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Acetylcholine receptors of the *Drosophila* brain: a 900 bp promoter fragment contains the essential information for specific expression of the *ard* gene in vivo

Norbert Hess[a], Barbara Merz[a], Eckart D. Gundelfinger[a,b,*]

[a]*Center for Molecular Neurobiology, University of Hamburg, Martinistr. 52, D-20246 Hamburg, Germany*
[b]*Federal Institute for Neurobiology, Department of Neurochemistry and Molecular Biology, Postfach 1860, D-39008 Magdeburg, Germany*

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Abstract

The *ard* gene encodes a β-subunit of *Drosophila* nicotinic acetylcholine receptors specifically expressed in a subset of neurons. To identify the *cis*-regulatory region responsible for this cell-specific expression, various 5′ fragments of the *ard* gene were fused to a *lacZ* reporter gene and introduced into the *Drosophila* genome. A DNA fragment spanning ~760 bp upstream and ~140 bp downstream of a cluster of putative transcription start sites produced a pattern of β-galactosidase activity that resembles the distribution of ARD transcripts. Both in embryos and adults the levels of *lacZ* RNA were similar to those of endogenous ARD transcripts, suggesting that the 900 bp fragment harbors all essential elements for proper expression of the *ard* gene.

Key words: Nicotinic acetylcholine receptor; β-subunit; Insect; Nervous system; Transcription; P-element

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are multimeric glycoproteins of the cell membrane. They are involved in acetylcholine-mediated signal transmission between nerve cells or from nerve cells to muscle fibers. Subunits of the various subtypes of nAChRs are encoded by a large gene family [1,2]. To express a particular nAChR subtype the genes contributing to this receptor must be regulated in a highly coordinate way, i.e. they must be expressed in the same cell, at the same time and possibly even in a defined ratio.

nAChRs are the predominant class of excitatory neurotransmitter receptors in the insect central nervous system (CNS) [3]. To date, five different nAChR genes have been identified from the fruitfly *Drosophila melanogaster*. Three of these genes encode ligand-binding α-subunits, called ALS, Dα2/SAD and Dα3, and two code for structural β-subunits, ARD and SBD (for review see [4,5]). Three of the subunits, i.e. ALS, Dα2 and ARD, share a widespread and very similar distribution in synaptic neuropil regions of the *Drosophila* CNS ([6,7] and Jonas et al., in preparation) suggesting that they may be expressing an overlapping, if not identical, set of neurons. Immunoprecipitation experiments have shown that ALS and ARD, indeed, are components of the same receptor complex [8,9]. To understand coordination of biosynthesis of nAChRs in the fly CNS we began to isolate the genes of various nAChR subunits [10,11] and to functionally study their promoter regions. Here we report an initial characterization of the genomic region responsible for the physiologically correct expression in vivo of the *ard* gene encoding the ARD structural subunit of *Drosophila* nAChRs.

2. Materials and methods

2.1. Oligonucleotides

The following oligonucleotides derived from *ard* and *lacZ* sequences were used in this study: ARDPRX, CCGCTGAAACGCTTCGAATCGTAGTTGACAAACTTGTCCACATCC; ARDRACE1, GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT; ARDRACE2, CGGTACCTGCAGAATTCTTACTCCAGGGACATG; ARDPCR1, GACGGCTCCAGTTCAAGACGGAAAGTTCGG; ARDPCR2, GACATTGATTAGCTGTACGAACGCCAAACCAAATC; ARDPCR3, GATGGCAACTACGAGGTGCGCTACAAGTCCARDPCR4, GGTGCCGGACTTCCAGTAATCCGACAGATCCAC; LACZPCR, GGGATGTGCTGCAAGGCGATTAAGTTGGGTAAC; ARD1HYB, TTCCCTGAAAAATCAGTGAAA; ARD2HYB, ACAAGTCCAACGTGCTGAT.

2.2. Primer extension and RACE experiments

Primer extension experiments were performed as described in [12]. End-labeled oligonucleotide ARDPRX was annealed to 5 μg poly(A) RNA from 1-day-old w^{1118} flies. Complementary DNA was synthesized in a standard reaction at 42°C for 1 h with MMLV reverse transcriptase (Gibco BRL). Extension products were separated on a 4% polyacrylamide gel and sized by comparison to appropriate DNA sequencing reaction products.

