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Abstract: The effects of the N-*ras* oncogene on the transcription levels of c-*myc*, p53, c-K-*ras* and β-actin genes have been investigated by Northern blotting in a cell line which normally differentiates into myotubes. Treatment of cells with dexamethasone activates N-*ras* gene, then cells proliferate and form foci. The transcription level of c-*myc* gene was generally high during transformation whereas c-K-*ras* mRNAs levels were high during differentiation. Following induction of the N-*ras* oncogene, the level of c-myc mRNA first decreased in two days and then increased in three and four days. During differentiation, c-K-*ras* mRNA decreased in three days and increased following day. The level of β-actin mRNA was slightly lower during transformation, and there were no changes in the mRNA levels of p53 and c-K-*ras*. An increase in the level of c-*myc* mRNA in four days of differentiation when myotubes form, shows that expression of c-*myc* may not have an effect after this critical period of differentiation.

Key Words: oncogene, myoblast, ras, myc, differentiation.

Introduction

Expression or cooperation of cellular oncogenes, such as *ras*, p53, c-*myc* and c-*fos* were implicated in several human cancer (1,2). The mechanisms of transformation of cells by oncogenes also involved altered function of these regulatory oncogenes by stimulating or repressing their expression (3,4).

Transformation of fibroblast cells by v-abl oncogene involved overexpression of the c-*myc* (5) or of the cellular protein p53 (6). Two proto-oncogenes, c-*myc* and c-K-*ras*, have been shown to cooperate in the transformation of secondary rat embryo fibroblasts (7). Oncogenic activation of c-*myc* occurs mainly through constituve and elevated expression of its gene product and generally requires cooperation with at least one additional oncogene, such as *ras* or bcl-2 (8,9).

The family of *ras* oncogene proteins (ras p21) is located on the inner side of the plasma membrane, and activated by growth factors, therefore they appear to act as a molecular switch in signal transduction from growth factor receptors on the cell surface to effector molecules (10, 11). It is suggested that *ras*-activated kinases (MAP kinases) phosphorylate transcription factors and thereby modify their ability to induce gene expression (12).

Transformation of cells by *ras* oncogenes 1. reduced growth factor requirement for their proliferation (13); 2. increased the rate of abnormal mitosis and disorganization of actin; 3. lost their anchorage dependency and developed membrane ruffling with extensive microvillli on their plasma membranes (14,15); 4. diminished their ability to response PDGF stimulation (16,17); and 5. caused alterations in the expression of cellular gene products (18). The expression of c-*myc*, TGF-α and β, transin, glucose transporter genes (19), transcription factor genes jun B and c-jun (20), and *max* (myn in murine cells) were increased after activation of the *ras* gene (21).

Taken together, these observations suggest that alterations of additional proto-oncogenes such as c-*myc*, c-K-*ras* and p53 may play some role in differentiation or in transformation events mediated by activated oncogenes. By transfecting a mouse muscle cell line with a plasmid containing a mutationally activated human N-*ras* gene under transcriptional control of the steroid sensetive promotor of the mouse mammary tumour virus long terminal repeat (MMTV-LTR) as described by Gossett et al. (22), we have looked at the role of this gene in the transcription levels of a few cellular genes such as c-*myc*, p53, c-K-*ras* proto-oncogenes and β-actin genes during transformation and differentiation of CO25 myoblast cells.

Materials and Methods

Cells

The CO25 cells (donated by Dr. E. N. Olson, University of Texas) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Feotal calf serum (FCS) (Gibco Laboratories), 1%L-glutamine and penicillin/ streptomycin at 100 units/ml.

To initiate differentiation, cultures at ~80% confluence were transferred to the same medium containing 10% Horse serum (HS) (Gibco) as a fusion promoting medium, instead of FCS. The cells were incubated for four days, at which times fusion and formation of myotubes were observed.

To induce the expression of the N-*ras* oncogene, the cells maintained in the 10% HS were exposed to 1μM dexamethasone for various periods of time.

