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A 5′-Flanking Region of the Chicken Acetylcholine Receptor α-Subunit Gene Confers Tissue Specificity and Developmental Control of Expression in Transfected Cells

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The 5′ end and promoter region of the α-subunit gene of chicken muscle acetylcholine receptor was mapped and sequenced. It includes a TATA and a CAAT box and a potential Sp1-binding site. When inserted in front of the chloramphenicol acetyltransferase gene, this promoter (including 850 base pairs of upstream sequence) directed high transient chloramphenicol acetyltransferase expression in transfected mouse C2.7 myotubes but not in C2.7 myoblasts or nonmyogenic 3T6 cells.

The genes encoding the nicotinic acetylcholine receptor (AChR) of vertebrate muscle (and the closely related electric organ of some fishes) have recently been cloned from several species (reviewed in reference 41) and constitute a system of choice for studying the control of gene expression in higher eucaryotes. Indeed, muscle AChR is made up of four subunits assembled in an $\alpha_2\beta\gamma\delta$ pentamer (9, 41), and one expects the expression of the corresponding four genes to be coordinately regulated. Also, this AChR is synthesized exclusively in skeletal striated muscle (and fish electric organ) (9, 15). During embryogenesis, or in various culture systems (7), its expression starts, at least partly because of transcriptional activation (7a), when myoblasts fuse into multinucleated myotubes (15, 23). The expression of many contractile proteins follows a similar pattern (7), which also primarily reflects the transcription of the relevant genes (19, 25, 27, 30, 35). It is thus of interest to compare the regulation of the genes encoding contractile proteins and the much less abundant synaptic proteins, such as the AChR. Finally, muscle electrical activity represses the expression of the AChR (15), and this effect can be studied in vitro, e.g., by using tetrodotoxin to block the spontaneous electrical activity of cultured myotubes (39). Such changes in AChR expression have been correlated with variations of the mRNA levels for its α-subunit (24); similar results have been obtained after muscle denervation in vivo (24, 28).

As a first step in studying the various signaling pathways involved in these regulations, we defined and characterized the 5′ end and upstream flanking region of the chicken AChR α-subunit gene; this region contains elements sufficient for tissue-specific and developmentally regulated expression of a transfected gene under transient expression conditions.

Characterization of 5′ end of chicken AChR α-subunit gene. Several laboratories, including ours, had previously isolated and identified genomic clones encoding the chicken AChR α-subunit (2, 24) on the basis of its high degree of sequence conservation with its counterparts in other species (5, 12, 33, 34). To map the first exon, the length of mRNA between the sequence corresponding to the exon named P2 in the human gene (24, 33) and the mRNA capsite was assessed by primer extension with reverse transcriptase, using poly(A)$^+$ RNA (purified as described previously [1]) from chick leg muscle (Fig. 1A). The size of the elongated product demonstrates the presence of 66 to 67 nucleotides upstream of P2.

Sequence determination by the dideoxy chain termination method (37) revealed a DNA region (Fig. 2A) approximately 700 base pairs (bp) upstream from exon P2 that contained an ATG start codon consistent with Kozak's rules (26), followed by a sequence encoding a hydrophobic peptide interrupted by a donor splice site (6), such that exon P2 is in frame with the start codon. The closed triangles in Fig. 2A mark the position of the 5′ end of the gene that can be deduced from the primer extension experiment.

We further confirmed the size and positioning of this first exon, P1, by S1 mapping with a single-stranded probe (4) containing the genomic sequence indicated in Fig. 2A (marked by the horizontal arrowheads). Probe protection was expected to be somewhat heterogeneous, ranging from 35 to 36 nucleotides, as predicted from primer extension, and 40 to 42 nucleotides, because of a fortuitous homology between the M13 vector flanking sequence present in the probe and the relevant part of the mRNA (five of seven nucleotides; indicated by stars in Fig. 2A). This was indeed observed (Fig. 1B). The open triangles in Fig. 2A mark the position of the 5′ end of exon P1, as deduced from the S1 protection pattern. Other authors have reported the template-independent addition of one nucleotide by reverse transcriptase (40), which may account for the difference seen here in the two types of experiments. The capsite of the large majority of eucaryotic mRNAs is a purine (43). We thus consider the G residue marked +1 in Fig. 2A as the 5′ end of the chicken AChR α-subunit gene. Upstream, one finds features common to many eucaryotic promoters, such as TATA and CAAT boxes (43).