Primer extension products were used as template for the rapid amplification and cloning of 5′ ends following the RACE protocol [13]. Nucleotides and primer were removed on agarose gels and isolated fragments ranging from 150 to 400 nucleotides were A-tailed. The PCR reaction was performed with oligonucleotides ARDRACE1 and

*Corresponding author at [b]Federal Institute for Neurobiology. Fax: (49) (391) 616 160.

Abbreviations: nAChR, nicotinic acetylcholine receptor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; CNS, central nervous system.

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ARDRACE2 as 5′ and 3′ primers, respectively [13]. Amplified DNA was cloned into the Bluescript pKS⁺ vector (Stratagene) using the *Cla*I and *Eco*RI restriction sites of the PCR products. Several independent clones were isolated and sequenced using a T7 sequencing kit (Pharmacia).

2.3. Promoter constructs

Three different *ard* promoter constructs, P-171, P-177 and P-178, were cloned into the P-element vector pW8 [14] to take advantage of a *Hin*dIII restriction site at position + 133 and span ~760 bp (P-171) and ~ 4.3kb (P-177) of the 5′ flanking region of the *ard* gene. The third one (P-178) includes the first four introns of the *ard* gene in addition to the 4.3 kb upstream region (see Fig. 1A). For constructs P-171 and P-177 the restriction fragments were fused to the AUG-lacZ-SV40-poly(A)-containing *Eco*RI fragment from the vector pC4-AUG-βgal [15] and cloned into pW8. P-178 was constructed by fusing the *Xba*I–*Sst*I fragment of the *ard* gene in frame to the *lacZ* gene. The correct fusion of the reading frames in P-178 was confirmed by sequencing.

2.4. Transformation of flies

Primary transformants were generated using standard protocols [16] by injecting 0.6 μg/μl construct DNA and 0.3 μg/μl of the helper P-element pπ25.7wc [17] into w^{1118} *Drosophila* embryos. Additional lines with independent insertion sites were generated by mobilizing P-elements from existing lines using P[ry⁺ Δ2–3](99B) flies as jump starter [18]. New integration sites were detected by a variant eye colour and confirmed by genetic means to be located on a different chromosome. All experiments were done with transformant lines homozygous for individual P-element insertions.

2.5. X-gal staining

For the detection of the expression pattern of β-galactosidase, embryos were dechorionated in 2.4% sodium hypochloride and rinsed several times with water. The embryos were fixed for 20 min in *n*-heptane saturated with glutaraldehyde (1 ml 100 mM cacodylate buffer, pH 7.3; 1 ml 50% glutaraldehyde were mixed with 2.4 ml *n*-heptane, the phases were allowed to separate and the *n*-heptane phase was used). Fixed embryos were washed with phosphate buffered saline (PBS) and incubated for 30 min in PBS containing 0.03% Triton X-100. Subsequently, β-galactosidase activity was visualized by incubating overnight in staining solution (20 mM $K_3Fe[CN]_6$, 20 mM $K_4Fe[CN]_6$, 1 mM $MgCl_2$, 0.04% X-gal in PBS) containing 0.03% Triton X-100. CNS's were dissected from third instar larvae in PBS, fixed in 0.75% glutaraldehyde for 25 min on ice and stained as above. Cryosections (12–18 mm) of 1- to 6-h-old flies were fixed with 2% glutaraldehyde in PBS for 5 min on ice and incubated overnight in staining solution to detect for β-galactosidase activity.

