Positive autoregulation of *ras* genes expression in fibroblasts

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Abstract We have studied the effect of ectopic overexpression of a *ras* gene on the expression of the other two members of the *ras* gene family. We obtained NIH3T3 cell lines stably transfected with inducible H-*ras* and N-*ras* oncogenes. The expression of these genes is driven by a glucocorticoid-responsive promoter and the addition of dexamethasone resulted in a dramatic induction (10–20-fold) of H- or N-*ras* mRNA, peaking 4 h after hormone addition. The induction of the expression of *ras* oncogenes resulted in a transformed phenotype. In quiescent NIH3T3 cells transfected with inducible H-*ras* oncogenes, the induction of H-Ras was followed 12 h later by a 3-fold increase in the mRNA expression of endogenous K-*ras* and N-*ras*. Similarly, in NIH3T3 transfected with inducible N-*ras* oncogene, the induction of N-*ras* was followed by an increase in the expression of endogenous K- and H-*ras* genes. Interestingly, the effect was not limited to the mutated N-*ras*, as a similar result was obtained in cells transfected with N-*ras* proto-oncogene. The induction of *ras* genes expression was not linked to cell cycle progression as it was reproduced in cells arrested in S-phase by pretreatment with hydroxyurea. These results suggest the presence of a positive cross-regulation in the expression among the members of the Ras family. This effect could play a role in Ras-mediated carcinogenesis.

Key words: *ras* gene; Dexamethasone; Autoregulation; Gene expression; Fibroblast

1. Introduction

The mammalian *ras* gene family is composed of three members: H-, K-, and N-*ras*, that encode highly related 21 kDa proteins, termed p21s. The three proteins are almost identical except for the last 20 amino acids. p21s are membrane-associated proteins and bind and hydrolyze GTP [1,2]. Mutated forms of *ras* genes in codons 12, 13 or 61 are present in a significant fraction of human and experimental tumors. These mutants are constitutively in the activated (GTP-bound) state [3,4]. Ras-GTP but not the inactive form Ras-GDP interacts with a series of effectors, namely Raf, PI3 kinase and RalGDS, which mediate signal transduction towards the nuclei through different pathways [5].

There is ample evidence that Ras are involved in the control of cell proliferation: (i) Microinjection of oncogenic H-Ras and N-Ras proteins into quiescent fibroblasts stimulates DNA synthesis [6–8] and microinjection of anti-p21 neutralizing antibody or dominant inhibitory Ras mutant proteins inhibits the stimulation of DNA synthesis induced by growth factors or phorbol esters [9–11]. (ii) Mitogenic stimulus elicited by growth factors or serum results in a rapid increase in the fraction of GTP-bound p21 [12]. (iii) We and others have previously demonstrated that the expression of the three *ras* genes is up-regulated upon mitogenic stimulation of fibroblasts [13–15]. Therefore, the increased activity of Ras proteins by growth factors is due to two factors: increased biochemical activity (a higher fraction of GTP-bound Ras) and increased levels of Ras proteins.

Despite the amount of work carried out on *ras* function, the expression of *ras* proto-oncogenes has received little attention. In this work we have explored the possibility that a Ras protein could modify the expression of the other *ras* genes.

2. Materials and methods

NIH3T3 cells were obtained from ATCC and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% newborn calf serum (Biochrom) and 80 μg of gentamycin

