Regulation of Acetylcholine Receptor α Subunit Variants in Human Myasthenia Gravis

Quantification of Steady-state Levels of Messenger RNA in Muscle Biopsy Using the Polymerase Chain Reaction

Thierry Guyon,* Philippe Levasseur,‡ Frederique Truffault,* Corinne Cottin,* Cyril Gaud,‡ and Sonia Berrih-Aknin*
**CNRS URA-1159 and ‡Département de Chirurgie Thoracique Hôpital Marie Lannelongue 92350 Le Plessis-Robinson, France*

Abstract

Myasthenia gravis (MG) is an autoimmune disease mediated by auto-antibodies that attack the nicotinic acetylcholine receptor (AChR). To elucidate the molecular mechanisms underlying the decrease in AChR levels at the neuromuscular junction, we investigated the regulation of AChR expression by analyzing mRNA of the two AChR α subunit isoforms (P3A+ and P3A−) in muscle samples from myasthenic patients relative to controls. We applied a quantitative method based on reverse transcription of total RNA followed by polymerase chain reaction (PCR), using an internal standard we constructed by site-directed mutagenesis. An increased expression of mRNA coding for the α subunit of the AChR isoforms was observed in severely affected patients ($P < 0.003$ versus controls) but not in moderately affected patients, independently of the anti-AChR antibody titer. Study of mRNA precursor levels indicates a higher expression in severely affected patients compared to controls, suggesting an enhanced rate of transcription of the message coding for the α subunit isoforms in these patients. We have also reported that mRNA encoding both isoforms are expressed at an approximate 1:1 ratio in controls and in patients. We have thus identified a new biological parameter correlated with disease severity, and provide evidence of a compensatory mechanism to balance the loss of AChR in human myasthenia gravis, which is probably triggered only above a certain degree of AChR loss. (*J. Clin. Invest.* 1994. 94:16–24.) Key words: acetylcholine receptor isoforms • regulation of mRNA • quantitative RT PCR • autoimmune disease • clinical correlations

Introduction

Myasthenia gravis (MG)[1] is an autoimmune disease mediated by antibodies directed against the nicotinic acetylcholine receptor (AChR). MG is characterized clinically by muscle weakness enhanced by physical effort. The anti-AChR antibodies detected in > 85% of patients sera interact with the receptor at the neuromuscular junction (1, 2), resulting in a loss of functional receptors and an impairment of neural transmission at the motor endplate (3). The nicotinic AChR is the main autoantigen in MG. It is a transmembrane glycoprotein of 270 kD that forms an oligomeric complex of four homologous subunits present in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ during early embryonic stages or after denervation (4, 5). In the adult form of the receptor, an ϵ subunit replaces the γ subunit (6). The α subunit contains both the site for ACh binding (7) and the main epitopes recognized by autoantibodies in MG (8, 9). The human muscle AChR α subunit exists as two isoforms (10), that arise from alternate RNA splicing, one with (P3A+) and the other without the P3A exon (P3A−). The P3A− variant was described in 1981, but the isoform including the P3A exon was only recently identified by Beeson et al. (10). This additional exon comprises 75 bp and is located between exons 3 and 4. Messenger RNA (mRNA) for the two isoforms (P3A+ and P3A−) are found in equal proportions in innervated and denervated human muscle. It seems that P3A exon is conserved throughout primate evolution. Expression of these two isoforms has recently been examined in several human tissues; interestingly, P3A+ mRNA was detected in all tissues, including muscle, while P3A− was found exclusively in muscle (11), suggesting a regulation of P3A exon splicing by a muscle-specific factor. Differential expression of the two isoforms may have clinical implications in MG.

The pathogenicity of anti-AChR antibodies has been clearly demonstrated by their ability to transfer the disease to normal animals (12, 13) and to reduce α-bungarotoxin binding sites in a myotube culture system (14–16). Several studies, including work in our laboratory, failed to show a correlation between the severity of the disease and either antibody titers or the functional activity of anti-AChR antibodies in myotube cultures, whereas the ability of patients sera to degrade AChR does correlate with the antibody titer (15, 16).

Variability in the severity of MG seems to be related not only to the absence or presence of antibodies against AChR but also to other individual factors. First, clinical signs may appear only above a certain threshold of AChR loss, which could vary from one patient to the next. Second, the AChR α subunit gene from normal individuals and patients with MG shows some polymorphism (17). In particular while the two isoforms of the α subunit mRNA and the expression of both protein variants has been reported (18), it is not yet known whether the autoantibodies equally recognize both types. Third, autoantibodies directed against targets other than AChR but involved in the stability of AChR or other structures of the endplate could be present in some patients (19), namely in seronegative ones.

Address all correspondence to Sonia Berrih-Aknin, Ph.D., CNRS-URA-1159-Hôpital Marie Lannelongue, 133, avenue de la résistance, 92350 Le Plessis-Robinson, France.

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; EAMG, experimental autoimmune MG; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MG, myasthenia gravis; RT, reverse transcription.

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Finally, there may be a compensatory mechanism that increases the amount of protein synthesized from preexisting mRNA and/or enhances AChR subunit gene expression. The efficiency of the regulation process could also present individual variability.

Recently, studies on experimental autoimmune myasthenia gravis (EAMG) have shown a significant increase in mRNA of AChR subunits in leg muscle of myasthenic rabbits, rats, and mice (20, 21). An increase in AChR α subunit mRNA was observed during development in mouse and calf (22, 23) and in response to muscle denervation in chicks, rats, and mice (24). A model of mouse embryonic development has been proposed by Changeux (25), in which trophic factors play a major role in the regulation of AChR expression. All these studies have been carried out in animals and little is known about the regulation and expression of these genes in humans. This is partly due to the difficulties in obtaining sufficient quantities of viable tissue samples for RNA extraction. The development of the PCR, an extremely sensitive method to investigate small amounts of DNA and mRNA, has made it possible to study the expression of genes in very small tissue samples such as human biopsies (26).

