A member of the MAP kinase phosphatase gene family in mouse containing a complex trinucleotide repeat in the coding region

A. M. Theodosiou1,+ , N. R. Rodrigues1,+ , M. A. Nesbit1, H. J. Ambrose1, H. Paterson2, E. McLellan-Arnold2, Y. Boyd3, M. A. Leversha4, N. Owen1, D. J. Blake1, A. Ashworth2 and K. E. Davies1,*

1Genetics Laboratory, Biochemistry Department, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK, 2CRC, Centre for Cell and Molecular Biology, Chester Beatty Laboratories, The Institute of Cancer Research, Fulham Road, London, SW3 6JB, UK, 3MRC, Mammalian Genetics Unit, Harwell, Didcot, OX11 0RD, UK and 4The Sanger Centre, Hinxton Hall, Cambridge, CB10 1RQ, UK

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We have identified a novel mouse gene encoding a protein that shows high homology to the dual-specificity tyrosine/threonine phosphatase family of proteins. The gene encodes a 5 kb transcript which is expressed predominantly in brain and lung and contains a translated complex trinucleotide repeat within the coding region. Using interspecific mouse backcross analysis, the gene has been localised to distal mouse chromosome 7. In human, homologous sequences are located in the syntenic region on distal chromosome 11p as well as to chromosome 10q11.2 and 10q22. The presence of a CG-rich trinucleotide repeat in the coding region provides a target for mutation which might result in loss of function or altered properties of this phosphatase.

INTRODUCTION

Pathways utilising the MAP (Mitogen Activated Protein) kinase family of protein kinases are involved in the transduction of a diverse range of extracellular signals in eukaryotes. These pathways display a high level of evolutionary conservation, and examples include sexual differentiation in yeast, eye development in Drosophila, and mitogenic signalling in mammals (1–4). Substrates of the MAP kinases include cytosolic phospholipase A2, (5) the protein kinases p90rsk (6) and MAPKAPK2 (7), and several transcription factors including c-Myc (8), c-Jun (9) and Elk-1 (10). The best characterised of the mammalian MAP kinases are ERK1 and ERK2 (also known as 44 kDa and 42 kDa isoforms respectively) (11). These enzymes are activated by phosphorylation on both threonine and tyrosine residues by the dual-specificity kinase, MAP kinase kinase (MAPKK) (12,13). MAPKK is in turn activated by serine/threonine phosphorylation by MAP kinase kinase kinase (MAPKKK), one form of which is the proto-oncogene c-raf (14). Recently, the MAP kinase family has been extended by the characterisation of the stress activated protein kinases (SAPKs). These show 40–45% sequence identity to the MAP kinases and include the jun kinases and a kinase, known as p38, RK or CSBP (15), which is related to the yeast MAP kinase HOG-1 (16). These MAP kinases are also activated by phosphorylation and are part of kinase cascades.

Although the mechanisms of activation of the MAP kinases are becoming well understood, the deactivation of these enzymes has received less attention. Recently however, a family of mammalian genes encoding dual-specificity tyrosine/threonine protein phosphatases has been reported (17). These phosphatases have some homology with the intracellular type of protein tyrosine phosphatases (PTPases) in the catalytic domain but clearly form a distinct sub-family (17–19). The PTPases regulate cell growth and proliferation, the cell cycle and cytoskeletal integrity in response to a variety of external stimuli (20). The prototypical member of the dual-specificity tyrosine/threonine phosphatase family, CL100 in human (21) and MKP-1/3CH134 in mouse (22) was isolated on the basis of inducibility by growth factors or cellular stress. Subsequently, other genes encoding potential MAP kinase phosphatases have been isolated. These include the human T-cell specific gene Pac-1 (Phosphatase of Activated Cells) (23) and its murine homologue, and the human VHR gene [Vaccinia Homology (VH1)-Related phosphatase] (24). Many of these genes have been shown to dephosphorylate and deactivate MAP kinase isoforms in vitro and/or in vivo, usually displaying high substrate specificity. All of the MAP kinase phosphatase proteins that have been described so far have been localised exclusively to the nucleus (23,25).