Isolation and Analysis of RNA

Extraction of total cellular RNA was performed by the method described in Chomczynski and Sacchi (23). Samples were resuspended in 0.5% sodium dodecyl sulphate (SDS) and stored at -20°C.

RNA samples were denaturated and electrophoresed as described in Maniatis et al. (24). Twenty μg of total RNA was electrophoresed through a 1% agarose gel containing 40 mM morpholino-propanesulfonic acid, pH 7.0; 10 mM sodium acetate; 1 mM EDTA, pH 8.0; 6% formaldehyde after addition of 1μg/ml Ethidium bromide. RNA was transferred on to nitrocellulose filter (Bio- Trace N.T. Gelman Sciencies) and hybridized with each probe. The gel and the filter were checked under U.V. after transfer in order to assess the efficiency of the transfer.

Prehybridization, hybridization and washes were performed as described in Williams and Mason (25). The filter was exposed to a pre-flashed Kodak X-OMAT X-ray film with an intensifying screen for different periods of time at -80°C. The filters were reused by removing the probe in DEPC treated distilled water at 100°C for 5-10 minutes or until it cooled to room temperature and hybridization was performed by the same method above.

Densitometric scans: We analysed the density of the image of the autoradiograms with a Transmittance/Reflectance scanning densitometer (Bio. Tech. Lt.).

DNA Probes

Probes were used for measurement of individual mRNAs were as follows: c-*myc*, a 2.3 kb Eco RI fragment from pUC plasmid containing full-length human c-*myc* gene (26); K-*ras*, a 1.1 kb Pst I fragment from human cDNA (Amersham) (27); p53, a 1.8 kb Xba I fragment of human cDNA (28); β-actin, a 1.15 kb Pst I fragment from pBR322 plasmid containing the mouse β-actin gene (29); and rRNA, a pXLrI0I plasmid (a gift from Dr. W. James, Oxford) containing the whole transcription unit from Xenopus.

Probes were isolated from a low melting temperature agarose (LMT) gel and labelled using a Random Primer Kit (Stratagene) by the method of Feinberg and Vogelstein (30).

Results

To investigate whether *ras*-dependent inhibition of myogenic differentiation was accompanied by effects on normal cellular gene expression including the proto-oncogenes c-*myc*, p53, c-K-*ras* and the structural gene β-actin, cellular RNAs were extracted from CO25 cells cultured in 10% HS with or without 1μM DEX for various times. Samples were analysed by Nothern blotting as described in the Materials and Methods.

C-*myc* mRNA levels

To examine the levels of c-*myc* mRNA in CO25 cells grown in the presence or absence of DEX, appropriate samples were assayed using a 2.3 kb Eco RI fragment from pUC plasmid containing full-length human c-*myc* gene as a probe.

C-*myc* mRNA bands were recognized above an 18 S rRNA marker (Fig.1.b). During differentiation of CO25 cells growing in 10% HS, the c-*myc* mRNA levels fell in 2 days, disappeared by 3 days, then began to reaccumulate and returned to the basal level by 4 days. During transformation of CO25 cells growing in the presence of DEX, the levels of c-*myc* mRNA were high on day 1, a slight decrease was observed in 2 days, a rise was observed in 3 days and finally it returned to the basal level in 4 days. In these cells, c-*myc* mRNA levels were generally higher than in the cells grown in the absence of DEX. The decrease in mRNA levels occurred in 2 days in both control and DEX treated cells. Thus, initiation of the differentiation programme was accompanied by a decline in the c-*myc* mRNA level, and a return to the basal level dur-