2.6. PCR-based semi-quantitative determination of transcript levels

Single-stranded DNA was synthesized from 5 μg of DNase-treated total RNA with a first-strand cDNA synthesis kit (Pharmacia). Three primers were used in each PCR reaction to simultaneously amplify the cDNAs from transcripts of both the endogenous *ard* gene and the transgene. For P-171- and P-177-transformed fly lines oligonucleotide ARDPCR1 (1 μg) was used as sense-strand (5′) primer for both kinds of transcripts and the oligonucleotides ARDPCR2 (0.5 μg) and LACZPCR (0.5 μg) as antisense (3′) primers for *ard* and *lacZ* transcripts, respectively. For P178-transformed strains a similar strategy was applied using oligonucleotides ARDPCR3 (1 μg), ARDPCR4 (0.5 μg) and LACZPCR (0.5 μg). Reaction conditions for cDNA synthesis were adopted from the manufacturer's instruction booklet. Samples were amplified in 20 or 30 cycles (1 min 94°C, 1 min 60°C, 2 min 72°C). PCR products were separated on agarose gels and hybridized to radiolabeled oligonucleotides ARDHYB1 and ARDHYB2 which recognize both endogenous and transgene transcripts of P-171-/P-177-transformed lines and P-178-transformed lines, respectively.

3. Results

3.1. The 5′ end of the ard *transcription unit*

The structural organization of the *ard* gene was reported previously [10,19] (see Fig. 1A). A primer extension experiment was performed to locate the start site of transcription. Multiple extension products were detected, a major one ending at a position defined as +1, and at least three minor ones ending at positions −9, +4 and +13 (Fig. 1B). Neither a TATA box nor any other canonical promoter element were found at appropriate distances from these positions. Therefore we decided to clone and sequence the primer extension products employing a RACE protocol to rule out the possibility that additional introns are located in the 5′ untranslated region of the *ard* gene. All analyzed RACE clones were co-linear with the genomic sequence and ended in the vicinity of the determined cluster of primer extension endpoints (Fig. 1B). This suggests that the *ard* gene lacks a TATA box and has multiple transcription start sites.

3.2. Characterization of ard *promoter fragments in vivo*

To identify DNA regions essential for the expression of the *ard* gene three different promoter fragments were fused to the *lacZ* gene from *E. coli* as a reporter gene (Fig. 1A) and introduced into the *Drosophila* genome. At least two independent lines of transgenic flies were analysed for each construct (Table 1). The P-177- and P-171-transformed lines showed significant β-galactosidase expression in the central nervous system of late embryos, third instar larvae and adults (Fig. 2). The line P-171-30-1 expresses the transgene in many tissues outside the CNS. This may be caused by activating sequences at the genome integration site, as mobilization of the insert from this line using P[ry⁺ Δ2–3](99B) flies as jump starter yielded a transformed line with an *ard*-specific *lacZ* expression pattern (Table 1). No X-gal staining was detectable in P-178-transformed lines, although transcript levels from the transgene are normal (see below). A PCR-based assay revealed that at least the fourth (155 bp) intron of the *ard* gene is poorly removed from transcripts of the fusion gene (data not shown), suggesting that ineffecient splicing is responsible for the failure to express β-galactosidase in P-178-transformed flies.

The *lacZ* expression patterns produced by the 0.9 kb and the 4.3 kb genomic 5′ fragments of the *ard* gene are virtually identical (Fig. 2): in the embryo the entire CNS is stained with X-gal. In the CNS of third instar larvae, weak *lacZ* expression is observed in the brain hemispheres and the anterior part of the ventral nervous system, whereas a subset of prominently stained neurons is located in the posterior part of the ventral ganglion (Fig. 2C). This restricted expression in the larval CNS is consistent with the low ARD transcript level observed in larval stages [20]. In the adult CNS X-gal staining is detected in all perikaryal regions of the brain and the thoracic ganglionic center (Fig. 2D–F) except for the lamina of the visual system. Both in the embryo and the adult CNS, the distribution of β-galactosidase activity coincides well with the distribution of ARD transcripts