Here we describe a cDNA that shows high homology to the members of the MAP kinase phosphatase family, which encodes a protein with several novel features. The protein is much larger than previously described members and is located in the cytoplasm, at least in some cell types. In addition, a complex trinucleotide repeat is present within the coding region of the...
cDNA. The presence of this repeat may mean that this part of the gene is particularly prone to mutation by repeat number variation. Given that MAP kinase phosphatases are likely to be important in switching off signal transduction pathways, it may be that mutations in this gene could have profound phenotypic consequences.

RESULTS

Isolation of the mouse PTPase cDNA clone

Approximately 200 000 recombinants from an adult mouse brain cDNA library were screened with probes from the Spinal Muscular Atrophy (SMA) candidate region on chromosome 5 (26–27). One of the 14 positive clones isolated cross-hybridised to genomic DNA from chromosome 5 but also to DNA from elsewhere in the genome. Sequencing of this 2.5 kb cDNA, designated M3/6, revealed an open reading frame with high homology to the family of dual-specificity phosphatases. The cDNA encoded a protein with an open reading frame of 663 amino acids which would predict a protein of approximately 69 kDa (Fig. 1a). In this open reading frame, the sequence has a potential ATG initiation codon at nucleotide number +1, identified by sequence comparison to the canonical sequences of a mammalian initiation site as described by Kozak (28). The presence of adenine at position –3 and guanine at position +4, which is characteristic of the initiator methionine is evident. The consensus AUUUA that can confer mRNA instability (29) is repeated in the 3′ untranslated region. However, the cDNA does not contain a polyadenylation sequence or a poly(A) tail. The cDNA also contains the active site signature motif [I/V][HCXAGXXR][S/T] (amino acids 244–253) that is present, without exception, in all PTPases where the conserved cysteiny1 residue participates in the catalytic mechanism (18). In addition, closer observation of the residues surrounding the signature sequence has revealed a further area of homology, characteristic for a subset of dual-specificity PTPases (including hVH-5, CL100, PAC-1 and VHR). The active site motif for these proteins is thus extended to _XXVH[CXAG][XSRS][Q][XXXAY][IV][LM]_ (where the underlined residues are conserved). Amino acid residues 29–49 and 117–136 revealed two motifs which are conserved within the dual-specificity tyrosine/threonine subset of PTPases. These two regions have been referred to as CH2 domain (cdc25 homology domain 2) (30–31). In cdc25, a dual-specificity PTPase involved in the cell cycle, these two motifs flank the active site sequence. In M3/6, as well as other PTPases of the tyrosine/threonine subfamily (including CL100, PAC-1 and hVH-5) they are found proximal to the catalytic domain. It has been suggested that these regions of homology could be involved in the interactions of these enzymes with their substrates.

The cDNA sequence shows high homology to several PTPases in the EMBL/GENBANK sequence database. Comparison of this sequence of M3/6 with the published human sequence hVH-5 (32) which has homology to the VH1 phosphatase gene (33) reveals 81% identity at the nucleotide level. Interestingly, the M3/6 sequence possesses an in-frame set of CG-rich triplet repeats in the 3′ end distal to the catalytic domain (nucleotides 1662–1799). This complex trinucleotide repeat spans 138 bp and consists of (CAG)₃GCG(TGG)ₐp(CAG)₃CAa(CAG)₃(TAG)₃. The human clone hVH-5 does not contain such a complex repeat. Instead, the above murine sequence is replaced by (GGC)₃AGC-GACCTG(CGG)₃. However, the region flanking the repeat is conserved at the amino acid level. Figure 1b is an alignment of the protein encoded by M3/6 with hVH-5, and this shows that there is 90% identity between the mouse and human genes excluding the trinucleotide repeat. Despite this high degree of identity between these mouse and human genes, the absence of the trinucleotide repeat from the human sequence suggests that they may not be true orthologues.

