Trans-regulation of myogenin promoter/enhancer activity by *c-ski* during skeletal-muscle differentiation: the C-terminus of the c-Ski protein is essential for transcriptional regulatory activity in myotubes

Koji ICHIKAWA^{*†}, Takahiro NAGASE[‡], Shunsuke ISHII[‡], Akira ASANO^{*} and Naotoshi MIMURA^{*1}

^{*}Institute for Protein Research, Osaka University, 3-2, Yamadaoka, Suita, Osaka 565, Japan, [†]AGENE Research Institute, 200 Kajiwara, Kamakura, Kanagawa 247, Japan, and [‡]Laboratory of Molecular Genetics, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan

c-ski gene product is a nuclear protein with myogenesis-promoting and transforming activities. We have analysed the effects of *c-ski* transfection on the promoter/enhancer activity of the upstream region of the myogenin gene during *in vitro* myogenesis using CAT reporter assay. When co-transfected with *c-ski* into myogenic C2C12 cells, promoter/enhancer activity was efficiently suppressed in proliferating cells, but the myogenesis-induced increase in activity was potentiated approximately ten times more (150-fold in the *ski*-transfected cells) than the ordinary increase (12-fold in the mock) 48 h after induction of differentiation. In non-myogenic 10T1/2 cells, *c-ski* transfection caused persistent suppression of promoter/enhancer activity in both proliferating and growth-arrested (i.e. myogenesis-inducing)

conditions. Thus the *ski*-dependent potentiation of myogenin gene transcriptional activity appears to be specific for myogenesis. The C-terminal region (amino acids 595–663) of the c-Ski protein was essential for the potentiating activity in myotubes. Other members of the *ski*-gene family, *snoN* and *snoA*, were ineffective in transactivation, possibly because of the defect in the corresponding C-terminal region. c-Ski protein underwent a mobility shift on SDS/PAGE after *in vitro* myogenesis which may explain the conversion of the activity from suppressive in myoblasts to potentiating in myotubes. Deletion analysis of the upstream region of the myogenin gene revealed that a responsive element to *c-ski* in myotubes is located at a distinct site upstream of the basal promoter/enhancer region.

INTRODUCTION

The *ski* oncogene (*v-ski*), which was originally isolated from Sloan-Kettering avian retroviruses, has been shown to cause anchorage-independent growth and morphological transformation and to induce myogenesis *in vitro* [1–4]. *c-ski*, the cellular counterpart of *v-ski*, is also capable of inducing not only expression of MyoD and myogenin in otherwise non-myogenic quail embryo cells but also morphological transformation of chicken embryo cells when overexpressed [5]. Consistent with these findings, members of the *ski*-gene family are expected to be present at a check point in the G₀ phase to direct cells toward differentiation or into the cell cycle [6] according to their competence. It has been reported that *c-ski* transcripts are expressed in dermomyotome from early on in myogenic commitment to terminal differentiation of skeletal muscles [7]. In transgenic mice, *c-ski* transgene expression was found to be muscle-specific and caused muscular hypertrophy with increases in type-IIb fast-muscle mass [8]. *In situ* analysis in transgenic mice also revealed that *c-ski* transcript was less abundant at birth and then increased temporarily in fast-muscle fibres between 5 and 12 days post partum resulting in a hypertrophic effect on the formation of first muscle IIb fibres [9]. In addition, atrophy of fast-muscle fibres by denervation caused *c-ski* mRNA to decrease significantly [9]. Thus *c-ski* gene product may have a role in the process from commitment to differentiation/maturity leading to formation of the specialized muscle phenotype.

Analysis of the myogenic process is required to elucidate the

mechanisms controlling the antagonism between cell growth and differentiation because growth cessation is a prerequisite for progression to muscle differentiation. The finding of four basic helix–loop–helix-class myogenic factors, called the MyoD family, has provided a clue to the molecular basis of acquisition of myogenic competency and muscle phenotype through subordinate muscle-specific gene activation [10–12]. Gene-targeting experiments on the myogenin gene resulted in a severe reduction in differentiated skeletal-muscle cells; other myogenic factors failed to compensate for the disrupted myogenin gene, and functional redundancy was observed between MyoD and myf5 [13,14]. Thus myogenin is an indispensable component of skeletal muscle differentiation. It would therefore appear to be impossible to understand the regulatory mechanisms of myogenic differentiation without clarifying how the expression of myogenic factors such as myogenin are regulated.

Expression of muscle-specific genes in non-myogenic quail embryo cells has been induced by transfection of *v-* or *c-ski* [4], and a muscle-specific enhancer has been reported to be activated by *c-ski* [15]. These results support the idea that *c-ski* is involved in transcriptional regulation of muscle differentiation.

To examine the physiological significance of the effect of *c-ski* on myogenesis, the present study focused on the following two points: (i) the effect of *c-ski* transfection on promoter/enhancer activity of the myogenin gene and a typical muscle gene, muscle creatine kinase (MCK), in cultures of a previously ‘determined’ mouse muscle cell line, C2C12, and non-myogenic 10T1/2 cells; (ii) identification of the part of the Ski protein responsible for the

Abbreviations used: CAT, chloramphenicol acetyltransferase; MCK, muscle creatine kinase; MLC, myosin light chain; SV40, simian virus 40.

