A transient increase of snoN transcript by growth arrest upon serum deprivation and cell-to-cell contact

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Abstract To analyze the possible involvement of c-ski and c-sno during the course of in vitro myogenesis, expression of their transcripts during differentiation of a murine muscle cell line (C2C12) was monitored by competitive reverse transcription-polymerase chain reaction (RT-PCR). The transcripts of c-snoN were temporarily increased 25-fold above basal level at 12 h prior to the onset of transcription of muscle-specific gene, e.g. myogenin and muscle creatine kinase, whereas c-ski was expressed invariably. The transient increase of c-snoN was blocked when myogenesis was interrupted by the presence of fetal calf serum in culture medium, probably due to growth factors being included; basic fibroblast growth factor (b-FGF) blocked the transient increase whereas epidermal growth factor (EGF) did not, consistent with the inhibitory effect of b-FGF and no effect of EGF on myotube formation of C2C12. In fibroblastic C3H10T1/2 cells, snoN exhibited a similar transient increase of transcript when growth arrested under the same conditions as for in vitro myogenesis, indicating that the expression of snoN is not sufficient to induce the onset of muscle differentiation and an unknown factor involved in myogenic cells is necessary. The transient increase of snoN transcript may represent a common entrance step of cells into the G0 phase where muscle differentiation is substantiated, considering that it was observed upon growth arrest of fibroblastic C3H10T1/2 cells and prior to the elevation of MCK in C2C12 but undetected when entry into G0 was blocked by b-FGF.

Key words: c-ski; c-snoN; Growth arrest; G1/G0 phase; EGF; b-FGF; In vitro myogenesis

1. Introduction

The nuclear oncogene, v-ski was isolated from the Sloan-Kettering viruses (SKV) which are acute transforming avian retroviruses[1]. The 1.3 kb v-ski cDNA encodes a truncated form with deletion of both the N- and C-termini of the cellular counterpart, c-ski [2]. Like v-ski, c-ski and c-snoN (ski-related gene) induce not only morphological alteration and anchorage-independent growth but also terminal muscle differentiation in quail embryo cells when overexpressed [3–5], although c-ski and c-snoN mRNAs are not exclusively expressed in muscle cells but widely expressed in many different tissues [6,7]. The muscle differentiation induced by overexpression of c-ski accompanies the induction of MyoD and myogenin expression and fusion of myoblast to form multi-nucleated myotubes [8]. The hypertrophic effect on the skeletal muscle of transgenic mice by a transgene of a truncated c-ski cDNA also suggests the possible involvement of ski in the process of myogenesis [9]. However, little is known about the physiological implication of c-ski and c-snoN in the prolonged process leading to myogenesis.

In vitro myogenesis provides a suitable model for analysis of the regulatory mechanism controlling the antagonism between cell growth and differentiation. Entry into quiescent state (G0) is necessary for myogenic cells to express muscle-specific proteins [10]. MCK expression during myogenesis could be, therefore, regarded as a molecular marker indicating that the cells are present in the G0 portion [10].

The aim of this study was to examine whether c-ski or c-snoN regularly participates in the process leading to muscle differentiation. The mRNA levels of c-ski and c-snoN were monitored during in vitro myogenesis of the skeletal muscle cell line C2C12 and in a quiescent state of fibroblast C3H10T1/2 cells. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect low abundance of the c-ski and c-snoN mRNAs. Analysis revealed that the amount of c-ski mRNA remained at a constant level throughout the myogenic process, while that of c-snoN mRNA increased temporarily prior to the onset of transcription of myogenin and muscle creatine kinase, followed by rapid down-regulation to basal level. b-FGF suppressed the transient expression of snoN and myotube formation of C2C12 cells whereas EGF suppressed neither. Consequently, transient expression of snoN was observed prior to the expression of MCK (G0 marker) in differentiation-induced C2C12 and growth-arrested 10T1/2 cells but was lost when entry into G0 was blocked by b-FGF. The signal mediating the G1 to G0 transition remains unknown. The possible involvement of snoN in the G1 to G0 transition will be discussed.

2. Materials and methods

2.1. Cell culture and growth factors

The C2C12 [11,12] and C3H10T1/2 cells were maintained and differentiated as described elsewhere [13]. EGF (Upstate Biotechnology Inc.) and b-FGF (Austral Biologicals) were added to differentiation medium at 10 nM where indicated.

