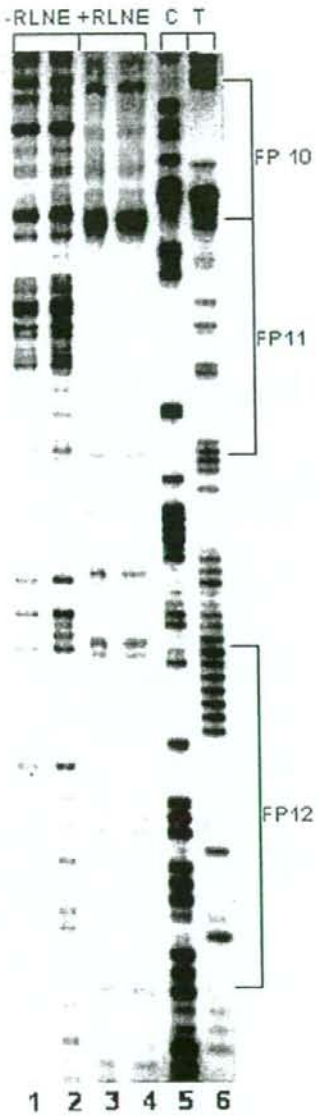

Figure 3
DNase I footprinting analysis of SMP30 promoter between -2028 to -1626 bp respectively: *Lane 1–2* represent DNA treated with DNase I in absence of RLNE. *Lane 3–4*, represent DNA treated with DNase I in presence of 50 μg RLNE *Lane 5–6*, represent sequencing ladder C and T.

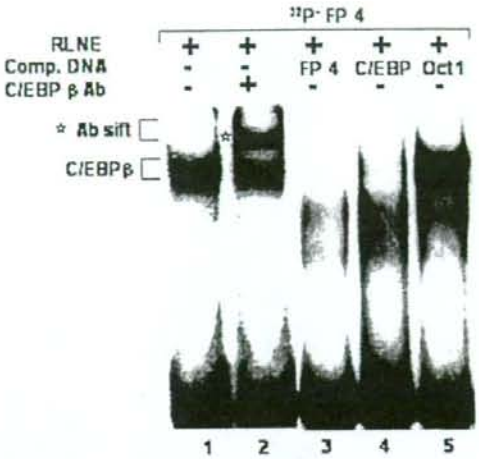

Figure 4
Electrophoretic mobility shift assay to confirm the binding of C/EBP transcription factor to DNase I protected site FP 4: *Lane 1*, labeled FP 4 oligonucleotide duplex with 6 μg RLNE; *Lane 2* C/EBPβ antibody; *Lane 3–5*, describe the competition with 100 fold molar excess of unlabeled homologous self, C/EBP consensus, and nonspecific Oct 1 oligonucleotide duplex respectively. Antibody shift is seen with C/EBPβ antibody.

factor binding site caused a significant increase in reporter activity (~23%) (Figure 5A). Individual mutation of only Sp1 and C/EBPβ did not contribute to any significant change in reporter activity. Mutation of Sp1 site reduced the reporter activity by only 16% and mutation of C/EBPβ lead to enhancement of reporter activity by only 14% (Figure 16). Binding of Sp1 to the region between -172 to -148 bp is confirmed by competitive EMSA done in presence of 100 fold molar excess of cold Sp1 consensus (Figure 17). EMSA was also carried out using labeled mutated Sp1 oligonucleotide, which showed no DNA-protein interaction, thus confirming the inability of Sp1 to bind to the mutated site. Binding of C/EBPβ to the region -190/-177 bp is confirmed by EMSA and antibody shift experiments using C/EBPβ antibody (Figure 5C). EMSA study carried out with labeled mutated C/EBP oligonucleotide also yielded a DNA-protein complex. But this complex is not due to binding of C/EBPβ as confirmed by competition with C/EBPβ consensus and antibody shift experiments (Figure 18).

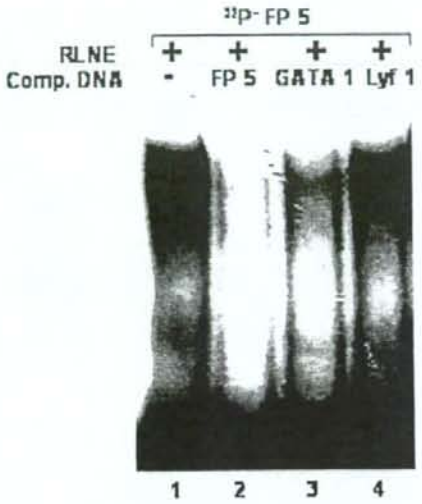

Figure 5
Electrophoretic mobility shift assay to confirm the binding of GATA1 transcription factor to DNase I protected site FP 5: *Lane 1*, labeled FP 5 oligonucleotide duplex with 6 μg RLNE; *Lane 2–4*, describe the competition with 100 fold molar excess of unlabeled homologous self, GATA1 consensus and Lyf-1 consensus.

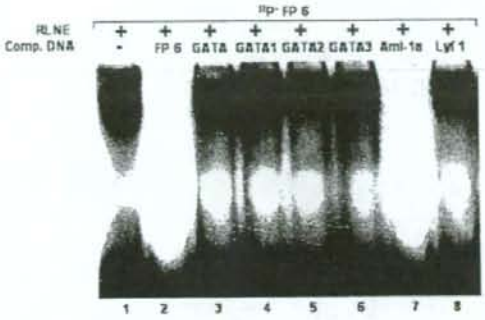

Figure 6
Electrophoretic mobility shift assay to confirm the binding of Aml-1a transcription factor to DNase I protected site FP 6: *Lane 1*, labeled FP 6 oligonucleotide duplex with 6 μg RLNE; *Lane 2–8*, describe the competition with 100 fold molar excess of unlabeled homologous self, GATA consensus, GATA1 consensus, GATA2 consensus, GATA3 consensus, Aml-1a and nonspecific Lyf-1 oligonucleotide duplex respectively.

Discussion

The multiple biological functions of SMP30 in diverse target cells require its expression to be regulated precisely. It is suggested that the transcriptional regulation of a particular gene is a complex process which usually involves interaction between multiple *cis*-acting regulatory elements and their cognate protein factors [8,9]. A growing list of transcription factors has been shown to function as either transcriptional activator or repressor in different gene promoter. In this study we analyzed the transcriptional regulation of SMP30 gene by DNase I footprinting, EMSA and functional characterization by transient transfection, reporter assay of 5' and 3' -serially deleted promoter reporter constructs and site-directed mutagenesis. We have earlier reported eight nuclear factor binding sites on SMP30 gene promoter [7]. In this report twenty eight new DNase I footprinting sites were identified using rat liver nuclear extract. We also demonstrate that the 5' -flanking regions of SMP30 gene possess a functional promoter when transfected into RAG cells. The results of 5' and 3' -deletion analysis illustrated the region -128/+157 bp possesses significant reporter activity. The presence of a TATA sequence (-29 ATAAAA -25) and a CAAT box (-72 CCAAT -68) were previously reported respective to the transcription start site [2]. Our results suggest that the TATA and CAAT box located between -128 bp and +157 bp plays an important role in determining the promoter activity and sufficient to drive SMP30 gene expression. 3' – deletion from +157 bp to +104 bp resulted in ~28% decrease in basal promoter activity, thus indicating that this region is essential for SMP30 gene expression. An interesting feature of SMP30 promoter is the presence of C/EBPβ binding site adjacent to Sp1 binding site. Sp1 is a ubiquitous DNA-binding protein with three zinc finger at its C-terminal that activates the transcription of many cellular and viral genes [10]. SMP30 promoter possess a Sp1 binding site between -172 bp to -148 bp. C/EBPβ belongs to CCAAT-enhancer-binding protein family of transcription factors, involved in different cellular response like in control of cellular proliferation, growth and differentiation, metabolism, immune response and many others. C/EBPβ binding site spans between -190 bp to -177 bp on SMP30 promoter. This spatial arrangement of C/EBPβ and Sp1 is critical as Sp1 is known to recruit C/EBPβ to cryptic C/EBP site [11]. Presence of these two sites in the minimal promoter region represented as Luc-5 did not show any significant change in luciferase activity as compared to the Luc-6, but mutation of both Sp1 and C/EBPβ significantly enhanced the reporter gene activity to about 23%. Thus, it is reasonable to believe that direct or indirect interaction between Sp1 and C/EBPβ in presence of some other regulatory factor occurs at transcriptional level

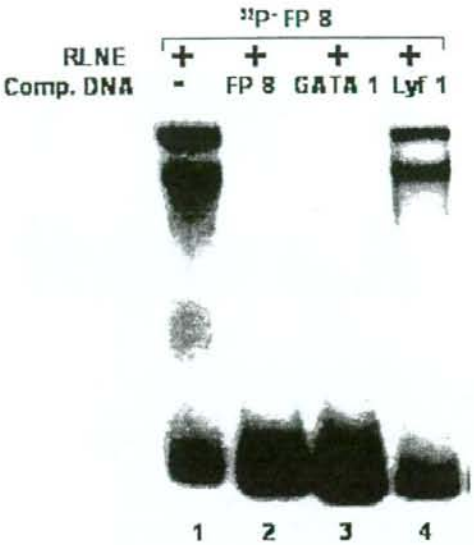

Figure 7
Electrophoretic mobility shift assay to confirm the binding of GATA1 transcription factor to DNase I protected site FP 8: *Lane 1*, labeled FP 8 oligonucleotide duplex with 6 μg RLNE; *Lane 2–4*, describe the competition with 100 fold molar excess of unlabeled homologous self, GATA1 consensus, and Lyf-1 consensus.