¹ To whom correspondence should be addressed.

activity as a transcriptional factor. Here, we show that *c-ski* functions as a suppressor of promoter/enhancer activity of the myogenin gene in proliferating myoblasts and as a potentiator in postmitotic myotubes. Consistent with this finding, *c-Ski* undergoes a change in mobility on SDS/PAGE after myogenesis. Deletion analysis revealed the functional portions of the *c-Ski* protein required to potentiate transactivation of promoter/enhancer activity in myotubes. In addition, deletion analysis of the upstream region of the myogenin gene suggests the presence of a distal *c-ski*-responsive element upstream of the basic promoter/enhancer region.

MATERIALS AND METHODS

Cell cultures

C2C12 mouse myoblast cells [16] were proliferated in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C, 5% CO₂ and 95% humidity. The culture medium was exchanged every 48 h. *In vitro* myogenesis was induced by replacement of the growth medium (10% fetal bovine serum/Dulbecco's modified Eagle's medium) with differentiation medium, COSMEDIUM-001 (CosmoBio Co.) when the cell population reached confluence. Mouse C3H10T1/2 fibroblast cells were maintained in the same way and exposed to the same differentiation conditions at confluence which caused growth-arrest without myogenesis.

Constructions

For expression of *c-ski* and *c-sno* genes in C2C12 and 10T1/2 cells, two distinct mammalian expression vectors, pACT1 and pKCRH2PL, were used. The expression plasmids with β -actin promoter were constructed by subcloning full-length *c-ski* or *c-sno* cDNA [17] into pACT1 at the *NcoI* and *XbaI* sites and designated pActski and pAct sno respectively. The pACT1 plasmid contains the 1.3 kb fragment of the 5' regulatory sequence of the chicken cytoplasmic β -actin gene [18] and simian virus 40 (SV40) polyadenylation signal. For construction of expression plasmids with SV40 early-gene promoter, the entire protein-coding sequence of the *c-ski* or *c-sno* cDNAs was excised from pActski or pAct sno by digestion with *NcoI* and *XbaI*, and then recloned into the *HindIII* and *XbaI* sites located in the cloning sites of the pKCRH2PL plasmid and designated pKCRski and pKCRsno respectively. pKCRH2PL has the SV40 early-gene promoter, donor and acceptor splice sites derived from the rabbit α -globin gene, and polyadenylation sites originated from the rabbit α -globin gene and SV40 early gene [19]. Plasmids used to express a series of deleted *c-ski* mutants, Ski Δ 1 to Ski Δ 8, were generated as described previously [20,21] by deleting the *StuI*–*ApaI* fragment (Δ 1, nucleotides 209–853; nucleotide numbers are as in ref. [17]), the *ApaI*–*EcoRI* fragment (Δ 2, nucleotides 848–1543), the C-terminal fragment downstream of the *EcoRI* site (Δ 3, downstream of nucleotide 1548), the *StuI*–*EcoRI* fragment (Δ 4, nucleotides 209–1543), the *ApaI*–*MluI* fragment (Δ 5, nucleotides 848–1854), the *ApaI*–*ScaI* fragment (Δ 6, nucleotides 848–2059), a combination of Δ 1 and Δ 3 (Δ 7), and the *MluI*–*ScaI* fragment (Δ 8, nucleotides 1859–2059) respectively, and then filling up by Klenow polymerase followed by religation with *Bg/III* linker of appropriate length to adjust the reading frame.

A chloramphenicol acetyltransferase (CAT) reporter plasmid under the control of 3.7 kb upstream of mouse myogenin gene, pMYO3700CAT, was a gift from Dr. E. N. Olson [11]. The pMYO184CAT, pMYO1102CAT and pMYO1700CAT con-

structs were made by digestion of pMYO3700CAT with *PstI*, *SstI* and *HindIII* respectively and religation.

A human MCK–CAT gene was a gift from Dr. J. J. Billadello [22]. It was made by insertion of 2620 nt of the human MCK 5'-flanking sequence including the transcription-start into the *HindIII* site of pSVOCAT in a 5' to 3' orientation upstream of the CAT gene. The resulting reporter plasmid was designated pMCKCAT2620.

Transfections and CAT assays

C2C12 and 10T1/2 cells were transfected by the calcium phosphate precipitation technique [23]. All plasmid DNAs used in our experiments were purified twice by CsCl centrifugation.

A 1.0 ml sample of calcium phosphate solution containing 25 µg of DNA was added to a 10 cm plate in which 4×10^5 cells were inoculated a day before transfection. The transfected DNAs were composed of 5 µg of effector plasmid (pACTski/*snoN/snoA* or pKCRski/*snoN/snoA*), 10 µg of CAT reporter plasmid, 2 µg of β -galactosidase expression vector (pKCRlacZ) and 8 µg of salmon sperm DNA as carrier unless otherwise indicated. Cells were exposed to DNA for 15 h at 35 °C and 3% CO₂, recovered in the growth medium for 24 h after transfection at 37 °C and 5% CO₂ and used to prepare the growing-cell extracts (G) for CAT assays. The transfected cells, which were recovered and cultured to near confluence, were differentiated for 48 h at 37 °C and 5% CO₂ and then used to prepare the differentiated-cell extracts (D) for CAT assays.

CAT assays were carried out in a total volume of 120 µl using 0.125 µCi of [*dichloroacetyl*-1-¹⁴C]chloramphenicol (Amersham) as described previously [24]. Cell extracts used for CAT assay were the supernatants prepared from C2C12 cells lysed in 0.25 M Tris/HCl, pH 7.8, by three cycles of freezing in liquid nitrogen and thawing at 37 °C and centrifuged at 10000 g for 5 min. The radioisotopic scanner BAS2000 system (Fuji Film Co.) was employed to determine the ratio of acetylated to non-acetylated chloramphenicol after separation by TLC (silica-gel 60TLC; Merck). Levels of CAT activity were normalized to β -galactosidase activity in the respective cell extracts.