2.2. RNA isolation and Northern blot analysis

Total RNA was isolated and Northern blot analysis was performed as described [13–15]. 20 μg of glyoxalated RNAs were electrophoresed and transferred to Hybond N+, a positively charged nylon membrane (Amersham). The following probes were used for hybridizations: (1) for the mouse muscle creatine kinase (MCK) cDNA probe, a 1143 bp *BalI*/*BalI* fragment from pMCKm36 [16]; (2) for the mouse MyoD1 probe, an about 800 bp *HpaII*/*Eco*RI fragment encoding the 3′ end

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Abbreviations: EGF, epidermal growth factor; b-FGF, basic fibroblast growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; MCK, muscle creatine kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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sequence of pEMC11s cDNA [17]; and (3) a rat myogenin probe generated by *Pst*I digestion of rat myogenin cDNApBSm13-MGN#11 [18]. cDNA probes were radiolabeled to a specific activity of 5×10^8 cpm/μg with [α-^{32}P]dCTP (10 μCi/μl, Amersham) by the random priming method [19].

The probes were stripped from the membranes for reuse by boiling for 20 min in 0.01×SSC 0.1% SDS. Filters were exposed to an imaging plate of a BAS2000 system (Fuji Film Co.) and quantified.

2.3. RT-PCR and DNA sequencing

Oligonucleotide primers specific for human c-ski, snoN, snoA [7], and mouse c-ski and snoN were prepared on a DNA synthesizer (Applied Biosystems, model 381A). The primers employed were the following oligonucleotides: 5′ oligo, 5′-GAGGTGGAAGTTGAAAGCAGG-3′ and 3′ oligo, 5′-TCATGCAGGAACTTCTCTTTGG-3′ for human ski; 5′ oligo, 5′-GAGAAGTTTAGCATGAGAAGTGG-3′ for human snoA and snoN; 3′ oligo, 5′-GTTCCTCAAGTGAGACATCTGG-3′ for human snoN and 3′ oligo, 5′-CTGCAATACAAATATATCAATCGG-3′ for human snoA. The mouse primers were as follows: 5′ oligo, 5′-TTGTCCTCGCTGTCCTCTCCG-3′ and 3′ oligo, 5′-TGCCGCAGGTGCTCCAACTCT-3′ for mouse ski; 5′ oligo, 5′-GAGAATCCAATCAAAGACAGATACAC-3′ and 3′ oligo, 5′-GCTGACTACTCTCATGAGGCTT-3′ for mouse snoN. 1 μg of total RNA from the indicated culture was reverse-transcribed using Molony murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL) and random hexamer primers. An aliquot of the first strand of cDNA was amplified using Amplitaq DNA polymerase (Perkin Elmer) in a Programmable Incubator (Rikoh Kagaku Lab., Inc). The reaction conditions were as follows: one cycle of denaturation for 10 min at 94°C, 35 cycles of amplification (1.7 min denaturation at 94°C, 1.7 min primer annealing at 60°C and 1.8 min polymerization at 72°C for each step) and extension at 72°C for 10 min.

For competitive PCR, competitor DNA was constructed from snoN-PCR product (325 bp) derived from C2C12 cells by insertion of an irrelevant short stretch of nucleotides (185 bp, intervening sequence obtained from the β-lactamase gene carried by pBluescriptII KS(+) plasmid). PCR-mediated amplification of snoN using specific primers generated the 257 bp DNA fragment from endogenous template and the 442 bp fragment from competitor DNA which was added before the start of the PCR reaction. The ratios of the 257 bp to 442 bp product were compared with the time course of in vitro myogenesis of C2C12. Amplified DNA fragments were cloned into pBluescriptII KS(+) (Stratagene) for double-stranded sequencing with Sequenase Ver. 2.0 (USB Co.). GAPDH included in cDNA was also determined using a commercially available PCR primer (Clontech Lab.) as an internal control for normalization of snoN products.