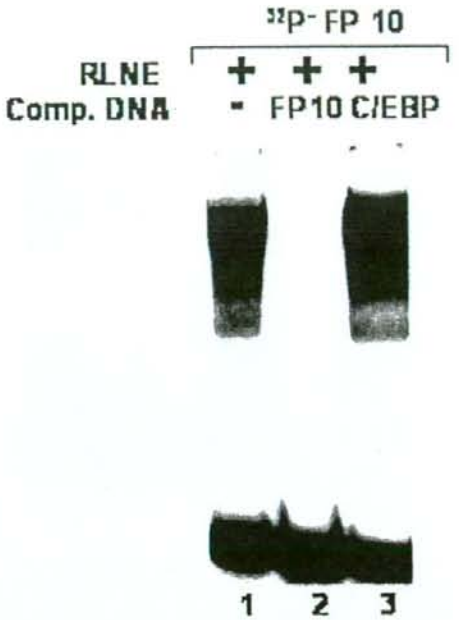

Figure 8
Electrophoretic mobility shift assay of DNase I protected site FP 10: *Lane 1*, labeled FP 10 oligonucleotide duplex with 6 μg RLNE; *Lane 2–3*, describe the competition with 100 fold molar excess of unlabeled homologous self, and non specific C/EBP consensus. Binding of no transcription factor was observed in TFSEARCH data base to this site.

in SMP30 promoter which causes a repression in SMP30 promoter activity. Transient transfection of 5' -deletion fragments revealed the presence of a repressor element between -513 to -352 bp, as deletion of this region caused 41% decrease in reporter activity. Our DNase I footprinting study showed three putative transcription factor binding sites within this region (Figure 12). In order to confirm the potential repressor among these DNase I protected sites, we carried out site directed mutagenesis studies of these three sites and subsequent transfection along with wild type (Luc-3). This result suggested a significant enhancement of reporter activity of Luc 3–3 mutated fragment by ~59%, Luc3-2 by 27% and Luc 3–1 by 29%. (Figure 13). TFSEARCH indicated the binding of SRY to wild type site Luc 3–3, GATA-2 to Luc 3–2 and CdxA to Luc 3–1 sequences, which is confirmed by competitive EMSA. Earlier reports depicted the tissue specific expression of SRY in testes [12] where it involve in testes determination and differentiation in mammals. Though expression of SRY in substantia nigra of adult male rodents in tyrosin hydroxylase expressing neurons has also been reported but its expression in liver and kidney is still obscure [13]. So the identified transcription factors might be SRY like proteins which bind to a similar binding site as SRY. The affinity of SRY for double-stranded DNA varies with DNA sequence and shares a conserved DNA binding domain (HMG-box) NACAAT [14]. SRY is reported to bind and negatively regulates the androgen receptor gene promoter [15]. We also suggest that GATA-2 and CdxA might be interacting directly or indirectly with SRY to bring about repression of SMP30 gene.

Conclusion

Transcription factors Sp1, C/EBPβ, SRY, GATA-2 and CdxA, binding within -513 of SMP30 promoter, have significant role in regulation of SMP30 gene expression.

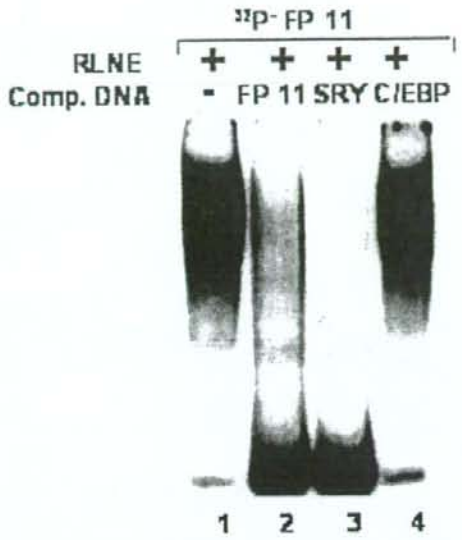

Figure 9
Electrophoretic mobility shift assay to confirm the binding of SRY to DNase I protected site FP 11: *Lane 1*, labeled FP 11 oligonucleotide duplex with 6 μg RLNE; *Lane 2–4*, describe the competition with 100 fold molar excess of unlabeled homologous self, SRY and non specific C/EBP consensus.

Methods

Preparation of nuclear extract

Nuclear extract from liver of adult (5 months) male rats (Fisher 344) were prepared as described previously [16]. Briefly, liver slices were homogenized in 4 volumes (w/v) of ice-cold buffer containing 0.25 M sucrose, 15 mM Tris-HCl (pH 7.9), 16 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.15 mM spermine, and 0.15 mM spermidine; supplemented with the following protease inhibitors: 0.1 mM PMSF, 2 μg/ml leupeptin, 5 μg/ml aprotonin. After centrifugation for 10 minutes at 2000 × g, the pellets were resuspended in 4 volumes of ice-cold buffer (10 mM HEPES; pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and protease inhibitors). The nuclei were pelleted down by centrifugation for 10 minutes at 4000 × g, and resuspended in ice cold buffer of 10 mM HEPES (pH 7.9), 0.75 mM $MgCl_2$, 0.5 M KCl, 0.5 mM EDTA, 12.5% glycerol and protease inhibitors. After incubation on ice for 30 minutes with continuous agitation, the supernatants containing the nuclear extracts were collected by centrifugation for 30 minutes at 14,000 × g, frozen in liquid nitrogen and stored in -70°C until used. All manipulations were carried out at 4°C. Protein concentrations were determined by the Bradford protein assay reagent (Sigma, USA).

DNase I footprinting

DNase I footprinting was carried out as described before [7]. Briefly, end-labeled DNA fragments (50 fmoles) were incubated with 50 μg of rat liver nuclear extract and 2 μg of poly (dI-dC) in binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5%

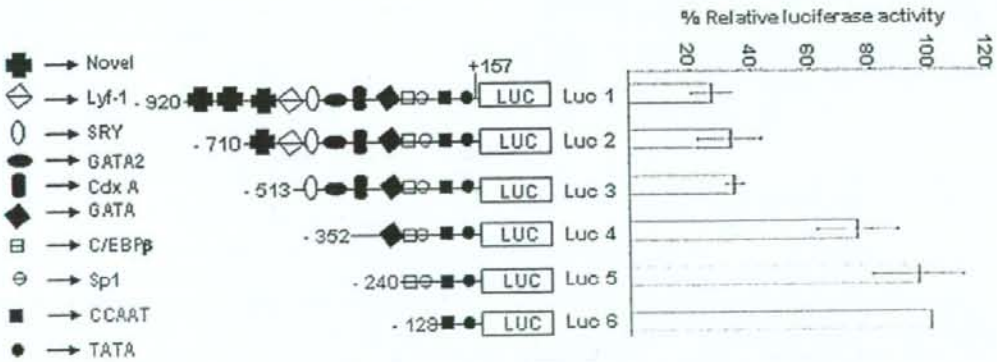

Figure 10
Relative luciferase activity of different 5' -serially deleted SMP30 promoter-reporter constructs were transfected in RAG cells. The results were obtained after normalization with β-galactosidase activity. All transfections were repeated in duplicates and the results are expressed as the mean of five different experiments ± S.D. On left, a schematic representation of all the 5' -deleted luciferase constructs used for transfection is depicted. Approximate locations of transcription factor binding sites are shown.