Expression of M3/6 in mouse and human tissues

To determine the pattern of expression of M3/6, the cDNA was hybridised to Northern blots of poly(A)⁺ RNA derived from a selection of adult mouse tissues. M3/6 detects a transcript of 5 kb in mouse eye and brain (Fig. 2a, lanes 3 and 5) as well as mouse heart and skeletal muscle (data not shown). A 1.8 kb transcript was also detected in mouse lung along with a faint transcript at 5 kb (lane 7). A similar 5 kb transcript was seen when a subclone of the cDNA, designated M3/6–4e was used (Fig. 2b). This subclone does not contain the trinucleotide repeat and therefore is a more specific probe than the whole of M3/6. In this case the 5 kb band in lung was much stronger (lane 7). Figure 2c shows the hybridisation of M3/6–4e to a Northern blot of human fetal tissues which shows the 5 kb transcript expressed in brain and lung (lanes 3 and 4). The lung also had a transcript of 2.5 kb. We confirmed the expression of M3/6 in different tissues by RT-PCR using various primers across the cDNA (data not shown). Furthermore, we amplified across the trinucleotide repeat in mouse RNA from several tissues using primers that spanned up to a kilobase showing that the trinucleotide was part of the transcript. Amplification across the repeat in mouse genomic DNA was never successful, presumably due to presence of exon-intron boundaries close to the repeat or the CpG richness of the region.

In vitro transcription/translation of M3/6

In order to determine whether the cDNA was transcribed and translated into the predicted protein from the amino acid sequence including the in-frame trinucleotide repeats, we performed an in vitro transcription/translation assay using a reticulocyte assay as described in Materials and Methods. The result of the analysis is shown in Figure 3. The protein product was consistent with the size predicted of the open reading frame suggesting that the translation of the mRNA must extend through and beyond the trinucleotide repeats. The slight aberrant migration of the protein is probably due to its proline rich nature or to the presence of the poly-glycine and poly-serine stretches in the trinucleotide repeat. Expression of M3/6–4e, the deletion construct (–97 to 907 bp) which does not contain the repeat, resulted in a smaller protein product of 36 kDa which was of the predicted size (data not shown).
Figure 2. Expression of M3/6 transcripts in mouse and fetal human tissues. (a) Expression of M3/6 in poly(A)⁺ RNA from adult mouse tissues. The Northern blot was probed with M3/6. Lane 1: marker; lane 2: blank; lane 3: mouse eye; lane 4: mouse spleen; lane 5: mouse brain; lane 6: skeletal muscle; lane 7: lung; lane 8: mouse small intestine; lane 9: mouse liver. (b) Northern blot of adult mouse poly(A)⁺ RNA probed with M3/6–4e. Lane 1: marker; lane 2: blank; lane 3: brain; lane 4: skeletal muscle; lane 5: mouse small intestine; lane 6: liver; lane 7: lung; lane 8: spleen; lane 9: heart; lane 10: kidney. (c) Northern blot of human fetal poly(A)⁺ RNA (Clontech) probed with M3/6–4e. Lane 1: fetal kidney; lane 2: fetal liver; lane 3: fetal lung; lane 4: fetal brain.

Figure 3. In vitro transcription/translation assay with M3/6. Lane 1: control; lane 2: M3/6 with T7 polymerase; lane 3: M3/6 with Sp6 polymerase; lane 4: random cDNA with T7 polymerase; lane 5: random cDNA with Sp6 polymerase; lane 6: no DNA with T7 polymerase; lane 7: no DNA with Sp6 polymerase.

Chromosomal localisation of M3/6

To localise M3/6 in the mouse, we initially used mouse × Chinese hamster somatic cell hybrids. Analysis of the results obtained from 18 mouse × Chinese hamster somatic cell hybrids revealed that the highest concordance values were obtained between M3/6–4e and mouse chromosomes 7 and 13 (data not shown). To confirm and extend these results, M3/6–4e was hybridised to TaqI digests of DNA prepared from interspecific backcross progeny from the European Collaborative Interspecific Backcross, EUCIB (34). Analysis of results obtained from typing 46 animals using MBx (34) revealed that M3/6–4e was linked to the anchor locus, D7Mit15 (three recombination events were scored between D7Mit15 and M3/6–4e). Inspection of the data stored in MBx for D7Mit15 and a more proximal marker D7Mit49 suggested that M3/6–4e lay proximal to D7Mit15 (Fig. 4a).