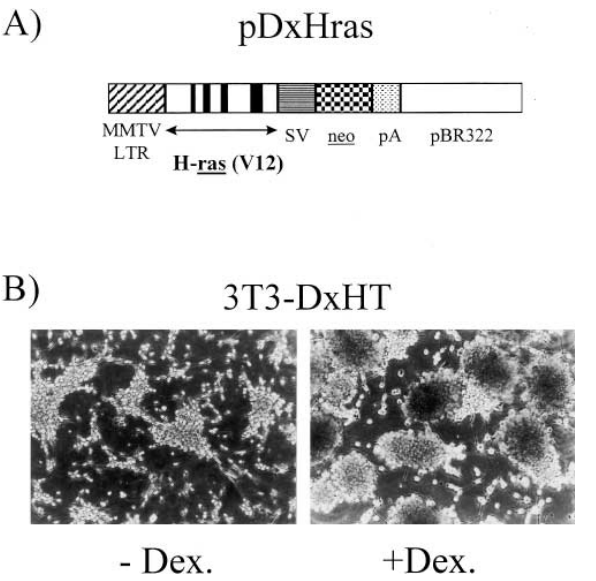

Fig. 1. A: Schematic representation of pDxHras. The boxes from left to right represent the MMTV LTR promoter (inducible by glucocorticoids), 2.9 kb from the human H-*ras* oncogene (black boxes indicate exons), the SV40 early promoter (SV), the neomycin/G418 resistance gene (*neo*), the SV40 early polyadenylation signal (pA) and sequences derived from pBR322, containing the β-lactamase gene. The total size of pDxHras is 11.3 kb. B: Phase-contrast micrographs of 3T3-DxHT cells untreated (right) and treated with 0.5 μM dexamethasone for three days (left). Original magnification: ×100.

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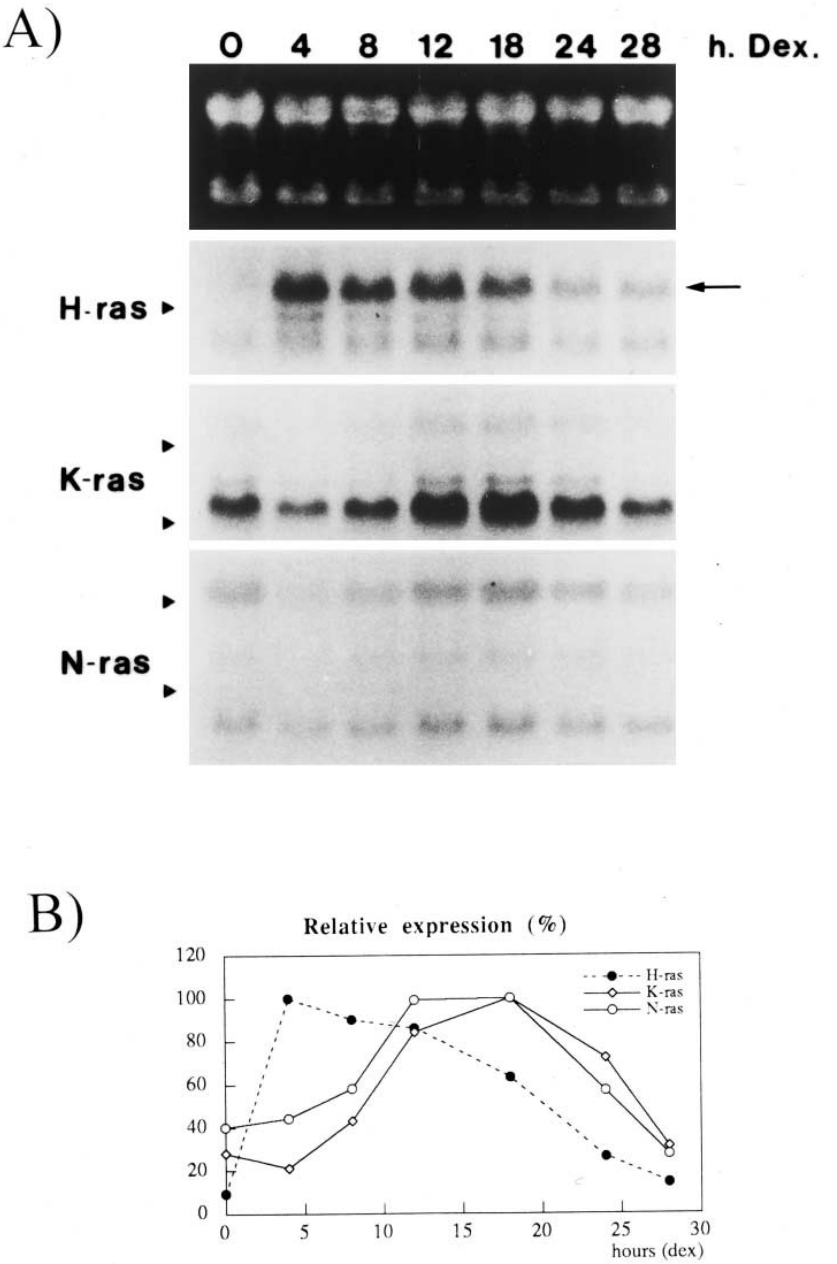