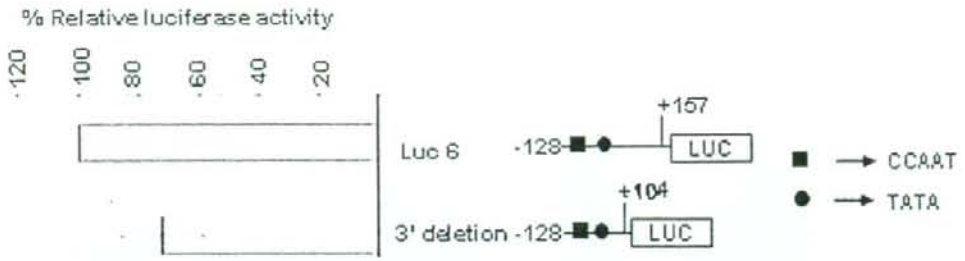

Figure 11
Luciferase analysis of 3' -deleted Luc 6 constructs as compared to wild type Luc 6 construct showing ~ 28% reduction of reporter activity. All transfections were repeated in duplicates and the results are expressed as the mean of two different experiments ± S.D. On right, a schematic representation of 3' deleted luciferase construct used for transfection is depicted along with wild Luc 6. Approximate locations of transcription factor binding sites are shown.

glycerol at room temperature for 30 minutes. Subsequent to binding reaction 7.5 mM $MgCl_2$ and 5 mM $CaCl_2$ were added and samples were incubated at room temperature with DNase I (0.25 U, Roche, USA) for 60 s. Ten to twenty folds less DNase I was used for control experiments without nuclear extracts. DNase I enzyme digestion was stopped by the addition of an equal volume of 1% SDS, 20 mM EDTA, 400 mM NaCl, 100 μg/ml yeast tRNA and 200 μg/ml proteinase K. Following incubation at 45°C for 60 minutes, samples were extracted twice with phenol/chloroform, precipitated with ethanol and electrophoresed on 6% polyacrylamide sequencing gel. After electrophoresis, gels were dried on Whatman filter paper and autoradiographed. Primers used for generating end labeled DNA fragments for footprinting of the region -513/-352 bp are: 5' -GCCTCATGCAAGGAAGCAAG-3' SS and 5' -GATAATGGCAGGTATGAGGG-3' AS. Primers used for footprinting from -2750 bp to -777 bp are shown in table 1.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides (both strands) corresponding to identified DNase I protected sites (table 2, 3, 5 and 6) were synthesized. For each site, one strand was end-labeled with [γ-^{32}P] ATP using T_4 polynucleotide kinase and annealed to its complementary unlabeled strand. Nuclear extracts (4–6 μg) were incubated with 20 fmoles of radiolabeled oligonucleotide duplex in 30 μl reaction containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 % glycerol and 1.0 μg poly (dI-dC) for 20 minutes at room temperature. In competition experiments, 100-fold molar excess of unlabeled oligonucleotide duplexes were added during preincubation period. For antibody shift assay, C/EBPβ antibody (Santacruz, USA) was added after addition of nuclear extract and incubated at 4°C for 10 min. Free DNA and protein bound DNA was separated on 5% non-denaturing polyacrylamide gel in 0.5 X Tris-boric acid-EDTA (TBE). After electrophoresis, gels were blotted onto filter paper, dried and autoradiographed.

Construction of 5' and 3' -serially deleted SMP30 fragments and it's cloning into pGL3-Basic vector

To construct 5' -serially deleted SMP30 fragments Luc-SMP-XhoI reverse primer and the forward primers as mentioned in table 4, containing Kpn1 sites were used. The PCR amplification was carried out using step cycles (94°C for 30 s, 62°C for 30 s, 72°C for 30 s) for 35 cycles with a final extension at 72°C for 10 minutes. Then the PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, USA). The serially deleted fragments and pGL3-Basic vector were digested with KpnI and XhoI enzyme (MBI Fermentas). The digested 5' -serially deleted fragments were then ligated into restriction enzyme digested pGL3-Basic vector using DNA ligase (USB, USA). The cloned fragments were then confirmed by vector specific PCR using RV and GL2 primer, and also by sequencing.

Site-directed mutagenesis

Five to six bases of the transcription factor core binding site were mutated as shown in the table 5 (bases in small letter represents mutated base). For mutagenesis of transcription factors two sets of PCR were carried out using the following combination of primers: For Sp1: MutSp1 sense/Luc-SMP-XhoI antisense and Luc 5 sense/Mut Sp1 antisense; for C/EBP: Mut C/EBP sense/Luc-SMP-XhoI antisense and Luc 5 sense/Mut C/EBP antisense; for Mut Luc 3–1: Mut Luc3-1 sense/Luc-SMP-XhoI antisense and Luc-SMP-3 sense/Mut Luc 3–1 antisense; for Mut Luc 3–2: Mut Luc 3–2 sense/Luc-SMP-XhoI antisense and Luc-3 sense/Mut Luc 3–2 antisense; for Mut Luc 3–3: Mut Luc 3–

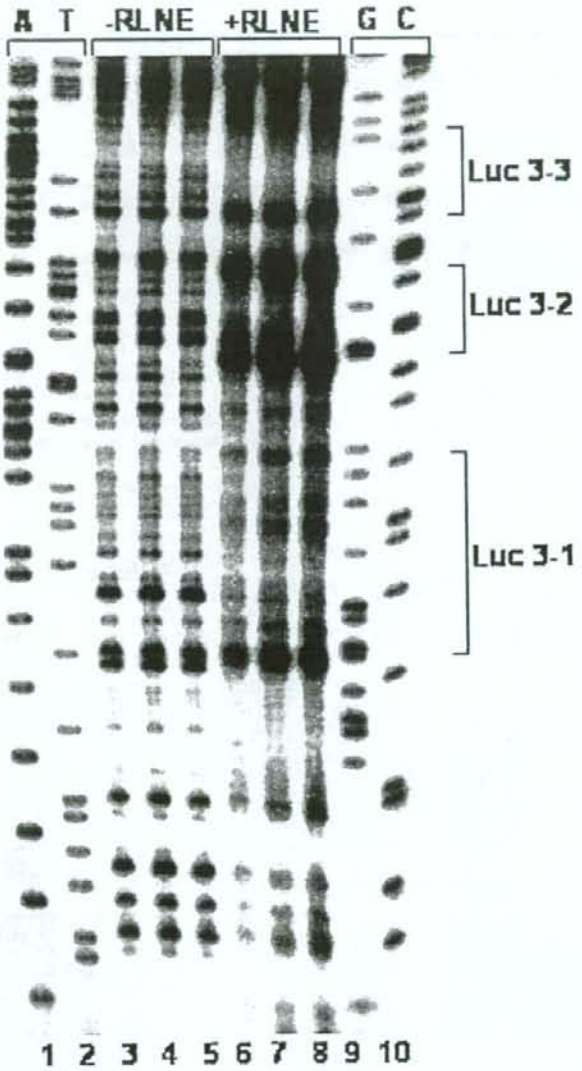

Figure 12
DNase I footprinting pattern of the repressor region (-513 to -352). *Lane 1,2 and 9,10* refer to sequencing reaction ladder obtained by taking the same labeled primer used in PCR for DNase I footprinting. *Lane 3,4,5* represent DNA treated with DNase I in absence of nuclear protein. *Lane 6,7,8* represent DNA treated with DNase I in presence of 50 μg rat liver nuclear extract (RLNE). The DNase I protected sites are marked on right site and the sequences are given in table 3. DNase I footprinting was carried out with fragments generated by labeled forward primer.

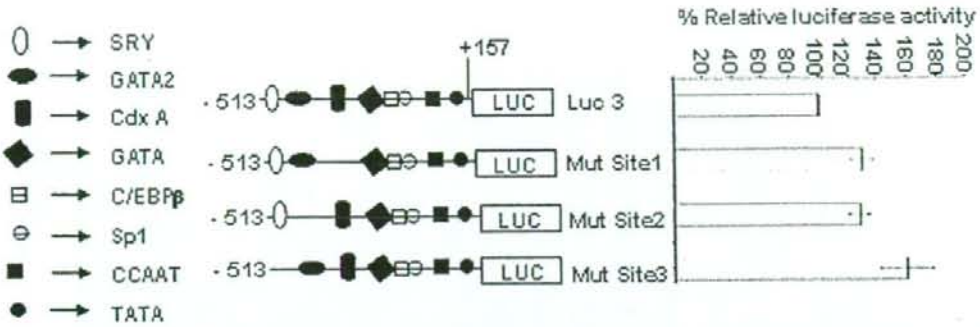

Figure 13
DNase I protected sites, Luc 3–1, Luc 3–2 and Luc 3–3 were mutated at their core binding sites. Reporter activity of the three mutated construct along with wild type Luc 3 construct is represented on the right site and the schematic representation of the mutated sites along with wild type Luc 3 on left site. All transfections were repeated in duplicates and the results are expressed as the mean of three different experiments ± S.D Approximate location of the transcription factor binding to wild type Luc 3 and site directed mutated construct are shown. Mutation of site 3 shows a significant (~59%) increase in luciferase activity as compared to wild type Luc3.

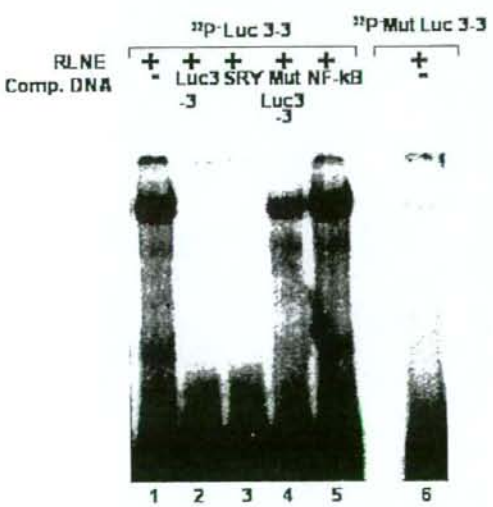

Figure 14
Electrophoretic mobility shift assay (EMSA) for site Luc 3–3 to confirm the binding of SRY transcription factor. *Lane 1*, labeled oligonucleotide duplex with 6 μg RLNE; *Lane 2–5*, 100 fold molar excess of unlabeled homologous self, SRY consensus, mutated Luc 3–3, nonspecific oligonucleotide duplex (NFkB). *Lane 6*, labeled Mut 3–3 oligonucleotide duplex with 6 μg RLNE.