M3/6–4e was used to isolate a cDNA clone from a human fetal brain library. Partial sequencing of the human clone HB5a reveals 81% identity at the nucleotide level with the mouse clone, but does not contain the complex trinucleotide repeat (unpublished data). HB5a shows 100% sequence identity with hVH-5 (32) in the regions that were sequenced. This clone was used for fluorescence in situ hybridisation (FISH) analysis on human metaphase chromosomes. The sequence mapped to three locations with the principal peaks being on 10q11.2 and distal 11p15, with a further peak on 10q22 (Fig. 4b). This suggests there are multiple loci for this sequence in the human genome.

Subcellular localisation of M3/6 protein

Proteins encoded by the MAP kinase phosphatase genes Mkp1 and Pac-1 are located exclusively in the cell nucleus (23,25). To determine the subcellular location of the protein encoded by M3/6, we cloned M3/6 cDNA into an eukaryotic expression vector such that the N-terminus was fused with an epitope MEQKLISEEDLK which is recognised by the monoclonal antibody 9E10 (35). The expression construct was introduced into (Swiss 3T3, MDCK or PC12) cells by microinjection. Subsequently, the M3/6 protein was detected by immunofluorescence with the 9E10 antibody and...
Functional analysis of the M3/6 gene by transfection

We wished to determine whether the M3/6 gene expressed in COS cells was able to dephosphorylate ERK2. To do this we analysed the phosphorylation and activation state of ERK2 by gel shift analysis. Phosphorylation of ERK2 leads to a reduction of mobility of the protein on gel electrophoresis which can be monitored by Western

confocal microscopy. In Swiss 3T3 fibroblast cells the protein was restricted to the nucleus (Fig. 5a). However, in MDCK and PC12 cells, M3/6 was cytoplasmically located in approximately half of the cells analysed. In the rest of the cells the protein was located in the nucleus (Fig. 5b). Thus, the M3/6 protein appears to be distinct from the MKP-1 and PAC-1 proteins which are located exclusively in the nucleus.
Figure 5. Cell type dependent subcellular localisation of M3/6. A DNA construct encoding a myc-tagged version of M3/6 in an expression vector was introduced into Swiss 3T3 (a) or MDCK cells (b). The myc-tagged M3/6 protein was detected by immunofluorescence. Phase-contrast (left) and immunofluorescence (right) images are shown. The immunofluorescence background in non-injected cells is negligible. Scale bar = 25 μm.

blotting and subsequent detection with the ERK2 antibody, 122 (36). We transfected COS cells with a construct containing the M3/6 cDNA cloned into a eukaryotic expression vector. The transfected cells were serum starved overnight and then serum stimulated for 20 min to induce ERK2 phosphorylation. Figure 6 shows that a MAP kinase phosphatase gene called TYP1 (37) can dephosphorylate ERK2. However, the M3/6 cDNA is apparently unable to do this under these conditions. This suggests that in vivo M3/6 may dephosphorylate substrates other than ERK2, such as the SAPKs.

DISCUSSION

The analysis of the cDNA M3/6 presented here suggests that it may encode a novel PTPase, member of the dual-specificity
tyrosine/threonine subfamily. Members of this subclass include dual-specificity PTPases have been recently reported (32,38), 5q13, which has yet to be cloned. Alternatively, there could be a homologous sequence on human SMA probes from chromosome 5, which were also very CG-rich.

Isolated due to its high CG-content by cross-hybridisation to the mouse transcript. It is not as yet clear whether M3/6, was the homologue of hVH-5. Despite the high homology, hVH-5 does not contain the active site signature motif of this dual-specificity subfamily has revealed homology over comparison of the amino acid sequence of M3/6 with other members of this dual-specificity subfamily has revealed homology over two motifs known as the CH2 domain. It is unlikely that these motifs confer dual substrate specificity to these proteins, since they are absent in some of these PTPases, including VHR and V1H which retain the ability to dephosphorylate both phosphoserine and phosphothreonine. However, it has been suggested that members of this subfamily possessing this region, may be more ‘selective’ for their substrates (31,30).

M3/6 contains repeated mRNA destabilisation sequences within the 3’ untranslated region. It has been proposed that the AUUUA consensus is the recognition signal for the degradation of mRNAs of transiently expressed genes including certain AUUUA consensus is the recognition signal for the degradation of mRNAs of transiently expressed genes including certain cytokines and proto-oncogenes (29). It is noteworthy, as these destabilisation signals were also found in other dual-specificity PTPases, including PAC-1 and hVH-5 which are immediate-early gene products.