In an attempt to analyze the mechanisms that control AChR gene expression in human myasthenia gravis, we developed a quantitative reverse transcription–polymerase chain reaction technique (RT-PCR) to evaluate mRNA levels of the two α-subunit AChR isoforms in muscle biopsy samples from myasthenic patients relative to controls. We constructed an internal standard by site-directed mutagenesis, which differed from the P3A+ α subunit AChR gene by a single base pair (bp), creating a new restriction site. We demonstrate an increase of the steady-state level of pre-mRNA as well as mature mRNA coding for AChR α subunit isoforms in severely affected patients, that suggests an accelerated rate of transcription of receptor messagers. In addition, analysis of differential expression of the two α subunit isoforms in muscle from MG patients shows an approximate 1:1 ratio. This new approach provides a precise and sensitive measurement of mRNA levels from small biopsy samples and proves to be interesting for the understanding of the regulation mechanisms involved in the AChR expression in myasthenia gravis in response to the antibody attack.

Methods

Patients. MG was diagnosed on the basis of clinical features, electromyographic decrement, and the positive effect of anticholinesterase. We studied 20 muscle samples from MG patients (13 women and 7 men) and 6 non-MG patients (sex- and age-matched). The clinical and immunological data are summarized in Table I. The anti-AChR antibody titer was determined using human muscle AChR complexed with ^{125}I–α-bungarotoxin as antigen (27). Functional activity in MG serum was tested on rat myoblast cultures as previously described (15) and data are expressed as the percentage loss of AChR relative to normal serum (Table I). The severity of MG was graded according to Osserman's classification (28). Patients presenting grade I MG have a purely ocular form of the disease. Patients presenting severity grade IIA have moderately impaired functional activity and weakness of the limb or eye muscles. Patients with grade IIB MG have a strong impairment of functional activity and strong signs of weakness of the limb and bulbar musculature. At the date of serum sampling, 7 patients had a mild form (I and IIA) and 13 had a severe form (IIB). Steroid, veinoglobulin, and plasmapheresis treatment is indicated in Table I. Thymic hyperplasia, defined according to Levine and Rosai (29) by the presence of germinal centers, was present in 10 patients. Two patients had a thymoma.

Muscle biopsy samples. Biopsies (100–200 mg) were obtained from skeletal muscle during thymic surgery at the Hôpital Marie Lannelongue with the patients' informed consent. All biopsies were flash-frozen in liquid nitrogen and stored at −80°C (in RNase-free conditions).

Plasmids. The plasmid pSP19 containing the human 1,667 bp cDNA α subunit AChR gene was a generous gift from David Beeson (Institute of Molecular Medicine, University of Oxford, UK). The mutant human α subunit AChR gene fragment of 704 bp, obtained by site-directed mutagenesis PCR, was subcloned in the Bluescript II KS$^-$ plasmid (Stratagene, La Jolla, CA) under the control of the T7 RNA polymerase promoter.

Total RNA preparation. Total RNA was isolated from 100 to 200 mg of frozen muscle by acid guanidinium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (30). The frozen tissue was homogenized in 1 ml of denaturing solution (guanidinium thiocyanate 4 M, sodium citrate 25 mM, pH 7, 0.5% *N*-lauryl-sarcosyl and 2-mercaptoethanol 0.1 M) with a Polytron (Luzern, Switzerland). After extraction, total RNA was purified with 0.5 volumes of ammonium acetate 7.5 M and 2.5 volumes of 100% ethanol, and then centrifuged at 15,000 rpm for 30 min at 4°C. The RNA pellet was washed in 75% ethanol, dried under vacuum, and stored at −80°C after dissolution in DEPC-treated water.

The total RNA concentration was determined by absorbance at 260 nm using a Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The purity of the RNA preparation was checked by measuring the 260/280 nm ratio. The quality of the preparation was checked by electrophoresis of 5 μg of RNA in 1.5% formaldehyde-agarose minigel. A 28/18S band intensity ratio of around 2 demonstrated the quality of the RNA.

Construction of the internal standard. The mutant fragment of α subunit AChR cDNA used as internal control was constructed by oligonucleotide overlap extension and amplification by PCR. Our procedure was similar to that published by Higuchi et al. (31). Site-directed mutagenesis was performed using mismatched primers to introduce a new BstUI restriction site (Table II). The primers used had a GG clamp and a BamHI restriction site to improve subcloning efficiency. After construction, the cDNA fragment was subcloned into Bluescript II KS$^-$ vector linearized at the BamHI site. Mutated RNAs were transcribed in vitro from 2 μg of HindIII-cut DNA plasmid, used as the template for transcription by T7 polymerase according to the transcription protocol of SP6/T7 Boehringer Transcription Kit (Boehringer Mannheim, GmbH, Mannheim, Germany). The resulting complementary RNA product was separated from the DNA template using phenol-chloroform extraction and ethanol precipitation after 1 h of incubation at 37°C with 10 U of RNase-free DNase (New England Biolabs, Beverly, MA). The cRNA product was quantified by its absorbance at 260 nm and stored at −80°C. The size and homogeneity of the product were checked by agarose gel electrophoresis.

Oligonucleotides used for amplification. Based on the known cDNA sequence of the P3A+ α subunit AChR gene, we designed a set of primers (sense and antisense) with the help of Oligo software (Med Probe, Oslo, Norway), for the amplification of the α subunit AChR gene in the region containing the P3A exon. The oligonucleotide primers were purchased from Genset (Paris, France) (Table II).