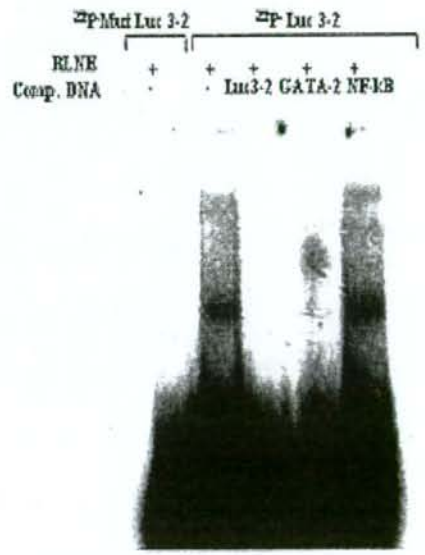

Figure 15
EMSA for site Luc 3–2 to confirm the binding of GATA-2 transcription factor. *Lane 1*, labeled mutated oligonucleotide duplex with 6 μg RLNE; *Lane 2*, labeled oligonucleotide duplex with 6 μg RLNE; *lane3 -5*, 100 fold molar excess of unlabeled homologous self, GATA-2 consensus, and nonspecific (NFkB) oligonucleotide duplex (NFkB).

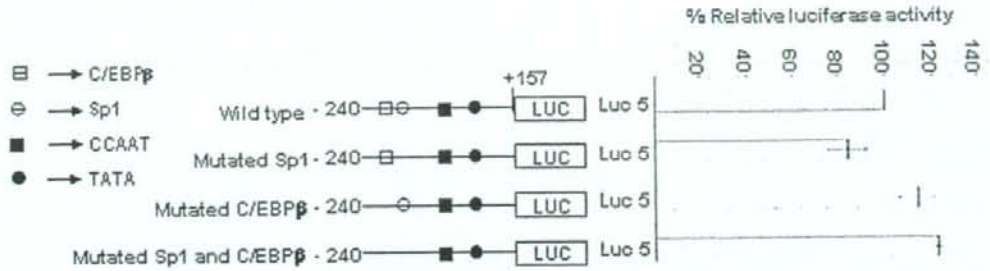

Figure 16

Reporter activity of site direct mutated Sp1, site direct mutated C/EBP and site direct mutated Sp1 and C/EBP sites reporter constructs along with wild type Luc 5 on right site and the schematic representation of the mutated Sp1 and C/EBP sites along with wild type Luc 5 sites on left site. All transfections were repeated in duplicates and the results are expressed as the mean of three different experiments ± S.D Approximate location of the transcription factor binding to wild type Luc 5 and site directed mutated construct are shown. A significant decrease (~16%) in reporter activity of mutated Sp1 construct is seen as compared with wild type Luc 5. There is no significant change in reporter activity with mutated C/EBP construct. Double mutation of Sp1 and C/EBP enhance the reporter activity by 23% as compared to wild type Luc 5.

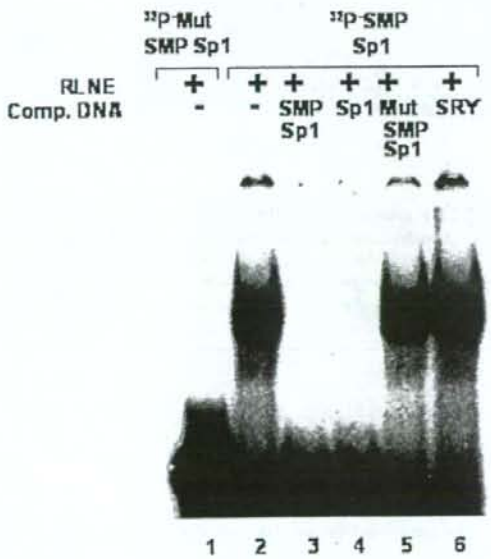

Figure 17

Electrophoretic mobility shift assay to confirm the binding of Sp1 transcription factor. *Lane 1*, labeled mutated SMP-Sp1 oligonucleotide duplex with 6 μg RLNE; *Lane 2–6*, labeled SMP-Sp1 oligonucleotide duplex with 6 μg RLNE; *Lane 3–6*, describe the competition with 100 fold molar excess of unlabeled homologous self, Sp1 consensus, Mut SMP-Sp1 and nonspecific SRY oligonucleotide duplex respectively.

3 sense/Luc-SMP-XhoI antisense and Luc-SMP-3 sense/Mut Luc 3–3 antisense. The PCR amplification was performed using step cycles (94°C for 1 min, 62°C for 30 s, 72°C 30 s) for 35 cycles with a final extension at 72°C for 10 minutes. Both the PCR products were purified using QIAquick Gel Extraction Kit. DNA was eluted using 30 μl of autoclaved deionised water. 5 μl of each PCR product was used as a template for the second round of PCR. For example: for construction of mutant Sp1 site: 5μl each of the PCR product Mut Sp1 sense/Luc-SMP-XhoI antisense and Luc-5 sense/Mut Sp1 antisense was used as template. For construction of mutant Sp1 and mutant C/EBP, Luc-5 and Luc-SMP-XhoI was used as forward and reverse primers. For construction of mutant Luc 3–1, Luc 3–2 and Luc 3–3, Luc-3 and Luc-SMP-XhoI was used as forward and reverse primers. PCR amplification was carried out using the same parameters as mentioned above. Then the PCR products were purified using QIAquick Gel Extraction Kit. The fragments with mutated transcription factor binding sites, having KpnI and XhoI restriction sites and pGL3-Basic vector were digested with KpnI and XhoI enzyme. The fragments were then ligated into restriction enzyme digested pGL3-Basic vector using DNA ligase. The cloned fragments were then confirmed by vector specific PCR using RV and GL2 primer, and the mutation was confirmed by sequencing.

Transient transfection and luciferase assay

Transient transfections were carried out using RAG cells (mouse renal adenocarcinoma cell line) as SMP30 is also expressed in kidney. The cells were plated at a density of 2 × 10^5 cells per well in six well plates, 18 h before transfection. For transient transfection 2 μg of respective reporter plasmid DNA and 0.5 μg of pSV-β-gal control vector

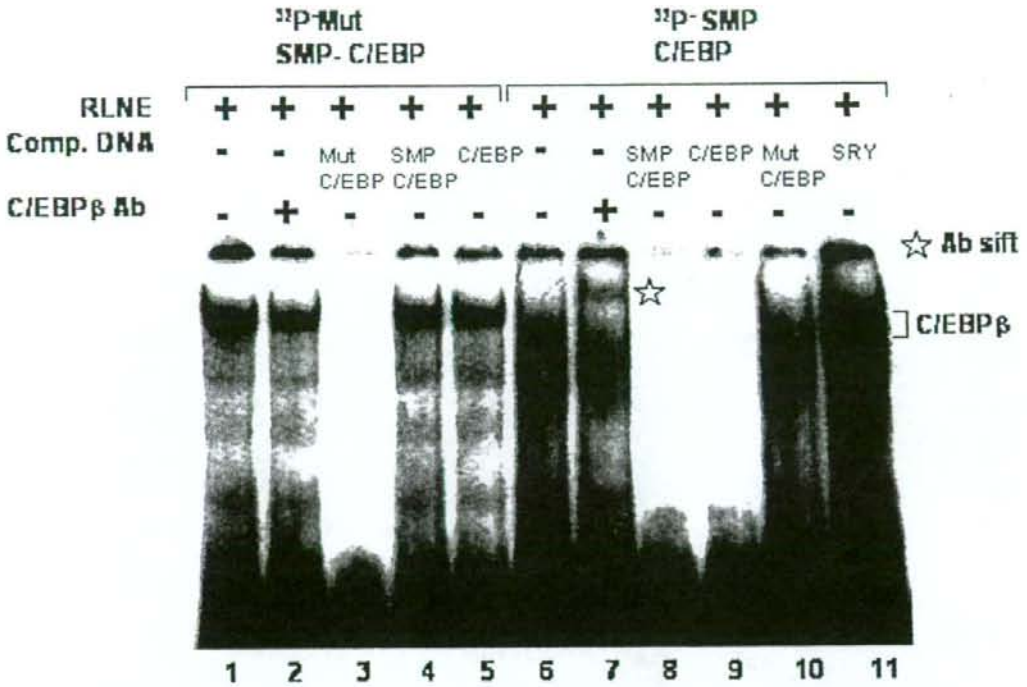

Figure 18

Electrophoretic mobility shift assay to confirm the binding of C/EBP transcription factor. *Lane 1–5*, labeled mutated C/EBP oligonucleotide duplex with 6 μg RLNE; *Lane 2*, C/EBPβ antibody; *Lane 3–5*, describe the competition with 100 fold molar excess of unlabeled homologous self, SMP-C/EBP and C/EBP consensus. *Lane 6–11*, labeled SMP-C/EBP oligonucleotide duplex with 6 μg RLNE; *Lane 7*, C/EBPβ antibody; *Lane 8–11*, describe the competition with 100 fold molar excess of unlabeled homologous self, C/EBP consensus, Mut SMP-C/EBP and nonspecific SRY oligonucleotide duplex respectively. Antibody shift is seen with SMP-C/EBP only and not with mutated SMP-C/EBP.