Immunoprecipitation, immunoblotting and immunofluorescence

To detect the expression level of Ski Δ 8 mutant, transfected C2C12 cells were lysed in 0.25 M Tris/HCl, pH 7.8, by three cycles of freezing in liquid nitrogen and thawing at 37 °C. After centrifugation at 10000 g for 5 min, the supernatants were kept for assay of CAT and β -galactosidase activities and the pellets were extracted with buffer containing 1% Nonidet P40, 0.1% sodium deoxycholate, 400 mM NaCl, 1% Trasyol, 1 mM PMSF, 10 mM Na₄P₂O₇, 10 mM NaF, 4 mM EDTA, 2 mM Na₃VO₄ and 50 mM Tris/HCl, pH 7.5, for 30 min at 4 °C and then immunoprecipitated. For detection of mobility shift of *c-Ski* protein on SDS/PAGE, C2C12 cells were lysed with lysis buffer containing 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂ and 0.5% Nonidet P40 supplemented with 1 mM PMSF and 1% Trasyol for 15 min on ice, and then centrifuged at 2000 g for 5 min at 4 °C. The supernatants were re-centrifuged at 10000 g for 30 min and the resultant supernatants were kept as the cytosolic fraction (C). The precipitates were washed twice with lysis buffer and the supernatant was kept as the washed supernatant (W). Washed precipitates were extracted with extraction buffer containing 1% Nonidet P40, 0.1% sodium deoxycholate, 400 mM NaCl, 10 mM Na₄P₂O₇, 10 mM NaF, 4 mM EDTA, 2 mM Na₃VO₄ and 50 mM Tris/HCl, pH 7.5, supplemented with 1 mM PMSF and 0.1% Trasyol in a rotatory shaker for 30 min at 4 °C and centrifuged at 10000 g for 30 min

at 4 °C. The resultant supernatants were kept as the nuclear extracts (N). Nuclear extracts were diluted with 2 vol. of 20 mM Tris/HCl, pH 7.6, and centrifuged again at 10000 *g* for 15 min. Diluted nuclear extracts, washed supernatant and cytosol fractions were precleared by incubation with Protein A–Sepharose beads (Pharmacia LKB Biotechnology) for 1 h at 4 °C, followed by centrifugation at 10000 *g* for 5 min and then subjected to immunoprecipitation.

Immunoprecipitation was performed with rabbit polyclonal antibody against bacterially produced human *c-Ski* [21] for 2 h at 4 °C, followed by incubation with 20 μ l of a 1:1 slurry of Protein A–Sepharose with excessive capacity for a further hour at 4 °C. The beads were washed with 50 mM Tris/HCl, pH 7.6, containing 0.5% Nonidet P40, 10 μ g/ml leupeptin, 1% Trasylol and 1 mM PMSF, boiled in 40 μ l of SDS sample buffer for 5 min and then resolved by SDS/PAGE [25]. The proteins were electrophoretically transferred to a nitrocellulose membrane sheet [26] and immunoblotted using mouse monoclonal antibody against human *c-Ski* protein produced in *Escherichia coli*, which recognizes an epitope on the N-terminal half (amino acids 46–260) of *c-Ski* protein. Anti-mouse IgG (Wako Chemical Co.) and mouse PAP (Zymed Laboratories) were used to detect the immunoreactive bands.

For immunofluorescence, C2C12 cells were grown at the growing phase on gelatin-coated coverslips, rinsed with PBS, fixed and permeabilized with a 1:1 mixture of methanol and acetone for 2 min. After being rinsed three times with PBS, the samples were blocked with 3% BSA/PBS for 5 min at room temperature, and then subjected to immunofluorescent staining by incubation with rabbit anti-Ski antibody followed by Texas Red-conjugated anti-rabbit immunoglobulin (Amersham) and mounted with 10% (w/v) glycerol in PBS.

RESULTS

Transcriptional regulation of myogenin promoter/enhancer activity by *c-ski* during C2C12 myogenesis

To test the effect of *c-ski* on trans-regulation of myogenin gene transcription, CAT-reporter gene activity of the 3.7 kb upstream region of the myogenin gene was determined when *c-ski* was overexpressed by co-transfection. The expression vector harbouring human *c-ski* cDNA (pKCR*ski*) was co-transfected into myogenic C2C12 cells with the pMYO3700CAT reporter gene carrying the 3.7 kb upstream region of the myogenin gene containing its promoter and enhancer [11]. In the mock transfection, CAT activity was increased in postmitotic myotubes (mock in panel D) to more than ten times that in the growing phase (mock in panel G) (Figure 1). As no myogenin gene transcript was detected in the proliferating C2C12 cells [6], the transcription in the growing phase could be regarded as leaky or background activity in the artificial CAT assay and was defined as 1.0 (Table 1). In proliferating C2C12 cells, the leaky transcription was efficiently suppressed to approx. 20% of the mock on *c-ski* overexpression. In contrast, myogenin promoter/enhancer activity increased over 2.5-fold more than the ordinary enhancement in postmitotic myotubes on *c-ski* transfection (compare '*ski*' with 'mock' in differentiated C2C12 cells in Table 1). These observations indicate that the *c-ski* gene product also functions as a transcriptional regulator, reversing the effect on the promoter/enhancer activity of the myogenin gene from suppression to potentiation upon myogenic differentiation; this leads to overall enhancement of myogenin promoter/enhancer activity during myogenesis from no more than 12-fold in the mock transfection (compare 'mock' in the differentiated state with 'mock' in the growing state) to more than 150-fold when

Figure 1 pMYO3700CAT reporter activity was suppressed in proliferating but potentiated in differentiated C2C12 cells by co-transfection with *c-ski*

Myogenin promoter/enhancer-driven CAT reporter plasmids were transfected into C2C12 cells with expression vectors harbouring *ski* (pKCR*ski*), *snoN* (pKCR*snoN*), *snoA* (pKCR*snoA*), a combination of *ski* and *sno* or the mock plasmid (pKCRH2PL) as indicated. Panels G and D represent CAT activity in growing and differentiated C2C12 cells (at 48 h after induction of myogenesis) respectively.