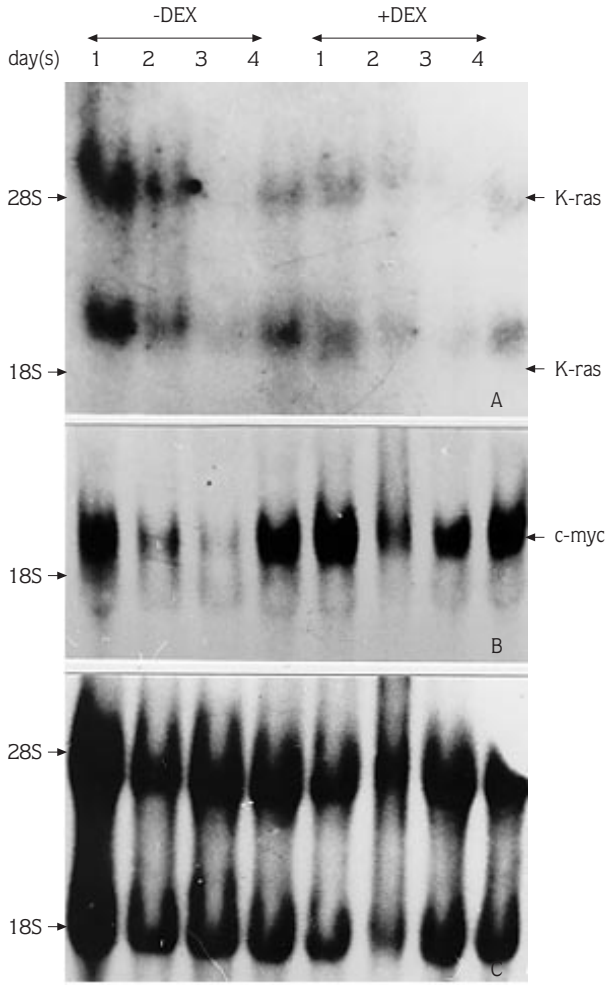

Figure 1. Northern blot hybridization analysis of c-K-ras mRNA and c-myc mRNA levels in CO25 myoblasts treated or untreated with DEX over 1-4 days. 20 µg of cytoplasmic RNA was analysed as described in the Materials and Methods. The migration of rRNA size markers is indicated.
Autoradiogram of c-K-ras mRNA levels, exposure time is 48 hours. (a); c-*myc* mRNA, the same blot was reprobed with c-*myc* after stripping of the c-K-*ras* probe, exposure time is 96 hours (b); and rRNA showing total RNA levels for each track, the blot was stripped from the c-*myc* probe and then reprobed with an rRNA probe, exposure time is 3.5 hours (c).

