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The cellular response to induction of the p21$^{c\text{-}Ha\text{-}ras}$ oncoprotein includes stimulation of *jun* gene expression

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We have studied the effects of c-Ha-*ras* oncogene in mouse NIH 3T3 fibroblasts by DNA transfection and analysis of gene expression at the mRNA and protein level in a heat- and heavy metal-inducible model system. The human c-Ha-*ras* proto-oncogene and oncogene were cloned under the hsp70 heat-shock promoter. Clonal lines of cells with negligible basal expression of the hsp-c-Ha-*ras* oncogene construct were chosen on the basis of the inducibilty of p21$^{c\text{-}Ha\text{-}ras}$ protein and several transformation parameters. We demonstrate that the expression of ornithine decarboxylase (ODC) mRNA is enhanced ~4-6 h after the induction of the p21$^{c\text{-}Ha\text{-}ras}$ oncoprotein. This increase was reversible upon cessation of c-Ha-*ras* mRNA and protein synthesis, while constitutively elevated ODC was characteristic for stably c-Ha-*ras*-transformed cells. The high-level expression of ODC in *ras*-transformed cells was insensitive to tumour promoter stimulation. A similar mRNA induction by c-Ha-*ras*$^{Val\text{-}12}$ was also observed for two other serum- and tumour promoter-regulated genes associated with the transformed phenotype: transin (stromelysin) and the glucose transporter. This prompted us to examine also potential changes in the expression of the serum- and tumour promoter-induced transcription factor genes *jun*B and c-*jun* after induction of the hsp-c-Ha-*ras* construct. The *jun*B mRNA was enhanced ~10-fold and the c-*jun* oncogene mRNA to a lesser degree in the hsp-c-Ha-*ras*-transfected cells after zinc activation of the hsp70 promoter. These effects were not seen in similarly treated control cells. These results provide a link between the expression of the c-Ha-*ras* and *jun* oncogenes and deregulation of certain genes characteristic of the transformed phenotype and offer a new model for the study of the effects of p21$^{c\text{-}Ha\text{-}ras}$ on gene expression in cultured cells.

Key words: c-Ha-*ras* oncogene/heat shock response/ornithine decarboxylase/transformed phenotype

Introduction

Deregulation of the expression of some genes, such as ornithine decarboxylase (ODC), transin and the glucose transporter, is characteristic of many tumours and tumour cell lines *in vitro* (Matrisian *et al.*, 1985, 1986a,b; Birnbaum *et al.*, 1987; Flier *et al.*, 1987; Pegg, 1988). For example, both chemically and virally transformed cells show increased ODC activity and polyamine levels (Yuspa *et al.*, 1976; Bachrach, 1978; Pegg, 1988; Redmond *et al.*, 1988). Elevated expression of these genes is also associated with growth stimulation, and it may be that constitutive mitogenic signals activated in general by oncogenesis upregulate, for example, ODC-gene expression, but that ODC activity is only of secondary importance for the transformed phenotype. However, the mechanisms responsible for this kind of cell transformation gene program have now become amenable to molecular analysis using oncogenes such as c-Ha-*ras* as effectors, and DNA clones as probes.

One way to study the *ras*-induced transformed phenotype is to characterize the changes occurring in gene expression upon introduction of the p21$^{c\text{-}Ha\text{-}ras}$ oncoprotein into normal cells. Although the targets for the action of *ras* proteins in cell transformation are cytoplasmic (Trahey and McCormick, 1987; Calés *et al.*, 1988), *ras*-induced signals are transduced to the nucleus resulting in changes in gene expression and increased DNA synthesis (for a review, see Barbacid, 1987). We have previously shown that transfection of the c-Ha-*ras* oncogene into fibroblastic cells is associated with an augmented ODC content which can be accounted for largely, but not solely, by an enhanced accumulation of ODC mRNA (Sistonen *et al.*, 1987; Hölttä *et al.*, 1988). Moreover, our experiments indicate that a similar overexpression of the normal c-Ha-*ras* proto-oncogene does not lead to constitutively activated ODC (Hölttä *et al.*, 1988). However, studies on ODC and other transformation-associated genes in *ras*-transformed cells have been performed with cell clones, which may be biochemically heterogeneous and may develop secondary or distinct, non-*ras*-related alterations. Therefore, the studies on gene deregulation by *ras* must also include analysis of clonal lines of cells, where *ras* expression can be manipulated.

Until recently, no temperature-sensitive c-Ha-*ras* mutants have been available and studies have relied on the use of inducible promoters and DNA vector transfection to obtain cell clones where oncogene expression can be regulated. Several such promoters have already been used in conjunction with c-Ha-*ras* or N-*ras*; such as the glucocorticoid-inducible mouse mammary tumour virus (MMTV) LTR (Jaggi *et al.*, 1986; McKay *et al.*, 1986; Wakelam *et al.*, 1986; Owen and Ostrowski, 1987) and the mouse metallothionein (MT) I promoter (Trimble *et al.*, 1986; Reynolds *et al.*, 1987; Li *et al.*, 1988) which is induced by low concentrations of heavy metals.