(Promega, USA) or 100 ng of pRL-TK control vector were cotransfected into cells using FuGENE reagent (Roche, USA). After 24 h post transfection, the cells were harvested, lysed, centrifuged and the lysate was used for luciferase assay using the luciferase assay system (Promega, USA). The colorimetric β-galactosidase assay was performed using β-Gal assay kit (Invitrogen, USA) and luciferase activity was divided by the β-galactosidase activity to normalize for transfection efficiency. For transfection of mutated constructs Renella was used as an internal control and dual luciferase assay was preformed to measure the luciferase reading as per manufacturer's instruction

Table 5: Primers and oligonucleotide used for site- directed mutagenesis.

Mut Luc-3-1	GCTGgAGGCcTAGCTCTGTAGCAGAgTACAccCAAG
Mut Luc-3-2	CAgGGTCCTcGTTCcATtCCaG
Mut Luc-3-3	CCAGTgCAgACgAGCAAGCggCTGTATATgC
SP1-SMP	GCTCCCCCCCCCCGCCCCCCCCCAGGG
Mut-SP1	GCTCCtCCtCCtCGtCtCCCtCCAG
C/EBP-SMP	ACTGATGTACACATTCCTAAAACTGGC
Mut-C/EBP	ACTGgTGgACACAggCCTAggACTGGC

Table 6: Oligonucleotide of the three footprints between -513 to -352 bp used for EMSA.

Footprint	DNase I protected region	Transcription factor
Luc3-1	GGAGCTGGAGGCATAGCTCTGTAGCAGAATACATTCAAGGT	CdxA
Luc3-2	TTCAAGGTCCTAGTTCTATCCCAG	GATA-2
Luc3-3	AACTACCAGTACAAACAAGCAAGCAACTGTATACAT	SRY

(Promega, USA). All the transfections were repeated in duplicates in three to five independent experiments. The number of independent experiments is being mentioned in respective figure legends.

Abbreviations

SMP30: Senescence Marker Protein 30; EMSA: Electrophoretic Mobility Shift Assay; RLNE: Rat Liver Nuclear Extract.

Authors' contributions

PCS and NM conceived the idea, designed and planned the experiments. BR and PCS wrote the manuscript. BR and RSP were involved in all experimentations. PRD was involved in designing and preparation of promoter-reporter constructs. All authors have analyzed the data and agreed with the final version of the manuscript.

Acknowledgements

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Myasthenia Gravis Experimentally Induced with Muscle-specific Kinase

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Here we present the first evidence that muscle-specific kinase (MuSK) antigen can cause myasthenia in animals. MuSK is expressed at the postsynaptic membranes of neuromuscular junctions (NMJ) and forms complexes with acetylcholine receptors (AChR) and rapsyn. MuSK is activated by agrin, which is released from motoneurons, and induces AChR clustering and subsequent formation of NMJ in embryos. Notably, autoantibodies against MuSK were found in a proportion of patients with generalized myasthenia gravis (MG) but without the characteristic AChR autoantibodies. However, MuSK autoantibodies had no known pathogenic potential, and animals immunized with purified MuSK proteins did not develop MG in former studies. In contrast, we have now injected rabbits with MuSK ectodomain protein *in vivo* and evoked a MG-like muscle weakness with a reduction of AChR clustering at the NMJ. Our results showed that MuSK is required for maintenance of synapses and that interference with that function by MuSK antibodies causes myasthenic weakness. *In vitro,* AChR clustering in myotubes is induced by agrin and agrin-independent inducers, which do not activate MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty, but MuSK autoantibodies in myasthenic animals inhibited both agrin and agrin-independent AChR clustering. MuSK plays multiple roles in pre-patterning of the postsynaptic membrane before innervation and formation of NMJ in embryos. Some of these mechanisms may also participate in the maintenance of mature NMJ. This model system would provide new knowledge about the molecular pathogenesis of MG and MuSK functions in mature NMJ.

Key words: **myasthenia gravis (MG); experimental autoimmune MG (EAMG); muscle-specific kinase (MuSK); acetylcholine receptor (AChR); neuromuscular junction (NMJ); congenital myasthenic syndromes (CMS)**

Introduction

Although autoantibodies against muscle-specific kinase (MuSK) have been found in patients with myasthenia gravis (MG),[1] any pathogenic contribution of MuSK antibodies to the muscle weakness that typifies MG has remained in dispute. That is, until now, MuSK

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antibodies have not produced experimental autoimmune MG (EAMG).[2,3] Here we describe the recent progress toward understanding this phenomenon.

Autoantibodies against MuSK

About 80% of patients with MG have autoantibodies against acetylcholine receptor (AChR). A seminal experiment by Patrick and Lindstrom demonstrated the pathogenicity of autoantibodies to AChR about 30 years ago.[4] Although a number of studies have documented that AChR antibodies cause structural and functional damage to the neuromuscular junction (NMJ), autoantigens, in the nearly 20% of MG patients without such antibodies, remained obscure.[1] Then, in 2001, Hoch *et al.* identified antibodies against MuSK in a proportion of patients with generalized MG.[1] MuSK is required for clustering of AChR during the formation of NMJ and is expressed predominantly at the postsynaptic membrane in mature NMJ.[5,6] In MuSK knockout mice, AChRs fail to cluster opposite to growing motoneuron terminals on the surfaces of myotubes.[7] Additionally, a case of heteroalleric MuSK mutations that caused the reduction of MuSK expression has been associated with congenital myasthenic syndrome (CMS).[8] Further, the reduction of MuSK expression in rat muscles *in vivo* upon RNA interference induced disassembly of synapses.[9] Even though the function of MuSK in mature NMJ is still uncertain, a causal relationship between MuSK autoantibodies and MG has been proposed.[1,10,11]

Recent studies by Vincent and others showed that the frequency of MuSK antibodies in MG patients who were AChR seronegative (lacked autoantibodies to AChR) varied from 4 to 50%.[11–17] We detected MuSK antibodies in 29% of seronegative MG patients but not in any MG patients with AChR antibodies (seropositive MG) or with other autoimmune diseases.[16] Previously, we identified antibodies against a recombinant MuSK fusion protein with human alkaline phosphatase (AP) in seropositive MG patients[15] and later revealed that 8.8% of seropositive MG patients had autoantibodies to AP but not to MuSK.[16] We are currently studying the clinical significance of the autoantibodies to AP in seropositive MG.

Clinical features of patients with MG and MuSK antibodies are distinctive. Such patients often have severe bulbar dysfunctions that can be difficult to treat effectively with immunosuppressive and immunomodulatory strategies, and atrophy of facial and tongue muscles is common.[12,13,18,19] After the identification of MuSK antibodies in MG patients, laboratory quantification of these antibodies is now required to confirm the diagnosis of MG, the appropriate clinical treatment, as well as the presence of AChR antibodies.[18,20,21]

Experimental Autoimmune MG

Although MuSK antibodies are present in some seronegative MG patients and the clinical features are distinctive, proving the pathogenicity of MuSK antibodies has been difficult because these antibodies did not induce myasthenia in experimental animals. Formerly, the pathogenicity of AChR antibodies was shown when rabbits injected with AChR protein purified from electric eels developed muscle weakness and paralysis.[4] Injection of eel AChR protein stimulates the production of antibodies that cross-react with rabbit AChR at the NMJ. Electrophysiological studies confirmed that the flaccid paralysis in this animal model resembled that in MG patients. Similarly, EAMG appeared in other species after immunization with purified AChR protein. In addition, the antibodies to AChR in human MG patients could passively transfer disease to mice.[22] Therefore, creating an EAMG model induced by MuSK antibodies was indispensible for proving the pathogenicity of MuSK antibodies and investigating their pathogenic mechanisms in MG.[10,20,21]

To pursue this objective, we recently immunized rabbits with MuSK ectodomain, which caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG, as shown by Patrick and Lindstrom.[23] The extracellular segment of MuSK comprises five distinct domains, i.e., four immunoglobulin-like domains and one cysteine-rich region.[5,6] The fusion protein expression constructs we generated consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag and were used to transfect COS-7 cells.[23] The recombinant MuSK-Fc and MuSK-His proteins secreted were purified by using protein-A Sepharose and histidine affinity columns, respectively (FIG. 1). New Zealand white rabbits were then immunized with 100–400 mg of this purified MuSK recombinant protein. After three to four injections of MuSK protein, all six treated rabbits manifested flaccid paralysis (FIG. 2). Sera from the paretic rabbits contained high titers of MuSK antibodies that reacted specifically with MuSK molecules as observed by testing sera from MG patients with MuSK antibodies.[1,24] The paretic rabbits developed severe muscular exhaustion revealed by histological studies showing alterations in muscle fibers