3. Results

Since the amounts of the c-ski and c-snoN transcripts were not sufficient to be detected by Northern blot analysis, competitive RT-PCR was applied to detect and analyse the expression profile. To design PCR primers for mouse c-ski and c-snoN mRNAs, the cDNAs of which have not yet been sequenced, known sequences of human counterparts were used for making the putative PCR primer [7]. For prevention of cross-amplification between ski and snoN sequences, the ski- and snoN-specific primers used in this RT-PCR reaction were designed to generate specific portions encoded in exon 6 of c-ski and in exon 1–5 of snoN, respectively. An alternatively spliced form of *sno* gene, snoI mRNA was reported to be accumulated specifically in human skeletal muscle [20]. The specific primers for mouse snoN used in this study were available to detect mouse snoI, but the predicted-sized PCR product from snoI insertion could not be detected as was the case for chicken sno [21]. The sequences of the PCR products using this putative primer and the cDNA template from mouse C2C12 cells were compared with the corresponding sequences of the human counterparts. The DNA sequence homology with human counterparts was found to be 85.4% identical for c-ski and 82.9% for c-snoN, respectively (data not shown). Considering the high score of the identity, the DNA sequences of the PCR products were regarded as representing endogenous mouse c-ski and c-snoN, respectively. These sequences were therefore used to design the authentic primers of PCR for endogenous c-ski and c-snoN in mouse skeletal muscle cell line C2C12 as indicated in Section 2. A transient rise of snoN expression abruptly took place with a peak at 12 h and had disappeared by 36 h after induction of muscle differentiation of C2C12, while ski was being expressed invariably (Fig. 1) and snoA was not detected (data not shown).

Muscle differentiation is blocked by the presence of 10% fetal bovine serum (FBS) in culture medium even after growth arrest at confluence [22]. Along with interruption of myogenesis in the presence of 10% FBS, the specific expression of snoN was also absent while ski remained to be expressed constantly (Fig. 2). Some growth factors included in serum may represent the inhibitory effect on both myogenesis and snoN expression. To assess this possibility, the effects of growth factors of which the receptors have tyrosine kinase activity on the mutinucleated myotube formation of C2C12 and snoN expression were determined. EGF and basic FGF have a typical effect on myoblast fusion (Fig. 3A) and snoN expression (Fig. 3B). When myotube formation was interrupted by b-FGF, snoN ceased to appear. Prevention of myogenesis by b-FGF is due to the block of entry into G0 [10], suggesting that snoN expression may be linked to the G1 to G0 transition. EGF had no effects on either of the two events, myoblast fusion (Fig. 3A) and snoN-expression (Fig. 3B) with a delay in peak formation. Thus, a correlation is suggested to exist between snoN expression and myotube formation.

The fibroblastic cell line C3H10T1/2 is incapable of differentiation toward myogenesis, unless *MyoD* family gene is overexpressed under the strong promoter. When growth-arrested C3H10T1/2 cells at confluence were exposed to the same differentiation conditions as for myogenesis of C2C12, typical snoN expression was observed with a delayed peak at 24 h relative to the case for C2C12 cells (Fig. 4). This result means that snoN expression is insufficient to direct cells toward myogenesis in contrast to MyoD family protein. Some unknown factors downstream of snoN are needed for myogenesis, existing in myoblasts but not in fibroblasts. Furthermore, snoN expression is not exclusively observed in myogenesis but is also evident in the process leading to the quiescent state (G0) of non-myogenic 10T1/2 cells.

The expression profiles of c-ski and c-snoN in the course of in vitro myogenesis of C2C12 cells were compared with those of mRNAs of MyoD, myogenin, MCK and pyruvate kinase detected by Northern blot analysis (Fig. 5). While a housekeeping gene, pyruvate kinase retained a constant expression level in every case examined (data not shown), muscle-specific genes being up-regulated during C2C12 differentiation. MyoD was expressed even in growing cells. By contrast, myogenin transcript was not detected until differentiation had been triggered by the depletion of serum mitogens. A peak in the transient snoN expression emerged prior to the expression peaks of the muscle-specific transcription regulators, myogenin and MyoD as well as muscle-specific marker, MCK which is expressed during G0 [10].