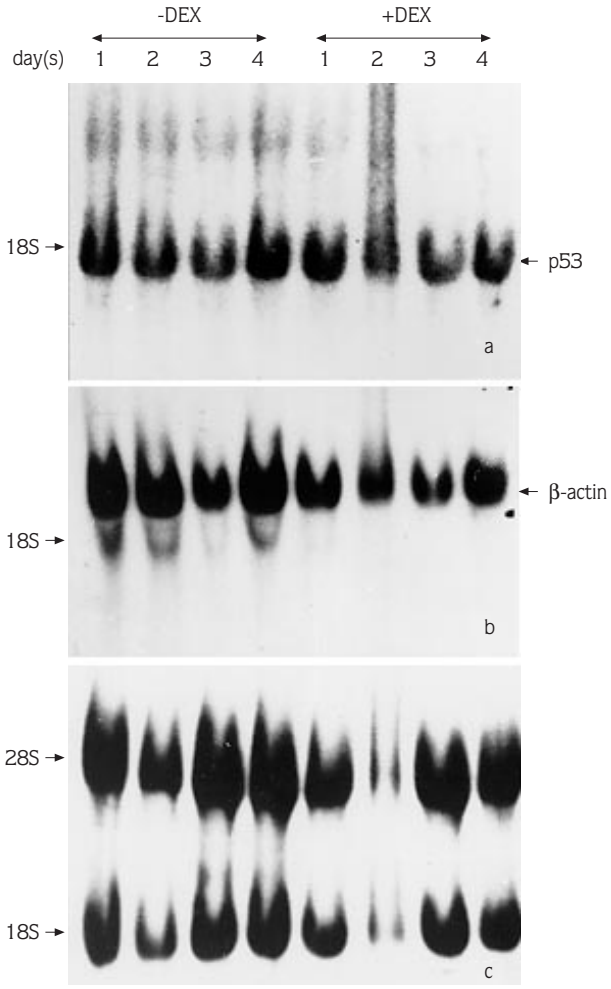

Fig.ure 2. Northern blot analysis of p53 mRNA and β-actin mRNA levels in CO25 myoblasts treated or untreated with DEX over 1-4 days. 20 µg of cytoplasmic RNA were analysed. The migration of rRNA size markers is indicated.
Autoradiogram of p53 mRNA levels, exposure time is 19 days (a); β-actin mRNA levels, the same blot was reprobed with β-actin after stripping of the p53 probe, exposure time is 6 hours (b) and rRNA showing total RNA levels for each track, the blot was stripped of from the β-actin probe and then reprobed with an rRNA probe, exposure time is 3.5 hours (c).

ing terminal differentiation after 4 days growth. However, during transformation, c-*myc* mRNA levels increased and remained at this level.

c-K-*ras* mRNA levels

The c-K-*ras* mRNA levels analysed in CO25 cells grown in the presence or absence of DEX as described in the Materials and Methods.

A c-K-*ras* probe, 1.1 kb Pst I fragment from human cDNA, detected two mRNA bands on the blot containing the RNA samples (Fig.1.a). Similar transcription patterns of the c-K-*ras* gene have been reported by other workers (31). Both transcripts of c-K-*ras* appeared at a similar level in each sample. During differentation and transformation of CO25 cells, the expression of the c-K-*ras* gene revealed different levels. c-K-*ras* mRNA levels were decreased in 2 days

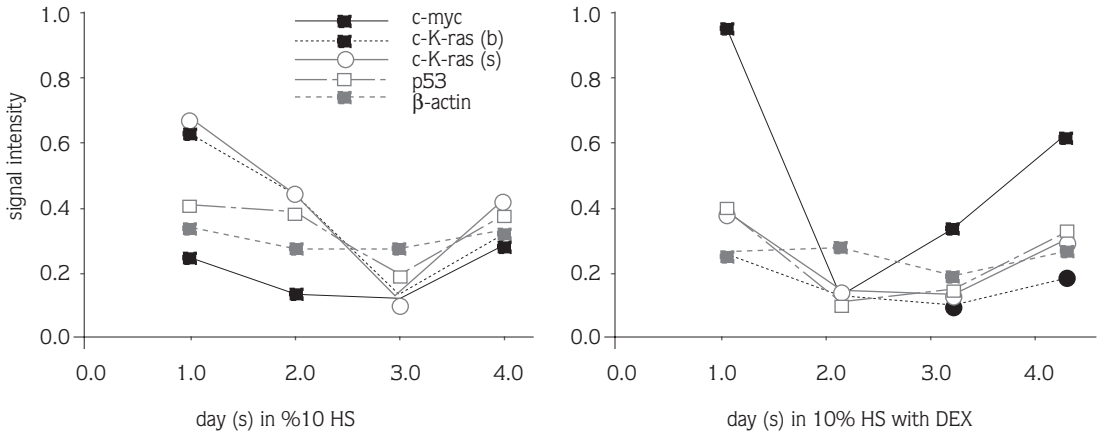

Figure 3. Comparative levels of RNA species during differentiation (a) or transformation (b) of CO25 cells. Autoradiograms were scanned with a Transmittance/Reflectance scanning densitometer (BIOTECN.Lt.). The peaks were integrated and graphically expressed in arbitary units which are ratios of the intensity of mRNA/rRNA. (b), large size; (s), small size K-ras.

and disappeared in 3 days. They then rose in 4 days to 50% of the initial level during cell differentiation. In the transformed cells, the levels reached 80% of the initial levels. However, the levels of both c-K-*ras* mRNAs were generally high during differentiation.

p53 mRNA and β-actin mRNA levels

A p53 probe in a 1.8 kb Xba I fragment of human cDNA was used to analyse the level of p53 mRNA. A single mRNA species was detected and it migrated just ahead of the 18S rRNA marker (Fig.2.a) as previously reported by Reich and Levine (32). We have found that CO25 cells express a small amount of p53 mRNA as seen in Fig.5.a, needing a very long exposure time when compared with exposure times of other blots.