The sense primer QPCR1 corresponds to the α subunit AChR cDNA sequence at position 106–125 and the antisense primer QPCR2 corresponds to the cDNA sequence at position 720–739. Using these primers, the amplified fragment containing P3A+ α subunit cDNA was 704 bp, whereas the P3A− fragment amplified was 633 bp. The amplification product of the internal standard gave rise to two fragments of 552 and 152 bp after digestion with the restriction enzyme BstUI (see Fig. 1). For analysis of pre-mRNA we used two intronic primers P5P6 A and P5P6 B described in Table II and located in the intron between P5 and P6 exons. The amplification products gave rise to one fragment of 440 bp. Primers G1 and G2 used to amplify a 573-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene are also described in Table II.

RT-PCR of α subunit AChR mRNA. Total RNA was reverse tran-

Table I. Characteristics of the Patients: Clinical Data and mRNA Levels of AChR α Subunit Isoforms

Patient	Sex	Age	Treatment*	Disease severity‡	Anti-AChR Ab titer	Serum activity§	Thymic histology	Onset of disease	α subunit mRNAs‖	P3A+ mRNAs
		yr			*nM*	%		*Mo*		%
1	M	21	—	I	0.5	0	Hyperplasia	18	4.64	56.90
2	M	56	—	I	> 310	ND	Thymoma	6	1.77	54.80
3	M	34	—	I	0	ND	Normal	84	1.50	45.00
4	F	45	—	IIA	0	13	Hyperplasia	6	1.89	56.08
5	F	24	—	IIA	2.4	ND	Hyperplasia	12	1.31	53.40
6	M	18	—	IIA	21.3	ND	Normal	36	1.76	54.55
7	F	46	—	IIA	1137	ND	Normal	144	3.65	52.05
8	F	34	P	IIB	0	0	Hyperplasia	12	38.15	45.95
9	F	21	—	IIB	1.8	9	Hyperplasia	4	54.30	55.99
10	M	24	P	IIB	1.1	9	Hyperplasia	0.5	3.74	46.73
11	F	30	—	IIB	> 15	21	Hyperplasia	4	5.20	46.15
12	F	22	—	IIB	7.3	28	Hyperplasia	96	6.70	44.78
13	F	20	P	IIB	6.8	56	Hyperplasia	9	5.46	52.20
14	F	33	C	IIB	> 29	62	Hyperplasia	9	7.30	60.27
15	M	47	C	IIB	2.6	12	Thymoma	16	21.67	50.02
16	F	5	V	IIB	0	0	Normal	12	10.00	49.00
17	F	29	C	IIB	0	0	Normal	22	6.20	54.84
18	F	36	—	IIB	0	ND	Normal	7	2.20	54.55
19	F	12	V	IIB	0.2	ND	Normal	6	13.90	46.04
20	M	65	C	IIB	> 11.5	ND	Normal	72	4.86	41.77

* MG treatment; *P*, Plasmapheresis; *C*, Corticotherapy; V, Veinoglobulins. ‡ According to Osserman classification. § Serum functional activity was assessed in a rat culture myotube system as described in Methods. Results are expressed as percentage of AChR loss evaluated by α-Bgt binding. ND, not done. ‖ α subunit mRNAs are expressed as number of molecules per μg of total RNA × 10^6.

scribed into first-strand cDNA by using the Stratagene First Strand Synthesis Kit (Stratagene). A 50 μl reverse transcription reaction mixture containing 2 μg of total RNA, known amounts of internal standard, 10 μl of 5× RT buffer, 10 mM dithiothreitol, 1.5 mM dNTPs, 10 U of RNase Block II, 50 pmoles of 3′ primer (QPCR2) and 20 U of Moloney murine leukemia virus reverse transcriptase was incubated at 37°C for 60 min and then quickly chilled on ice.

PCR was carried out in a total volume of 100 μl containing 10 μl of RT reaction mix, 10 μl of PCR buffer (50 mM KCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 1% Triton X-100), 200 μM each dNTP, 50 pmoles of each primer (QPCR1 and QPCR2) and 2.5 U of Taq polymerase (Bioprobe Systems, Montreuil, France). A trace amount of ^{32}P-labeled 5′ primer was added (2–3 × 10^6 cpm/tube). The mixture was overlaid with mineral oil and then amplified using the PHC3 thermal cycler (Techne, Cambridge, UK) as follows: 94°C, 1 min; 58°C, 1 min; 72°C, 2 min. The final elongation step lasted 10 min.

Table II. Couples of Oligonucleotides Used in the Study

Application	Name	Strand	Sequence	Position	Temperature
					°C
Mutagenesis	M′	−	5′-ATCAACGCGGAAAGCGACCA-3′ (★)	580/599	62
	M	+	5′-TGGTCGCTTTCCGCGTTGAT-3′ (★)	580/599	62
PCR-subcloning	C1	−	5′-[GGGGATCC]TCCGAACATGAGACCCG-3′	108/125	54
	C2	+	5′-[GGGGATCC]AGTCAGGCGCTGCATGACG-3′	723/738	62
RT-PCR of α subunit mRNA	QPCR 1	−	5′-GGCTCCGAACATGAGACCCG-3′	106/125	66
	QPCR 2	+	5′-GAAGCAGTACGTCGCGGACG-3′	720/739	66
RT-PCR of pre-mRNA	P5P6 A	−	5′-GGTGGCCACATATGATAAGG-3′	Intronic*	60
	P5P6 B	+	5′-AGGCAGGTCACCCTGATGAG-3′	Intronic*	64
RT-PCR of GAPDH mRNA	G1	−	5′-ATCACCATCTTCCAGGAGCG-3′	3396/3415‡	62
	G2	+	5′-CCTGCTTCACCACCTTCTTG-3′	4454/4473‡	62

The sequence in brackets in C1 and C2 oligonucleotides represents the clonage site and GG clamp. The star indicates the mutated nucleotide, that leads to a new restriction site to BstUI (CGCG) in M and M′ oligonucleotides. * Primer locations correspond to intronic sequence between P5 and P6 exons. ‡ The position of the primers are numbered from GAPDH gene sequence.