It should be noted that the same mRNA start site is used in both innervated and denervated muscle, as judged by primer extension (Fig. 1A, lanes a and b) and S1 mapping (data not shown for innervated muscle). This is consistent with, yet does not prove, a transcriptional control of AChR gene expression by muscle innervation.

Construction of a CAT recombinant vector, pαAChCAT+, that contains AChR α-subunit upstream sequences sufficient for muscle-specific and developmentally regulated gene expression. DNA fragments that included approxi-

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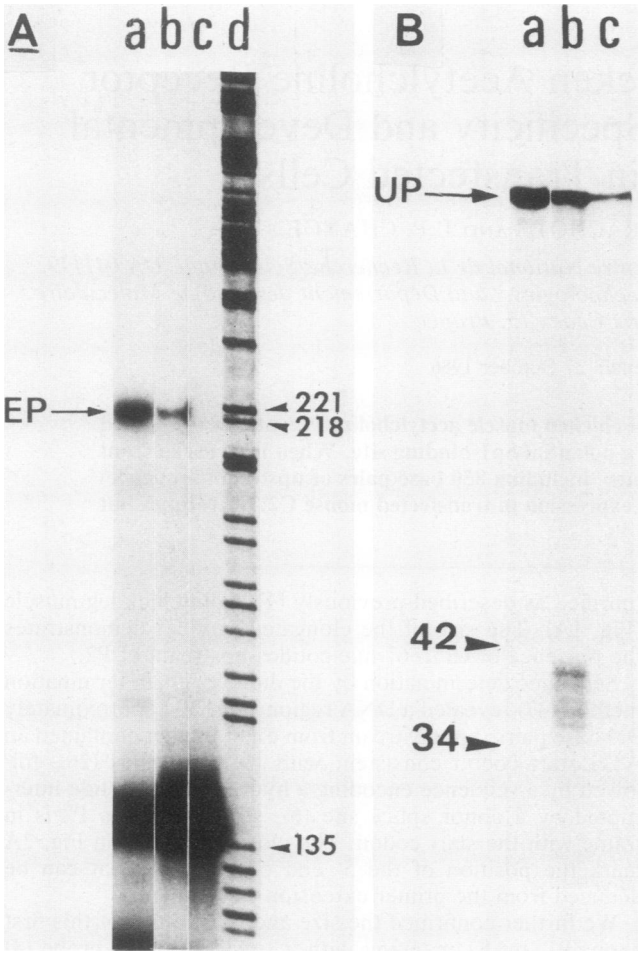

FIG. 1. Primer extension study (A) and S1 mapping (B) of the 5′ end of chicken AChR α-subunit mRNA. (A) Single-stranded labeled probe (110 nucleotides long) containing 82 nucleotides of exon P2 (33) was hybridized to 2 μg of poly(A)$^+$ RNA and extended with reverse transcriptase. Note that such probes also contain M13 primer and polylinker sequences (4). Reaction products were analyzed on a 6% denaturing polyacrylamide gel which was then dried and autoradiographed. Lanes: a, poly(A)$^+$ RNA from chick leg muscle denervated for 4 days; b, poly(A)$^+$ RNA from the contralateral innervated muscle; c, *E. coli* tRNA; d, molecular weight markers with sizes expressed in nucleotides. All lanes are from the same gel, with an 18-h exposure for lane a and a 3-day exposure for the others (without intensifying screens). EP, Extended product. An unspecific smear around 135 nucleotides can be seen. It has the same intensity with all three RNA sources and presumably represents self-priming of the probe. (B) The probe contained the 138 nucleotides complementary to the sequence indicated by horizontal arrowheads in Fig. 2A (and some adjacent M13 sequence, see panel A). Reaction products were analyzed on a 12% denaturing polyacrylamide gel which was then dried and autoradiographed. Lanes: a, pattern obtained with 20 μg of *E. coli* tRNA; b, pattern obtained with 20 μg of total RNA from denervated muscle; c, undigested probe (20 times less than in the S1$^-$ digested samples). Sizes are in nucleotides, as deduced from sequencing reactions run in parallel. UP, Undigested probe. Exposure was for 18 h without intensifying screens.