M3/6 has a high proline content as observed also from its homology to hVH-5 (90% at the amino acid level, Fig. 1b). However, it is not clear whether M3/6 represents the mouse homologue of hVH-5. Despite the high homology, hVH-5 does not contain the complex trinucleotide repeat, which was shown to be part of the transcript. It is not as yet clear whether M3/6, was isolated due to its high CG-content by cross-hybridisation to the SMA probes from chromosome 5, which were also very CG-rich. Alternatively, there could be a homologous sequence on human 5q13, which has yet to be cloned.

The two clones, M3/6 and hVH-5 show a similar pattern of expression on Northern analysis (32). A mRNA of approximately 5 kb was highly abundant in brain, and also present in eye and skeletal muscle (Fig. 2). According to Martell et al., (32) analysis of mouse tissue mRNA demonstrated that hVH-5 was also present in mouse lung. Using M3/6 as a probe, it was shown that in mouse lung the main transcript was of 1.8 kb (Fig. 2a). This could be due to alternative splicing since the size is smaller than expected given the length of the cDNA clone. Alternatively, this could represent the transcript of a closely related gene. Further experiments are currently underway, aiming to elucidate the origin of the larger 5 kb and the smaller 1.8 kb transcripts.

The FISH data presented in this paper show that the human cDNA, H55a, hybridises to homologous loci on human 11p15, 10q11.2 and 10q22 (Fig. 4b). The reported hVH-5 clone, was isolated from a gene sequence hVH-4 which was localised to chromosome 10q11 (38). These authors did not report a signal on chromosome 11p but suggested that the locus on chromosome 10q11 was a pseudogene (32). Our preliminary data on chromosome 11p indicate that the gene sequence at this position has an intron/exon structure and contains the cognate bands seen on Southern blot using the cDNA as a probe (Nesbit and Davies, unpublished observations). Which, if any, of the human loci contain a trinucleotide repeat, remains to be determined.

The mapping of M3/6 to mouse chromosome 7 is consistent with the mapping to the human syntenic region on 11p15. Few genes have been mapped to this distal region of the human chromosome 11 (39). In addition, this is one of the regions recently implicated in non-small-cell lung cancer (40). Thus the expression of an isoform of this gene in the lung is intriguing. Given the potential role of deactivating members of the MAP kinase family, it is possible that the human homologue of M3/6 which is located at 11p15 may act as tumour suppressor gene.

Another PTPase, PTPε has been mapped to distal region of chromosome 7 in the mouse (41). On comparing the sequence of the human homologue of PTPε to M3/6 no homology was found, therefore implying that this is another PTPase mapping to the distal region of chromosome 7 in mouse (42). The two genes (M3/6 and PTPε) map in close proximity, as shown by the linkage maps of the distal region of mouse chromosome 7 (Fig. 4a) (41). This region of distal chromosome 7 is one of the imprinted regions in mouse where there is a clustering of imprinted genes, including H19, Igf2 and Ins2 (43,44). It would be interesting to find out whether M3/6 is also subject to imprinting. The human PTPRE gene has been localised by FISH, to human chromosome 10q26 (45) a region distinct to the localisation of the sequence reported here at 10q11.2 and 10q22.

Some members of the dual-specificity tyrosine/threonine phosphatase family have been shown to be capable of deactivating MAP kinase. The MAP kinases are part of a protein kinase cascade responsible for transducing signals from the cell surface to the nucleus in a diverse range of eukaryotes from yeast to man. Thus the dual-specificity phosphatases may play a key role in regulating many cellular processes. Several of this subfamily of phosphatases have been shown to dephosphorylate ERK2 both in vitro, using recombinant proteins, or in transfection experiments in mammalian cells. We have failed to demonstrate any activity of the M3/6 protein towards ERK2 when expressed in mammalian cells. This was not due to poor expression of the protein as demonstrated by Western blotting. In contrast to these results Martell et al., (32) showed that a highly truncated, both at the