Quantitative analysis. To ensure that quantification took place in the exponential phase of PCR, amplification products of the same reaction mixture were amplified for 36 cycles and aliquots of 7.5 μl were taken at various times (22 to 36 cycles). To discriminate DNAs formed from wild-type and mutant RNAs, the aliquots of PCR products were subjected to BstUI digestion and electrophoresed on 1.5% agarose gel containing ethidium bromide, in Tris borate EDTA buffer. Gels were visualized under UV light and photographed. The appropriate bands were cut out of the gel and radioactivity was determined by Cerenkov counting. The amount of radioactivity (cpm) recovered from the excised gel bands was plotted against the number of PCR cycles. In all experiments, the presence of contaminants was checked for by amplification of a control sample in which reverse transcriptase was omitted from the reverse transcription reaction mixture.

Statistical analysis. Nonparametric Mann-Whitney test was used throughout.

Results

Quantitative RT-PCR (RT-QPCR) is a powerful technique to accurately quantify the expression of mRNA, even when only small amounts of tissue are available (32, 33). However, because of tube-to-tube variability in amplification efficiency, an internal control must be coamplified in the same tube. This control must have several characteristics. First, it should be amplified with the same efficiency as the natural mRNA target. Second, the different PCR products must be distinguishable. For these reasons, we constructed a fragment template of 704 bp which differed from the P3A+ α subunit AChR gene by a single base pair, creating a new restriction site. This minor modification would not be expected to affect amplification efficiency, but it allowed us to distinguish the amplified product of the competitor from the P3A+ target template, after restriction enzyme digestion. In addition, since we used the same primers to coamplify the two α subunit cDNA variants and the internal standard, the ratio of products remained constant throughout the exponential phase of amplification. The introduction of a known quantity of the internal RNA standard in the reverse transcription assay serves first as an internal mRNA control for the reverse transcription reaction, and second to generate a standard curve for quantifying the specific target α subunit mRNA variants from experimental samples.

Optimal amount of internal standard for quantitative PCR. In the quantitative analysis by PCR, the amount of standard RNA in the reverse transcription reaction has to be adjusted so that the experimental sample and the internal standard undergo amplification with the same efficiency. To determine the optimal ratio, various concentrations of synthetic RNA were added to a constant amount of muscle RNA. The experimental and control RNAs, as well as their cDNAs, compete for substrate in both reactions (reverse transcription and PCR). The results obtained with the sample from one patient are presented in Fig. 1. Although the concentration of muscle RNA was constant in all the samples, the PCR product of muscle RNA clearly decreased as a function of the concentration of internal standard. Conversely, the amount of PCR product of the standard cDNA increased as a function of its original concentration (Fig. 1, lanes *6–9*). In the case shown in Fig. 1, the optimal conditions for similar amplification efficiency of the α-subunit RNA variants and standard cDNA were obtained with 1–5 pg of internal standard, corresponding to 2.4–12.1 $\times$ 10^6 molecules.

For all muscle samples, the amount of internal standard RNA to be added was estimated by direct visualization of the ethidium bromide-stained PCR products from two or three different standard RNA concentrations.

Quantitative analysis of α subunit mRNA variants. For each sample from MG patients and each control, 2 μg of total RNA extracted from muscle biopsy, as well as a predetermined amount of internal standard, were reverse transcribed and amplified in the same reaction in the presence of the α subunit ^{32}P-labeled 5′ primer. To ensure that quantification occurred in the exponential phase of the amplification, an aliquot was harvested every two or three cycles between cycles 22 and 36.

After BstUI enzyme digestion, the reaction products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining (Fig. 2). The amount of PCR products increased as a function of the number of PCR cycles, and a plateau was reached after ~ 30 cycles. The amounts of radioactivity recovered from the excised gel bands were plotted as a function of the number of PCR cycles. In the case shown in Fig. 3, the amplification was exponential between 22 and 28 cycles. After 30 cycles, the rates decreased drastically and approached a plateau. Within the exponential phase, the three curves (the two isoforms and the internal standard) were parallel, indicating that the amplification efficiency was the same for the coamplified targets. Thus, the amounts of α-subunit mRNA variants could be calculated by comparison with the internal standard, in the exponential phase. Thus for this patient, the amounts in 1 μg of total muscle RNA calculated by this method were 4.8 and 2.7 $\times$ 10^6 molecules of P3A+ and P3A− isoform mRNA, respectively.

The total amount of mature α subunit mRNA is increased in severely affected MG patients. We examined 20 biopsy samples from MG patients, 7 with a mild form of the disease and 13 with a severe form. Fig. 4 and Table I show the results expressed as the total number of molecules of α subunit mRNA variants (P3A+ and P3A−) per μg of total RNA. The mean number of AChR α subunit molecules was 3.08 $\times$ $10^6/\mu$g of mRNA in control samples versus 2.36 and 13.82 $\times$ $10^6/\mu$g in patients with moderate and severe forms, respectively. Of note, the values obtained for control muscle were uniform (range: 2.6 to 3.44 $\times$ 10^6 molecules/μg of total RNA), whereas in the patients they were more heterogeneous. The mean increase in AChR α subunit mRNA variants in patients with severe forms was about 4.5-fold ($P < 0.003$ versus controls), whereas levels were normal in patients with moderate forms. Also of interest, 12 of the 13 severely affected patients had a total number of α subunit mRNA molecules above the highest control value, whereas this was the case of only 2 of the 7 moderately affected patients. It thus appears that the increase in α subunit mRNA correlates with MG severity.

Analysis of immunological and immunohistological parameters in the severely affected patients indicated that those with thymic hyperplasia or a thymoma had a higher number of α subunit variant mRNA molecules than those with a normal thymus (mean 17.81 versus 7.43 $\times$ $10^6/\mu$g of mRNA) although the difference was not statistically significant.