mately 850 bp of 5′-flanking sequence from the AChR α-subunit gene were obtained by short *Bal* 31 digestion of a 900-bp *Sst*I fragment from genomic clone λαch20 (24). This removed the ATG codon of the α-subunit gene to avoid possible interference with the initiation codon of the

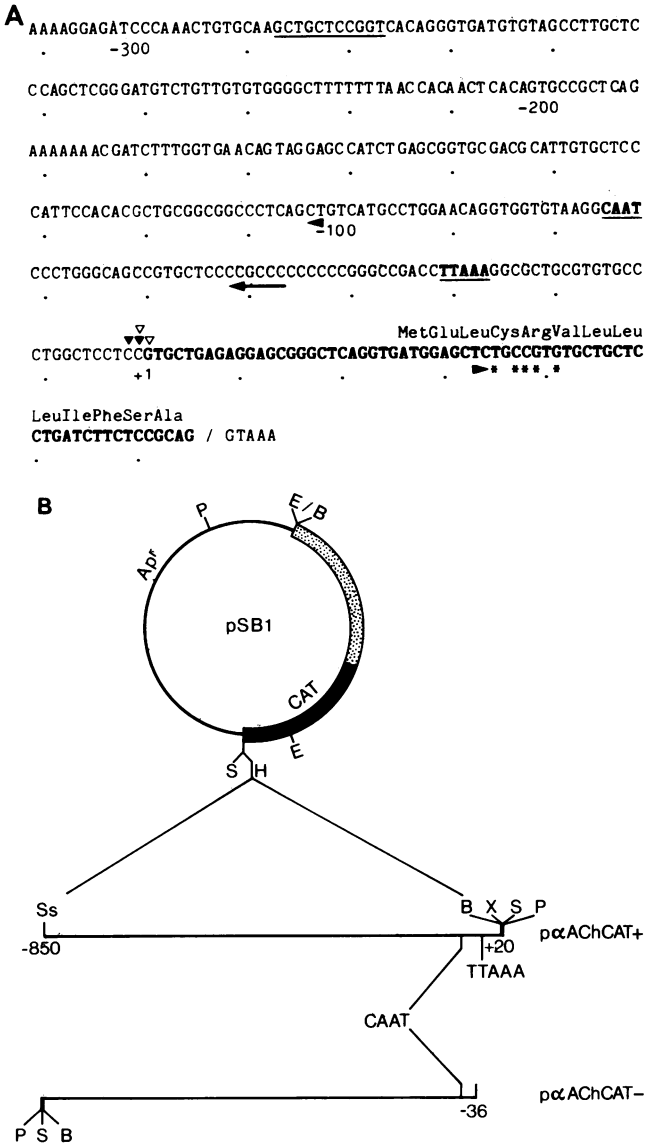

FIG. 2. (A) Sequence of the first exon and 5′-flanking region of the chicken AChR α-subunit gene. The closed and open triangles represent the 5′ end of the gene as deduced from primer extension and S1 mapping experiments, respectively. The extent of chicken genomic DNA contained in the probe used for S1 mapping is shown by horizontal arrowheads. The fortuitous homology of the mRNA with adjacent M13 sequence, present in this probe, is indicated by an asterisk below the corresponding nucleotides. The proposed start for transcription is the purine (G) nucleotide marked +1. Exon sequence is in bold type, and the exon-intron boundary is shown as a shill (/). It splits a codon in the same position as in the human gene (33), so that exon P1 and exon P2 are in the same reading frame (33; data not shown). The TATA and CAAT boxes are underlined and printed in bold type. The Sp1 core is underlined by an arrow to indicate its orientation (i.e., the consensus GGGCGG is actually found on the other DNA strand). Also underlined is an 11-bp motif that occurs in the chicken cardiac actin gene in which it overlaps the first exon-intron boundary, at position +32 (8). (B) Structure of the CAT recombinants used in transfection experiments. Plasmid pSB1 is drawn according to Ott et al. (36). Thin line, pBR322 sequences; dotted box, simian virus 40 sequences. The insert in pαAChCAT+ is derived from a subclone in M13mp10, and that in pαAChCAT− is from a subclone in M13mp9. Both inserts result from *Bal* 31 deletion performed on a 900-bp *Sst*I fragment. The sites for the following restriction enzymes are shown: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H),