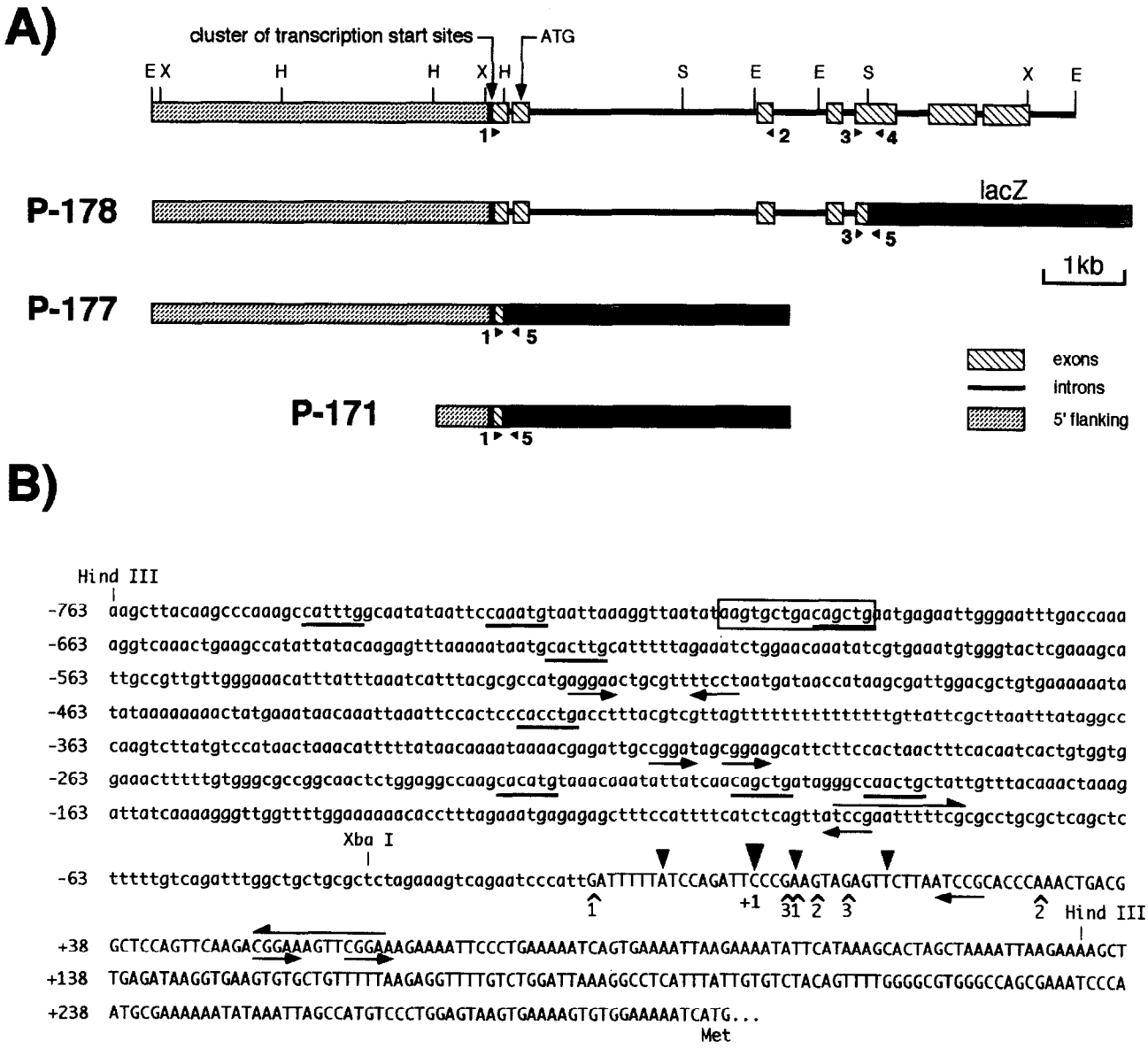

Fig. 1. Promoter constructs and DNA sequence of the promoter region of the *ard* gene. (A) Structure of the *ard* gene and gene fragments fused to the *lacZ* gene from *E. coli*. Relevant cleavage sites for restriction endonucleases, transcription and translation start sites, and positions of oligonucleotides (1, ARDPCR1; 2, ARDPCR2; 3 ARDPCR3; 4 ARDPCR4; 5, LACZPCR) used for the assessment of transcript levels are indicated. (B) DNA sequence of the region between the *Hin*dIII site at position −763 and the translation start site. The 5′ extensions of primer extension products (arrow heads above the sequence) and RACE products (arrow heads underneath the sequence, numbers give the number of independently isolated clones) are indicated. E-boxes are underlined. Ets-like protein binding sites and the inverted repeats are indicated by arrows underneath and above the sequence, respectively. The Dα2 similarity region is boxed. The sequence will appear in the EMBL/GenBank database under the accession number X78591.

detected by in situ hybridization [19,21] and is in good agreement with the widespread occurrence of ARD-like immunoreactivity [6].