Precursors of α subunit mRNA are also increased in severely affected MG patients. To further study the transcription level of AChR α subunit gene, we investigated the expression of α subunit mRNA precursors in three controls and three severely affected patients (Nos. 12, 16, and 19) by using a RT-PCR assay with primers located in the intronic region between P5 and P6 exons. The RT-PCR products were visualised on agarose gel (Fig. 5 *A*). The level of expression of pre-mRNA is higher

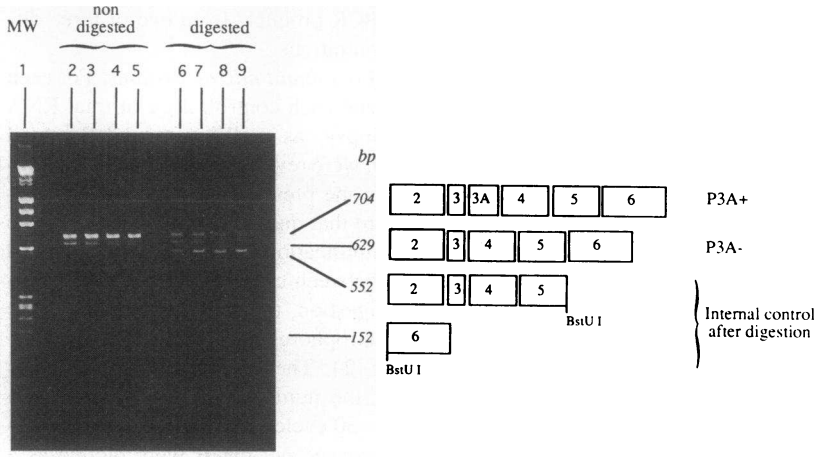

Figure 1. Determination of the optimal amount of internal standard to be used in the quantitative PCR. 2 μg of total RNAs extracted from a muscle sample from a myasthenic patient was amplified after reverse transcription with serial dilutions of internal standard. The PCR products were loaded on a 1.5% agarose gel. Lane *1* corresponds to the molecular weight marker (ΦX174 HaeIII/λ HindIII). Lanes *2–5* correspond to nondigested PCR products while lanes *6–9* show PCR products digested by BstUI enzyme. Only two bands are visible in the nondigested products. The first one (704 bp) consists of two products, the P3A+ isoform, and the internal standard, different from the P3A+ isoform by a single base pair. The second is the P3A− isoform (629 bp). In the digested products, four distinct fragments are visible: 704 bp (P3A+ isoform), 629 bp (P3A− isoform), 552 and 152 bp (not visible on the gel); these two latter are the products obtained after BstUI digestion of the standard control. Band intensity increased with the amount of internal standard: 1 pg (lanes *2* and *6*); 5 pg (lanes *3* and *7*); 10 pg (lanes *4* and *8*); 15 pg (lanes *5* and *9*). Raising the amount of internal standard from 1 to 15 pg progressively inhibited amplification of both muscle isoforms although the original amount of total muscle RNA was constant (lanes *6–9*). The optimal amount of internal standard was that giving the same amplification efficiency of the two isoforms and the internal standard. On electrophoresis gels it corresponds to a band intensity ratio of ~ 1 (lanes *6* and *7* with 1 to 5 pg of internal standard).

in MG patients presenting a high grade disease than in controls. This was not due to experimental variations of RNA in the samples since the signals observed when amplifying GAPDH housekeeping gene were nearly similar for patients and controls (except for patient No. 19). Of note, patient 19 who shows a low expression of GAPDH, presents a higher α subunit mRNA precursors expression compared with controls. To avoid amplification of possible genomic contaminations, extracted muscular RNA samples were pretreated with DNase I. The digestion efficiency of the nuclease enzyme was checked in our samples by the absence of amplification product after PCR assay performed on digested RNA samples (data not shown).

To estimate the difference in the expression level of mRNA precursors between patients and controls, a dilution assay of pre-mRNA RT-PCR products was performed. As shown in Fig. 5 *B*, the ratio between the expression level of mRNA precursors in patient 19 and one control (Fig. 5 *A*, lane *3* is 3–4-fold,) is approximately similar to the one obtained when analyzing, for

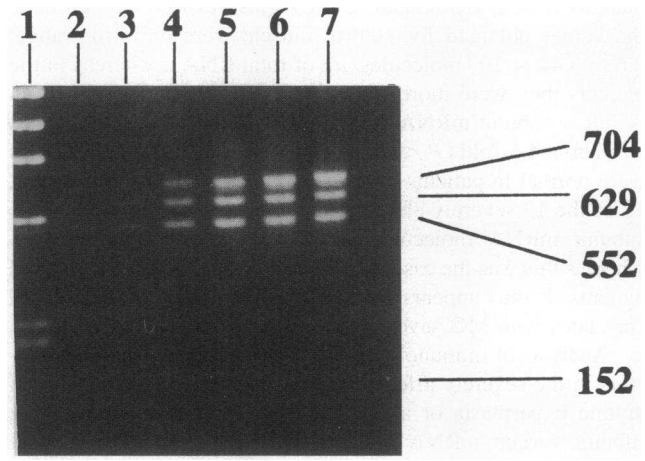

Figure 2. Quantitative analysis of RT-PCR products in one representative muscle sample. 2 μg of total RNA and an appropriate amount of internal standard (5 pg) were reverse transcribed and the resulting cDNA was amplified for various numbers of cycles in the presence of the QPCR primers labeled with γP^{32} ATP. At various steps of PCR, aliquots were taken and loaded on 1.5% agarose gel. Lane *1* shows the molecular weight marker (ΦX174 HaeIII/λ HindIII). Lanes *2–7* represent PCR products harvested at cycles 22, 24, 27, 30, 33, and 36. The three expected bands (704, 629, and 552 bp) were visible after BstUI digestion and were cut from the gel to be quantified by Cerenkov counting.