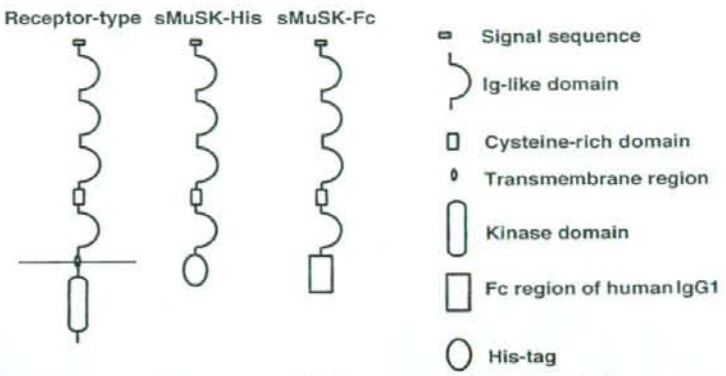

FIGURE 1. Schematic representation of the muscle-specific kinase (MuSK) domain structure and expression of secretory MuSK proteins in COS-7 cells. The domain structures of recombinant secretory MuSK protein (MuSK-His and MuSK-Fc) and receptor-type MuSK are shown. The whole coding region of the MuSK extracellular domain was fused with the His-tag or Fc region of human IgG1 as shown.

ranging from subtle to angular atrophy intermingled with normal muscle. Atrophic changes of this type can result from MG, reduced mechanical activity of muscles, or cachexia. Repetitive electromyograms of a paretic rabbit were then done to measure the result of stimulating the retroauricular branch at 20 Hz and recording responses from the retroauricular muscle. The compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG.[23] However, the injection of ACh esterase inhibitor did not significantly offset the CMAP decrement or decrease the symptoms. Importantly, the induction of EAMG by MuSK antibodies is not limited to rabbits, i.e., we and others have also produced EAMG in mice by injecting MuSK protein (FIG. 2).[25]

AChR Clustering and Structure of NMJ in Rabbits with EAMG and MuSK Antibodies

The clustering of AChR necessary for NMJ formation is completely abolished in MuSK knockout mice,[7] and AChR clustering at the NMJ is reduced in subjects with CMS and MuSK mutations.[8] In a previous RNA interference experiment, injection of double-stranded RNA (dsRNA) targeting MuSK diminishes the expression of MuSK protein and AChR clusters in rat muscle fibers *in vivo*, whereas dsRNA targeting nonessential proteins does not have any effect (RNA-interference experiment).[9] Therefore, we examined the expression of AChR at NMJ in soleus muscles of paretic and normal rabbits by fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, a-bungarotoxin. Images of AChR clustering were then recorded by using a digital camera.[23] The sizes and optical densities were measured using National Institute of Health (NIH) image analysis software with unprocessed digitized NIH images (version 1-62; http://rsb.info.nih.gov/nih-image). The results unequivocally pictured a significantly reduced area and intensity of AChR fluorescence in the paretic rabbits compared with their normal counterparts. In addition, a structural examination showed that the size and branching of the NMJ were significantly diminished in paretic rabbits. Similar changes of NMJ structure were observed in rats with reduced expression of MuSK evident by RNA interference,[9] in a patient with CMS and MuSK mutations, and in mice expressing the missense mutation by electroporation experiments.[8] Our results demonstrated that MuSK antibodies also elicited synaptic changes in EAMG, including the reduced expression of surface AChR at postsynaptic membranes of NMJ. Further examination of MuSK knockout mice disclosed presynaptic defects in addition to postsynaptic ones,[7] indicating that MuSK is also required for presently unidentified retrograde signals to maintain the presynaptic structure in mature NMJ.

Pathogenic Mechanisms of MuSK Antibodies in AChR Clustering at NMJ

MuSK plays multiple roles in clustering AChR during development of the postsynaptic membrane of NMJ. Contact of the motor-nerve growth cone with

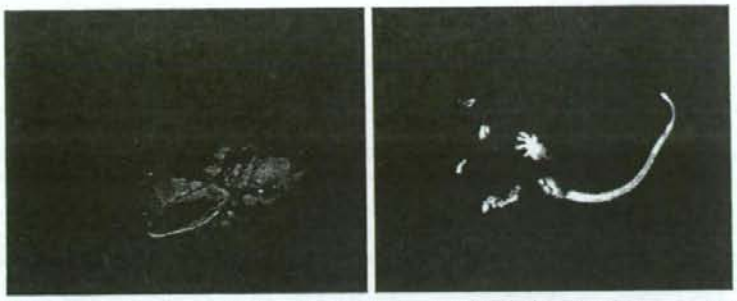

FIGURE 2. Manifestation of muscle weakness after injection of purified MuSK proteins in experimental animals (*left*, a paretic rabbit; *right*, a paretic mouse).

the muscle induces a narrow, distinct, endplate zone in the mid-muscle that is marked by a high density of AChR clustering.[26–29] In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites. However, a direct physical interaction between MuSK and agrin has so far not been demonstrated, despite many efforts to do so.[27] Thus, the mechanisms of MuSK activation and the following events remain obscure, although a co-receptor of MuSK, co-ligand of agrin or either post-translational modification of agrin or MuSK have been postulated. Intriguingly, MuSK is also required for organizing a primary synaptic scaffold to establish the post-synaptic membrane.[30,31] Preceding muscle innervations, AChR clusters form at the central regions of muscle fibers, creating an endplate zone that is somewhat broader than that in innervated muscle. Thus, MuSK is required for pre-patterning of AChR clustering in the absence of motor innervation. The scenario of MuSK's roles in the process is somewhat complicated; possibly an element other than agrin achieves activation of MuSK and triggers postsynaptic specialization at the NMJ, and/or MuSK acts as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at NMJ development could also require the maintenance of mature NMJ. Studies performed *in vivo* have shown that synaptic AChRs intermingle completely over a period of approximately 4 days and that many extrasynaptic AChRs are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears to be macroscopically stable.[32,33] Therefore, the mechanisms at play in AChR clustering during NMJ development are also required in mature NMJ when postsynaptic complexes, including AChR and MuSK, are dynamically turning over for maintenance.

To elucidate the mechanisms of AChR clustering at NMJ, a number of studies were performed using cultured C2C12 myotubes. Agrin induces clustering of AChR in C2C12 myotubes following MuSK autophosphorylation.[26,27,29] This event *in vitro* represents a major cascade of AChR clustering at the NMJ after innervation by motoneurons.[27,34–36] Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) can induce AChR clustering in C2C12 myotubes without activation of MuSK.[34,37–40] Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. However, these mechanisms may also play important roles in the maintenance of NMJs via agrin-independent pathways and in their formation, as shown by genetic studies.[30,31]

In their previous study, Hoch *et al.* observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes.[1] We also found that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect.[23] Thus, MuSK antibodies were responsible for inhibiting the formation of agrin-induced AChR clustering. We also perceived that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself.

Conclusions

In our experimental model of myasthenia, MuSK antibodies routinely mediated pathogenesis in rabbits and mice.[23,25] Consequently, we now believe that MuSK antibodies cause MG in patients. However,

the pathogenic mechanisms of these antibodies entail multiple events in which MuSK acts as a multifunctional platform from which to regulate synapse formation and maintenance. These are reflected in a diversity of clinical features ranging from typical MG to a multitude of variants.[12,13,18,19]

AChR antibodies have been shown to affect neuromuscular transmission by three main mechanisms: (a) binding and activation of complement at the NMJ; (b) accelerated degradation of AChR molecules cross-linked by antibodies (antigenic modulation); and (c) functional AChR block.[20,21] Intriguingly, MuSK antibodies in MG patients are mainly of the IgG4 subclass, which does not activate complement.[41] Electron microscopic observations of NMJ in the EAMG rabbits demonstrated a significant reduction of synaptic folds but no destruction, thus our EAMG model resembles the phenotypes of MG with MuSK antibodies. MuSK antibodies against compound antigenic determinants on the extracellular domain may elicit pathogenic effects through antigenic modulation and/or restraint of MuSK functions,[41] and the consequences of these effects range from a partial to entire loss of MuSK functions.