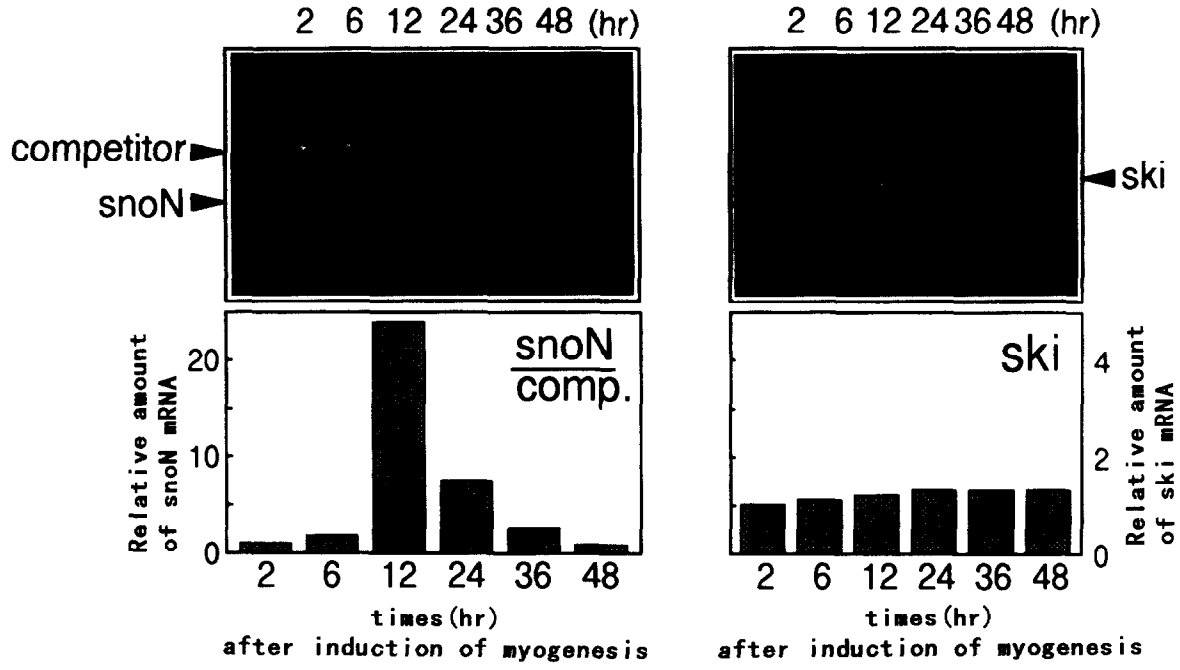

Fig. 1. Expression patterns of mouse endogenous c-ski and c-snoN during C2C12 differentiation. RT-PCR analysis of mouse c-ski and c-snoN expression during differentiation of C2C12 cells. Total RNA (1 μg) was extracted from C2C12 cells harvested at the indicated time of differentiation. cDNAs were obtained by reverse transcription, and then an aliquot was subjected to PCR as described in Section 2. PCR products were analyzed by 6% PAGE (upper panel: left, snoN; right, ski). Molecular weight standard (marker 4, Nippon Gene Co.) was applied on the rightmost and leftmost lanes. To compare m-snoN expression in independent reaction tubes, each amount of m-snoN products normalized with that of control fragment (designated as a competitor). Relative amounts at each time were determined against that at 0 h which is regarded as 1 (lower panel: left, snoN; right, ski).