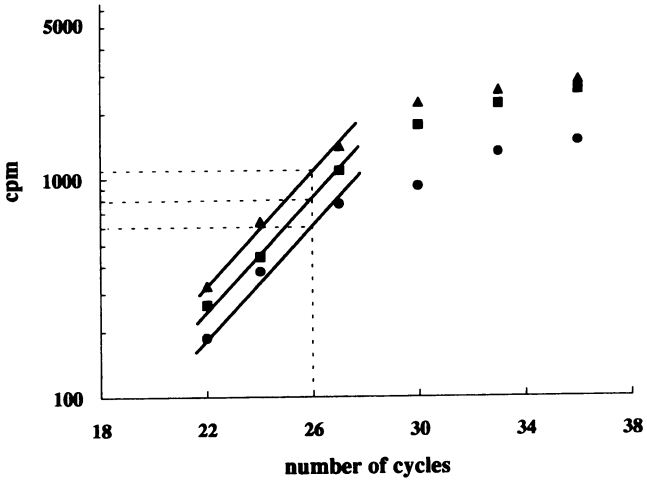

Figure 3. Quantitative determination of AChR α subunit mRNA variants in a representative muscle sample from a MG patient. The number of cycles was plotted against cpm (logarithmic scale). The first part of the curve is linear, indicating that the amplification is exponential. In addition, the three curves are parallel, demonstrating a similar degree of amplification. Since the amount of standard control is known (5 pg of internal standard corresponds to 12.1×10^6 molecules), we can estimate (in the linear part of the curve) the number of RNA molecules in the experimental muscle sample. The curves represent the linear regression calculated from experimental values. Here, P3A+ and P3A− isoforms were estimated at 2.7 and 4.8×10^6 molecules/μg of total RNA. (■) P3A+ isoform; (●) P3A− isoform; (▲) internal standard.

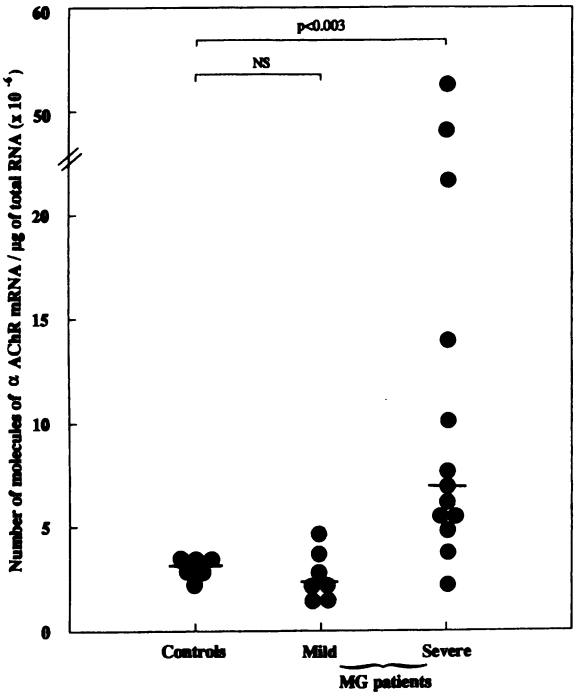

Figure 4. Individual analysis of α subunit AChR transcripts in MG patients and controls. A striking increase in total α-subunit transcripts was observed in severely affected patients compared with moderate MG ($P < 0.001$) and controls ($P < 0.003$). Each patient is represented by a point.

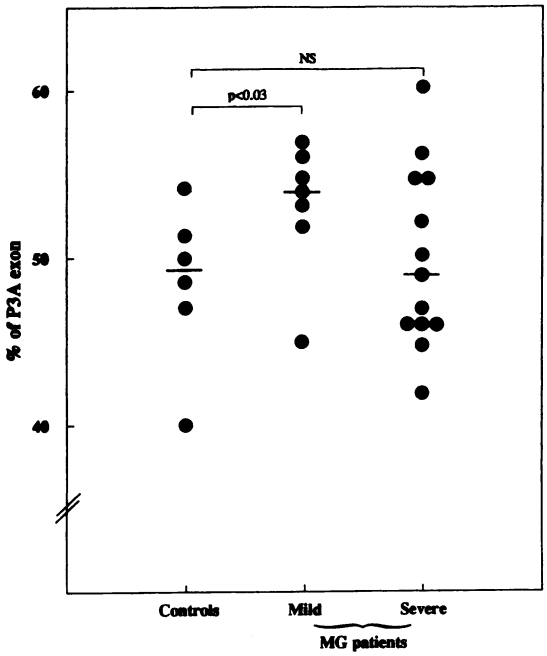

Figure 6. Differential expression of P3A+ isoform. The amount of P3A+ variant was determined as a percentage of total mRNA in the three groups. No difference in the relative expression of P3A+ and P3A− α subunit mRNA was observed between severely affected patients and controls (49.86% of P3A+ variant versus 48.55%), but there was a significant difference between the patients with moderate MG and controls (53.25% of P3A+ variant versus 48.55%, $P < 0.03$). Each point represents one patient.

the same patient, the steady state level of α subunit mRNA compared to controls. Estimation of difference in the pre-mRNA expression between controls and patients 12 and 16 indicate a ~ 2–3-fold increase level of pre-mRNA, respectively (data not shown). All these results show evidence of a similar increase level of α subunit mRNA precursors and mature mRNA compared to controls.