Figure 6. Expression of M3/6 protein in COS cells. (a) The myc-tagged M3/6 expression vector utilised in Figure 5 was transfected into COS cells and cell lysates were electrophoresed on a SDS polyacrylamide gel and transferred to nitrocellulose. The myc-tagged protein was detected by ECL. Lysates prepared from cells transfected with M3/6 or TYP-1 expression plasmids, either serum starved or serum starred and then serum stimulated, were analysed by Western blotting and probed with the ERK2 antibody 122 (36). The upper shifted band represents the phosphorylated form of ERK2.
N- and C- termini, recombinant version of M3/6 was active against phosphorylated ERK2 in an in vitro assay. Thus it seems possible that the activity observed by these authors is due to the protein being highly truncated or that, in vivo, the enzyme is unable to access its potential substrate. A further difference with other members of the dual-specificity PTPase family is that the M3/6 protein is partially cytoplasmic, at least when introduced in some cell types. Hence, at present it is unclear what the in vivo targets of the M3/6 enzyme might be, but given the strong homology to the dual-specificity phosphatases it is likely to be a MAP kinase phosphatase and experiments aimed at identifying substrates for M3/6 are underway.

Given the high homology of this clone to the dual-specificity tyrosine/threonine phosphatases, we propose the name Ntp1 (Neuronal Tyrosine/Threonine Phosphatase 1) which has been approved by the Mouse Nomenclature Committee.

**MATERIALS AND METHODS**

**Isolation of M3/6**

M3/6 was isolated from a mouse adult brain cDNA library from oligo dT and random primed cDNA (46), cloned into the EcoRI site of the vector pcDNAII (Invitrogen). Approximately 200 000 colonies were screened using a combination of probes from the SMA candidate region. These probes were radiolabelled with α[32P]dCTP (3000 Ci/mmol) using Amersham megaprime labelling kit. The M3/6 cDNA was isolated from the XL1-Blue host cells, by standard alkaline lysis method of preparing plasmid DNA. The 2.5 kb insert containing the entire M3/6 cDNA was released from the vector by digestion with the restriction enzyme EcoRI. M3/6–4e is a deletion derivative of M3/6 generated by the Erase-a-Base system (Promega) which can be released from the vector using EcoRI and XbaI. It encompasses nucleotides –97 to 907 of M3/6.

**In vitro transcription-translation assay**

The pcDNAII vector utilises Sp6 and T7 promoters which can be directly used for in vitro transcription-translation assays. One µg of RNase-free circular plasmid DNA containing the insert was used for each reaction. The assay was performed according to the instructions provided with the Promega TNT Coupled Reticulocyte Lysate Systems using instructions provided with the Promega. The assay was performed according to the instructions provided. The protein being highly truncated or that, in vivo, the enzyme is unable to access its potential substrate. A further difference with other members of the dual-specificity PTPase family is that the M3/6 protein is partially cytoplasmic, at least when introduced in some cell types. Hence, at present it is unclear what the in vivo targets of the M3/6 enzyme might be, but given the strong homology to the dual-specificity phosphatases it is likely to be a MAP kinase phosphatase and experiments aimed at identifying substrates for M3/6 are underway.

**Sequencing**

A nested set of deletion clones spanning the whole length of the 2.5 kb cDNA was generated using the Erase-a-Base System (Promega). These clones were sequenced using a standard sequencing protocol from USB (Amersham). Sequencing reactions were resolved on a 6% acrylamide gel and visualised by autoradiography after overnight exposure at room temperature. Thus, a complete sequence of the M3/6 clone was generated in one direction. The sequence in the opposite direction was obtained using insert specific oligonucleotides with the ABI (Applied Biosystems, Inc.) sequencing protocol. Sequence analysis was done using the GCG Wisconsin package version 8.

**RNA extraction and Northern analysis**

RNA was extracted from mouse tissue following the method of Chomczynski and Sacchi (47). Poly(A)+ RNA was prepared from 100 µg of total RNA using the Dynabeads mRNA purification kit (Dynal). Northern blots were prepared according to Current Protocols in Molecular Biology, with each lane containing 2 µg of poly(A)+ RNA. The human fetal tissue Northern was obtained from Clontech. The blots were prehybridised for 4 h in formamide buffer (5× SSPE, 10× Denhardt’s solution, 100 µg/ml salmon sperm, 50% formamide, 2% SDS). Hybridisation was carried out at 42°C in the same buffer and the blots were washed to a stringency of 0.1× SSC, 0.1% SDS at 55°C. The blots were visualised by autoradiography after exposure for one to two days at –70°C.

