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A Rapid and Simple Method for the Isolation of Mutant Variants Regulating Tissue-Specific Expression of the *TnI* Gene Through Drug Selection

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ABSTRACT

TnINEO fusion gene was constructed by fusing 3.4-kbp of quail *TnI* genomic DNA sequences spanning the promoter to exon 5 and a *neo* gene in frame. A myoblast cell line was established after transfection of pTnINEO. Since this cell line was passaged several times, a high frequency of neomycin (G418) sensitivity conversion was detected. Two drug-resistant variants were analyzed through genomic Southern blot and S1 nuclease protection assay. One variant has a mutation(s) in the regulatory element that activated the dormant *TnI* promoter-enhancer in myoblast, and the other has shown the genomic rearrangement. This result presented the possibility of isolating factor(s) that activate the muscle-specific *TnI* promoter simply by screening drug-resistant cells having appropriate mutations.

Index Entries: Fast troponin I gene; stage-specific expression; myoblast; drug selection; mutant; genomic rearrangement.

Abbreviations: bp, base pair(s); kbp, kilobase pair(s); *TnI*, troponin I gene; *neo*, neomycin resistance gene.

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INTRODUCTION

The development of skeletal muscle tissues depends on a series of discrete regulatory events (*1,2*). One of the major regulators in these events is exerted at the level of transcription (*3*). The molecular mechanism underlying this transcriptional control involves specific regulatory factors that interact with defined cis-acting elements in the control regions of the genes. Previously, Lee and Emerson (*4*) have identified a cis-acting regulatory element of the quail fast troponin I gene (*TnI*) through deletion analysis. This element, being localized in the first intron of the *TnI* gene, controls the tissue-specific expression of the gene under both the homologous and heterologous promoters, and turned out to be composed of multiple regulatory motifs that interact with specific DNA binding factors in myoblast, as well as in muscle cells.

In this article, we attempted to develop an experimental system in which the mutant variants regulating tissue-specific expression of the *TnI* gene could be selected by using a drug. For this purpose, a fusion gene containing the troponin I promoter and its intragenic control region (enhancer), and the neomycin resistance gene (*neo*) as a reporter gene was constructed. This fusion construct can provide a very sensitive drug selection assay system through activation of the troponin I regulatory element fused to the *neo* gene; that is, 10T1/2 fibroblasts and 23A2 myoblasts stably transfected with this fusion gene construct are sensitive to G418, a neomycin analog leading to cell death, since the transfected *TnI* gene is not expressed in fibroblasts and myoblasts. However, differentiation activates transcription of the *TnI* gene and results in neomycin resistance of the cells. Therefore, a G418-sensitive myoblast cell line transfected with this fusion vector is theoretically converted to a G418-resistant cell line when they differentiate into myofibers. However, we frequently observed G418-resistant fibroblast and myoblast cells with high passage numbers when plated on drug-containing media. Because these drug-resistant fibroblast and myoblast cells somehow activated the expression of muscle-specific *TnI* gene, tissue-specific expression of the *TnI* gene might be understood by analyzing these cells.

MATERIALS AND METHODS

Cell Culture

The mouse multipotential 10T1/2 cells and 23A2 myoblasts were maintained in basal medium-Eagle medium (BME, GIBCO), supplemented with 10 and 15% fetal calf serum (FCS), respectively. Myoblasts proliferate as undifferentiated cells at low density. These cells, however, spontaneously differentiate into myofibers by fusing at high density. On the other hand, 10T1/2 cell lines were treated with 3 μM 5-azacytidine (Sigma, St.

Louis, MO) for 20–24 h, rinsed with saline and maintained in BME supplemented with 10% FCS for a few days, and then finally passaged and maintained in BME supplemented with 15% FCS to differentiate into myofibers.

Plasmid Constructions

The plasmid pTnINEO was constructed by inserting the 3.4-kbp *Bam*HI fragment containing the 5′ half of the quail fast *TnI* gene into the *Bam*HI site of pUCNEO, which had been made by inserting a 1.8-kbp fragment of neomycin resistance gene-SV40 small t intron, as well as a polyadenylation signal after adding *Eco*RI linkers to this fragment into *Eco*RI site in pUC19.