Table 1 Qualification of pMYO3700CAT activity in differentiated C2C12 cells by co-transfection with *c-ski*

The conversion ratios to the acetylated form of chloramphenicol in Figure 1 were determined and normalized to β -galactosidase activity generated by co-transfection with pKCR*lacZ*. The results are means $\pm$ S.D. for four independent experiments.

Transfection	Effector construct	CAT activity relative to control	
		Growing C2C12 cells	Differentiated C2C12 cells
Mock	pKCRH2PL	1.00	12.17 $\pm$ 3.12
<i>ski</i>	pKCR <i>ski</i>	0.22 $\pm$ 0.04	34.46 $\pm$ 6.37
<i>snoN</i>	pKCR <i>snoN</i>	0.52 $\pm$ 0.17	14.71 $\pm$ 3.18
<i>ski</i> + <i>snoN</i>	pKCR <i>ski</i> + pKCR <i>snoN</i>	0.24 $\pm$ 0.05	31.85 $\pm$ 8.47
<i>snoA</i>	pKCR <i>snoA</i>	0.51 $\pm$ 0.08	11.66 $\pm$ 1.91
<i>ski</i> + <i>snoA</i>	pKCR <i>ski</i> + pKCR <i>snoA</i>	0.30 $\pm$ 0.09	21.91 $\pm$ 1.72

c-ski was transfected (compare '*ski*' in differentiated cells with '*ski*' in growing cells).

Two types of *sno* cDNA (*ski*-related novel gene) of the *ski* gene family were subjected to CAT analysis with the same expression vector (pKCR*snoN* and pKCR*snoA*) to compare the transcriptional regulatory activity with that of *c-ski* (Figure 1). Overexpression of *snoN* or *snoA* resulted in weaker suppression in the growing phase but failed to potentiate the reporter activity of pMYO3700CAT in the differentiated cells (Table 1). This is consistent with the fact that *snoN* is inferior to *c-ski* with respect to competence of both transforming and myogenesis-promoting activities [27]. SnoA protein lacks the C-terminal region from

Table 2 Effect of *c-ski* and *c-snoN* transfection on myogenin promoter/enhancer activity in non-myogenic 10T1/2 fibroblasts

Non-myogenic C3H10T1/2 fibroblasts were transfected with the same set of effector and reporter plasmids as shown in Figure 1. The culture conditions for growing and growth-arrested 10T1/2 fibroblasts correspond to the stages of G and D in Figure 1 respectively. The results are means for three independent transfections.

Transfection	Effector construct	CAT activity relative to control	
		Growing 10T1/2 cells	Growth-arrested 10T1/2 cells
Mock	pKCRH2PL	1.00	1.00
<i>ski</i>	pKCR <i>ski</i>	0.40	0.23
<i>snoN</i>	pKCR <i>snoN</i>	0.72	0.57
<i>snoA</i>	pKCR <i>snoA</i>	0.90	0.83
<i>ski</i> + <i>snoN</i>	pKCR <i>ski</i> + pKCR <i>snoN</i>	0.31	0.30
<i>ski</i> + <i>snoA</i>	pKCR <i>ski</i> + pKCR <i>snoA</i>	0.52	0.25

amino acid 324 to the end (amino acid 728) of the c-Ski protein, suggesting that the C-terminus of c-Ski is important for the potentiation of myogenin gene transcription. On the other hand, members of the *c-ski* gene family share 54% homology in the N-terminal region from amino acid 1 to 324 of c-Ski, and weak suppression in myoblasts is common for all three types of *ski*, *snoN* and *snoA*. Thus the N-terminus of the c-Ski protein may participate in the suppression observed in myoblasts. *ski*-dependent potentiation in postmitotic myotubes was not effected by co-expression of *c-sno* genes ('*ski* + *snoN*' and '*ski* + *snoA*') despite a proposed interaction between c-Ski and c-SnoN [28], suggesting that the interaction with the *c-snoN* gene product is not necessary for *ski* function in this case.

Although the *ski*-dependent increase in myogenin promoter/enhancer activity in differentiated muscle cells is not inconsistent with the myogenesis-inducing activity of *c-ski*, we cannot rule out the possibility that such *ski*-dependent activation was induced simply by signals transmitted through cell-cell contacts or growth cessation rather than myogenesis. Therefore the same reporter assays were carried out using non-myogenic 10T1/2 fibroblasts, which are not competent to undergo myogenesis *in vitro* unless a member of the MyoD gene family is introduced. The differentiation conditions for C2C12 cells entrapped 10T1/2 cells in the growth-arrested state without expression of muscle-specific genes such as myogenin. As shown in Table 2, effective suppression of CAT activity was observed in growing 10T1/2 cells on overexpression of *c-ski*, as was the case for growing C2C12 cells. However, even after 48 h of exposure to the same differentiation conditions as for the C2C12 cells, myogenin promoter/enhancer activity remained suppressed in *ski*-transfected 10T1/2 cells that were growth-arrested. These observations suggest that the *ski*-related potentiation of myogenin promoter/enhancer activity is specific for muscle differentiation and not induced by growth arrest.