Fig. 2. A: Expression of *ras* genes in quiescent 3T3-DxHT cells after dexamethasone addition. Cells were grown to confluence, and the medium was replaced with DMEM containing 0.5% calf serum. After 72 h dexamethasone was added to a concentration of 0.5 μM. Total RNA was isolated at the indicated times and analyzed by Northern hybridization. The same filter was consecutively hybridized to the indicated probes. Arrowheads signal the position of 28S and 18S rRNA. The arrow indicates the position of the exogenous H-*ras* transcript. A photograph of the filter after transfer is shown to assess loading and integrity of RNAs. Exposure times were 3 h for the H-*ras* hybridization and 3 days for K- and N-*ras*. B: Relative expression of *ras* gene mRNA. The intensity of the signals of *ras* transcripts from the Northern blot of A was determined by densitometric analysis. The graph represents the expression of the smaller mRNA of *ras* genes normalized with respect the 18S rRNA. The highest expression for each gene was taken as 100% value.

per ml. To construct the plasmid pDxHras, a 2.9 kb *Sac*I genomic fragment from human T24 oncogene (plasmid pT24-C3 [16]) was first cloned into the *Sac*I site of pUC1813 [17]. The fragment *Sal*I of the resultant plasmid containing H-*ras* was cloned in the *Sal*I site of the polylinker of pMAMneo (Clontech). The resulting plasmid, pDxHras, expresses the human H-*ras* oncogene (mutated in codon 12) under the control of the mammary tumor virus long terminal repeat (MMTV LTR), inducible by dexamethasone (Fig. 1A). Plasmid pDxNras contains murine N-*ras* oncogene (mutated in codon 61) inducible by dexamethasone. It was constructed by cloning a *Sma*I-*Pst*I fragment containing the MMTV LTR and N-*ras* oncogene from the plasmid pMMTV-N-ras [18] into the *Bam*HI site of the vector pNeo3, which carries the G418-resistance gene [19] (Fig. 4A). The plasmid pDxNras/N is the equivalent to pDxNras but carrying the normal murine N-*ras* proto-oncogene and with the MMTV LTR-N-*ras* fragment inserted in the opposite orientation into pNeo3. Transfections were carried out by electroporation (Bio-Rad Gene Pulser, 300 V, 0.5 mF) and transfected cells were selected with G418 (400 μg/ml). Individual colonies were picked and expanded. Total RNA was isolated from cells by the acid guanidine thiocyanate method [20]. Northern blots were prepared and hybridizations were carried out essentially as described [14]. The probes for murine H-, K- and N-*ras* and for human H-*ras* genes were as described [14]. Semi-quantitative determination of mRNA levels was carried out by densitometric reading of short exposure films

with a HP ScanJet Plus densitometer and Quantiscan program (Biosoft). Filters were consecutively hybridized to different probes and signals from the previous hybridization were stripped out by boiling the filters for 5 min in water. Immunoblots were performed as described using anti-N-Ras antibody (Oncogene Science) [14].

3. Results and discussion

We constructed an expression vector, termed pDxHras which contains the human H-*ras* oncogene (Val12 mutant) under the control of MMTV promoter and the G418-resistance gene, which is schematically depicted in Fig. 1A. pDxHras was transfected by electroporation into NIH3T3 and several G418-resistant clones were analysed for H-ras mRNA overexpression after addition of 0.5 mM dexamethasone. A clone showing a clear H-*ras* mRNA induction and transformed phenotype after drug addition, termed 3T3-DxHT (for transforming H-*ras*), was selected for further studies. 3T3-DxHT cells exhibited a transformed phenotype when growing in the absence of added dexamethasone, a finding due to the well documented leakiness of the MMTV promoter. However, after addition of the hormone (0.5 μM, 3 days) the cells showed a markedly transformed phenotype with formation of numerous foci (Fig. 1B). To study *ras* genes induction, 3T3-DxHT cells led to quiescence by incubation for 48 h with medium containing 0.5% calf serum. This treatment reduced the levels of endogenous *ras* mRNA levels [15]. The cells were then treated with 0.5 μM dexamethasone and RNA was prepared after different times of treatment. The expression of the three members of the Ras family is shown in Fig. 2A. As expected, H-*ras* expression was rapidly and dramatically induced by dexamethasone. The exogenous H-*ras* transcript can be distinguished from the endogenous one by its larger size. The maximum level of H-*ras* mRNA was detected 4 h after hormone addition. To study the expression of the other *ras* genes we reprobed the Northern blot with specific murine K- and N-*ras* probes. Interestingly, the expression of endogenous K-*ras* and N-*ras* genes was also clearly induced 8–14 h after the peak of H-*ras* mRNA, i.e. 12–18 h after hormone induction (Fig. 2A). Densitometric analysis of the films and rRNA compared with the rRNA present in the filters revealed a maximal induction 3.5-fold and 2.5-fold for K-*ras* and N-*ras* respectively (Fig. 2B). To rule out a possible effect of dexamethasone on the regulation of *ras* genes in NIH3T3 cells, we determined the mRNA levels of the three *ras* genes after dexamethasone addition to arrested NIH3T3 cells. As shown in Fig. 3, the treatment of NIH3T3 cells with 1 μM dexamethasone did not result in any increase in the mRNA levels of H-, K- and N-*ras* genes. Therefore we conclude that the up-regulation of N- and K-*ras* in 3T3-DxHT cells was not due to the hormone but to *ras* gene expression.