TABLE 1. CAT activity normalized to beta-galactosidase activity in pre- and postfusion C2.7 cells and in 3T6 nonmyogenic cells

Transfected DNA	CAT activity[a] in:		
	C2.7 myotubes	3T6 cells	C2.7 myoblasts
pSV2CAT	890; 300 (100)	190 (100)	220; 63 (100)
pαAChCAT+	100; 170–180[b] (35)	0.1 (<0.1)	1.3; 0.7–1[b] (1.0)
pSB1	0.2; 0.4 (0.07)	0.1 (<0.1)	2.6; 0.4 (0.9)
pαAChCAT−	0.9; 1.8 (0.35)	<0.1 (<0.1)	2.1; 1.2 (1.4)

[a] CAT activity was expressed as the percentage of chloramphenicol acetylated by 10 μl of extract in a 5-min reaction (using 0.5 μCi of [^{14}C]chloramphenicol), and beta-galactosidase activity was expressed as the optical density at 420 nm extrapolated to the whole 150-μl extract in a 30-min incubation. CAT activity was then normalized to 100 beta-galactosidase units. Values obtained in independent experiments are separated by a semicolon. All CAT measurements were done under conditions in which no diacetylated chloramphenicol was detectable on the chromatograms. Normalized CAT activity was set to 100 for pSV2CAT in all cell types, for easier comparison. The corresponding relative values are given in parentheses. They are averaged over two experiments for C2.7 myoblasts and myotubes.

[b] Two independent dishes were transfected in these experiments.

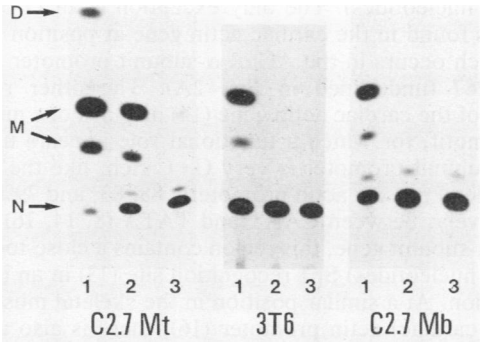

FIG. 3. Chromatograms of CAT assays. CAT activity was assayed in extracts from C2.7 myotubes and myoblasts and 3T6 cells after transfection with various CAT constructs. Lanes 1, pSV2CAT; lanes 2, pαAChCAT+; lanes 3, pαAChCAT−. For C2.7 cells, half of the extract (75 μl) was used in these assays, with 2.5-h-long incubations. For 3T6 cells, 35 μl of extract was used, with 15 min of incubation for pSV2CAT and 3 h for pαAChCAT+ and pαAChCAT−. N, Nonacetylated chloramphenicol; M, monoacetylated forms of chloramphenicol; D, diacetylated chloramphenicol.

chloramphenicol acetyltransferase (CAT) gene. The deleted fragments were cloned into M13 sequencing vectors to map the position of their promoter-proximal end. Two such fragments were then selected for insertion into the *Hin*dIII site of plasmid pSB1 (22), upstream of the CAT gene. DNA ends were made compatible by filling in with the Klenow fragment of *Escherichia coli* DNA polymerase I.

One of the recombinants, pαAChCAT+, contained the TATA box and the capsite of the gene, whereas another, pαAChCAT−, lacked both (Fig. 2B). These recombinants were transfected into a mouse muscle cell line, C2.7 (a subclone [10] of the C2 line originally isolated by Yaffe and Saxel [42]), or into a mouse fibroblast cell line, 3T6, by the standard calcium phosphate technique (18). Extracts from C2.7 cells were made from either the myoblast or the myotube stage, 30 to 72 h after transfection. In the experiments involving myotubes, similar results were obtained when cells were transfected before or after fusion.