DNA Transfection

DNA transfections were carried out using the calcium phosphate precipitation method described by Wigler et al. (*5*) with slight modification (*6*). For transfection, 30 μg of carrier DNA and 30 ng of pSV2-gpt plasmid DNA (*7*) mixed with 1 μg of pTnINEO DNA were used/plate of cells. Twenty-four hours following transfection, cells of each plate were split into five dishes (100 mm) in nonselective medium, and the next day, cultures were changed to the medium containing 25 μg/mL of mycophenolic acid and HATX (100 μg of xanthine, 15 μg of hypoxanthine, 10 μg of thymidine, and 2 μg of aminopterin/mL of medium). After approx 2 wk, drug-resistant individual clones were picked, and the remaining clones were pooled for further analysis.

Southern Blot Analysis

Genomic DNAs extracted from the transfected cell lines were digested with either *Eco*RI or *Bam*HI, and separated by agarose gel electrophoresis. A 1.2-kbp *Eco*RI-AvaI *neo* DNA fragment was radioactively labeled by Nick translation method as probes and the filters were hybridized to these probes under stringent conditions (5X Denhardt, 6X SSC at 65°C). Filters were subsequently washed in 1X SSC, 0.1% SDS for 30 min, and 0.1X SSC, 0.1% SDS for 2 h at 65°C.

S1 Nuclease Protection Analysis

The levels of transcripts of the transfected quail *TnI*, the *neo*, and the endogenous mouse α-actin gene were determined by S1 nuclease protection analysis using ^{32}P-end-labeled DNA as probes, a 3.4-kbp of *Bam*HI *TnI* fragment from pTnINEO and 1.43-kbp of *Bam*HI/*Ava*I DNA fragment from plasmid ptkneo (Nevis Fregien, personal communication) were used as probes for the *TnI* and *neo* genes, which resulted in the 109-bp and 1-kbp protected fragments, respectively (*see* Figs. 1 and 4). For the α-actin gene probe, a 1.8-kbp *Bam*HI fragment from p91 (*8*) was used and resulted in a 730-bp protected fragment (*see* Fig. 4). For the reaction, 35 μg of RNA

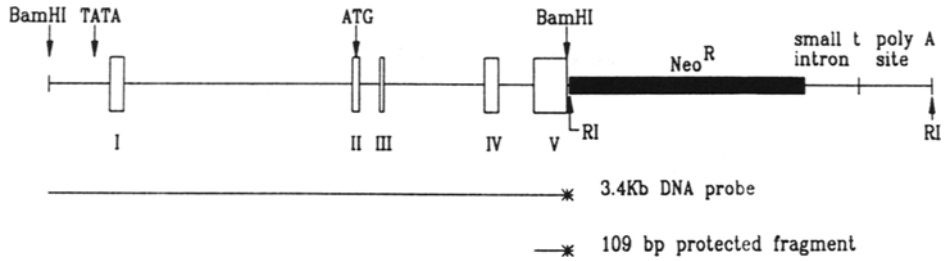

Fig. 1. Schematic diagram of the TnINEO fusion gene construct and the probe used for S1 analyses. The fusion gene contains the 3.4-kbp *Bam*HI fragment harboring the promoter to the fifth exon of the *TnI* gene, and the 1.8-kbp *Eco*RI (RI) fragment containing neomycin resistance gene (Neo^R), and SV40 small t intron as well as the polyadenylation signal (poly A site). Exons of the *TnI* gene are indicated as open rectangles with the Roman numerals underneath. A TATA box and an ATG initiation codon are marked. The probe used for the S1 analyses and the resulting protected fragment are drawn below as the lines with the asterisk.

were annealed with 40,000 cpm of probe and hybridized at 49°C. Nuclease digestion was carried out at 37°C for 30 min with 200 U of S1 nuclease (Pharmacia) per reaction. The samples were extracted with phenol/chloroform, ethanol-precipitated, and resolved by electrophoresis on denaturing 5–8% polyacrylamide gels.

RESULTS AND DISCUSSION

Construction and Characterization of Fusion Plasmid pTnINEO

To understand tissue-specific expression of the *TnI* gene, we developed a system in which the undifferentiated myoblast cells that can activate the transfected *TnI* promoter somehow are specifically selectable. For this purpose, plasmid pTnINEO was constructed as described in Materials and Methods. It contains 3.4-kbp quail *TnI* genomic DNA sequences spanning from the promoter to exon 5, which are followed by the *neo* gene as a selectable marker and SV40 small t intron, as well as a polyadenylation signal (Fig. 1). Since the *neo* gene is fused in frame under the *TnI* promoter, drug sensitivity is dependent on the muscle cell differentiation.