Some reports suggest that there are differences in the biochemical activities of the three *ras* genes [21–23]. To study whether the effect described above with H-*ras* was reproduced with another *ras* gene, we transfected NIH3T3 cells with pDxNras, an expression vector carrying an inducible murine N-*ras* oncogene (mutated at codon 61) (Fig. 4A). After transfection and selection with G418, we selected a cell clone termed 3T3-DxNT (for transforming N-*ras*). As in the case of H-*ras*-transfected cells, the 3T3-DxNT cells in the absence of dexamethasone also showed a more transformed morphology than parental cells, but the addition of dexamethasone resulted in a dramatic enhancement of this phenotype (Fig. 4B). The treatment of quiescent 3T3-DxNT cells with 0.5 μM

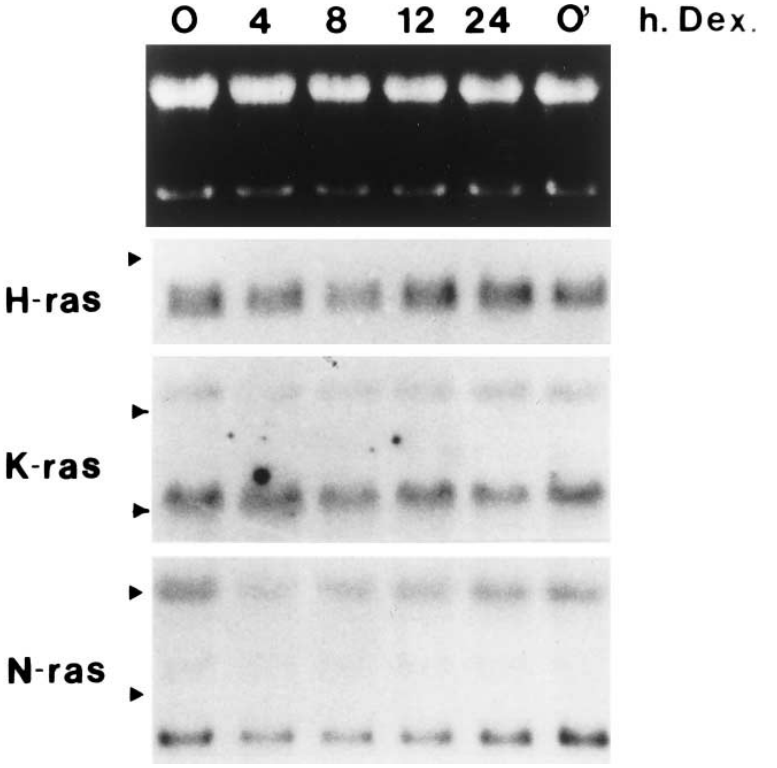

Fig. 3. Expression of *ras* genes in NIH3T3 after the indicated times of exposure to 1 μM dexamethasone. The experiment was performed as described in the legend to Fig. 1.