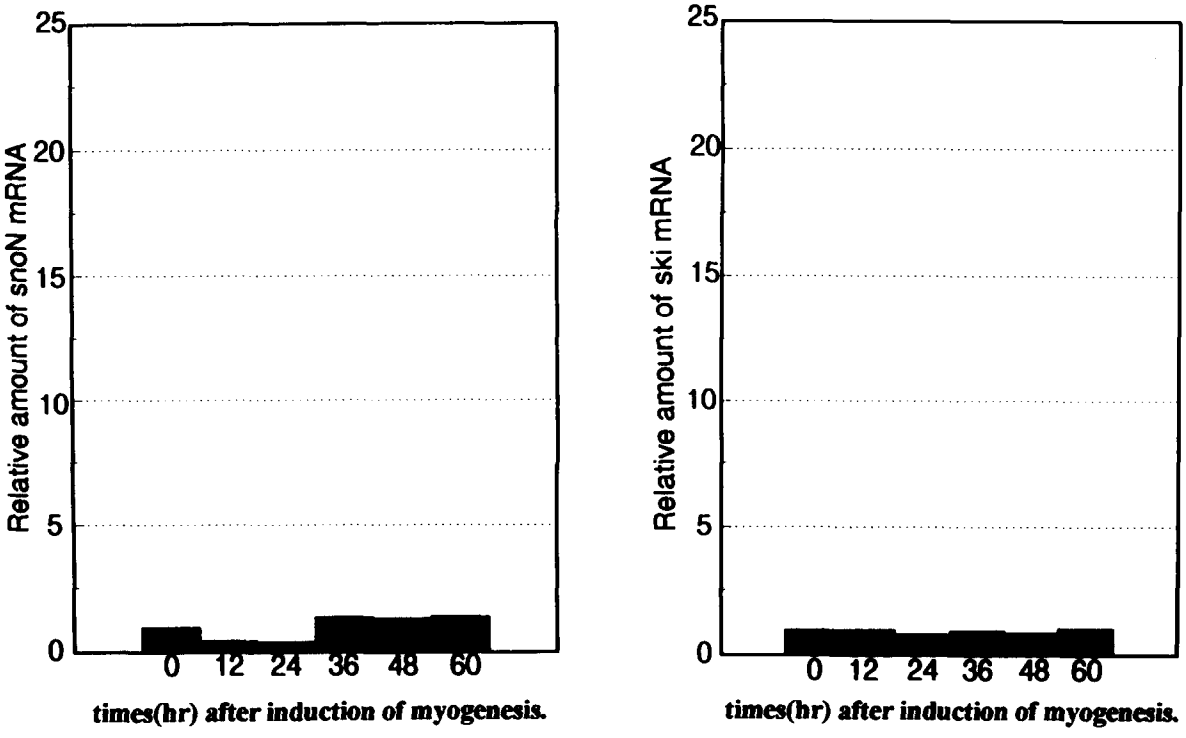

Fig. 2. Expression patterns of endogenous c-ski and c-snoN in C2C12 cells when differentiation was blocked in the presence of 10% FBS. C2C12 cells were allowed to grow to confluent cell density and growth-arrested, then exposed to fresh 10% FBS-containing growth medium (DMEM) in place of differentiation medium. Time (h) after exposed to fresh medium is indicated on the bottom. Competitive PCR was carried out under the same condition as described in Fig. 1 and Section 2. Endogenous mouse GAPDH was amplified by PCR as an internal standard. Each amount of the PCR product of snoN (left) or ski (right) was normalized with those of endogenous GAPDH and competitor DNA (snoN). Relative amounts at each time were determined against that at 0 h which is regarded as 1.