We have now cloned the c-Ha-*ras* oncogene and proto-oncogene under the control of the promoter of the hsp70 gene, which encodes the major human heat-shock protein of M_r 70 000 (Hunt and Morimoto, 1985; Pelham, 1986). This construct allows a rapid and substantial induction of c-Ha-*ras* expression but maintains minimal expression in the absence of inducers. The construct has been used to demonstrate directly that the c-Ha-*ras* oncogene is capable of increasing the mRNA levels of ODC, transin and the glucose transporter in a few hours after the onset of synthesis of its encoded p21 protein. The changes observed are reversible

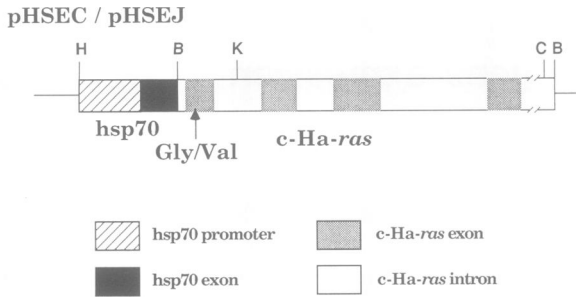

Fig. 1. Diagram of pHSEC and pHSEJ expression vectors. The c-Ha-*ras* proto-oncogene and oncogene were obtained from the pLTR H-*ras*(N)/(A) expression vectors (Jaggi *et al.*, 1986) as *Bam*HI restriction fragments and ligated to the *Bam*HI site of the plasmid containing a *Hin*dIII–*Bam*HI fragment including 300 bp of promoter and 150 bp of leader mRNA sequences of the hsp70 gene (Hunt and Morimoto, 1985). The arrow indicates the location of the activating point mutation (Gly/Val) at the 12th codon of the c-Ha-$ras^{Val\text{-}12}$ oncogene. Abbreviations for the restriction endonuclease cleavage sites are: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I.

after cessation of $p21^{c\text{-}Ha\text{-}ras}$ synthesis and turnover of the oncoprotein. Interestingly, a rapid increase of the transcription factor *jun*B and c-*jun* mRNAs is also obtained after c-Ha-*ras* induction, suggesting that *jun* genes may be involved in some of the changes in gene regulation caused by c-Ha-*ras*.

Results

Construction of the hsp–c-Ha-ras expression vector

For rapid induction of c-Ha-*ras* expression in NIH 3T3 cells, we cloned the c-Ha-*ras* oncogene (Val-12) and proto-oncogene (Gly-12) sequences under the promoter of the human heat shock gene hsp70 (Hunt and Morimoto, 1985). This was done by excising the 4.8 kb c-Ha-*ras* oncogene and proto-oncogene inserts from the pLTR H-*ras* expression vectors (Jaggi *et al.*, 1986; a kind gift from Drs R.Jaggi and B.Groner, Ludwig Institute for Cancer Research, Bern, Switzerland) and ligating each one separately to the *Bam*HI site of a plasmid carrying a 450 bp *Hin*dIII–*Bam*HI insert from the promoter of the hsp70 gene (a kind gift from Dr Richard I.Morimoto), which can be induced by a variety of stimuli including heat and heavy metals (Wu and Morimoto, 1985; Morgan *et al.*, 1987; Wu *et al.*, 1987). Figure 1 shows a diagram of the vector plasmids constructed. As is indicated in the figure, separate expression vectors were completed for the normal (pHSEC) and Val-12-activated (pHSEJ) forms of c-Ha-*ras*.

We used the pHSEJ and pHSEC constructs to transfect NIH 3T3 cells with the calcium phosphate precipitation method followed by neomycin selection. Drug-resistance selection was used to isolate cell clones, which in normal growth conditions would show very little basal expression of the oncogene, and would therefore display normal cell morphology and growth characteristics. Thus, these cells would not show up as colonies on top of normal cell monolayers or in a semisolid medium. These clonal lines of neomycin-resistant cells obtained after transfection were tested for heat-inducibility of a transformed cell morphology and $p21^{c\text{-}Ha\text{-}ras}$ expression. Clones HAj and HAl (expressing the pHSEJ oncogene construct) and HN6 (expressing the pHSEC proto-oncogene construct) were selected on the basis of these criteria for subsequent experiments.

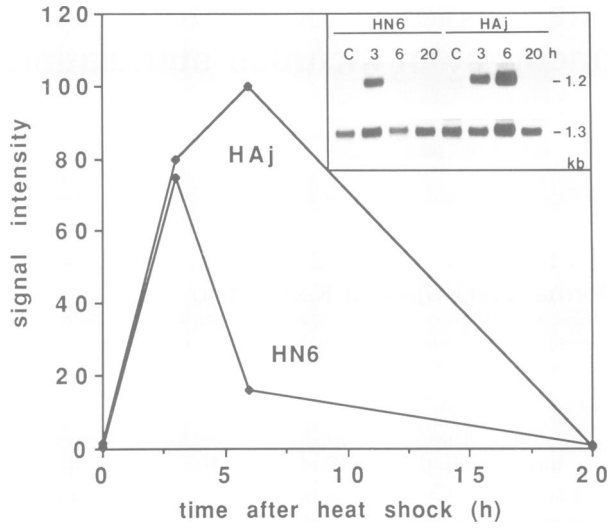

Fig. 2. Expression of the hsp–c-Ha-*ras* constructs in heat-shocked HN6 and HAj cells. Polyadenylated RNA was isolated from heat-shocked HN6 and HAj cells after 3, 6 and 20 h of recovery at 37°C. RNA samples of 8 μg were used for Northern filter hybridization analyses with the c-Ha-*ras* (1.2 kb mRNA) and GAPDH (1.3 kb) probes (see the two panels of the inset). Autoradiographic signals were quantitated with a densitometer and normalized to the GAPDH mRNA values obtained from the same samples. Values for the relative autoradiographic signal (*Y* axis) were obtained by comparing the signal intensities with the maximal signal intensity (relative value 100) seen in HAj cells 6 h after the heat shock. 'C' indicates untreated cells harvested at 3 h. In the stringent hybridization and washing conditions used the mouse mRNA does not give a signal with the human c-Ha-*ras* probe.