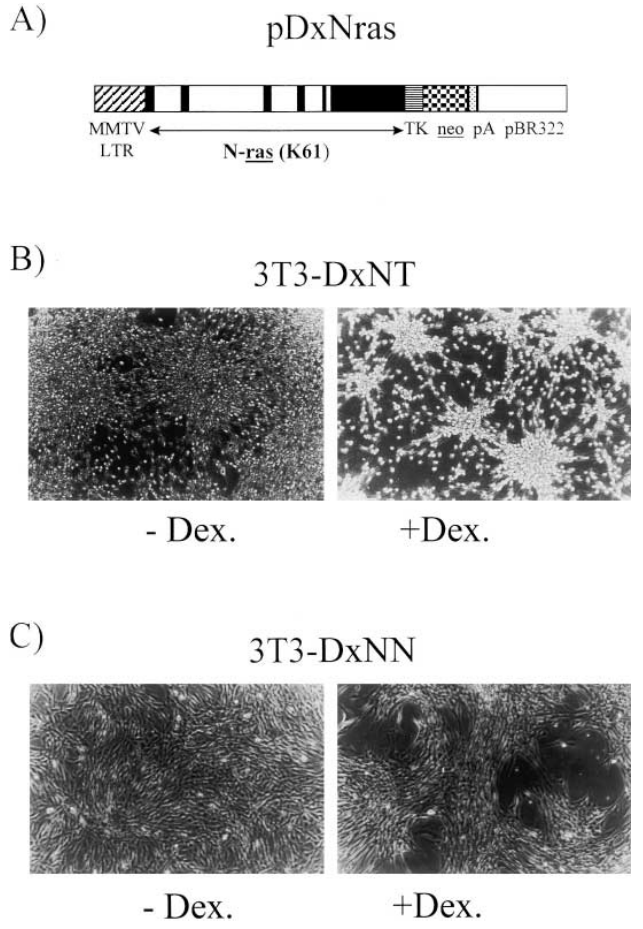

Fig. 4. A: Schematic representation of pDxNras. The boxes from left to right represent the MMTV LTR promoter inducible by glucocorticoids, 7.1 kb from the murine N-*ras* oncogene mutated in codon 61 (black boxes indicate exons), the herpes thymidine kinase promoter (TK), the neomycin/G418 resistance gene (*neo*), the herpes thymidine kinase polyadenylation signal (pA) and sequences derived from pBR322, containing the β-lactamase gene. The total size of pDxNras is 13.2 kb. B: Phase-contrast micrographs of 3T3-DxNT cells untreated (right) and treated with 0.5 μM dexamethasone for three days (left). Original magnification: ×100. C: Phase-contrast micrographs of 3T3-DxNN cells untreated (right) and treated with 0.5 μM dexamethasone for three days (left). Original magnification: ×100.

dexamethasone resulted in a rapid overexpression of murine N-*ras* mRNA (Fig. 5A). This was confirmed at the protein level by immunoblot, using a specific anti-N-Ras antibody (Fig. 5C). Reprobing of the Northern blot with H- and K-*ras* probes revealed that the mRNA expression of these genes also increased, peaking 20 h after hormone addition. The increase in the expression of these genes (normalized with respect to rRNA amount), was about 5-fold, as determined by film densitometry (Fig. 5B). Thus, ectopic overexpression of N-*ras* oncogene reproduced the effect observed for H-*ras*, i.e. an augmented mRNA level of the other two *ras* genes.

In order to know whether the cross-regulation of *ras* can also be induced by a normal *ras* gene, we obtained NIH3T3 cells transfected with the plasmid pDxNras/N, which encodes the inducible normal murine N-*ras* proto-oncogene. We chose a transfected cell line, 3T3-DxNN (for normal N-*ras*). These cells were not transformed, although they showed some transformed morphology upon dexamethasone addition (Fig. 4C). This finding is in agreement with the transforming effect of the overexpression of N-*ras* proto-oncogene in vitro [24] and in vivo [25]. In 3T3-DxNN cells we also observed an up-regulation of the endogenous H-*ras* and K-*ras* genes, thus reproducing the results obtained with cells transfected with the activated N-*ras* gene (Fig. 6A). Densitometric analysis revealed an increase of 10-fold for K-*ras* and 3-fold for H-*ras*, normalized to rRNA amount (Fig. 6B). It is noteworthy that the dexamethasone-induced expression of exogenous normal N-*ras* did not decline as rapidly as mutated H-*ras* (Fig. 6A) and mutated N-*ras* (Fig. 2A). This difference has been previously described for normal and mutated H-*ras* and explained as an oncogene-mediated repression of MMTV LTR, although the mechanism for this effect remains unclear [26].

The results of this work show that overexpression of a particular *ras* gene up-regulate the expression of the other two *ras* genes, peaking 18–20 h after dexamethasone addition. In the three transfectant cell lines studied, the highest up-regulation was observed for the endogenous K-*ras* gene. In all systems tested, including NIH3T3, there is a correlation between levels of *ras* mRNA and protein, and therefore we suggest this is also the case in our transfectants. Commercial antibodies specific for Ras proteins tested by us showed some cross-reactivity among them, making difficult to confirm the former hypothesis. We do not know the mechanism by which Ras exerts this up-regulation of the other gene family members. Further work is required to establish whether it is due to an increase in gene transcription or in the stability of *ras* mRNA.

