–] (Stratagene). The coding region of the CAT gene was PCR amplified from the plasmid pBLCAT2 (Tsonis et al., 1988) using the primers 5'-GGGGCGAGATTTTCAGGAGCTAAGG-3' and 5'-GCACCAGGCGTTTAAGGGCA-3' and was cloned as a 731 bp fragment into the *Sma*I site upstream of the 3' *T1* gene region. A 7 kb *Eco*RI fragment from the plasmid pSH4.9, spanning 6.2 kb of 5' *T1* gene flanking sequence, the first exon and intron and part of the second exon (Trüb et al., 1994) was inserted into the *Eco*RI site upstream of the CAT fragment. This plasmid was cotransfected with pSV2neo into NIH3T3 cells and transfected clones were selected in medium containing G418.

Note added in proof

TNF α and IL-1 mediated accumulation of T1 mRNA has recently also been reported by Kumar et al. (Kumar S, Tzimas MN, Griswold DE and Young PR (1997). *Biochem. Biophys. Res. Comm.*, **235**, 474–478.

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