Heat-shock induction of c-Ha-ras expression

In order to assess the induction of the pHSEC and pHSEJ constructs in the transfected cell clones, we performed riboprobe protection analysis of RNA isolated from untreated and heat-shocked cells. These data showed that the RNA expressed from the transfected oncogene constructs is correctly initiated in the cells (data not shown), and responsive to heat-shock induction. This was also shown by Northern blot analysis. For the latter experiment, polyadenylated RNA was isolated from 5×10^7 cells 3, 6 and 20 h after the heat shock. Cells maintained at 37°C served as controls. The time-course of induction of c-Ha-*ras* mRNA after the heat-shock treatment is shown in Figure 2. In HN6 cells the c-Ha-*ras* proto-oncogene reached its maximal expression 3 h after the heat shock, while in HAj cells the levels were still higher ~3 h later. By 20 h, expression of both constructs had returned to undetectable levels. Some variation was evident in the timing of induction in separate experiments, probably due to slight differences in the parameters of heat treatment, but in general the induction of the oncogene mRNA was somewhat stronger than the proto-oncogene mRNA induction, presumably because of increased stability of the former mRNA (Cohen and Levinson, 1988, see also Figure 4). We are also studying the possibility that the $p21^{c\text{-}Ha\text{-}ras}$ protein enhances the heat-shock response.

Induction of $p21^{c\text{-}Ha\text{-}ras}$ protein in oncogene-expressing cells

For estimation of the levels of the $p21^{c\text{-}Ha\text{-}ras}$ protein, immunoprecipitations were performed from lysates of heat-

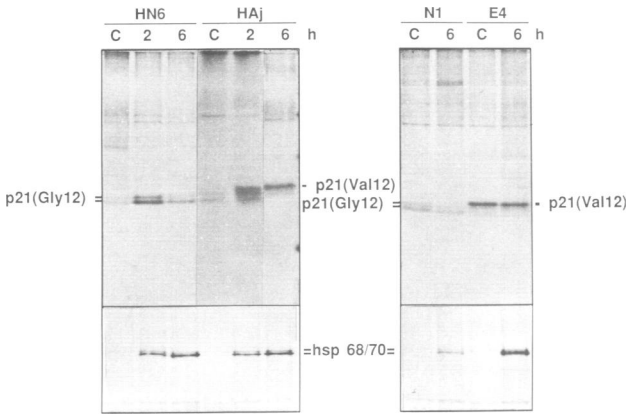

Fig. 3. Induction of synthesis of p21$^{c\text{-}Ha\text{-}ras}$ proteins during recovery from heat shock. The synthesis of c-Ha-*ras*-encoded p21 proteins in heat-shocked HN6 and HAj cells was followed by metabolic labelling and immunoprecipitation with specific antibodies. The N1 and E4 serve as controls for neomycin-resistant NIH 3T3 cells and stably c-Ha-*ras*-transformed NIH 3T3 cells respectively (see the text for details). Lanes maked C represent samples from cells maintained at 37°C. p21(Gly-12) and p21(Val-12) indicate the mobilities of the c-Ha-*ras* proto-oncogene- and oncogene-encoded proteins respectively. As is evident from the figure, these two forms of p21 protein can be distinguished by SDS–PAGE (Furth *et al.*, 1982). The lower panels show immunoprecipitation of the hsp68/70 proteins from the corresponding samples. Only the hsp68 form is significantly induced after heat shock.

shocked cells pulse-labelled with [^{35}S]methionine for 2 h. The results are shown in Figure 3. They demonstrate abundant p21$^{c\text{-}Ha\text{-}ras}$ protein biosynthesis in the HN6 and HAj cells ~2 h after heat shock (~6-fold elevated when compared with the level of biosynthesis of the endogenous mouse p21). The synthesis of p21 stayed at a high level during a prolonged period in the HAj cells expressing the oncoprotein (still ~5-fold elevated at 6 h after heat shock). In contrast, the biosynthesis of p21$^{c\text{-}Ha\text{-}ras}$ decreased more rapidly in HN6 cells expressing the normal *ras* protein ~3-fold elevated at 6 h, Figure 3). However, the heat-shock treatment did not affect the synthesis of the endogenous p21^{ras} protein in neomycin-resistant normal cells (N1) or the constitutively high levels of p21$^{Val\text{-}12}$ in E4 cells carrying multiple copies of the c-Ha-*ras* oncogene (Sistonen *et al.*, 1987, Figure 3).

Comparison was also made with the synthesis of the hsp70 protein. The cells were heat-induced and allowed to recover at 37°C for varying lengths of time, whereafter a 2 h radioactive pulse of [^{35}S]methionine was given, followed by lysis and immunoprecipitation of the cells with a hsp68/70-specific antiserum. The results are shown in the lower panel of Figure 3. Enhanced synthesis of hsp68, the main inducible heat-shock protein in mouse cells, occurred 2–6 h after the heat shock and returned to basal levels by 24 h (Figure 3 and data not shown) as has been reported for normal cells by Welch and Suhan (1986). According to the densitometric scanning of the fluorograph, the hsp68/70 polypeptide was ~12-fold enhanced over background in the HAj cells 4–6 h after the heat shock. Similarly, the increase of hsp68/70 expression in the proto-oncogene-expressing HN6 cells was ~10-fold. The magnitude of hsp70 induction was also greater in the E4 cells stably transformed with the c-Ha-*ras* oncogene than in the N1 cells, which express only the neomycin resistance gene. We are examining these differences further.