We and others have shown that serum stimulation of quiescent cells results in up-regulation of *ras* genes peaking in late G1-phase [13–15]. Therefore, the results presented here with the inducible *ras* genes could be explained if the overexpression of a *ras* gene provokes the transit from quiescence to proliferation which in turn would increase the expression of the other two *ras* genes. However, the data do not exclude the possibility that the overexpression of a particular *ras* gene induces the expression of the other members of the *ras* family through a pathway independent of cell cycle progression. To explore this latter possibility we induced Ras expression by dexamethasone in 3T3-DxHT cells previously treated with hydroxyurea, a DNA synthesis inhibitor. In these conditions, cell cycle progression is abolished. As shown in Fig. 7, an increase of N- and K-*ras* mRNA is detected 12 h after hormone addition in the absence of serum and in the presence of hydroxyurea. As expected, DNA synthesis was undetected by thymidine incorporation assays (not shown). Although this possibility cannot be formally excluded, the former results are consistent with the idea that H-Ras protein can induce the expression of the other two *ras* genes by a mechanism not linked to DNA synthesis and cell cycle progression.

Many animal and human tumors with activation of *ras* genes show overexpression of mutated [27–29] or normal *ras* genes [30–33]. In the present work we have found an autoregulatory positive effect on the expression of the *ras* gene family. To our knowledge this is the first report of a regulatory interaction between *ras* genes. It is noteworthy that this effect has been observed both for mutant and normal N-*ras* genes, as well as for H-*ras* oncogene. Thus, these results support the hypothesis that the overexpression of a *ras* gene in tumors may contribute to carcinogenesis by increasing in turn the expression of the other *ras* genes.