A PCR-based strategy was designed to get a rough estimate of the abundance of transcripts produced from the *lacZ* reporter gene as compared to the level of endogenous ARD transcripts. The results are shown in Fig. 3. In the embryo as well as in the adult head, RNA levels transcribed from the transgene are in the same range as endogenous transcript levels. Line P-171-30-1 which expresses the *lacZ* gene non-specifically (Table 1) appears to produce higher *lacZ* transcript levels than other transformant lines (Fig. 3 and not shown). Both the overlap of the distribution of *ard* gene products with the X-gal staining pattern and the comparable abundance of endogenous and transgenic transcripts suggest that all es-

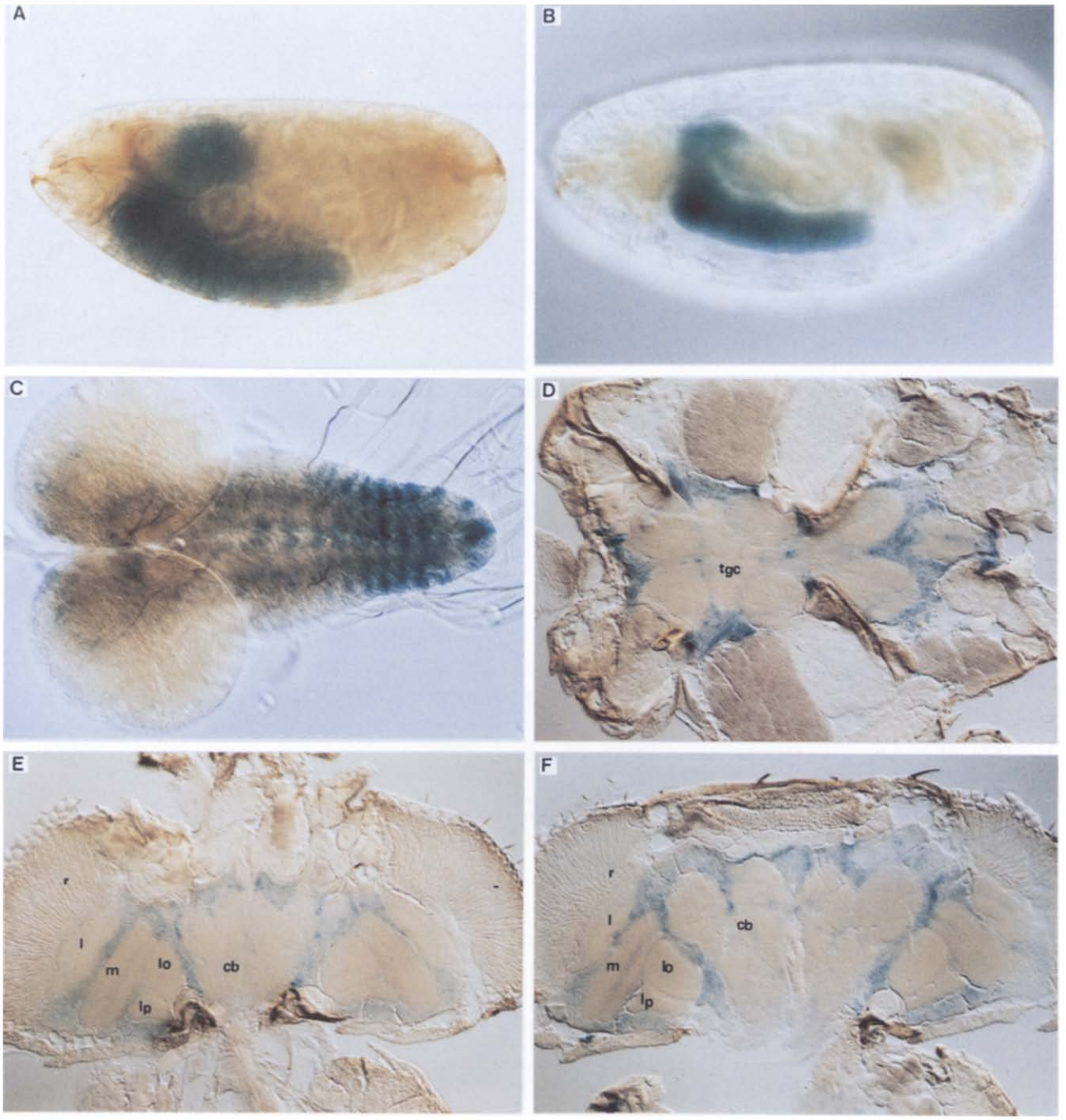

Fig. 2. Distribution of β-galactosidase activity in P-171- and P-177-transformed *Drosophila* lines. The X-gal staining patterns in whole mount preparations of the late embryos (A,B) and the CNS of a third instar larva (C), as well as horizontal sections of the adult thoracic ganglionic center (D) and the adult brain (E,F) of transformant lines P-171-9-1 (A,E) P-177-1-2/4 (B,C,D,F) are shown. cb, central brain; l, lamina; lo, lobula; lp, lobula plate; m, medulla; r, retina; tgc, thoracic ganglionic center.

sential *cis*-acting elements responsible for the spatial and temporal regulation of *ard* gene expression are located between nucleotides −763 and +138.

4. Discussion

An initial step to understand the temporal and spatial regulation of nAChRs in the CNS is to define the genomic region responsible for specific gene expression. For the *ard* nAChR gene no identifiable promoter elements were found upstream of several isolated cDNA clones. Primer extension and RACE cloning experiments have suggested that the *ard* gene has multiple transcription start sites and lacks classical promoter elements like a TATA box or a CAAT box. This is in common with

a number of vertebrate nAChR genes, like the murine genes encoding the α- and β-subunits of the muscle receptor [22,23], the chicken muscle δ-subunit gene [24], and the genes for the chicken neuronal α2- and α7-subunits [25,26].