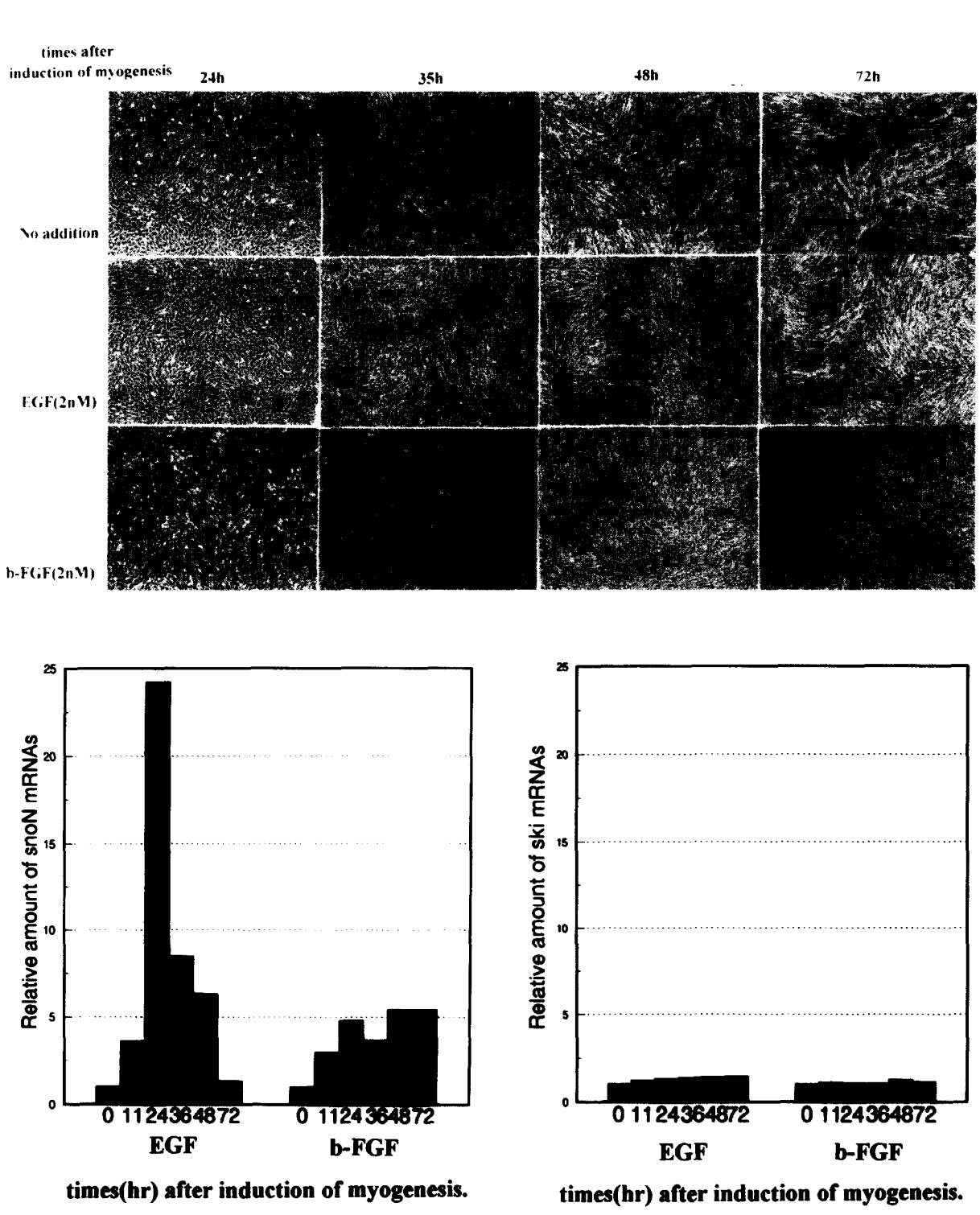

Fig. 3. Effects of growth factors on c-ski and c-snoN expression. After C2C12 cells were allowed to grow to confluent density, culture medium was changed from growth medium to differentiation medium containing 2 nM EGF or 2 nM FGF. (A) Phase-contrast microscopic observations of C2C12 cells in the presence of EGF or b-FGF. Times (h) after exposure to differentiation medium with or without 2 nM b-FGF or 2 nM EGF are indicated at the top. (B) Competitive PCR was carried out and each amount of the PCR product of snoN (left) or ski (right) was normalized with those of GAPDH and competitor DNA (snoN). Relative amounts at each time (hours after exposure to differentiation medium) were determined against that at 0 h which is regarded as 1.

4. Discussion

Although c-ski and the related c-snoN proteins possess activities to induce in vitro myogenesis of fibroblastoid embryo cells [3,4] and a truncated version of the c-ski transgene exerts a hypertrophic effect on skeletal fast muscle in transgenic mice [9], it remains unknown as to how endogenous ski or sno is expressed and functions during differentiation of the cells