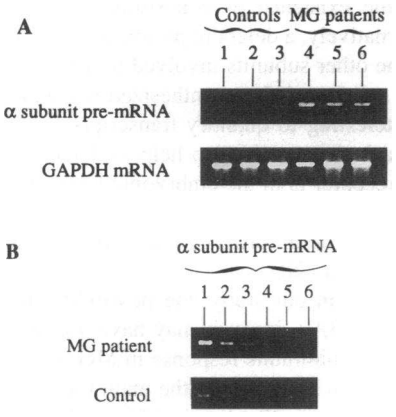

Figure 5. Compared levels of unspliced α subunit mRNA precursors in three controls and three MG patients. (*A*) Amplification products were obtained with DNAseI treated muscular RNA after 36 cycles of RT-PCR according to conditions described in the method section and were analyzed on agarose gel (PCR amplification was checked to be realised in an exponential phase). Lanes *4–6* correspond to patients No. 19, 16, and 12, respectively. Amplification products of GAPDH mRNA were also analyzed as standard for each mRNA extracted samples. (*B*) Estimation of the increased level of pre-mRNA between one control (corresponding to lane *3* of *A*) and MG patient 19 by using a dilution assay of the RT-PCR products. Lane *1* corresponds to undiluted products and lanes *2–6* to 2-, 4-, 8-, 16-, and 32-fold diluted products, respectively.

Differential expression of the two mRNA variants. The differential expression of the P3A exon was analyzed in both groups of MG patients and the controls. The amount of the P3A+ mRNA variant was expressed as a percentage of the total α subunit mRNA. Individual values are shown in Fig. 6 and in Table I. We found no evidence of different expression of the P3A+ mRNA variant in severely affected MG patients relative to controls (49.86±5.18% versus 48.55±4.37%). Of note, moderately affected patients show a slight but statistically significant increased expression of the P3A+ mRNA variant compared with controls ($P < 0.03$ versus controls), with 53.25±3.68% of the total α subunit transcripts containing the P3A exon.

Discussion

This is the first precise quantification of AChR α subunit mRNA variants in human muscle, showing a regulatory mechanism of AChR α subunit gene expression in human myasthenia gravis. Our data were obtained using a method that allowed absolute mRNA quantitation from limited amount of muscle biopsies (100–200 mg) by means of an RT-PCR assay.

Northern and dot blotting, and RNAse protection methods have commonly been used for quantifying mRNA. However, difficulties in obtaining sufficient human tissue samples have led to the use of a more sensitive method, PCR. Several methods for mRNA quantification by PCR have been published. Coamplification of sequences of a different gene, such as β-actin, has not been satisfactory, as primers different from that of the target sequence have to be used (34). The kinetics of primer hybrid-

ization may thus be rather variable, influence amplification efficiency and prevent accurate comparisons. In addition, this approach is problematic when the control gene is affected by physiological conditions. The alternative method used here, is the coamplification with a single pair of primers of the natural mRNA target, using a defined amount of specific RNA as internal standard. The use of only one set of primers ensures identical amplification efficiency of the wild-type mRNA and the internal standard. In each case, the amplification products of the endogeneous and mutant gene can be separated by gel electrophoresis since the internal standard has a single base substitution introducing a new BstUI restriction site in the cDNA. By including a known quantity of the internal standard RNA at the reverse transcription step, a standard curve can be generated for quantification of the target mRNA variants. Quantification has to be done during the exponential phase, since reassociation of DNA strands not used as templates could lead to heterodimeric DNA species (resistant to BstUI digestion) when the PCR conditions are limiting and could thus affect the results. For the two α AChR mRNA isoforms, the results can be expressed as the number of molecules per microgram of total mRNA.

Our data point to a compensatory mechanism for loss of AChR expression, and indicate a higher mean value of α subunit mRNA in severely affected patients than in moderately affected patients. Since loss of AChR is greater in the former group of patients (35, 36), the most likely explanation is that a transcriptional regulation exists in patients showing a high rate of degradation. This increase of AChR α subunit mRNA level could result either from enhanced transcription of the α subunit gene or from alterations in mRNA processing or degradation. Indications that regulation occurs at least in part, at the level of gene transcription have been provided by in situ hybridization with intronic probes by Fontaine and Changeux (37) who demonstrated that unspliced α subunit precursor mRNA accumulated in cultured myotubes after blocking spontaneous activity with tetrodotoxin. Our investigations concerning α subunit mRNA precursors are consistent with these data and indicate that in severely affected patients, in addition to mature mRNA level, pre-mRNA level is also enhanced compared to controls. These results are in accordance with data shown by Asher et al. (38) who demonstrate an increase of AChR α subunit transcripts associated with an increase of transcriptional factors such as myogenin and MRF4 in rat muscles after passive transfer of EAMG by anti-AChR antibodies. Thus, these data suggest that an accelerated rate of transcription of the message coding for the α subunit causes an increased message content and increases receptor synthesis, in response to the attack by auto-antibodies.

However, it was surprising that no increase in mRNA was observed in the patients with moderate MG, whereas degradation of AChR is known to occur (36). It is thus possible that the increase in AChR transcripts is triggered only above a certain threshold of AChR loss. This is consistent with studies in animals that showed a great reduction in AChR content associated with a large increase in AChR mRNA levels, whereas a small reduction in AChR content is not compensated for by an increase in AChR mRNA (20, 21). In their report, Asher et al. (21) showed that increases in AChR transcripts were only found in animals with disease manifestations. Interestingly animals immunized with modified AChR and presenting high anti-AChR antibody titers and no disease manifestations do not show an increase in AChR mRNA levels, even though they present a significant loss ($\sim$ 20%) of protein expression (21).