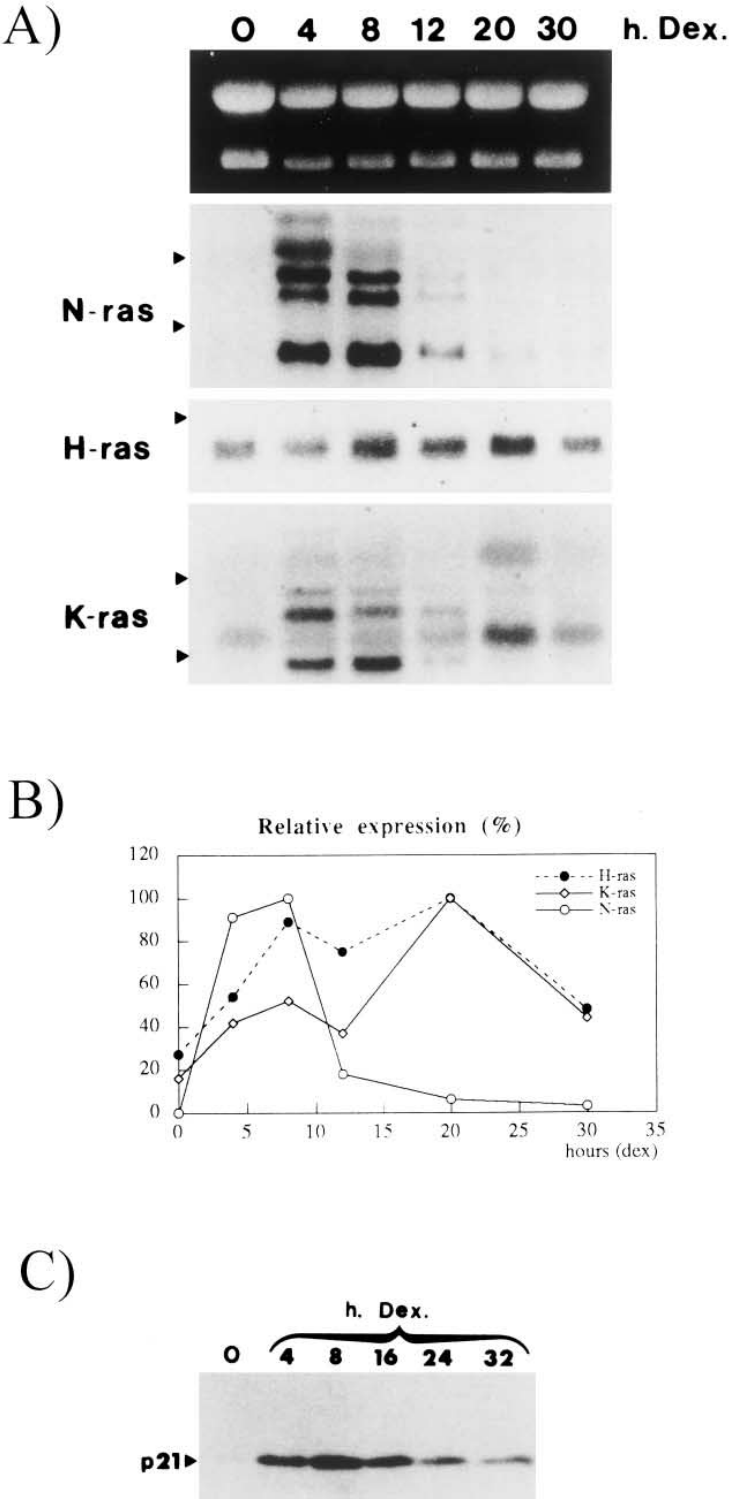

Fig. 5. A: Expression of the *ras* genes in quiescent 3T3-DxNT cells after dexamethasone addition. The experiment was performed as described in the legend to Fig. 1. Exposure times were 6 h for the N-*ras* hybridization and 5 and 7 days for H- and K-*ras* respectively. The vector used expressed several N-*ras* transcripts and the signal of the most abundant are also present in the K-*ras* hybridization, due to an incomplete removal of the signals after the hybridization with N-*ras* probe. B: Relative expression of the *ras* genes determined by densitometry, as indicated in the legend to Fig. 1. C: Accumulation of p21-N-Ras protein after the addition of dexamethasone to 3T3-DxNT determined by immunoblot with a specific anti-N-Ras antibody. The right lane contains bacterially expressed p21(Val-12) as a positive control. The mobility of this protein is lower than N-Ras.

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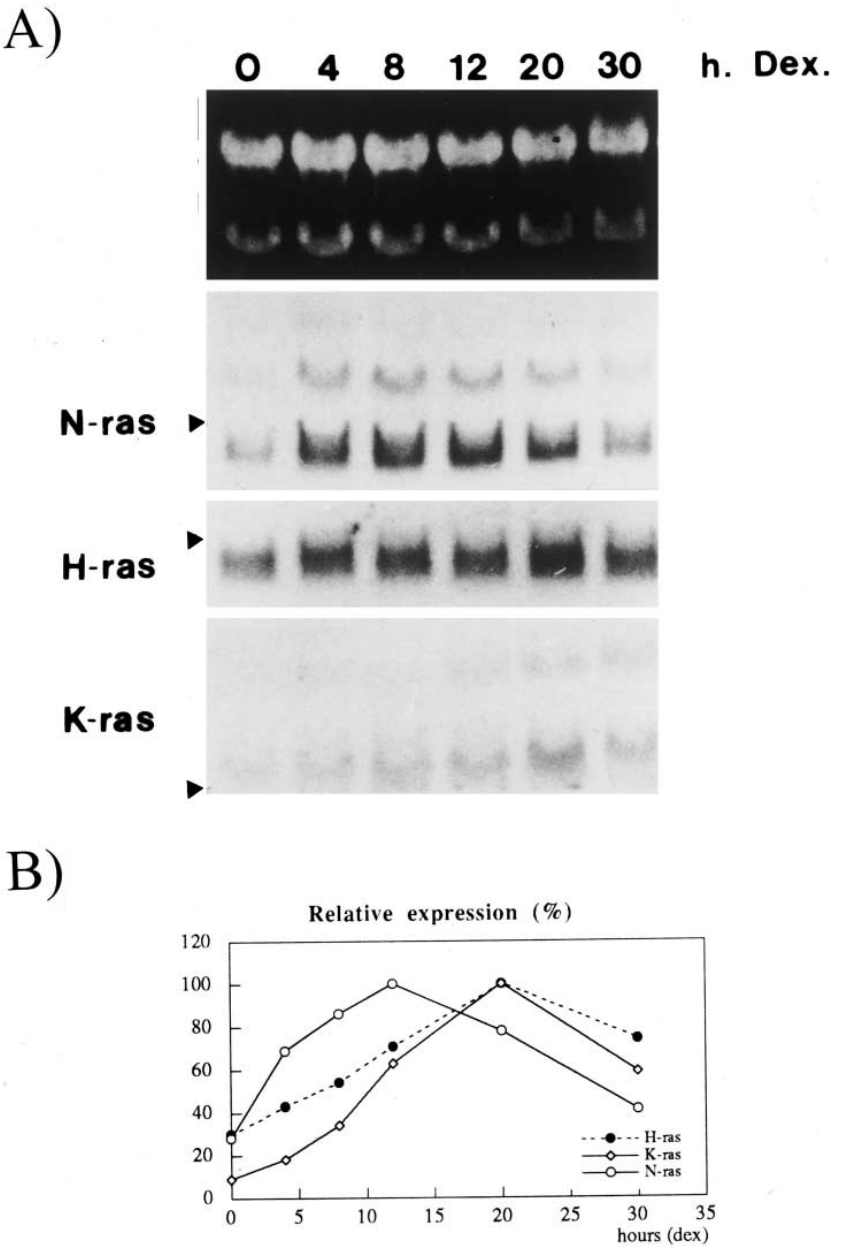