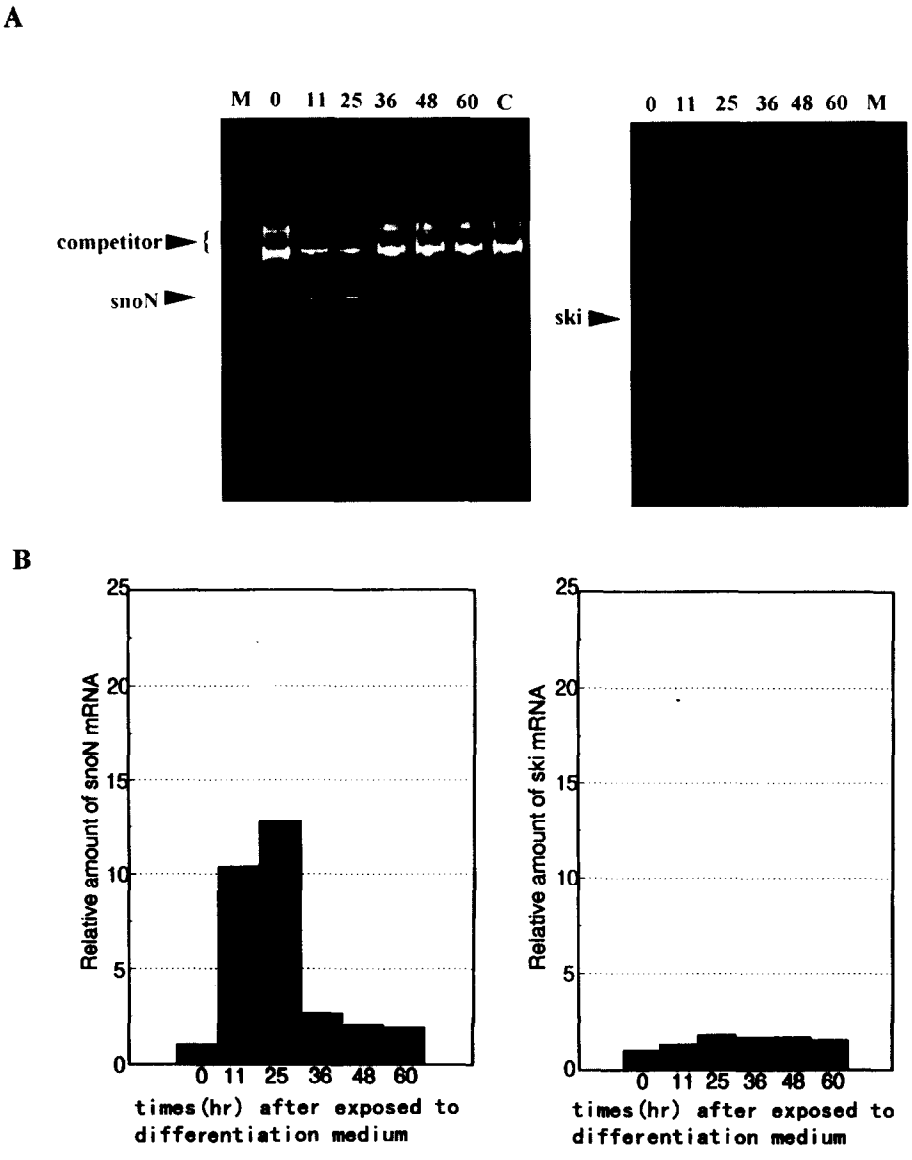

Fig. 4. Expression pattern of endogenous c-ski and c-snoN in C3H10T1/2 cells when exposed to differentiation condition. C3H10T1/2 cells at confluent density were exposed to differentiation condition. (A) Competitive RT-PCR was carried out using total RNA extracted from C3H10T1/2 cells harvested at the indicated time and analysed by 6% PAGE. The numbers at the top of the gel denote times (h) after the cells at confluent density were exposed to differentiation condition. Lane M, molecular weight standard; lane C, PCR products from competitor DNA alone. PCR product of competitor DNA showed ladder for unknown reason. (B) Each amount of the PCR products of snoN (left) or ski (right) was normalized with those of GAPDH and competitor DNA (snoN). Relative amounts at each time (hours after exposure to differentiation medium) were determined against that at 0 h which is regarded as 1.

naturally destined to become skeletal muscle. A considerable time is required to reach the onset of myogenesis, e.g. it takes 12 h for the expression of myogenin to begin (Fig. 5). Some unknown cryptic process must be included in the delay. This study describes time-dependent alterations of ski and snoN expression during the prolonged process leading to myogenesis of cultured myogenic cells.

By utilizing the RT-PCR technique, a transient increase of snoN mRNA upon serum depletion appeared earlier than the transcriptional regulator genes, e.g. MyoD and myogenin. Such transient expression is observed during phase transition of the cell cycle; cyclins [23], cdc2kinase [24] and c-fos [25] show similar transient expression. Therefore, the transient expression of c-snoN may represent an unknown phase transition state of the process from the growth to differentiation phase. Cells only arrested at G0 but not other points of G1 phase are competent to express muscle-specific protein [10]. The expression of MCK is, therefore, a marker to indicate the arrest of cells at G0 [10]. The expression of MCK as a G0 marker initiated approximately at the peak of snoN expression and did not accumulate maximally until the level of snoN mRNA had returned to basal, suggesting that snoN expression is positioned at the G1/G0 boundary where snoN may transmit a signal to advance the G1 to G0 transition. Exposure of myogenic cells to high concentrations of serum or the purified b-FGF blocked and repressed the progress of the differentiation program but failed to induce cell proliferation [26] since b-FGF functions to induce the exit of cells from the

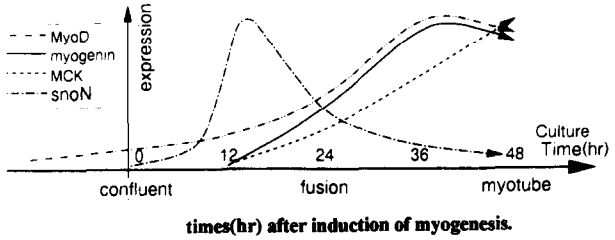

Fig. 5. Schematic representation of the expression pattern of snoN as compared with those of markers for myogenesis. Comparison between expression profiles of endogenous snoN mRNA and other muscle-specific gene transcripts during C2C12 differentiation schedule. Northern blot analysis was performed with 20 μg of total RNA from C2C12 cells harvested at the indicated times after the cultures were exposed to the differentiation condition.