A 900 bp genomic fragment which harbors the cluster of putative transcription start sites contains the essential signals for expression of the *ard* gene in the correct subset of neurons. This region was analyzed for sequence motifs of known *cis*-regulatory elements. Eight potential core binding sites for Ets-like proteins have been identified, six of which are arranged as tandem or inverted repeats (Fig. 1B). One of these tandem repeats is located 50–60 nucleotides downstream of the cluster of transcription start sites. Members of the Ets family have been reported to have a potential role in promoters lacking a TATA sequence and to occur downstream of the transcription start site [27]. Several E-boxes (CANNTG), binding sites for transcription factors containing the basic helix-loop-helix (bHLH) domain [28], were found within the 900 bp promoter fragment (Fig. 1B). Three of these E-boxes are clustered between nucleotides −744 and −689, another three between −225 and −184. BHLH proteins, such as MyoD, myogenin or MRF4, were reported to participate in the regulation of nAChR gene expression in the vertebrate muscle [23,29–34]. Moreover, bHLH proteins play an important role during development of the *Drosophila* nervous system (for review see [35]).

Other noticeable sequence elements are a pair of almost perfect 13-mer inverted repeats (nucleotides −91 to −79 and +52 to +64) which overlap with three of the Ets core binding sites and flank the cluster of transcription start sites (Fig. 1B). The functional significance of all potential *cis*-elements remains to be established.

Immunohistochemical and in situ hybridization studies, as well as promoter studies similar to those described here, have shown that the Dα2 gene encoding another nAChR subunit from *Drosophila* has an expression pattern very similar to that of the *ard* gene ([7] and Jonas et al., manuscript in preparation). Therefore we have compared the promoter regions of the *ard* and the Dα2 gene for possible sequence similarities. The only candidate for a common *cis*-element in the 5′ upstream regions has 14 out of 15 identical nucleotides and includes one of the E-boxes. It starts at position −703 of the *ard* promoter (Fig. 1B) and −676 of the Dα2 promoter. No significant sequence similarity has been found between the *ard* regulatory region and the promoter regions of other genes that are specifically expressed in the nervous system of *Drosophila*, including the *ace* gene encoding acetylcholinesterase [36], another component of the cholinergic system. Also, none of the elements responsible for the expression of the Dopadecarboxylase (*ddc*) gene in the CNS [37] was found in the *ard* promoter.