The 5'-upstream regulatory region of the MCK gene does not respond to *c-ski* transfection

To examine whether c-Ski directly regulates expression of subordinate muscle-specific genes, CAT reporter assays were performed by transfection to C2C12 cells with pMCKCAT2620 plasmid carrying the 2.6 kb of *cis*-elements upstream of the human MCK gene instead of pMYO3700CAT. Unlike myogenin, MCK promoter/enhancer activity in all transfectants was not detectable in proliferating C2C12 cells unless cultured

Table 3 Regulatory region of MCK gene is not directly responsive to *c-ski*

The pMCKCAT2620 reporter plasmid was co-transfected into C2C12 cells either with the expression vectors carrying the indicated DNA or with the mock construction. Relative CAT activities in transfected C2C12 cells after differentiation for 48 h are shown. The results are representative of duplicate independent experiments.

Transfection	Effector construct	CAT activity relative to control in differentiated C2C12 cells
Mock	pKCRH2PL	1.00
<i>ski</i>	pKCR <i>ski</i>	1.54
<i>snoN</i>	pKCR <i>snoN</i>	1.25
<i>snoA</i>	pKCR <i>snoA</i>	1.37
<i>ski</i> + <i>snoN</i>	pKCR <i>ski</i> + pKCR <i>snoN</i>	1.67
<i>ski</i> + <i>snoA</i>	pKCR <i>ski</i> + pKCR <i>snoA</i>	1.56

for 48 h under differentiation conditions (Table 3). In addition, there was no significant potentiation in the *ski*- and mock-transfected cells 48 h after differentiation, consistent with the results of Engert et al. [15] except that in our experiments no basal activity of MCK promoter/enhancer was detected in the myoblasts. These results mean that the region 2.6 kb upstream of the MCK gene is not susceptible to *c-ski*-dependent transactivation, i.e. *c-ski* is not able to activate MCK expression directly, unlike the myogenin gene.

A restricted C-terminal portion is essential for c-Ski activity as a transcriptional regulator

To determine the domains in the c-Ski protein required for up-regulation of myogenin promoter/enhancer activity in postmitotic myotubes, expression vectors harbouring various deletion mutants of human *c-ski* were co-transfected with the myogenin reporter gene, pMYO3700CAT, into C2C12 cells, and the resultant CAT reporter activities were examined (Figure 2).

First, we tested three mutants, Δ1, Δ2 and Δ3, to determine roughly which portion is essential for transcriptional control of the myogenin promoter. The three mutants lacked one-third of the N-terminus-proximal portion (Δ1; amino acids 46–260), the central portion (Δ2; amino acids 263–490) or the C-terminal portion (Δ3; amino acids 493–728). Deletion of the N- and C-terminal portions independently resulted in a significant reduction in potentiation of CAT reporter activity. Both portions correspond to domains required for DNA binding of c-Ski in association with unidentified nuclear factors [21] and also for homodimer formation [28]. The combined deletions Δ1 and Δ3 (Δ7) therefore resulted in an effective reduction in *ski*-dependent activation as compared with the combination Δ1 and Δ2 (Δ4). However, further deletion of the central portion from the Δ1 mutant resulted in an unexpected recovery of activity (compare Δ1 with Δ4). Thus the central portion may have a negative effect on the transcriptional regulatory activity of the C-terminal portion, and the N-terminal portion is not essential for the activity of the C-terminal portion but appears to neutralize the negative effect of the central portion. The difference between Δ5 and Δ6 suggests that one of the parts essential for Ski function lies in a small region (amino acids 595–663). As expected, SkiΔ8 lacking the corresponding C-terminal portion (amino acids 595–663) significantly reduced the potentiating activity.

It is necessary to preclude the possibility that the inability of the deletion mutants to activate the promoter/enhancer activity of the myogenin gene is due to less abundance of the protein than

Figure 2 Analysis of functional domains of Ski protein involved in the transactivation of myogenin promoter/enhancer

The histogram on the right shows relative values of pMYO3700CAT reporter activity in C2C12 cells after 48 h of differentiation when co-transfected with deletion mutants of *c-Ski*, constructs of which are shown on the left. Amino acid numbers are given on the constructs of *Ski* mutants. Expression vectors for a series of deletion mutants of *c-Ski* protein were constructed as described in the Materials and methods section. After transient transfections with 5 μ g of *ski*-mutant expression plasmid and 10 μ g of reporter plasmid, the transfected cells were recovered for 24 h and differentiated for 48 h, and then subjected to CAT reporter assay. CAT activities relative to those of the mock-plasmid (pACT1) transfection are indicated as means $\pm$ S.E.M. for four independent experiments.

Table 4 Effect of $\Delta 8$ mutant on pMYO3700CAT reporter activity when transfected with ten times more DNA than the wild-type

The *Ski* $\Delta 8$ mutant construct was transfected to C2C12 cells with 20 μ g of DNA, 10 times more than the wild-type pAct*ski* (2 μ g). Relative CAT activities in cell extracts from transfected C2C12 cells after differentiation for 48 h were normalized to co-transfected β -galactosidase activity. The results are representative of duplicate independent experiments.