Fig. 6. A: Expression of the *ras* genes in quiescent 3T3-DxNN cells after dexamethasone addition. The experiment was performed as described in the legend to Fig. 1. Exposure times of the filters were 8 h for the N-*ras* hybridization and 4 and 5 days for H- and K-*ras* respectively. B: Relative expression of the *ras* genes determined by densitometry as indicated in the legend to Fig. 1.

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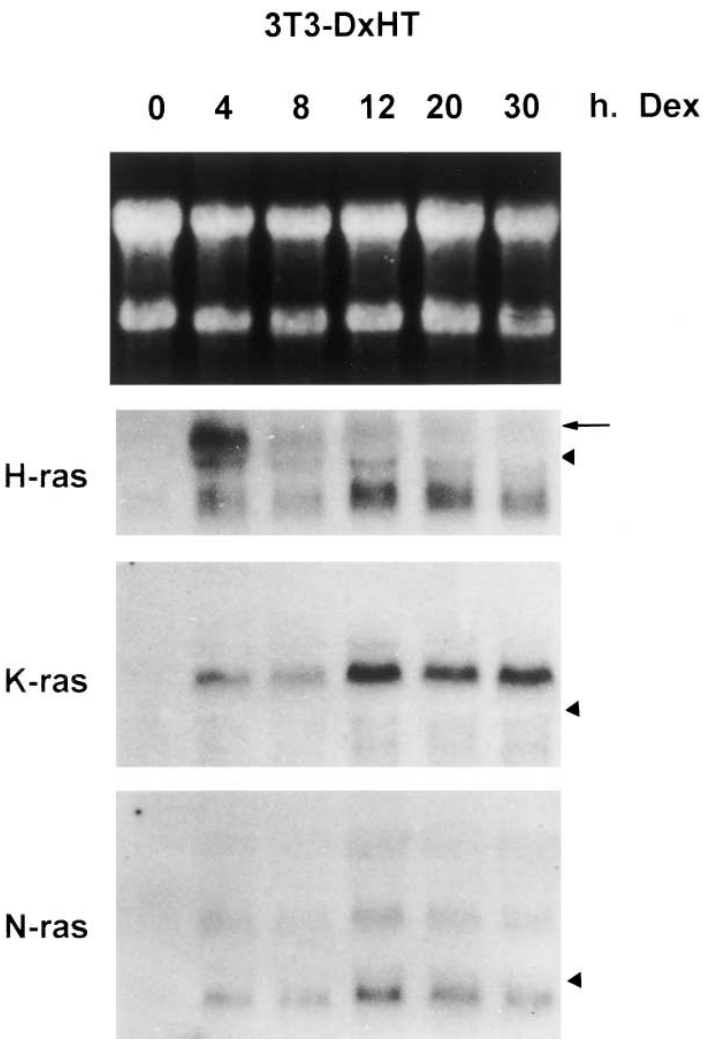

Fig. 7. Expression of *ras* genes in NIH3T3-DxHT cells in the presence of hydroxyurea after dexamethasone addition. Confluent cells were incubated for 18 h with DMEM supplemented with 0.5% CS. At this point hydroxyurea was added to 3 mM final concentration and the cells were incubated for another 18 h. Dexamethasone to 0.5 μM final concentration was then added and RNA samples prepared at the indicated times. Expression of the *ras* genes was determined by Northern blot as indicated in the legend to Fig. 1. The arrow marks the position of the main exogenous H-*ras* mRNA.

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