Recently, a new MuSK-interacting cytoplasmic protein, called Dok-7, has been discovered.[42] Dok-7 knockout mice underwent a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Mutations in Dok-7 caused a genetic form of limb-girdle myasthenia (CMS).[43,44] Some clinical features of these patients resemble the severe type of MG with MuSK antibodies[44]; therefore, the EAMG model with MuSK antibodies presented here promises to facilitate resolution of the pathogenic basis of MG and CMS at the molecular level and identification of beneficial treatment strategies against them.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Neurobiology

Accumulation of Citrullinated Proteins by Up-Regulated Peptidylarginine Deiminase 2 in Brains of Scrapie-Infected Mice

A Possible Role in Pathogenesis

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Peptidylarginine deiminases (PADs), which are a group of posttranslational modification enzymes, are involved in protein citrullination (deimination) by the conversion of peptidylarginine to peptidylcitrulline in a calcium concentration-dependent manner. Among the PADs, PAD2 is widely distributed in various tissues and is the only type that is expressed in brain. To elucidate the involvement of protein citrullination by PAD2 in the pathogenesis of brain-specific prion diseases, we examined the profiles of citrullinated proteins using the brains of scrapie-infected mice as a prion disease model. We found that, compared with controls, increased levels of citrullinated proteins of various molecular weights were detected in different brain sections of scrapie-infected mice. In support of this data, expression levels of PAD2 protein as well as its enzyme activity were significantly increased in brain sections of scrapie-infected mice, including hippocampus, brain stem, and striatum. Additionally, the expression levels of PAD2 mRNA were increased during scrapie infection. Moreover, PAD2 immunoreactivity was increased in scrapie-infected brains, with staining detected primarily in reactive astrocytes. Using two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry, various citrullinated proteins were identified in the brains of scrapie-infected mice, including glial fibrillary acidic protein, myelin basic protein, enolases, and aldolases. This study suggests that accumulated citrullinated proteins and abnormal activation of PAD2 may function in the pathogenesis of prion diseases and serve as potential therapeutic targets. *(Am J Pathol 2008, 173:1131–1144; DOI: 10.2353/ajpath.2008.080388)*

Accumulation of misfolded proteins, posttranslational modification of proteins, alteration of free ion distribution, and perturbation of cellular redox homeostasis are general features of progressive neurodegenerative disorders. These changes have been observed consistently as part of the neuropathogenesis and neuropathology of prion diseases. Prion diseases are characterized by various neurological symptoms and common histopathological features such as spongiform degeneration of the central nervous system, reactive gliosis, neuronal loss, and, in some cases, formation of amyloid plaques.[1] It has been reported that all prion diseases are associated with the aberrant metabolism of prion protein (PrP). Conversion of the cellular prion protein (PrP^{C}) into an abnormal, protease-resistant and infectious isoform (PrP^{Sc}) is believed to be a principal molecular basis of prion diseases,[2] and the accumulation of PrP^{Sc} in the central nervous system is thought to be responsible for neuronal loss and/or astrocytosis.[3] In general, the pathogenic mech-

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anisms of neurodegenerative disorders are not fully delineated; prion diseases are no exception to this uncertainty.

Alteration of intracellular calcium (Ca^{2+}) distribution and Ca^{2+}-related proteins have a critical role in synaptic dysfunction and neuronal cell death in neurodegenerative diseases. In cultured cells, prion infection induced abnormalities in Ca^{2+} homeostasis by altering receptor-mediated intracellular Ca^{2+} responses,[4,5] suggesting a possible role of Ca^{2+} in the neuronal cell death seen in prion diseases. Peptidylarginine deiminases (PADs) are known to be directly affected by Ca^{2+} homeostasis and convert peptidylarginine to peptidylcitrulline (protein citrullination or deimination) in a Ca^{2+} concentration-dependent manner.[6,7] This modification of proteins decreases their positive charge resulting in changing the functions of native proteins.[8] PADs are found as five different isoforms in various mammalian tissues such as brain, spinal cord, spleen, and skeletal muscle.[9] Among them, only PAD type II (PAD2) is expressed in adult rat brains and its cellular localization was found in glial cells.[10–12] In a very recent report, PAD2 expression was detected in cultured Schwann cells.[13] Previous reports indicate that PAD2 is involved in the citrullination of various cerebral proteins under neurodegenerative conditions.[14] Recently, it has been reported that the abnormal accumulation of citrullinated proteins including glial fibrillary acidic protein (GFAP) and vimentin were found in Alzheimer's disease (AD)-afflicted hippocampus; increased expression of PAD2 and its enzyme activity were detected during neurodegenerative changes and were accompanied by impairment of intracellular Ca^{2+} homeostasis.[15] In multiple sclerosis (MS) patients, previous studies have revealed that citrullinated myelin basic protein (MBP) was increased to 45% of total MBP compared to healthy adults[16] and has been implicated in the pathological mechanism of MS.[17] Therefore, PAD and citrullinated proteins can be used as important factors for the diagnosis of various human diseases.[7]

To our knowledge, there are no data available regarding citrullination by PAD2 in prion diseases. Here we report for the first time that increased citrullinated proteins including GFAP, MBP, and several newly identified proteins were found in the brains of scrapie-infected mice along with increased expression of PAD2 protein and its enzyme activity. These findings suggest a possible role of citrullination in the induction of pathological changes in the brains of scrapie-infected mice.

Materials and Methods

Animals and Scrapie Strain

C57BL/6J mice, 4 to 6 weeks of age, were obtained from the Experimental Animal Center of Hallym University. The original stock of ME7 scrapie strain was kindly provided by Dr. Alan Dickinson of Agriculture and Food Research Council and Medical Research Council Institute (Edinburgh, UK); this scrapie strain was maintained by serial intracerebral passage of brain homogenate from terminally affected mice. Mice were inoculated intracerebrally with 30 μl of 1% (w/v) brain homogenate in 0.01 mol/L phosphate-buffered saline (PBS) prepared from ME7-injected C57BL mice or from control mice that had been injected with normal brain homogenate. The mice were then sacrificed under 16.5% urethane at 150 ± 10 days after inoculation with ME7 scrapie strain, a time when clinical manifestations of disease were evident. To perform a time course study, brains were also collected at different time points (50, 100, and 150 days after inoculation). Mice inoculated with normal brain homogenate remained healthy throughout the same period. For immunohistochemistry, mice were perfused transcardially with PBS followed by 4% paraformaldehyde in PBS (pH 7.4). The brains were removed immediately, postfixed in the same fixative for 2 hours at room temperature, rinsed with PBS, dehydrated with ethanol, and embedded in paraffin.

Western Blot Analysis

Brains from control and scrapie-infected mice were homogenized in modified RIPA buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 1% Nonidet P-40, 0.25% sodium deoxycholate, and protease inhibitors.[18] The homogenates were rocked at 4°C for 1 hour and centrifuged at 18,000 × *g* at 4°C for 10 minutes to remove cell debris. The supernatant was collected and protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL). Citrullinated proteins were detected by Western blot analysis as described previously.[19] Briefly, equal amounts of protein (50 μg/lane) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane using an electrotransfer system (Bio-Rad, Hercules, CA). For detection of citrullinated proteins, citrulline residues on the polyvinylidene difluoride membrane were chemically modified by overnight incubation at 37°C in modification reagent [1 vol of 1% diacetyl monoxime/0.5% antipyrine/1 N acetic acid, and 2 vol of a mixture of 85% H_3PO_4/98% H_2SO_4/H_2O (20/25/55) containing 0.025% $FeCl_3$].[20] The membrane was then blocked with 5% nonfat dry milk in PBST (8 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, 138 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4, 0.05% Tween 20) for 2 hours at room temperature, then probed with an anti-modified citrulline antibody at 1:1000 (Upstate, Lake Placid, NY) in PBST overnight at 4°C. For the detection of other target proteins, the transferred polyvinylidene difluoride membranes were directly probed with either monoclonal anti-PAD2 antibody (1:2000), mouse monoclonal anti-neuron-specific enolase (1:2000) (Ab-Frontier, Seoul, Republic of Korea), rabbit polyclonal anti-aldolase C (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-MBP (1:3000) (Abcam, Cambridge, MA), or mouse monoclonal anti-β actin (1:10,000) (Sigma, St. Louis, MO) in 5% nonfat dry milk in PBST. These membranes were then incu-

bated with the appropriate secondary antibody-conjugated to horseradish peroxidase. Bound antibodies were visualized by chemiluminescent substrate as described by the manufacturer (Amersham Biosciences, Piscataway, NJ).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from brain samples of control and scrapie-infected mice using TRI-reagent (Sigma) according to the manufacturer's protocols. Complementary DNA (cDNA) was generated using the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the instructions of the manufacturer. RT-PCR was performed using primers specific for the PAD2 (665 bp; forward, 5'-CTGCGGTCTCTGGGTCCT-TCCTGTA-3' and reverse, 5'-GACCAGGCGAGAGAA-CAGAAATAGC-3') and β-actin (196 bp; forward, 5'-TGTGATGGACTCCGGTGACGG-3' and reverse, 5'- ACA GCTTCTCTTTGATGTCACGC-3') genes. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light.

Measurement of PAD2 Activity

The PAD2 activity was determined as described previously.[9] Briefly, the reaction mixture containing 100 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $CaCl_2$, 5 mmol/L dithiothreitol, with or without 10 mmol/L benzoyl-L-arginine ethyl ester (Sigma), and 0.5 mg of brain protein in a final volume of 120 μl was incubated at 50°C for 1 hour. After incubation, the reaction was stopped by adding final 1 mol/L perchloric acid. Samples were cooled down on ice for 20 minutes and then centrifuged at 18,000 × *g* for 5 minutes at room temperature. Aliquots of 120 μl of supernatant were mixed with 380 μl of dH_2O and 500 μl of color developing reagent and incubated at 95°C for 15 minutes. The samples were cooled to room temperature and then the absorbance was measured at 540 nm by enzyme-linked immunosorbent assay reader (VersaMax; Molecular Devices, Sunnyvale, CA). Quantification of citrulline was determined by comparison with appropriate standards. One unit of the enzyme is defined as the amount of enzyme that deiminates 1 μmol/L of the substrate (benzoyl-L-arginine ethyl ester) in 1 minute at 50°C.