During the differentiation and transformation of CO25 cells, there was no significant change in the levels of p53 mRNA, expressed at the similar levels during these processes. However, a very small decrease occurred in 3 days and increase in 4 days similar to the c-*myc*, c-K-*ras* and β-actin mRNA levels.

As shown in Fig.2.b, a probe for β-actin, a 1.15 kb Pst I fragment from a pBR322 plasmid containing the mouse β-actin gene detected a single band just above the 18S rRNA marker. After stripping off the p53 probe, the blot was reprobed with β-actin as described in the Materials and Methods. The levels of β-actin mRNA decreased slightly in 2-3 days, then returned to the initial level at 4 days during both the differentiation and transformation of CO25 cells. However, the β-actin mRNA levels were generally higher in differentiated cells than in the transformed.

Discussion

Induction of cell proliferation, transformation and differentiaiton appear to be mutually exclusive phenomena in various differentiating cell systems, including myogenic cells (22,33). In various cell, c-*myc* mRNA, c-K-*ras* mRNA, p53 mRNA and β-actin mRNA are induced at an early stage when quiescent cells stimulated to proliferate in response to growth factors, whereas their cytoplasmic levels decrease markedly in terminally differentiated cells (3,32,34-36). These observations suggest that a high level expression of these genes is necessary for the initiation of DNA synthesis and cell proliferation, and also their down-regulation is involved in the induction of specific genes for differentiation.

The relative transcription levels of protooncogenes, c-*myc*, c-K-*ras*, p53 and β-actin, which have been observed in this work were summerized in graphs as shown in Fig.3.a and b.

c-*myc* mRNA levels

The c-*myc* oncogene is expressed in the majority of proliferating normal cells, and altered expression of this gene has been implicated in the genesis of a wide variety of tumors (37). Also it is known that c-*myc* phosphoprotein heterodimerizes with Max and regulates differentiation and programmed cell death (apoptosis) beside cell proliferation (8,38). Induction of c-*myc* mRNA expression had an antimitogenic effect during differentiation of adrenocortical cells (39).

The results presented here demonstrate that the c-

myc mRNA decreased within 2 days and disappeared following 3 days after culturing CO25 myoblast cells in the fusion promoting medium (Fig.1.b). This decrease in c-*myc* expression seems to be required for the initiation of differentiation. After this critical period myotubes form within four days, and an increase in c-*myc* mRNA does not inhibit differentiation. This observation is in agreement with the results of Endo and Nadal-Ginard (34) who showed that c-*myc* transcription was regulated independently of muscle-specific gene (Myosin heavy chain) transcription. Our observation is also consistent with the results of Leibovitch et al. (3) and Denis et al. (33) who showed that in myogenic subclone lines, c-*myc* is expressed at high levels.

Previous studies involving the expression of exogenous c-*myc* constructs suggest that the rapid suppression and subsequent reexpression of c-*myc* may be involded in determining the kinetics of commitment to terminal differentiation (33,36). The expression of mck, Ach receptors (40) and functional sodium and calcium channels which are characteristic of skeletal muscle cells (41) can coexist with the c-*myc* expression.

Endo and Nadal-Ginard, (34) reported that insulin and IGF-1 induce c-*myc* mRNA in the quiescent myogenic cells L6E9-B. It has been shown that the levels of growth factors IGF-I and IGF-II, and their receptors dramatically increased during differentiation of C2 myoblasts (42,43). For that reason, c-*myc* reaccumulation by 4 days of differentiation may be regulated by an autocrine mechanism and involves the expression of growth factors and their receptors at this stage in the differentiation process.