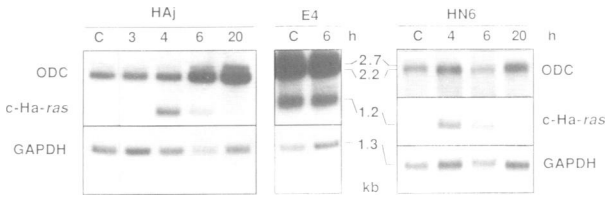

Fig. 4. Northern hybridization analysis of c-Ha-*ras* and ODC RNA in heat-shocked HAj and HN6 cells. Polyadenylated RNA was isolated from heat-shocked HAj, E4 and HN6 cells after various recovery periods at 37°C (3, 4, 6, 20 h); lanes 'C' indicate RNA from non-heat-shocked cells. Eight micrograms of poly(A)$^+$ RNA was used for analysis of ODC and c-Ha-*ras* mRNA content. The HAj and E4 panels are a composite from one autoradiogram and simultaneous analysis, but the mRNA signals in the HN6 panel cannot be compared with these due to differences in the specific activity of probes and in exposure times.

p21$^{Val\text{-}12}$ increases ornithine decarboxylase mRNA

We have earlier shown that the expression of ODC mRNA is elevated in c-Ha-*ras* oncogene-transformed cells when compared with normal cells (Sistonen *et al.*, 1987; Hölttä *et al.*, 1988). Exploiting the cells with the inducible p21$^{Val\text{-}12}$ protein we now tested the role of p21$^{Val\text{-}12}$ oncoprotein in increasing the steady-state levels of ODC mRNA in single cell clones. The HAj cells were heat-shocked and RNA samples were isolated after various recovery periods at 37°C, electrophoresed, blotted and hybridized with the c-Ha-*ras* and ODC probes. The results are shown in Figure 4. They demonstrate maximal c-Ha-*ras* mRNA amounts at 4–6 h after heat shock. Although the c-Ha-*ras* mRNA levels declined thereafter, immunoprecipitation studies showed an ~6-fold increased biosynthesis of p21$^{c\text{-}Ha\text{-}ras}$ protein at 2 and 6 h after heat shock (see above). These data are consistent with the relatively long half-life of the p21$^{c\text{-}Ha\text{-}ras}$ protein, ~20 h (Ulsh and Shih, 1984). Expression of the ODC mRNA was ~6-fold elevated 6 h after heat shock (Figure 4). The ODC mRNA level was maintained at a high level until 20 h after the heat shock (4-fold elevated) but it returned back to normal levels by 30 h (data not shown). Heat shock did not significantly alter the levels of ODC mRNA in the E4 cells, which maintained a high level of ODC and c-Ha-*ras* mRNA expression (Figure 4). The effect of p21$^{c\text{-}Ha\text{-}ras}$ on ODC expression was apparently also specific for the oncoprotein, since a similar induction of the proto-oncogene mRNA (Figure 4) and the corresponding p21$^{Gly\text{-}12}$ protein (Figure 3) was associated with only a small increase in ODC mRNA content of the HN6 cells (Figure 4; see also Figure 6).

ODC mRNA is not induced by TPA in c-Ha-ras-transformed cells

The above as well as our earlier results suggested that the induction of the c-Ha-*ras* oncoprotein might cause a signal which was similar to that delivered by serum and tumour promoters like tetradecanoyl-phorbol acetate (TPA) which are known to elevate the expression of ODC only transiently in normal cells (Verma *et al.*, 1986; Katz and Kahana, 1987) but constitutively in the case of tumour development (Gilmour *et al.*, 1987). In agreement with this data we found that TPA caused a transient increase in ODC mRNA in the normal N1 cells (Figure 5). Interestingly, however, the ODC mRNA in the c-Ha-*ras* oncogene-expressing E4 cells was

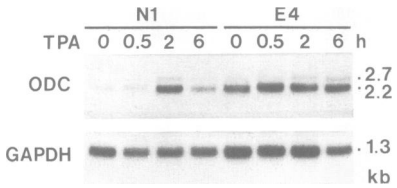

Fig. 5. Analysis of ODC mRNA in TPA-treated N1 and E4 cells. The cells were treated with tetradecanoyl phorbol acetate (TPA; 50 nM) beginning 48 h after subculture, when ODC mRNA had declined (see Hölttä *et al.*, 1988). After the incubation periods, poly(A)$^+$ RNA was isolated and analysed as in Figure 4.

constitutively elevated and refractory to stimulation with TPA (Figure 5).

Induction of transin and glucose transporter mRNAs by the c-Ha-ras oncoprotein

Results similar to the ODC data were obtained for two other transformation-associated mRNAs which are TPA-responsive in normal cells: transin and the glucose transporter (Figure 6). Transin mRNA encoding a major neutral proteinase (stromelysin) is present at significantly higher levels in cells transformed by polyoma virus, Rous sarcoma virus and c-Ha-*ras* oncogene than in the corresponding normal cells (Matrisian *et al.*, 1985). Variable, but very low levels of a 1.9 kb poly(A)$^+$ RNA, hybridizing with the rat transin cDNA were seen in HAj cells not subjected to heat shock. The amount of transin RNA was, however, >20-fold increased at its maximum, 6 h after the heat shock, i.e. ~4 h after the maximal biosynthesis of p21$^{Val\text{-}12}$ in the HAj cells. In HN6 cells, on the contrary, no expression of transin mRNA occurred regardless of the level of p21$^{Gly\text{-}12}$ (Figure 6).

It has been known for a long time that an accelerated rate of glucose transport is among the most characteristic biochemical markers of the transformed phenotype. The main biochemical mechanism responsible for increased glucose transport in c-Ha-*ras*-transformed cells is an increase in the expression of the glucose transporter mRNA (Birnbaum *et al.*, 1987; Flier *et al.*, 1987). In agreement with this we found that the amount of the 2.8 kb glucose transporter mRNA was increased ~2- to 3-fold 7 h after hsp–c-Ha-*ras* induction and it persisted at an elevated level for at least 24 h (Figure 6). The magnitude of this change was similar to the difference reported by Birnbaum *et al.* (1987) between stable lines of c-Ha-*ras*-transformed cells and normal cells. In addition, a minor 3.6 kb mRNA, which hybridized with both the human and rat glucose transporter cDNAs, was induced in HAj cells. The 3.6 kb mRNA was not expressed in the HN6 cells which also maintained stable expression of the 2.8 kb mRNA throughout the heat-shock experiment (Figure 6).