Transfection	Effector construct	Amount of transfected DNA (μ g)	CAT activity relative to control in differentiated C2C12 cells
Mock	pAct1	5	1.00
<i>ski</i>	pAct <i>ski</i>	2	2.54
<i>ski</i> $\Delta 8$	pAct <i>ski</i> $\Delta 8$	20	1.17

in the wild-type. Since no appropriate antibody is available to detect all the deletion mutants, immunochemical analysis was focused on the inactive mutant with the most restricted deletion ($\Delta 8$), the gene products of which are detectable with our antibodies. Unfortunately, the abundance of the products of the N-terminal deletion mutant $\Delta 1$ gene failed to be confirmed because of lack of reactivity with our anti-Ski antibodies. The detailed analysis on the $\Delta 8$ mutant (Table 4 and Figure 3), however, confirmed the results on the mutants with more restricted deletions at the C-terminus, including the 595–663 sequence, independent of their expression levels, and also confirmed the significance of the C-terminus for potentiating the promoter/enhancer activity of the myogenin gene. When $\Delta 8$ was transfected with ten times more DNA than the wild-type, $\Delta 8$ protein appeared to accumulate in greater amounts than the

Figure 3 Expression level and subcellular localization of $\Delta 8$ *ski* mutant

(A) The amount of protein synthesized on transfection of *c-ski* or mutant $\Delta 8$; 20 μ g of $\Delta 8$ (lane 1), 2 μ g of *ski* (lane 2) or 5 μ g of vector DNA pAct1 (lane 3) was transfected to C2C12 cells. Nuclear extracts prepared from the transfected C2C12 cells were immunoprecipitated. Resultant immunoprecipitates were analysed by immunoblot using monoclonal anti-Ski antibody. Lane 4 shows the band corresponding to *c-ski* by loading bacterially produced *c-Ski*. kd = kDa (B) Immunostaining of $\Delta 8$ or *c-Ski* in transfected C2C12 cells. $\Delta 8$ and *c-ski* were transfected with the pMYO3700CAT reporter gene into C2C12 cells with 5 μ g of DNA. After recovery for 48 h in growth medium, cells fixed with methanol/acetone (1:1, v/v) were exposed to anti-Ski antibody and visualized with Texas Red-labelled anti-rabbit immunoglobulin. The arrowhead and arrow show the nucleus of the cell with overexpressed *c-ski* (left) or $\Delta 8$ (right) and the nucleus of a DNA-unincorporated cell with very faint staining respectively.

wild-type (Figure 3A, compare lane 1 with lane 2). Nonetheless, $\Delta 8$ still failed to potentiate CAT activity, suggesting that it is functionally deficient (Table 4). Immunocytochemical analysis showed one positive *c-Ski* per four or five nuclei when *c-ski* was transfected transiently into C2C12 cells (Figure 3B), suggesting that endogenous *c-Ski* is not abundant enough to be visualized by immunofluorescence, and transfection efficiency is almost as low as 20%. This efficiency is not uncommon for transient transfection experiments. Nuclear transport of $\Delta 8$ protein was also substantiated in the DNA-incorporated cell (Figure 3B right panel, arrowhead). Therefore $\Delta 8$ had lost the ability to function as a transcriptional regulator of the myogenin gene in the nucleus, i.e. the functional domain resides on the portion deleted in the $\Delta 8$ mutant. Taken together the results indicate that the C-terminal (amino acids 595–663) portion containing a predicted α -helical structure is essential for the transcription-potentiating activity of the *c-Ski* protein in postmitotic myotubes and the N-terminal region may sustain the activity by blocking the inhibitory effect of the central portion.

Analysis of the *c-ski*-responsive element in the upstream region of the myogenin gene

The upstream region of the myogenin gene was deleted at nucleotides –1700, –1102 and –184 with appropriate restriction enzymes and religated to the CAT reporter gene to examine whether the *c-ski*-responsive element is located inside

Figure 4 Deletion analysis of myogenin 5'-flanking region responsible for Ski-dependent transcriptional activation

The 5' deleted pMYO3700CAT constructs were transfected with either pKCRH2PL (mock transfection; solid bars) or pKCRski (ski transfection; shaded bars) into C2C12 cells. After recovery for 24 h and differentiation for 48 h, cells were subjected to CAT reporter assay. The relative values are indicated as means $\pm$ S.E.M. for three or more independent transfections and shown as a histogram. The numbers in bp denote the distance relative to the transcriptional-start site. The basal elements responsible for the muscle-specific expression of myogenin are depicted in the scheme of the pMYO184CAT construct.

or outside of the basic promoter/enhancer region. The *c-ski*-dependent potentiation of myogenin gene promoter/enhancer activity was sustained in the pMYO1102CAT construct but lost in the pMYO184CAT construct (Figure 4). This result indicates the presence of a *c-ski*-responsive *cis*-element between nucleotides -1102 and -184 which is outside the basic promoter/enhancer region composed of E-boxes, MEF site and TATA box [11]. In addition, the loss of the *c-ski*-induced potentiation caused by

Figure 5 Mobility shift of c-Ski protein on SDS/PAGE after *in vitro* myogenesis of C2C12 cells

C2C12 cells transfected with the *c-ski* gene were harvested at 0 and 48 h after induction of muscle differentiation, and then separated into the cytoplasmic fraction (C), washed supernatant (W) and nuclear extracts (N). Each fraction was subjected to immunoprecipitation, and resultant immunoprecipitates were analysed by Western blotting with monoclonal antibody against Ski. Immunoreactive bands of c-Ski (arrowheads) were detected only in nuclear fraction. Lane W confirmed no release from the nuclear homogenate by prewashing. An additional more slowly migrating band (asterisk) emerged in the nuclear fraction after differentiation for 48 h. This result is representative of duplicate independent experiments.