The induction of the mutated N-*ras* oncogene in CO25 myoblast cells following treatment with DEX, caused an increase in the expression levels of c-*myc* gene (Fig.1.b). However, a small decrease by 2 days occurred similar to the decline observed during the differentiation of CO25 cells, in the absence of DEX. In contrast with the disappearence of c-*myc* mRNA in 3 days during differentiation, c-*myc* mRNA was reaccumulated in 3 days and was expressed at the same level in 4 days in the N-*ras* induced cells. The reason for this decline in these cells in 2 days may be dependent on serum deprivation as found during the initiation of differentiation. At the later stages, the presence of increased *ras* p21 levels may overcome the effects of low serum conditions and interfere with the overexpression of c-*myc* mRNA. Thus the elevated expression of the c-*myc* gene in 3 days may have a negative effect on the initiation of differentiation, leading instead to the transformed state.

The increase in the level of c-*myc* mRNA after induction of the N-*ras* oncogene is in agreement with the results of Olson et al. (35). They showed that c-*myc* mRNA declined in abundance during differentiation of C2 cells and that activated H-*ras* and N-*ras* oncogenes prevented the normal decline in this mRNA species. But, they obtained their results from the cells which had been grown for 5 days in the presence and in the absence of DEX, and did not analyse the levels of c-*myc* expression from day 1.

Taken together, these results suggest that a decrease in c-*myc* expression in CO25 myoblasts must occur at an early stage to allow differentiation, and activation of the N-*ras* oncogene inhibits the decline of c-*myc* expression, but does not inhibit its initial decline at the early stages of transformation process, and confirms the important role of c-*myc* in the transformation-differentiation switch.

c-K-*ras*, p53 and β-actin mRNA levels

An early decline was observed in the levels of c-K-*ras*, p53 and β-actin mRNAs in 2-3 days during both differentiation and transformation stages of CO25 cells.

In the *ras*-induced cells, reaccumulation of c-K-*ras* mRNA reached 80% of the initial 1 day level. In differentiating cells it reached 50% of the same level. In contrast, Leibovitch et al. (3) showed that c-K-*ras* mRNA declined and became negligible throughout the process of L6a1 myoblast cell differentiation whilst the level of c-N-*ras* mRNA rose. The changes in c-K-*ras* expression during CO25 differentiation and transformation were in general agreement with the findings of Campisi et al. (31). They reported that the level of c-K-*ras* mRNA decreased after differentiation of F9 teratocarcinoma stem cells to endoderm. Both transcripts of this gene were elevated in transformed fibroblasts. They did not report any reaccumulation of c-K-*ras* mRNA during the differentiation processes of these cells. The levels of p53 and β-actin mRNA showed similar changes during differentiation and transformation of CO25 cells. Their mRNA trancripts decreased generally by day 3 and reaccumulated by day 4. The early changes in c-*myc* and p53 protein levels have been reported by Richon et al. (36) and they declined during the differentiation of MEL cells. Similar results were obtained Lachman and Skoultchi

(44) showing a decline in both β-actin and histone H3 mRNA. Olson and Capetanaki (45) reported that β-actin was downregulated in C2 myoblast differentiation, whereas oncogenic H-*ras* transfection of these cells prevented this downregulation as reported similar results by Leibovitch et. al. (3). The results here contrast with the observation since we showed a slight decrease in the levels of β-actin mRNA after induction of the N-*ras* oncogene.

It should be noted, however, that the type of RNA analysis used here measures steady-state levels. We cannot therefore distinguish whether the dramatic dissappearance of these mRNAs and their reappearance are the result only of a modulated rate of transcription or whether it reflects also modulation of the mRNAs stability.

These results reported here suggest that there may be a specific time in the differentiation process during which down-regulation of c-*myc*, c-K-*ras*, p53 and β-actin genes is necessary for commitment to terminal differentiation, and that once this stage is passed, reexpression cannot reverse the commitment process. An activated N-*ras* gene by a mutation of a regulatory domain can prevent the down-regulation of some genes, eg. c-*myc* in the absence of mitogens. This may be regulated by an indirect mechanism which may involve phosphorylation of a factor(s) capable of regulating transcription of these genes. Thus the oncogenic form of N-*ras* p21 may transform cells by constitutively activating early response proto-oncogenes such as c-*myc*.

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