G0 portion of the cell cycle and arrest at approx. 4–6 h in the G1 phase [10]. When the entry of C2C12 into G0 was blocked by b-FGF at 10 nM, a concentration which completely inhibited myotube formation, the transient expression of snoN disappeared (Fig. 2). In contrast, since EGF functions as a progression factor to mediate the G1- to S-phase transition but not to induce the G1/G0 phase transition [27] and does not inhibit myotube formation, the entry of cells into the G0 state occurs regularly and snoN is expressed normally, accompanying the delay and attenuation of the peak (Fig. 3). Transient expression of snoN was also observed when fibroblastic C3H10T1/2 cells became quiescent (entry to G0) by serum deprivation and contact inhibition of cell growth at confluent cell density. This suggests that the transient snoN expression is related to the G1 to G0 phase transition rather than the initial event for the programmed schedule of myogenesis. bFGF directly inhibits MyoD family proteins through protein kinase C-mediated phosphorylation [28]; therefore, there is no evidence that the loss of snoN expression is a direct cause of the failure to undergo myogenesis. Rather, it should be related to the inability to enter G0. The induction of in vitro myogenesis of quail embryo cells by overexpression of snoN [4] would be due to the increase in cells at G0. Considering a transient increase of snoN was absent when entry to G0 was blocked by exposure to b-FGF but is maintained when exposed to EGF which was unable to block the G1 to G0 phase transition, a transient increase characteristic of snoN expression upon serum deprivation may represent a molecular marker for entry to the G0 phase. c-fos was also transiently expressed upon reinitiation of cell growth (exit from G0 to G1) with an immediate early response within minutes of growth factor stimulation [29,30]. In contrast, it takes approx. 12 h for snoN to be expressed maximally, suggesting the entry from G1 to G0 is a time-consuming process relative to exit from G0. Two AUUUA sequences on the 3′ non-coding region in c-snoN mRNA (chicken) [4] may cause relatively rapid degradation. Endogenous snoA mRNA was undetected in C2C12 cells. SnoA has not been detected in transcripts and genomic DNA in chicken [4]. Therefore, snoN is a dominant form of sno in mouse as in chicken.

Consistent with the results demonstrated in avian myogenic QM7 cells and non-myogenic QM5 cell [31], no transient expression of c-ski was observed during myogenesis of mouse myogenic C2C12 cells and when non-myogenic C3H10T1/2 cells was exposed to the condition for myogenesis (right panels in both Figs. 1 and 4). In mouse myeloid cells, ski mRNA showed transient expression in mid G1 and snoN mRNA was expressed maximally in early to mid G1 [32]. Our results also showed that snoN transcript was expressed at a point of G1 prior to the entry to G0 although it is impossible to specify the point of snoN expression at G1 exactly. The transcripts of ski were expressed with a peak at mid G1 during hematopoiesis, in contrast to the invariable expression of ski mRNA during myogenesis (Fig. 1). But the invariably low abundance of c-ski mRNA during differentiation does not necessarily mean that c-ski is not involved in myogenic process of C2C12 cells. Posttranslational modification such as phosphorylation [33] and/or cooperative interaction with other factors [34,35] may be required on ski protein to confer a potential activity.

Muscle differentiation conditions also caused the transient expression of snoN in fibroblastic C3H10T1/2 cells, irrespective of whether they were defective for myogenesis. Thus, snoN expression is not sufficient to compel C3H10T1/2 to undergo myogenesis, consistent with the observation that overexpression of exogenous snoN in fibroblastic C3H10T1/2 cells did not induce myogenesis (unpublished observation) although overexpression of MyoD family proteins alone did [15,16,36]. An unknown factor downstream of snoN protein is needed for myogenesis to be triggered; the factor is not present in fibroblastic C3H10T1/2 cells but in myoblast C2C12 cells. In this context, it seems intriguing that snoN protein is localized in the nucleus [4]and possibly involved in transcriptional regulation by formation of a heterodimer complex with c-ski [37].

Investigation of the mechanism that mediates the exit from the cell division cycle to the quiescent state (G0) should lead to a knowledge of how to determine the fate of cells, growth or differentiation. The critical step in this process remains enigma and it remains unknown even whether a discrete boundary exists between the G1 and G0 phases. The biological consequence of the transient increase in snoN expression is also unknown at present, but may provide a clue to elucidation of the process underlying G0 entry.

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