deletion of the region from nucleotides -1102 to -184 appeared to be due to abolition of suppression in the mock transfection, suggesting that *c-ski* can mediate the release of suppression on myogenin gene transcription.

c-Ski protein undergoes alteration during *in vitro* myogenesis of C2C12 cells

Immunoblot analysis of the proteins immunoprecipitated with anti-Ski antibody showed a mobility shift of c-Ski protein to the upper band in the nucleus after *in vitro* myogenesis of C2C12 cells (Figure 5). This shift can be as much as 20 kDa, which would appear to be too large to be the phosphorylated form. This finding suggests that some unidentified modification or alternative splicing induces c-Ski to function as a myogenesis-related transcription regulatory factor, although it is not known what kind of alteration to Ski occurs during muscle differentiation.

DISCUSSION

Using CAT reporter assays, we have demonstrated that the Ski protein is able to regulate the promoter/enhancer activity of the myogenin gene upstream region during *in vitro* myogenesis. In addition, deletion mutants of *c-ski* revealed the regions responsible for potentiation of myogenin gene promoter/enhancer activity. From these findings, possible transcription regulatory activity of c-Ski protein during terminal differentiation of skeletal muscle will be discussed.

Overexpression of *c-ski* showed ambivalent activity on trans-regulation of myogenin promoter/enhancer activity, i.e. suppression in the proliferating myoblast but potentiation in the differentiated myotube. In fibroblastic 10T1/2 cells, overexpression of *c-ski* caused persistent suppression of basal promoter/enhancer activity of the myogenin gene. These findings may reflect the fact that *ski* has the dual property of serving as a transcriptional suppressor and a muscle-specific potentiator according to the biological environment. In C2C12 cells, the transcriptional level of pMYO3700CAT was increased by approx. 12-fold during myogenesis *in vitro*. As this value is rather low compared with the increase in the endogenous myogenin gene transcript [6], CAT reporter activities using pMYO3700CAT may not completely represent transcription of the endogenous myogenin gene in C2C12. In this context, the transfected *c-ski* appears to reproduce the physiologically significant enhancement of myogenin gene transcription (more than 150-fold) by suppressing basal transcription in myoblasts and potentiating myogenesis-induced transcription in myotubes. Thus overexpression of *c-ski* is required to achieve full CAT reporter activity, suggesting that the endogenous c-Ski protein expressed in C2C12 cells is insufficient to activate the transfected pMYO3700CAT reporter gene fully.

As the *c-ski* transcript is invariably expressed [6] or increased twofold at most [29] during terminal differentiation of the skeletal-muscle cell line *in vitro*, a post-translational modification of the c-Ski protein may underlie the conversion from suppression in myoblasts to potentiation in myotubes. In fact, an additional band of c-Ski protein with slower mobility was detected on SDS/PAGE after completion of *in vitro* myogenesis, suggesting that c-Ski is regulated in a myogenesis-dependent manner to acquire the competence to potentiate myogenin gene transcription. Detailed analysis of the cause (e.g. post-translational modification or alternative splicing) of the mobility shift is underway.

The promoter/enhancer activity of the MCK gene was not potentiated by *c-ski* transfection as much as that of the myogenin

gene in myotubes. *c-ski*-transfection failed to cause further enhancement of endogenous myogenin gene transcription (results not shown) probably because there is enough endogenous *c-ski* to cause full activation of the endogenous myogenin gene. Thus the failure of the *c-ski* gene to induce potentiation of MCK promoter/enhancer activity indicates that the *c-ski*-responsive element is not present in the upstream region of the MCK gene. The promoter/enhancer activities of MCK and myosin light chain (MLC) genes are activated by *c-ski* transfection in C2C12 myoblasts [15], whereas *c-ski* transfection caused suppression in C2C12 myoblasts and potentiation in C2C12 myotubes of the promoter/enhancer activity of the myogenin gene in this study. The difference between the effects of *c-ski* transfection on the promoter/enhancer activities of the MLC/MCK genes and the myogenin gene may reflect the independent and distinctive functions of *c-ski*, suggesting that it functions as a multifunctional transcriptional regulator. To examine this, we generated a series of deletions in the myogenin 5' upstream region to determine the element responsible for the *ski*-dependent potentiation of myogenin gene transcription. A comparison of these deleted upstream regions introduced into CAT constructs for reporter analysis showed the locus responsible for the *c-ski*-dependent potentiation to be upstream of, and distinct from, the basal promoter/enhancer region of the myogenin gene. More detailed analysis suggests that known consensus sequences such as an E box are not sufficient to respond to *c-ski* (K. Ichikawa, T. Nagase, S. Ishii, A. Asano and N. Mimura, unpublished work), although an E box is reported to be a responsive element for *c-ski* overexpression in myoblasts with respect to MLC/MCK genes [15].

Interaction with SnoN is not necessary for c-Ski to function as a transcriptional regulator in this case because co-transfection with *snoN* did not appear to increase the transcriptional activity of c-Ski. As c-Ski is unable to bind to DNA directly, some unidentified cofactor other than SnoN may be required for transcriptional activity of c-Ski. SnoN was also unable to increase myogenin gene transcription alone. SnoN shows 40% homology with the locus (amino acids 595–663) responsible for the transcriptional regulatory activity of *c-ski*. A major difference in the region is the basic-amino-acid-rich stretch preceding the helical structure in Ski. SnoN is missing such basic amino acid cluster, suggesting that the positively charged amino acids in the locus are of importance.