It is interesting to note that the patients with undetectable anti-AChR antibodies had the same levels of mRNA as seropositive patients, suggesting that AChR undergoes similar degradation in the two subgroups of patients. Possible explanations are a very high pathogenicity of anti-AChR antibodies present at a very low titer, or an indirect effect on other synaptic proteins that could in turn induce a loss of AChR expression. This last hypothesis is supported by reports of antibodies bound to endplates in these patients (19) and evidence of antibodies to other structures than AChR (39). Alternatively, increased mRNA levels could be due to attack by antibodies directed against a target involved in muscle activity or in the transduction of the electrical signal. Klarsfeld and Changeux (40) have shown that blocking of the spontaneous electrical activity of cultured chick myotubes with tetrodotoxin greatly increased the levels of mRNA coding for the α subunit of AChR.

Investigations of patients with similar clinical, immunological and therapeutic feature indicate that they can have different amounts of mRNA. For example, patients 9 and 12 (young females) had a low titer of anti-AChR antibody, severe MG, thymic hyperplasia, and no immunosuppressive treatment. However, patient 9 had 54.3×10^6 mol/μg of RNA compared with 6.7×10^6 mol/μg of RNA in patient 6, suggesting the existence of individual factors that could control AChR mRNA expression. These factors could play an additional role in the expression of the disease. Studies on denervated chicken muscle treated with cycloheximide have shown the need for protein synthesis to stimulate AChR gene expression (41), supporting the involvement of neural and myogenic factors in the regulation of transcriptional activity.

Nevertheless, the increase in mRNA levels of AChR α subunit observed in IIB MG patients could not predict whether a similar increase in protein expression occurs. Tetrodotoxin treatment of cultured chick myotubes led to a 13-fold increase in α subunit mRNA content, yet only a twofold increase in the number of surface AChR (40), indicating that protein expression is also regulated at other levels. The finding that treatment of cultured chick myotubes with the tumor promoter 12-0-tetradecanoyl phorbol 13 acetate (TPA) reduced the number of surface AChR receptors without affecting α-subunit mRNA levels (42) also supports the existence of a posttranscriptional control mechanism. Alternatively, a defect in protein expression could be due to one of the other subunits involved in the structural organization of the receptor, if it is synthesized in limited amounts. It would be interesting to quantify transcripts of the other subunits, an approach that would also help to determine if the newly transcribed receptor is in the embryonic form (γ), as is the case after denervation (24) and treatment of rats by α-bungarotoxin (43), or in the adult form (ϵ) as in experimental autoimmune myasthenia gravis (20).

We have also considered in our study, the possibility that abnormal expression of the P3A+ isoform may have a role in initiating or sustaining the autoimmune response in MG. Based on the fact that the α subunit is known to be the main immunogenic target for pathogenic auto-antibodies in MG, and given the location of the P3A exon near the main immunogenic region, a differential expression of the P3A exon could modulate the antibody attack on the AChR α subunit. To check this hypothesis we have analyzed the differential expression of the two α isoforms in muscle from MG patients with grade disease IIA and IIB. Our results have shown that mRNA encoding both AChR α-subunit isoforms are expressed at a ratio of approxi-

mately 1:1 in both myasthenic and normal muscles. Our data do not support the hypothetical role played by the P3A exon in the prevention of AChR degradation by auto-antibody attack. It is interesting to note that we have shown slightly increased expression of the variant including the P3A exon in muscle of patients with low grade disease versus controls (53.25 vs 48.55%, $P < 0.03$). The minor difference between the ratio of the two α isoforms in low and high grade disease seems to minimize the biological implication of P3A exon in modulation of the antibody attack. Nevertheless, P3A exon is found to be present in the genome of primates and conserved in species known to be susceptible to MG (44). This suggests a possible role for the additional 25 amino acids that it encodes. Further studies will be necessary to investigate this point. In addition, abnormal AChR expression in other muscles or tissues that express muscle AChR subunits and involved in the myasthenic autoimmune response cannot be excluded. Recent studies have provided evidence for expression of P3A+ variant mRNA in human thymus (11). We are currently investigating the differential expression of P3A+ and P3A− variants in the thymus of MG patients. We have already observed in our present study an association between the increased level of muscular α-subunit mRNA and thymus histology in thymectomized patients with MG. The mean level of muscular messengers coding for the α subunit gene was 2.5-fold higher in patients with hyperplastic thymus than in patients with normal thymus. The presence of muscle-type AChR or AChR like protein in the thymus has been also reported (45, 46) and these findings are compatible with the production of pathogenic antibodies in the hyperplastic thymus confirming the central role played by the thymus in the induction of the autoimmune response to AChR.

The constant ratio between the two isoforms of ~ 1 in myasthenic and nonmyasthenic muscle also suggests a regulatory mechanism involving alternative splicing. This is in accordance with recent data (11) showing exclusive expression of the isoform including the P3A exon in several human tissues, while both isoforms are expressed in muscle, suggesting the involvement of a muscle-specific factor. The nature of these putative alternative splicing mechanisms is not clear. Several mechanisms of regulation of alternative transcripts have been demonstrated, including *cis*- and *trans*-acting factors (47). *Cis*-acting elements could theoretically be determinant for this splicing, as demonstrated in other systems such as chicken β-tropomyosin transcripts (48) where the pattern of splicing depends on the state of differentiation of muscle cells; this led to the suggestion that an intronic sequence participates in the negative regulation of exon splicing in cooperation with a sequence-specific factor.

Although expression of P3A protein variants has been reported in the human cell line TE671 (18) the membrane insertion of the isoform including the P3A exon and its function have not yet been established. Thus, it is not clear whether AChR is packaged as P3A+ or P3A− α subunit homodimer or hybrid heterodimer, or whether these receptors are all functional. The observation that the two isoform mRNAs are equally expressed makes it tempting to speculate that each of the two α subunit isoforms is represented in the AChR pentamer.

In conclusion, we have identified a new biological parameter that correlates with the severity of human myasthenia gravis and obtained evidence of a compensatory mechanism for the loss of acetylcholine receptors. This pathophysiological model appears to be relevant to studies of AChR gene regulation and could contribute to the development of specific strategies for treating myasthenia gravis.

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