To characterize whether the TnINEO fusion gene is properly regulated in muscle cells during differentiation, multipotential 10T1/2 and 23A2 myoblast cells were cotransfected with the plasmids pTnINEO and pSV2-gpt. Since pSV2-gpt confers the resistance to mycophenolic acid and HATX by expressing the dominant selectable marker xanthine-guanine-phosphoribosyl transferase gene (*gpt*), the transfectants were selected for 2 wk in the medium containing mycophenolic acid as described in Materials and Methods. The *gpt*-positive cells were pooled and used to measure the

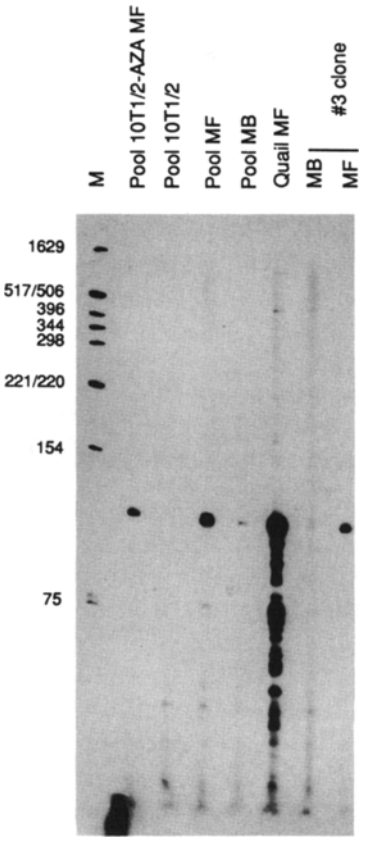

Fig. 2. S1 nuclease protection analysis of the transfected pTnINEO expression in multipotential 10T1/2 cells and in 23A2 myoblast. After transfection of cells with the plasmid pTnINEO, total cellular RNAs were extracted from the 10T1/2 cells (pool 10T1/2), 5-azacytidine derived-myofiber cultures of the pooled clonal populations (pool 10T1/2-AZA MF), and from myoblasts (MB) and myofiber (MF) cultures of an individual clone (#3 clone) and pooled clonal populations. RNAs were hybridized to the quail *TnI* genomic DNA probe (*see* Fig. 1). Quail myofiber RNAs were used as positive controls for the quail *TnI* transcripts. The molecular weight marker (M) is an *Hin*fI-digested pBR322 plasmid. A 109-nt fragment is predicted from S1 nuclease digestion. The samples were resolved on an 8% denaturing polyacrylamide gel.

RNA expression of TnINEO gene following the differentiation. In the case of 10T1/2 cells, 5-azacytidine was used to differentiate into the myoblasts. The TnINEO gene transcripts were analyzed by the S1 nuclease protection analysis using the 3.4-kbp *Bam*HI *TnI* genomic fragment of pTnINEO as a probe (Fig. 1), and the amounts of the nuclease-protected 109-bp fragment were compared between myoblasts and myofibers. As expected, the cells differentiated into myofibers have expressed TnINEO at a higher level compared to those of 10T1/2 and 23A2 myoblasts (Fig. 2). This result demonstrates that the transfected TnINEO gene is regulated properly,

which means that the differentiated myofiber is able to express the TnINEO fusion gene by providing a muscle-specific factor or factors that act on the *TnI* promoter-enhancer sequences cloned.

We next determined the differentiation specific expression of TnINEO fusion gene with the individual clone. Five individual *gpt*-positive 23A2 myoblast clones were picked randomly after cotransfection with pTnINEO and pSV2-gpt, and the RNA level of TnINEO was measured as described above using S1 nuclease protection analysis. Like the #3 clone shown in Fig. 2, all showed proper regulation of *TnI* gene expression during differentiation, which again confirmed that the transfected pTnINEO is regulated properly.

Isolation and Characterization of Drug-Resistant Myoblast Variants

Since the TnINEO fusion gene contains an intact *neo* gene following the *TnI* promoter (Fig. 1), undifferentiated 23A2 myoblast clonal lines are sensitive to the antibiotic G418. However, when cells are passaged several times, some cells lose their original drug sensitivity. Therefore, we can easily screen myoblast lines, which are G418-resistant and express the TnINEO fusion gene. In order to isolate the neomycin-resistant myoblast, the #3 myoblast clonal line was continuously passaged 12 times under nonselective media, and then screened on the media containing G418. When the G418-sensitive (1X = 278 μg/mL) #3 myoblast was exposed to 2X G418, several resistant colonies were detected with the frequency of 10^{-4}–10^{-5} (data not shown). In order to understand the possible mechanisms that lead to drug resistance, two colonies (sub-A and sub-B) were isolated and characterized further.