Reference CAT vectors were also used: (i) pSB1, the promoterless plasmid from which the pαAChCAT recombinants were constructed (22), or in some experiments the closely related pSV0CAT (18), or both, as negative controls; (ii) pRSVCAT (17) and pSV2CAT (18), in which CAT expression is under the control of the Rous sarcoma virus long terminal repeat or the simian virus 40 early promoter and enhancer, respectively, as positive controls. Cotransfection with pCH110, a beta-galactosidase-expressing vector (21), allowed us to correct for variations in transfection efficiency between the several dishes of a given cell type in any single experiment. CAT and beta-galactosidase assays were performed and quantified essentially as described previously (22).

Table 1 shows the measured CAT activities (normalized to beta-galactosidase) for C2.7 myoblasts and myotubes and 3T6 cells. Average values relative to pSV2CAT are given in parentheses. In all cell types, CAT activities for pRSVCAT and pSV2CAT were roughly equal (within a factor of 2, data not shown). In C2.7 myoblasts and 3T6 cells, pαAChCAT+ was as little active as the negative controls (Table 1; Fig. 3). However, transfection of pαAChCAT+ into C2.7 myotubes resulted in much greater CAT activities, 35% of pSV2CAT activity on average, and consistently at least 100-fold higher than either pSB1 or pαAChCAT− activity. This latter construct, lacking the TATA box and the capsite, always gave low levels of CAT activity. This strongly suggests that transcription of the hybrid CAT gene was directed by the chicken AChR α-subunit promoter. Thus, an 850-bp-long 5′-flanking region of this α-subunit gene contains elements that confer tissue-specific and developmental control of expression.

Similar results have been reported for the regulation of other muscle-specific genes, using stably transfected myogenic cells (25, 35). However, in some cases, developmental regulation was not observed (30, 38). This can depend on the cell line used: a human cardiac actin gene showed some degree of regulation in rat L8 cells, but not in C2C12 cells (30), although the endogenous cardiac actin (29) is correctly regulated in both cell lines. This difference was interpreted (30) in terms of two distinct regulatory steps, activation (which transfected genes would not require) and subsequent modulation, with "modulating factors" accumulating more precociously in C2C12 than in L8 myoblasts. This scheme can accomodate the present results in at least two ways: the AChR α-subunit gene is sensitive to the same modulating factor but, contrary to the cardiac actin gene, not at the lower concentrations expected in myoblasts; alternatively, its own specific modulating factor is not expressed as early. A similar reasoning might also be valid for troponin I (25). In any case, our results demonstrate that C2 cells can indeed support developmental regulation of a transfected chimeric gene in transient expression experiments.

Comparison with other muscle-specific gene promoters. The present results also confirm that tissue-specific and developmental regulation of gene expression involves evolutionarily conserved mechanisms (11, 25, 31, 35), since a chicken promoter and flanking region will display correct regulatory properties in mouse cells. However, sequence comparisons with other chicken muscle-specific genes (8, 14, 16, 20, 32) failed to reveal any significant homology (above 70% over at

*Pst*I (P), *Sal*I (S), *Sst*I (Ss), and *Xba*I (X), as well as the positions of the TATA and CAAT boxes. The original *Hin*dIII site in pSB1 has been lost owing to the fill-in procedure. Numbering is given taking the transcription start site of the α-subunit gene as +1 (panel A). The positions of the 5′ ends of the inserted chicken DNA were deduced from migration in agarose gels and can be considered accurate to approximately 30 bp.

least 15 nucleotides). The only exception is an 11-bp motif which is found in the cardiac actin gene at position +32 (8) and which occurs in the AChR α-subunit promoter at position −287 (underlined in Fig. 2A). The other reported version of the cardiac actin gene (14) displays one mismatch in this motif, for which a functional role appears unlikely. The α-subunit promoter is very G+C rich, like the cardiac and skeletal muscle actin promoters: 88, 83, and 79% G+C, respectively, between CAAT and TATA (8, 14, 16). In the AChR α-subunit gene, this region contains a close-to-perfect (9 of 10 nucleotides) Sp1 recognition site (13) in an inverted orientation. At a similar position in the skeletal muscle (but not the cardiac) actin promoter (16), there is also a match with the Sp1 sequence in the direct orientation. However, recent experiments suggest that it has no functional significance in that actin gene (3).

In the long run, the approach outlined here may allow the dissection, starting from the level of gene transcription, of the various regulatory pathways involved in the tissue-specific, developmentally regulated, and muscle activity-dependent expression of vertebrate muscle AChR.

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