**Localisation of M3/6**

M3/6–4e detected fragments of approximately 4.7 kb and 5.5 kb in EcoRI digests of DNA prepared from mouse (CBA/H) and approximately 6 kb in Chinese hamster respectively. Concordance values for the presence of the mouse specific fragment and individual mouse chromosomes were obtained by analysis of the hybridisation pattern revealed by M3/6–4e on EcoRI digests of DNA from a well-characterised panel of 18 mouse × Chinese hamster somatic cell hybrids (48). To confirm and extend these results, M3/6–4e was hybridised to TaqI digests of DNA prepared from interspecific backcross progeny from the European Collaborative Interspecific Backcross, EUCIB (34). M3/6–4e detected common bands of approximately 2.3 and 4.6 kb and a Mus spretus RFLV of 1.55 kb.

**Fluorescence in situ hybridisation**

HB5a DNA was nick translated with Digoxigenin–11–dUTP (Boehringer). Metaphase spreads were prepared from a normal male lymphoblastoid cell line by standard cytogenetic methods. Prior to hybridisation, slides were incubated in 0.06 mg/ml proteinase K (in 2 mM CaCl2, 20 mM tris pH 7.4) at 37°C for 7 min, rinsed in 2× SSC, and then denatured at 65°C for 2 min. The probe mix, comprising 40 ng labelled probe, 1 mg Cot1 DNA and 10 ml hybridisation buffer (50% formamide, 10% dextran sulphate, 2× SSC, 0.1% SDS, 1× Denhardt’s solution, 40 mM sodium phosphate pH 7) (49), was denatured at 65°C for 10 min and then pre–annealed at 37°C for 30 min. Hybridisation was performed at 37°C overnight. Stringency washes were in two changes of 50% formamide/50% 2× SSC (v/v) at 42°C for 5 min each (50). Hybridisation was detected using FITC–conjugated mouse anti–digoxin (Sigma) diluted 1:1000 in a blocking solution of 4× SSC, 0.05% Tween 20 and 5% non–fat milk powder, followed by a 1:250 dilution of FITC–conjugated goat anti–mouse antibody (Sigma). After staining in 0.08 mg/ml DAPI in 2× SSC for 2 min, the slides were mounted in antifade solution (Citifluor AF1) and examined using a Zeiss Axioskop fluorescence microscope with a Photometrics cooled CCD camera controlled by SmartCapture imaging software (Digital Scientific). The
distribution of fluorescent signals was scored for a total of 25 metaphases.

**Transient transfection of COS cells**

COS1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. Transfection, harvesting, and Western blotting of cell lysates were all carried out as described previously (14). In brief, transient transfection of COS cells (2 x 10^5 per 10 cm plate) was by DEAE dextran; cells were harvested after 3 days, using serum starved for the final 16 h then stimulated with serum for 20 min. Lysates were run on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with anti ERK2 antiserum 122 (36) or the 9E10 (anti-myc epitope) antibody. Bound antibody was detected by ECL (Amersham).

**Microinjection and immunofluorescence**

These methods were carried out as described previously (51). In brief, microinjections were performed on a Zeiss Microinjection Workstation (Carl Zeiss, Oberkochen). Areas of cells to be injected were delineated with a scalpel blade on the tissue-culture surface of 60 mm petri dishes. All plasmids were injected into the nucleus of target cells at a concentration of 60 µg/ml. After 16–20 h cells were fixed in 4% formaldehyde in Dulbecco’s phosphate buffered saline (PBS) for 15 min, washed in several changes of PBS for 30 min and permeabilized in 0.2% Triton X-100 in PBS for 15 min. The specimens were then quenched in 100 mM glycine in PBS for 10 min followed by 10% fetal bovine serum in PBS for 30 min. Primary antibody incubations were performed for 1 h at room temperature. Injected areas of cells were finally mounted under a glass coverslip in Moviol mountant containing 21. Keyse,S.M. and Emslie,E.A. (1992) Oxidative stress and heat shock induce a p-phenylene diamine (Sigma) and were examined using a Biorad MRC600 confocal imaging system.

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