In conclusion, the *ard* gene has a relatively compact promoter which contains all essential elements for the expression of the ARD protein in a wide variety of neurons at all developmental stages from late embryos through adult flies within a 900 bp genomic region. This is in contrast to the Dα2 promoter which is quite complex and contains functional elements responsible for Dα2 expression in a distinct subgroup of cells far upstream of the transcription start site ([7] and Jonas et al., manuscript in preparation). It will be very interesting to identify *trans*-acting factors for the two promoters and to understand what is in common and what distinguishes the transcription machinery for the two genes.

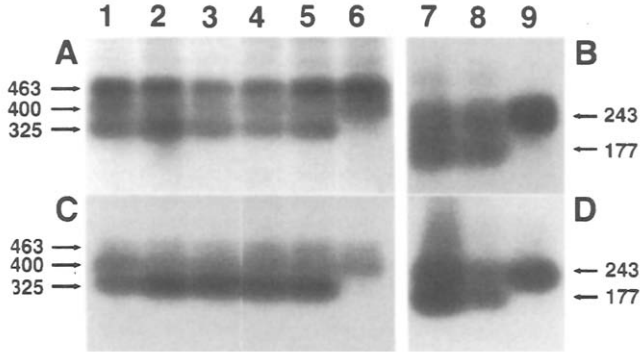

Fig. 3. PCR-based assessment of relative transcript levels produced from the endogenous *ard* gene and the transgenic *lacZ* fusion genes. Total RNA isolated from 10- to 22-h-old embryos (A,B) or adult heads (C,D) of transgenic *Drosophila* lines (lane 1, line P-171-9-1; lane 2, P-171-30-1; lane 3, P-171-30-17; lane 4, P-177-1-2; lane 5, P-177-29-1; lane 7, P-178-4-1-5; lane 8, P-178-113-5) or from the mutant strain w^{1118} (lanes 6 and 9) were transcribed into single-stranded cDNA and used as PCR templates (for details of the strategy see section 2.6.). PCR products were separated on agarose gels and Southern blotted. Blots in A and C were hybridized to the ^{32}P-labeled oligonucleotide ARDHYB1, and blots in B and D to ARDHYB2. In fly lines P-171 and P-177 (A,C) PCR products of 325 bp derive from *lacZ* transcripts; fragments of 400 bp and 463 bp from spliced and unspliced ARD transcripts. In P-178 lines (B,D) PCR products of 177 bp and 243 bp are produced from *lacZ* and ARD transcripts, respectively.

Table 1
Summary of X-gal staining data with transformant lines

Transformant line	Inserted chromosome	X-gal staining
P-171-9-1	III	+[2]
P-171-30-1	II	e[3]
P-171-30-17[1]	III	+
P-177-1-2/4	X	+
P-177-29-1	III	+
P-178-4-1	III	–
P-178-174-4	III	–
P-178-113-5	III	–

[1] Mobilized from P171-30-1
[2] +, specific expression pattern as shown in Fig. 2
[3] e, wide expression possibly caused by enhancer trapping.