Induction of jun mRNAs

The transcription of ODC, transin and glucose transporter genes is stimulated by TPA and serum in normal cells. One possibility to explain the stimulation of these mRNAs in the c-Ha-*ras*-induced cells is that transcription factors binding to TPA- and serum-regulated DNA promoter elements were activated by p21$^{c\text{-}Ha\text{-}ras}$. Induction of such a transcription factor has recently been reported in c-Ha-*ras*-transformed cells (Imler *et al.*, 1988; Wasylyk *et al.*, 1988). The *jun* genes (*jun*B, c-*jun*) encode proteins binding to the TPA-,

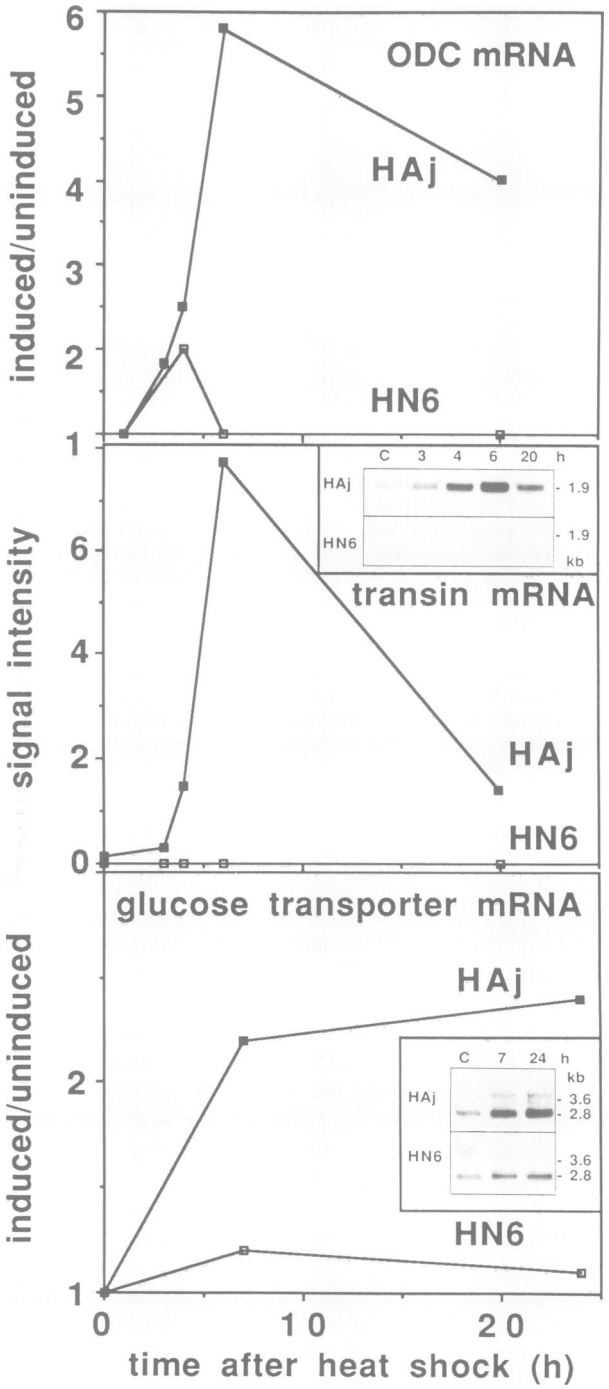

Fig. 6. Comparison of ODC, transin and glucose transporter mRNAs in heat-shocked HAj and HN6 cells. Numerical values for the fold of induction of ODC mRNA in comparison with the uninduced control mRNA level are plotted against time of recovery from heat shock. Autoradiographic signals were quantitated densitometrically and normalized to the GAPDH mRNA values. The results are plotted for the 2.2 kb ODC mRNA, 1.9 kb transin mRNA and for the 2.8 kb glucose transporter mRNA. Insets show the autoradiograms corresponding to the latter two genes. Note the induction of a 3.6 kb glucose transporter RNA. This may correspond to the mRNA of the recently described glucose transporter-like gene (Fukumoto *et al.*, 1988).

serum- and *ras*-regulated DNA sequences (Imler *et al.*, 1988; Wasylyk *et al.*, 1988; for a review, see Vogt and Tjian, 1988). Therefore, we tested the possibility that the expression of *jun* genes was activated at the mRNA level.

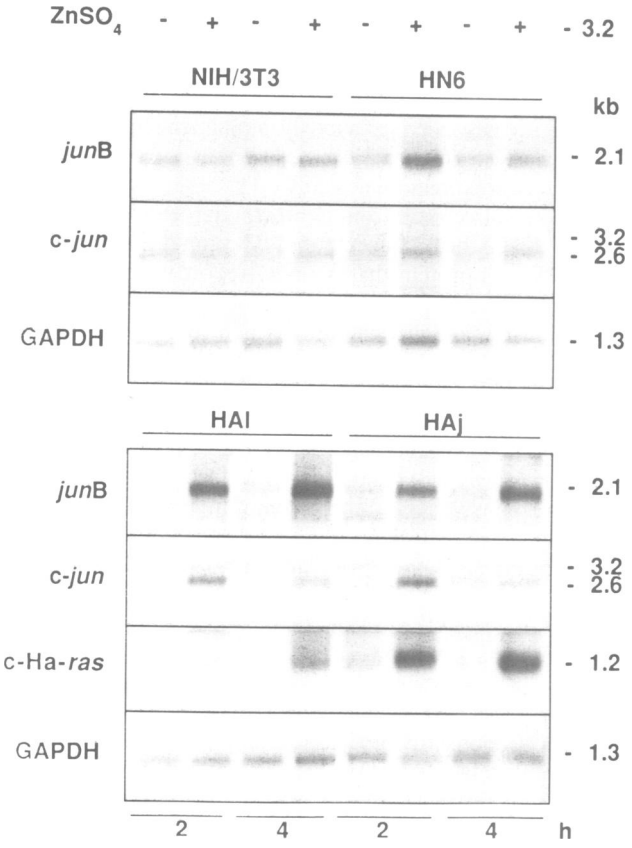

Fig. 7. Effect of Zn^{2+} on the expression of *jun* and c-Ha-*ras* mRNAs. RNA was analysed from the NIH 3T3, HN6, HA1 and HAj cells treated with 100 μM $ZnSO_4$ for 2 and 4 h. Note that the c-*jun* mRNA response begins to decline at 4 h of induction, before the *jun*B response (cf. Figure 8).