Immunohistochemistry and Double-Immunofluorescence Staining

Immunohistochemical procedures were performed using the ABC kit (Vector, Burlingame, CA) by a modification of the avidin-biotin-peroxidase method. Briefly, 6-μm sections of brain were deparaffinized with xylene and hydrated in a graded ethanol series, and then treated with 0.3% hydrogen peroxide in methyl alcohol for 20 minutes to block endogenous peroxidase. The sections were exposed to normal donkey serum (Jackson ImmunoResearch, West Grove, PA), and then incubated with mouse monoclonal antibody for PAD2 (1:500) overnight at 4°C. After washing, the sections were treated sequentially with biotinylated anti-mouse IgG and avidin-biotin-peroxidase complex, developed with diaminobenzidine-hydrogen peroxide solution (0.003% 3,3-diaminobenzidine and 0.03% hydrogen peroxide in 50 mmol/L Tris buffer), and finally counterstained with hematoxylin. For staining of citrullinated proteins, the sections were incubated in modification reagent for 2 hours at 37°C before initiation of the immunohistochemistry protocol. After three washes in PBS buffer, the sections were exposed to 10% normal donkey serum for 1 hour at room temperature and then rabbit polyclonal antibody to modified citrulline (1:4000) for 1 hour at 37°C. The subsequent procedures were performed as described above for PAD2 staining. For double-immunofluorescence staining, the sections were incubated in the following order: 10% normal donkey serum in PBS for 1 hour, rabbit polyclonal anti-PAD2 (1:100)[21] overnight at 4°C, lissamine rhodamine sulfonyl chloride (LRSC)-conjugated donkey anti-rabbit IgG (1:200) (Jackson ImmunoResearch) for 1 hour at room temperature, washed, and blocked with 10% normal goat serum in PBS for 1 hour at room temperature and then incubated with various primary antibodies as follows: mouse monoclonal anti-GFAP antisera (1:400; DAKO, Copenhagen, Denmark), mouse monoclonal anti-NeuN (1:50) (Chemicon, Temecula, CA), mouse monoclonal anti-MBP (1:3000) overnight at 4°C and finally washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:200) (Jackson ImmunoResearch). For microglia staining, the lectin *Griffonia simplicifolia* (GSA) was optimized by incubating the sections in 0.5 mg/ml of trypsin in 0.05 mol/L Tris-buffered saline containing 1 mmol/L $CaCl_2$ (pH 7.6) for 5 minutes at 37°C. Biotinylated lectin GSA B4-isolectin (Sigma) was then added for 1 hour at room temperature, followed by fluorescein isothiocyanate-labeled streptavidin (Zymed, San Francisco, CA). The sections were examined with a LSM 510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

Two-Dimensional Gel Electrophoresis (2-DE) and Proteome Analysis

Protein extraction and 2-DE were performed as reported previously.[22] Briefly, 200 μg of brain protein of control and ME7 scrapie-infected mice was dissolved in a rehydration buffer containing 8 mol/L urea, 2% CHAPS, 65 mmol/L dithiothreitol, 0.5% immobilized pH gradient (IPG) buffer (Bio-Rad), 40 mmol/L Tris-HCl, and 0.002% bromophenol blue. The brain homogenates were applied to the IPG Readystrip, 7 cm, pH 3 to 10 linear gradient (Bio-Rad). The IPG strips were rehydrated for 16 hours at 20°C using the PROTEAN isoelectric focusing cell (Bio-Rad) according to the manufacturer's instructions. Briefly, isoelectric focusing was conducted at 250 V for 15 minutes, linearly increased throughout 2 hours to a maximum of 4000 V, and then run to accumulate a total of 20,000 Vhours. The gel strips were equilibrated before second dimen-

sional electrophoresis for 15 minutes in 50 mmol/L Tris-HCl (pH 8.8) containing 6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.002% bromophenol blue, and 80 mmol/L dithiothreitol or 0.025% iodoacetamide. The gel strips were then separated in 12% polyacrylamide gels to perform the second dimensional electrophoresis. The 2-DE gels were then exposed to Coomassie Brilliant Blue G-250 (CBBG-250) or silver staining. Duplicated 2-DE gels were also transferred on polyvinylidene difluoride membrane and then used for the detection of citrullinated proteins using an antibody to modified citrulline. The protein spots of immunoblotting-matched citrullinated proteins were subjected to in-gel trypsin digestion, and digested peptides were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (the Proteomics Service by Genomine, Pohang, Republic of Korea). The obtained peptide mass fingerprints spectra were analyzed by searching the Genomine local database and the National Centre for Biotechnology Information, nonredundant protein database with ProFound (*http://prowl.rockefeller.edu/prowl-cgi/profound.exe*).

Statistical Analysis

Quantitative results were expressed as means ± SEM. The probability of statistical differences between control and scrapie-infected groups was determined by a one-way analysis of variance test as indicated. Differences were considered significant at $P < 0.001$, $P < 0.01$, and $P < 0.05$.

Results

Increased Accumulation of Citrullinated Proteins in Various Brain Regions of Scrapie-Infected Mice

We first used Western blot analysis as described in the Materials and Methods to examine the citrullinated proteins from different brain regions of control and ME7 scrapie-infected mice. Total proteins were extracted from whole brains as well as from various dissected brain regions including cerebral cortex, hippocampus, striatum, cerebellum, and brain stem of control and scrapie-infected mice. In general, citrullinated proteins in most brain sections of scrapie-infected mice were markedly increased compared to controls (Figure 1, asterisks). In all brain regions, most bands from 10 kDa to 100 kDa were increased in scrapie. Those bands between 100 kDa and 150 kDa in whole brain, cerebellum, and brain stem were increased during scrapie infection, whereas no difference in citrullination was seen in other regions.

Next, we performed immunohistochemical staining of citrullinated proteins in different brain sections of control and scrapie-infected mice. As shown in Figure 2, citrullinated proteins (indicated by arrows) were more prominent in the scrapie-infected brains (Figure 2, F–J) than in control brains (Figure 2, A–E). These results correlated with the increased citrullinated proteins observed in scrapie-infected mice using Western blot analysis (Figure 1). We also observed similar results in the brains of 87V scrapie-infected mice (data not shown), indicating the occurrence of increased citrullination after infection is a general phenomenon.

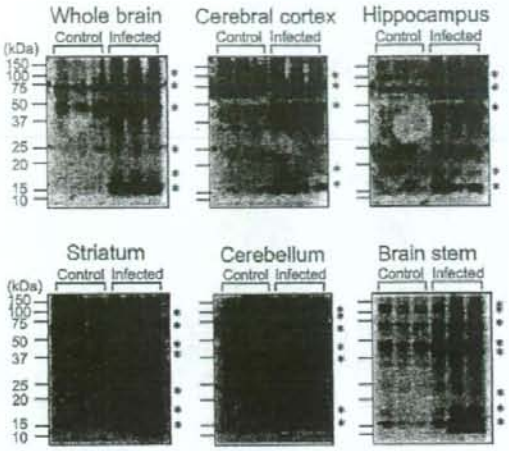

Figure 1. Detection of citrullinated proteins in brains from control and ME7 scrapie-infected mice. Whole brains and various brain sections of control (**lanes 1** to **3**) and ME7-infected mice (**lanes 4** to **6**) were homogenized, and then citrullinated proteins were detected by Western blot analysis using a polyclonal anti-modified citrulline antibody. Note that citrullinated proteins were increased in scrapie-infected regions compared to controls. Each lane shows the results for a homogenate obtained from brains of each individual mouse. **Asterisks** indicate the citrullinated proteins. These results are representative of at least three separate experiments.

Up-Regulation of PAD2 Expression in Various Brain Regions from Scrapie-Infected Mice

Posttranslational modification of protein arginine residues to citrulline is mediated by PAD2 enzyme in the brain. Therefore, we examined whether the expression levels of PAD2 in the brains are increased after ME7 scrapie infection. As can be seen in Figure 3A, the expression levels of PAD2 protein appeared to be increased in scrapie-infected mice in the following brain sections: cerebral cortex, hippocampus, striatum, and brain stem. However the expression level of PAD2 in cerebellum did not appear to be different between control and infected mice. Densitometric analysis of the gels showed that the relative intensity of the PAD2 bands was significantly higher in all sections of scrapie-infected brains compared to controls except in the cerebellum (Figure 3B).

In the next experiment, we measured expression levels of PAD2 mRNA in whole brains of both control and ME7 scrapie-infected mice using PAD2-specific primers as described in the Materials and Methods. As can be seen in Figure 3C, RT-PCR analysis showed increased PAD2