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References

[1] Claudio, T. (1989) in: Frontiers in Molecular Biology: Molecular Neurobiology (Glover and Hames, eds.) Oxford IRL Press, pp. 43–162.
[2] Sargent, P.B. (1993) Annu. Rev. Neurosci. 16, 403–443.
[3] Breer, H. and Sattelle, D.B. (1987) J. Insect Physiol. 3, 771–790.
[4] Gundelfinger, E.D. (1992) Trends Neurosci. 15, 206–211.
[5] Gundelfinger, E.D. and Hess, N. (1992) Biochim. Biophys. Acta 1137, 299–308.
[6] Schuster, R., Phannavong, B., Schröder, C. and Gundelfinger, E.D. (1993) J. Comp. Neurol. 335, 149–162
[7] Jonas, P. (1993) Ph.D. thesis, Fachbereich Biologie, Universität Hamburg
[8] Schloss, P., Betz, H., Schröder, C. and Gundelfinger, E.D. (1991) J. Neurochem. 75, 1556–1562.
[9] Schloss, P., Mayser, W., Gundelfinger, E.D. and Betz, H. (1992) Neurosci. Lett. 145, 63–66.
[10] Sawruk, E., Hermans-Borgmeyer, I., Betz, H. and Gundelfinger, E.D. (1988) FEBS Lett. 235, 40–46.
[11] Jonas, P., Baumann, A., Merz, B. and Gundelfinger, E.D. (1990) FEBS Lett. 269, 264–268.
[12] Mason, P.J. and Williams, G.W. (1985) in: Nucleic Acid Hybridisation: A Practical Approach (Hames and Higgins, eds.) IRL Press, Oxford, pp. 139–160.
[13] Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[14] Klemenz, R., Weber, U. and Gehring, W.J. (1987) Nucleic Acids Res. 15, 3947–3958.
[15] Thummel, C.S., Boulet, A.M. and Lipshitz, H.D. (1988) Gene 74, 445–456.
[16] Rubin, G.M. and Spradling, A.C. (1982) Science 218, 348–353.
[17] Karess, R.E. and Rubin, G.M. (1984) Cell 38, 137–146.
[18] Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K. and Engels, W.R. (1988) Genetics 118, 461–470.
[19] Wadsworth, S. C., Rosenthal, L. S., Kammermeyer, K. L., Potter, M. B. and Nelson, D. J. (1988) Mol. Cell. Biol. 8, 778–785.
[20] Hermans-Borgmeyer, I., Zopf, D., Ryseck, R.-P., Hovemann, B., Betz, H. and Gundelfinger, E.D. (1986) EMBO J. 5, 1503–1508.
[21] Hermans-Borgmeyer, I., Hoffmeister, S., Sawruk, E., Betz, H., Schmitt, B. and Gundelfinger, E.D. (1989) Neuron 2, 1147–1156.
[22] Prody, C.A. and Merlie, J.P. (1991) J. Biol. Chem. 266, 22588–22596.
[23] Prody, C.A. and Merlie, J.P. (1992) Nucleic Acids Res. 20, 2367–2372.
[24] Wang, X.M., Tsay, H.J. and Schmidt, J. (1990) EMBO J. 9, 783–790.
[25] Bessis, A., Savatier, N., Devillers-Thiery, A., Bejanin, S. and Changeux, J.P. (1993) Nucleic Acids Res. 21, 2185–2192.
[26] Matter-Sadzinski, L., Hernandez, M.-C., Roztocil, T., Ballivet, M. and Matter, J.-M. (1992) EMBO J. 11, 4529–538.
[27] Wasylyk, B., Hahn, S. and Giovane, A. (1993) Eur. J. Biochem. 211, 7–18.
[28] Blackwell, T. and Weintraub, H. (1990) Science 250, 1104–1110.
[29] Piette, J., Bessereau, J.-L., Huchet, M. and Changeux, J.-P. (1990) Nature 345, 353–355.
[30] Gilmour, B.P., Fanger, G.R., Newton, C., Evans, S.M. and Gardner, P.D. (1991) J. Biol. Chem. 266, 19871–19874.
[31] Numberger, M., Durr, I., Kues, W., Koenen, M. and Witzemann, V. (1991) EMBO J. 10, 2957–2964.
[32] Jia, H.T., Tsay, H.J. and Schmidt, J. (1992) Cell. Mol. Neurobiol. 12, 241–258.
[33] Bessereau, J. L., Mendelzon, D., Lepoupon, C., Fiszman, M., Changeux, J. P. and Piette, J. (1993) EMBO J. 12, 443–449.
[34] Dulcert, A., Savatier, N. and Changeux, J.P. (1993) Proc. Natl. Acad. Sci. USA 90, 3043–3047.
[35] Campos-Ortega, J. (1991) Int. Rev. Cytol. 124, 1–41.
[36] Fournier, D., Karch, F., Bride, J.M., Hall, L., Berge, J.B. and Spierer, P. (1989) J. Mol. Biol. 210, 15–22.
[37] Scholnick, S.B., Bray, S. J., Morgan, B.A., McCormick, C.A. and Hirsh, J. (1986) Science 234, 998–1002.