The tandem repeats of 25 amino acids present in the C-terminal region (amino acids 515–695) probably assume an amphipathic α -helix and probably mediate homodimer formation by a coiled-coil interaction as predicted by Heyman and Stavnezer [28]. The region deleted in the $\Delta 8$ mutant corresponds to the C-terminal half of the tandem repeats. In addition, a 150-residue stretch within the N-terminal 250 amino acids was suggested to be a weaker self-binding domain for homodimer formation [28] and also required for *c-ski* to potentiate myogenesis-induced myogenin gene transcription in our study. Therefore homodimer formation may be responsible for the transcription-potentiating activity of the c-Ski protein. As c-Ski is not competent to bind DNA directly, the loss of trans-regulation by $\Delta 8$ may be due to its failure to interact with DNA-binding protein endowed with transcriptional activity (possibly muscle specific). Consistent with this idea, the locus deleted in $\Delta 8$ also corresponds to the region required to bind unidentified cofactors in the nucleus [21]. Taken together the results suggest that homodimer formation of c-Ski may be required for interaction with unidentified DNA-

binding proteins and to mediate transcriptional regulation of the myogenin gene during *in vitro* myogenesis. The biological significance of dimerization of the c-Ski protein has already been pointed out [28]. However, the molecular basis of the biological activity mediated by homodimer formation of c-Ski remains unknown. We demonstrated that both regions probably involved in homodimer formation are indispensable for *c-ski* to potentiate myogenesis-dependent transcription of the myogenin gene. This may suggest that the transcriptional regulation of the myogenin gene described here is one of the molecular bases for the biological function of homodimer formation in c-Ski proteins.

We are indebted to Professor E. N. Olson and Professor J. J. Billadello for providing the CAT reporter plasmids pMYO3700CAT and pMCKCAT2620 respectively. We also thank Professor S. Tajima for helpful advice and suggestions.

REFERENCES

- 1 Stavnezer, E., Gerhard, D. S., Binari, R. C. and Balzas, I. (1981) *J. Virol.* **39**, 920–934
- 2 Stavnezer, E., Barkas, A. E., Brennan, L. A., Brodeur, D. and Li, Y. (1986) *J. Virol.* **57**, 1073–1083
- 3 Li, Y., Turk, C. M., Teumer, J. J. and Stavnezer, E. (1986) *J. Virol.* **57**, 1065–1072
- 4 Colmnares, C. and Stavnezer, E. (1989) *Cell* **59**, 293–303
- 5 Colmnares, C., Suttrave, P., Hughes, S. and Stavnezer, E. (1991) *J. Virol.* **65**, 4929–4935
- 6 Mimura, N., Ichikawa, K., Asano, A., Nagase, T. and Ishii, S. (1996) *FEBS Lett.* **397**, 253–259
- 7 Ambrose, M. R., Bottazzi, M. E. and Goodenow, M. M. (1995) *DNA Cell Biol.* **14**, 701–707
- 8 Suttrave, P., Kelly, A. M. and Hughes, S. H. (1990) *Genes Dev.* **4**, 1462–1472
- 9 Leferovich, J. M., Lana, D. P., Suttrave, P., Hughes, S. H. and Kelly, A. M. (1995) *J. Neurosci.* **15**, 596–603
- 10 Davis, R. L., Weintraub, H. and Lassar, A. B. (1987) *Cell* **51**, 987–1000
- 11 Edmondson, D. G., Cheng, T.-C., Cserjesi, P., Chakraborty, T. and Olson, E. N. (1992) *Mol. Cell Biol.* **12**, 3665–3677
- 12 Braun, T., Bushhausen-Denker, G., Bober, E., Tannish, E. and Arnold, H. H. (1989) *EMBO J.* **8**, 701–709
- 13 Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993) *Nature (London)* **364**, 501–506
- 14 Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. (1993) *Nature (London)* **364**, 532–535
- 15 Engert, J. C., Servaes, S., Suttrave, P., Hughes, S. H. and Rosenthal, N. (1995) *Nucleic Acid Res.* **23**, 2988–2994
- 16 Blau, H. M., Chiu, C. P. and Webster, C. (1983) *Cell* **32**, 1171–1180
- 17 Nomura, N., Sasamoto, S., Ishii, S., Date, T., Matsui, M. and Ishizaki, R. (1989) *Nucleic Acid Res.* **17**, 5489–5500
- 18 Seiler-Tuyns, A., Eldrige, J. D. and Paterson, B. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2980–2984
- 19 Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, Z., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M. and Numa, S. (1984) *Nature (London)* **307**, 605–608
- 20 Nagase, N., Nomura, N. and Ishii, S. (1993) *J. Biol. Chem.* **268**, 13710–13716
- 21 Nagase, T., Mizuguchi, G., Nomura, N., Ishizaki, R., Ueno, Y. and Ishii, S. (1990) *Nucleic Acids Res.* **18**, 337–343
- 22 Trask, R. V., Strauss, A. W. and Billadello, J. J. (1988) *J. Biol. Chem.* **263**, 17142–17149
- 23 Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
- 24 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
- 25 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 26 Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 27 Boyer, P. L., Colmnares, C., Stavnezer, D. and Hughes, S. H. (1993) *Oncogene* **8**, 457–466
- 28 Heyman, H. C. and Stavnezer, E. (1994) *J. Biol. Chem.* **269**, 26996–27003
- 29 Namciu, S., Lyons, G. E., Mikales, B. K., Heyman, H. C., Colmnares, C. and Stavnezer, E. (1995) *Dev. Dyn.* **204**, 291–300