The *jun*B gene was originally discovered as a growth factor-induced cDNA isolated by differential screening of resting and serum-stimulated cells (Lau and Nathans, 1987; Ryder *et al.*, 1988). The c-*jun* gene is a cellular homologue of the v-*jun* gene first found in a chick retrovirus (Maki *et al.*, 1987; Vogt *et al.*, 1987); it is also activated during the G_0/G_1 transition (Ryseck *et al.*, 1988). We used the *jun* cDNAs as probes to measure *jun*-specific mRNA levels in the c-Ha-*ras*-induced HAj and HAl cells. Our preliminary experiments indicated that the expression of the *jun* genes may be affected by heat shock even in normal cells (data not shown). We therefore took advantage of the property of the hsp70 promoter to be induced by heavy metals, e.g. by zinc (Wu *et al.*, 1986).

Addition of 100 μM $ZnSO_4$ to the cultures of the NIH 3T3 cells, their neomycin-resistant clonal derivatives, HN6 cells expressing the hsp−c-Ha-*ras* proto-oncogene or E4 cells expressing the c-Ha-*ras* oncogene produced only a relatively small increase in the *jun*B or c-*jun* mRNA levels (<3-fold; Figure 7 and data not shown). By contrast, a similar treatment of the c-Ha-*ras* oncogene-expressing HAl or HAj cells induced the *jun*B mRNA ~5- to 10- and 15- to 18-fold at 2 and 4 h of treatment respectively (Figure 7). The c-*jun* mRNA was induced 4- to 8- and 2- to 4-fold, respectively, during the same time period. Simultaneously, expression of the c-Ha-*ras* mRNA was elevated to levels seen in the corresponding heat-induced cells, whereas the GAPDH mRNA stayed at a constant level. The accumulation of *jun*B and c-*jun* mRNAs was observed already at 1 h of Zn^{2+} treatment, when the c-Ha-*ras* mRNA began to accumulate (*Figure 8*). Also, expression of *jun*B and c-*jun* began to decline already after 6 h of treatment, when the c-Ha-*ras* mRNA levels were accumulating, showing the transient nature of the induction.

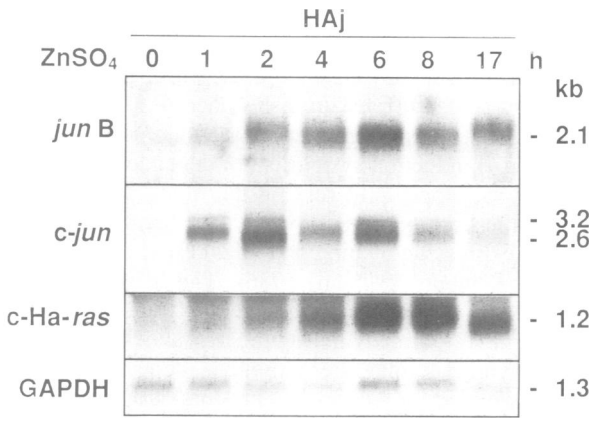

Fig. 8. Time-course of *jun* mRNA induction by the c-Ha-*ras* oncogene in HAj cells. The experiment was carried out for the indicated lengths of time and its analysis was as in Figure 7.

Discussion

The above results present a new experimental system for the analysis of the cellular, biochemical and genomic effects of the *ras* oncogene. The inducibility of the hsp constructs provides some distinct advantages which may help to complement data obtained using other inducible vector systems. A major problem with the commonly used MMTV LTR promoter element is that it is rapidly shut off by the c-Ha-*ras* oncoprotein (Jaggi *et al.*, 1986). Therefore, it is best suited for the analysis of the proto-oncogene only, being titratable by increasing concentrations of glucocorticoids (McKay *et al.*, 1986; Wakelam *et al.*, 1986). On the other hand, the maximum induction takes a long time to develop after the administration of dexamethasone. A similar slow induction is characteristic of the MT promoter, which is apparently also stimulated by *ras* proteins (Schmidt and Hamer, 1986; Reynolds *et al.*, 1987). Besides, in our hands, the uninduced levels of c-Ha-*ras* expression from the mouse metallothionein-I promoter (Durnam *et al.*, 1980) are substantially higher than that from the hsp70 promoter (L.Sistonen *et al.*, unpublished data). A disadvantage of the MT promoter is also a relatively low degree of induction (4- to 8-fold) obtained in most cell lines even under optimal conditions and with doses and periods of treatment that are nearly toxic for cells (Trimble *et al.*, 1986).

The basal activity of the hsp70 promoter cannot be totally eliminated either in continuous mouse cell lines (Greene *et al.*, 1987). However, by screening a large series of clonal cell lines where the hsp−c-Ha-*ras* construct was integrated into the chromosome we obtained some clones where *ras* sequences were expressed at very low levels and where no p21$^{Val\text{-}12}$ oncoprotein could be detected by immunoprecipitation. These cells appeared normal in their growth behaviour. As the hsp70 promoter is regulated also by the cell cycle and by serum, the oncogene induction experiments should ideally be performed in cultures arrested by serum deprivation and confluence. To avoid such limitations we

are experimenting with CCAAT-competing DNA templates and introduction of the constructs into primary cells in culture or also into tissues through the use of transgenic animals (M.Rassoulzadegan, F.Cuzin, L.Sistonen, P.Koskimem and K.Alitalo, unpublished data).