Since genomic rearrangement attributes the gene conversion in many cases (*9,10*), genomic Southern blot analysis was undertaken to test this possibility. Genomic DNAs were isolated from the #3 cells as well as the G418-resistant sub-A and sub-B cells, and digested with either *Eco*RI or *Bam*HI. DNAs were separated on the gel, transferred onto a nitrocellulose filter, and hybridized with the probe (*see* Materials and Methods). In both blots digested with either *Eco*RI or *Bam*HI restriction endonucleases, the original #3 line and sub-B line showed the same patterns of hybridized bands, whereas sub-A exhibited a different band pattern (Fig. 3). As reported by others (*9,10*), this result also implies that the genomic rearrangement has taken place during passages in the case of sub-A, which probably led to drug resistance. On the other hand, sub-B did not reveal genomic recombination, implying that another event (or events) leads to the conversion of drug sensitivity. In an attempt to understand the case of sub-B, the expression pattern of the *neo* as well as the *TnI* gene was examined.

Since the *neo* gene was fused in frame to the *TnI* gene under the *TnI* promoter, the expression of *neo* gene depends on the activation of the *TnI* promoter within the pTnINEO. Therefore, the expression levels of the

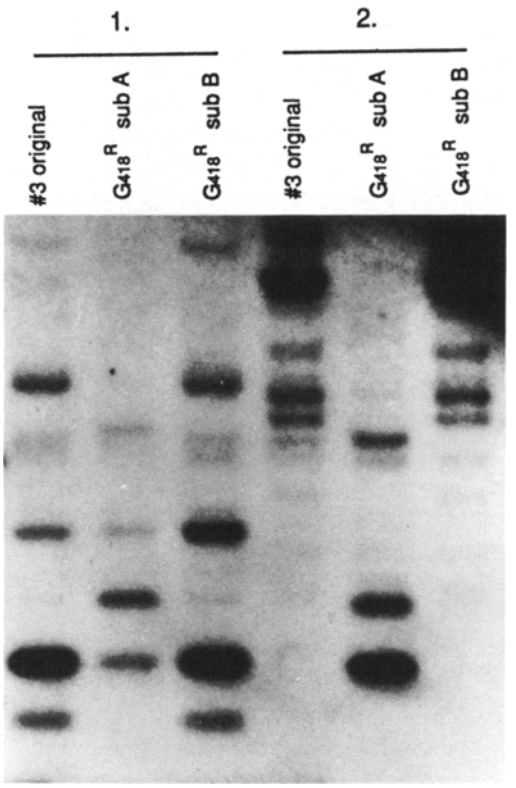

Fig. 3. Southern blot analysis of G418-resistant variants hybridized with the *neo* DNA fragment. G418-resistant colonies were rescued by growing G418-sensitive #3 myoblasts (23A2 cells stably transfected with the plasmid pTnINEO) in the media containing a high concentration of the antibiotic G418. Genomic DNAs from the G418-resistant variants (sub-A and sub-B) and original G418-sensitive #3 clonal line were digested with either *Eco*RI (1) or *Bam*HI (2) and hybridized to the 1.2-kbp *neo* gene sequences.

TnI and the *neo* genes among the #3 original lines and sub-A as well as sub-B lines were determined by S1 analysis using *neo* and quail genomic *TnI*, and mouse α-actin sequences as probes. Since the α-actin gene is expressed in differentiated muscle, this gene was used as an internal standard to estimate the relative levels of the *TnI* as well as *neo* transcripts in the different cell lines. Figure 4 shows that the expression of both the *TnI* and *neo* gene increases when cells are differentiated into myofibers in the case of sub-A. This indicates that the overall expression of the neomycin gene fused to the *TnI* gene is driven by the upstream *TnI* promoter. However, in myoblast, the *neo* gene is expressed at the detectable level, even though the *TnI* gene is not transcribed. This suggests that one or