The hsp70 induction system is not dependent on protein synthesis, but appears to rely on the modification of pre-existing transcription factors, which then directly upregulate hsp gene expression by binding to their characteristic DNA sequence elements (Pelham, 1986). The hsp leader RNA may provide a selective advantage for translation of the chimeric mRNA already during the heat shock, when other protein synthesis may be inhibited, depending on the temperature used for the shock (Theodorakis and Morimoto, 1987). The induction of hsp70 by heat is transient, which may be a disadvantage in some experiments, because the downregulation of hsp−c-Ha-*ras* expression cannot be controlled at will. Even the transient expression may be useful for several types of experiments in addition to those ones outlined in the present work. Further, this construct offers the possibility of initiating expression from the hsp70 recombinant by using various other inducers (Wu *et al.*, 1986; Figures 7 and 8). Wurm *et al.* (1986) amplified a silent *Drosophila* hsp−*myc* oncogene template in cultured cells followed by heat-shock induction and concluded that the excessive c-*myc* protein levels obtained may be cytotoxic for cells, at least after a heat shock. Use of the hsp70 promoter construct in our analysis of the cellular response to the c-Ha-*ras* oncogene did not result in cytotoxicity. Our studies showed that several transformation-associated mRNAs, among them, interestingly, the *jun*B and c-*jun* proto-oncogene and transcription factor mRNAs are transiently increased as an immediate effect of $p21^{c\text{-}Ha\text{-}ras}$ expression. In contrast, the c-*jun* and *jun*B mRNA levels were not constitutively elevated in our c-Ha-*ras* oncogene-transformed cell lines.

According to prevailing theories and experimental evidence, proto-oncogenes encode proteins involved in the transduction of signals from cell exterior to the nucleus. Oncogenic mutations may damage this signal transduction system sufficiently to cause a deregulation of the expression of genes involved in cell growth. A fundamental question, then, concerning the functions of proto-oncogenes and oncogenes, is the pathway of signal transduction and the characterization of the genes responsive for normal growth signals as well as those deregulated by oncogenic insults.

The protein products of the c-*ras* genes are located at the inner surface of the plasma membrane (Willumsen *et al.*, 1984), and are believed to be involved in the transduction of proliferative signals from cellular second messengers, such as the active metabolites of phospholipid breakdown, induced by growth factor−receptor interactions (Yu *et al.*, 1988). Several studies have analysed the genes activated by *ras* oncogene expression, and in line with the above deductions, overlaps have been noted between the set of *ras*-regulated genes and those stimulated by tumour promoters, which activate the protein kinase C, normally involved in the amplification and transduction of phospholipid-derived signals (Rabin *et al.*, 1986; Imler *et al.*, 1988; Wasylyk *et al.*, 1988). On the other hand, tumour promoters may complement transformation by the c-Ha-*ras* oncogene both in cell culture and *in vivo* (Dotto *et al.*, 1985; Brown *et al.*, 1986), by as yet unknown mechanisms.

Our present study shows that at least three other genes are also stimulated by heat-shock-induced c-Ha-*ras* oncogene expression. These are the genes for ornithine decarboxylase, transin (stromelysin) and the glucose transporter. None of these genes has been previously studied in an inducible c-Ha-*ras* transformation system, and thus their previously described overexpression in stably c-Ha-*ras*-transformed cell clones could also result from indirect, e.g. cell proliferation-related, effects of transformation. The rapid effects of the $p21^{c\text{-}Ha\text{-}ras}$ oncoprotein that we now report were measured at the steady-state mRNA level and normalized against the expression of GAPDH mRNA, which in our cells is not significantly affected by c-Ha-*ras* expression when analysed relative to total polyadenylated RNA isolated from the cells. Our present experiments do not distinguish between possible *ras*-effects on the stability of the mRNAs or on the promoters of the genes involved, but one common feature of these genes is their responsiveness to the potent tumour promoter TPA (Matrisian *et al.*, 1986a,b; Flier *et al.*, 1987; Katz and Kahana, 1987). Other *ras*- and TPA-responsive genes include cathepsin L, also known as the gene for the major excreted protein (MEP; Denhardt *et al.*, 1987; Troen *et al.*, 1988) and the polyoma virus enhancer (Wasylyk *et al.*, 1987).

TPA is known to stimulate the expression of a variety of enhancers of transcription, and several DNA binding proteins (transcription factors) have been implicated in the binding to the TPA-responsive sequence elements (Angel *et al.*, 1987; Lee *et al.*, 1987; Curran and Franza, 1988). During the preparation of this manuscript, a c-Ha-*ras*-responsive transcription element was reported from the polyoma virus and found to be identical with the sequence element which also mediates promoter activation by TPA (Imler *et al.*, 1988). Interestingly, the TPA-responsive genes c-*fos* and c-*myc* have been found to be refractory to TPA-induction in v-*ras*-transformed rat thyroid cells (Colletta *et al.*, 1987)−as we show here for the ODC mRNA in the E4 cells. Our hsp70−c-Ha-*ras* model provides a means to study these interrelationships further in a system that responds quickly to various inducers such as heat, heavy metals and even amino acid analogues (Wu *et al.*, 1986).