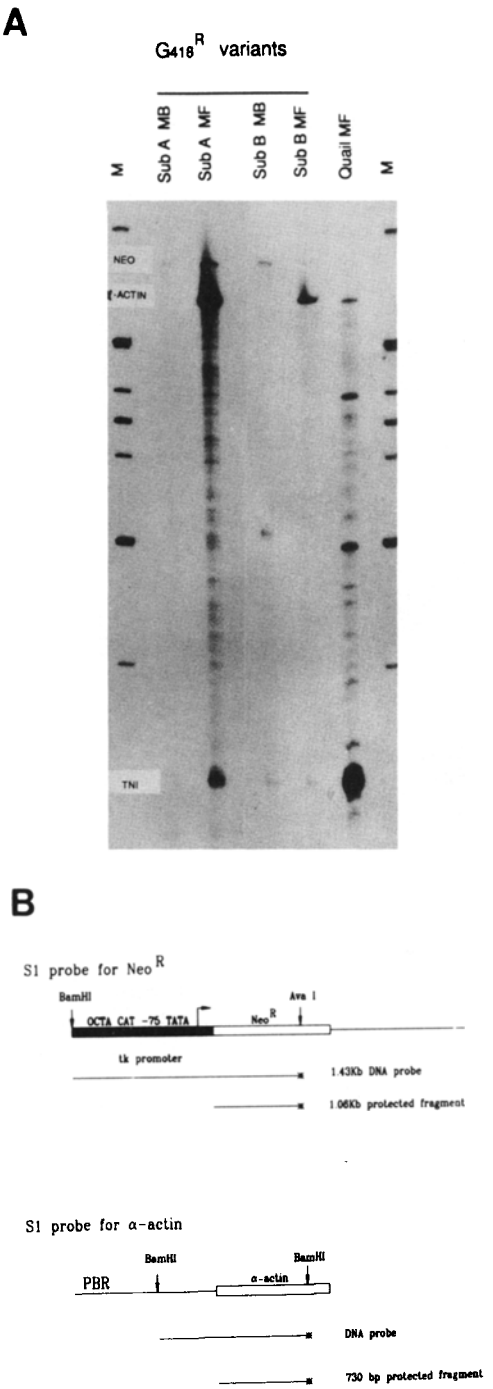

Fig. 4. (A) S1 nuclease analysis of G418-resistant variants derived from the G418-sensitive #3 clonal line. Total mRNAs from each cell line were hybridized to the *neo*, mouse α-actin, and quail *TnI* genomic DNA probes, which are shown in (B) and in Fig. 1; 1.06-kbp, 730-bp, and 109-bp protected fragments are predicted from S1 nuclease digestion for *neo*, α-actin, and *TnI* probes, respectively. Quail myofiber RNAs were used as positive controls for the quail *TnI* transcripts. Samples were resolved on a 6% denaturing polyacrylamide gel.

more copies of the *neo* gene is (are) regulated by other promoter(s), rather than by the upstream *TnI* promoter (enhancer). This result, together with the genomic blot data, implies that this specific clone arose by genomic rearrangement(s). A recombination event might have rearranged the dormant *TnI* promoter next to other enhancer, which then activated the dormant promoter. Alternatively, *neo* gene expression might be regulated by other promoters, rather than by the *TnI* promotor through rearrangement.

On the other hand, the sub-B variant has lost the capability to form myofiber. As shown in Fig. 4, a myofiber-specific α-actin message of this variant is not abundant, indicating a poor differentiation. S1 nuclease analysis shows that both the *TnI* and *neo* are more actively transcribed in myoblast than in myofiber, suggesting a possible mutation (or mutations) that led to the activation of the transfected gene in myoblast. Mutations in cis- or trans-acting elements involved in muscle-specific gene expression could activate the TnINEO gene; that is, the expression of the muscle-specific positive trans-acting factor in myoblast could activate the *TnI* promoter, or the inactivation of a repressor that regulates muscle gene expression in myoblast could lead to the activation of muscle-specific gene if muscle genes are under negative conctrol. However, the mutation in the sub-B line does not seem to be in the MyoD1-related gene, because the product of MyoD1-related gene has been shown to be sufficient to activate the muscle differentiation program (*2,11,12*). Further analysis of mutant myoblasts, such as sub-B in this particular experiment, could enable us to understand factors involved in tissue-specific expression of *TnI* gene during differentiation.

Although this experiment had been designed originally to understand muscle-specific gene expression of *TnI* and with a hope for isolating a transacting factor or factors that activate the *TnI* promoter, by simply isolating drug-resistant cells having mutation(s) that enable *TnI* gene to express many resistant cells having genomic rearrangement have been found (data not shown). This strategy, however, enriches cells having genetic recombinant through selection in the drug-containing media, and supplies good experimental sources to reveal the recombination mechanism.

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