The response of cells to mitogens involves the transcriptional induction of several genes (Lau and Nathans, 1987; Almendral *et al.*, 1988), some of which are constitutively deregulated by transformation. Of special interest are genes whose protein products function as transcription factors, prominent among them the c-*fos* and c-*myc* proto-oncogenes (Cochran *et al.*, 1983; Kelly *et al.*, 1983; Greenberg and Ziff, 1984). The expression of c-*fos* is induced soon after microinjection of the c-Ha-*ras* oncoprotein into cultured, serum-starved cells (Stacey *et al.*, 1987) and c-*fos* expression is essential for the transcriptional activation of collagenase gene expression by oncogenes and phorbol esters (Schöntal *et al.*, 1988). In serum- and growth factor-stimulated cells the c-*fos* protein is associated in transcriptional complexes with the protein product of the c-*jun* proto-oncogene, a component of the transcription factor AP-1 (Bohmann *et al.*, 1987; Bos *et al.*, 1988; Chiu *et al.*, 1988; Franza *et al.*, 1988; Lubicello *et al.*, 1988; Rauscher *et al.*, 1988a,b; Sassone-Corsi *et al.*, 1988), whose expression is also induced by serum and growth factors (Lamph *et al.*, 1988; Ryseck *et al.*, 1988; Ryder and Nathans, 1988). The AP-1 activity, measured as specific DNA-sequence binding protein

or as the activity of AP-1-sensitive, serum- and TPA-regulated promoter function, is increased in transformed cell lines and in short-term oncogene−reporter plasmid transfection experiments (Imler *et al.*, 1988; Wasylyk *et al.*, 1988). However, it is largely unknown how AP-1 is activated by c-Ha-*ras*. A likely possibility is that post-translational modifications, such as protein phosphorylation, play a role in the constitutive activity of AP-1 in transformed cells. Here we show that a sudden c-Ha-*ras* oncogene expression causes a rapid and transient enhancement of the *jun* mRNA levels, thus delivering a growth factor-like signal to the level of expression of immediate early genes. The induction of the *jun* and *fos* genes could then cause some of the secondary, early gene deregulations typical of the transformed phenotype shown here for the ODC, transin and glucose transporter genes.

Materials and methods

Cells and transfections

NIH 3T3 cells and their transfected clonal derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C. 4×10^5 cells were transfected with 18 μg of the c-Ha-*ras* proto-oncogene (pHSEC) or oncogene (pHSEJ) DNA (linearized with the *Cla*I restriction enzyme) together with 2 μg pSV2neo DNA (ATCC no. 37149, linearized with *Eco*RI) carrying the Tn*5* neomycin phosphotransferase gene according to the calcium phosphate precipitation technique described by Graham and van der Eb (1973). The neomycin analogue, G418 (Gibco Ltd, UK) was added to the culture medium at a concentration of 0.6 mg/ml 24 h after the removal of the DNA−$CaHPO_4$ precipitate. G418-resistant single cell clones were isolated and expanded.

N1 cells are NIH 3T3 cells transfected only with the neomycin resistance gene. E4 cells carry several copies of c-Ha-*ras* oncogene (pEJ6.6) and express constitutively high levels of $p21^{Val\text{-}12}$, as described previously (Sistonen *et al.*, 1987). TPA (phorbol 12-myristate 13-acetate, Sigma, St Louis, MO) was dissolved at 50 μM in absolute ethanol.

Molecular clones

The following cDNA clones were used as molecular probes: pODC 16 (mouse ornithine decarboxylase; Jänne *et al.*, 1984), pTR1 (rat transin; Matrisian *et al.*, 1985), pB1GT (human glucose transporter; Mueckler *et al.*, 1985) and pGT4-12 (rat glucose transporter; Birnbaum *et al.*, 1986). The *jun*B cDNA clone 465.20 (Lau and Nathans, 1987) was a kind gift from Dr Daniel Nathans (The Johns Hopkins University, Baltimore, MD). For the detection of the c-*jun* oncogene mRNA we used both a cDNA plasmid (ph-cJ-1; a kind gift from Dr Peter Angel, University of California, San Diego, CA) and a HPLC-purified 30mer antisense oligonucleotide (corresponding to nt 1321−1350 in the published human c-*jun* sequence; Angel *et al.*, 1988) labelled at its 5′-end with [γ-^{32}P]ATP using the T4 polynucleotide kinase (Maniatis *et al.*, 1982). The glyceraldehyde phosphate dehydrogenase (GAPDH) probe was the rat full-length cDNA insert from the pRGAPDH-13 (Fort *et al.*, 1985). Nick translations of DNA were carried out according to the manufacturer's instructions (Amersham).

Heat shock and zinc induction

Two days after subculture, the cells were heat shocked at 43°C for 1−2 h without change of the culture medium and allowed to recover at 37°C before analysis. For heavy metal induction 100 μM $ZnSO_4$ was added to the cultures. The lack of toxicity of the treatment was controlled using NIH 3T3 cells in parallel experiments.

Isolation of RNA and riboprobe protection analysis

Polyadenylated RNA was isolated by oligo(dT) chromatography from cell lysates (Schwab *et al.*, 1983). Aliquots (8 μg) of RNA were electrophoresed in 1.2% formaldehyde−agarose gels, transferred to Biodyne nylon filters in 20 × SSC and hybridized with nick-translated probes as described earlier (Sistonen *et al.*, 1987).

For the RNA protection assay the appropriate GEM plasmid (Promega, Madison, WI) was linearized with *Hin*dIII and ^{32}P-labelled single-stranded anti-sense RNAs were synthesized using T7 polymerase. After purification, the transcriptss were hybridized in solution with polyadenylated RNA overnight at 55°C in the presence of 80% formamide. Single-stranded RNA was then digested and the protected fragments were separated in a 5% polyacrylamide/7 M urea sequencing gel as described by Melton *et al.* (1984).

Metabolic labelling and immunoprecipitation

Cells were heat-shocked at 43°C for 2 h and were labelled during the recovery period beginning 0−4 h after heat shock with [^{35}S]methionine (250 μCi/ml, Amersham) for 2 h at 37°C and cell lysates were prepared as described by Furth *et al.* (1982). Immunoprecipitation of p21 was carried out by incubating cell lysates with the rabbit polyclonal antibody RS-1 against $p21^{ras}$ proteins (a kind gift from Dr R.Sweet, Swedeland, PA) and protein A−Sepharose particles.

Immunoprecipitation analysis of the constitutive (hsp70) and inducible (hsp68) forms of hsp70 was carried out using the monoclonal antibody specific for the 70 kd stress proteins (Amersham). The protein A−Sepharose particles were coated with rabbit anti-mouse IgG.

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