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The DNA binding activity of the paired box transcription factor Pax-3 is rapidly downregulated during neuronal cell differentiation

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Abstract Mutations in the murine P*ax*-3 gene lead to a range of developmental abnormalities including deficiencies in sensory and sympathetic neurones. We have investigated P*ax*-3 expression during neuronal differentiation and show levels of P*ax*-3 DNA binding decrease upon cell cycle arrest and morphological differentiation. The fall in P*ax*-3 DNA binding occurs within 1 h of the induction of differentiation and is mediated in part by a decrease in P*ax*-3 mRNA. This decrease in P*ax*-3 binding activity precedes any changes in cell proliferation or morphology, suggesting that the downregulation of this transcription factor may be an important prerequisite for the differentiation of neuronal progenitor cells.

Key words: Neuronal cell; Cell proliferation; Differentiation; *Pax*-3

1. Introduction

P*ax*-3 is a member of a family of evolutionarily conserved transcription factors, which share a conserved DNA binding motif of 128 amino acids, known as the paired-box motif [1,2]. In mice, nine paired-box encoding genes have so far been identified, and these have been named *Pax*-1 to *Pax*-9 [3]. The importance of this gene family in embryogenesis has been demonstrated by the finding that specific mutations within a number of the P*ax* genes lead to a range of developmental abnormalities in both humans and mice. Mutations in the PAX-6 gene lead to the human genetic disorder aniridia [4,5] and the *small eye* phenotype in mice [6]. The Pax-1 gene is mutated in *undulated* mice [7] and disruption of the PAX-3 gene results in the Waardenburg syndrome in humans [8–10] and the *splotch* phenotype in mice [11,12]. Homozygous *splotch* mice die in utero on embryonic day 14 (E14), and display severe neural tube defects, a lack of musculature and deficiencies in neural crest-derived cell types such as Schwann cells, sensory and sympathetic neurones.

Analysis of P*ax*-3 expression has revealed that it is expressed in a temporally and spatially restricted manner during embryogenesis [13]. Expression of P*ax*-3 is first detected in day 8.5 mouse embryos within the developing spinal cord and brain. Expression of P*ax*-3 is also observed within the neural crest, a transient embryonic stem cell population which upon differentiation gives rise to a multitude of cell types including Schwann cells and sensory and sympathetic neurones of the peripheral nervous system. During Schwann cell development P*ax*-3 expression is limited to Schwann cell precursors or embryonic Schwann cells [14]. No expression of P*ax*-3 is detected after the terminal differentiation of these cells into non-proliferative myelinating Schwann cells. Within the Schwann cell lineage the restriction of P*ax*-3 transcripts to immature Schwann cells has led to the hypothesis that P*ax*-3 may play an important role during the differentiation of Schwann cells by establishing and maintaining the phenotype of pre- differentiated Schwann cells prior to their terminal differentiation.

To determine whether P*ax*-3 may also be involved in the differentiation of neuronal cells, we have studied the expression and regulation of P*ax*-3 in the neuronal cell line ND7 [15]. ND7 cells normally proliferate indefinitely in the presence of 10% serum, but upon the addition of 1 mM cyclic AMP/0.1% serum undergo cell cycle arrest and morphological differentiation. The differentiated cells produce long neurite processes, secrete sensory neuropeptides such as the calcitonin gene-related peptide, and exhibit electrophysiological responses to bradykinin, all properties characteristic of a mature sensory neurone. In this study, we report that high levels of P*ax*-3 binding activity are associated with mitotically active immature neuronal cells. Within 1 h of the induction of differentiation, we found that the DNA binding activity of P*ax*-3 was rapidly downregulated, prior to any overt changes in cell morphology or cell proliferation.

2. Materials and methods

2.1. Cell culture

ND7 cells were grown in Leibovitz's 15 medium (L15) containing 10% bovine calf serum and either maintained in this medium or transferred to L15 medium containing 1 mM dibutyryl cyclic AMP and 0.1% serum.

2.2. Electrophoretic mobility shift assays

Oligonucleotides for use in electrophoretic mobility shift assays were annealed by heating to 85°C for 3 min and labelled using [γ-^{32}P]ATP and polynucleotide kinase (Promega). The oligonucleotides used were the e5 oligonucleotide [16] (5′-CTCAGCACCGCACGATTAGCACCGTTCCGCTTC-3′) and the DNA binding site for the Sp1 general transcription factor 5′-ATTCGATCGGGGCGGGGCGAGC-3′. Nuclear extracts were made using the method described by Dignam et al. [17] and electrophoretic mobility shift assays carried out as previously described [18]. Competitions were performed using 100-fold excess of unlabelled oligonucleotide which was incubated with the nuclear extracts prior to the addition of the probe. To confirm the identity of the retarded complex, nuclear extracts were also incubated on ice for 15 min with 4 μl of 1 μg/ml specific antiserum prior to the addition of the probe.

2.3. Antibodies

Anti-P*ax*-3 antiserum was made against a polypeptide containing amino acids 280–479 of *Pax*-3. This antibody has been shown to specifically recognise P*ax*-3 [19] and in Western blotting experiments the antibody recognises only one protein in ND7 cells which has a molecular weight of 56 kDa, the estimated molecular weight for P*ax*-3

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(data not shown). Anti-P*ax*-5 and B*rn3b* antibodies were obtained from Santa Cruz.

2.4. RT-PCR

Total cellular RNA was extracted using Trizol (Gibco) and used as a template to prepare complementary DNA (cDNA) using Moloney murine leukaemia virus reverse transcriptase (MoMLV RT) (Gibco). RNA concentrations were determined by slot-blot hybridisations. The cDNA was then amplified using Taq polymerase (Promega) with specific primers for *Pax*-3 and cyclophilin [20]. The PCR conditions in which the input cDNA was linearly proportional to the PCR product were initially established for each primer pair by taking aliquots at 15, 20, 25, 30 and 35 cycles to locate the exponential phase of the PCR cycle. On the basis of these initial experiments, one tenth of the cDNA sample was amplified for 25 cycles with the *Pax*-3 primers and 20 cycles with the cyclophilin primers. Samples were analysed on 2% agarose gels, followed by Southern blotting and hybridisation with radiolabelled probes. The PCR primers used for the cyclophilin PCR were (a) 5′-TTGGGTCGCGTCTGCTTCGA-3′, (b) 5′-GCCAGGACCTGTATGCTTCA-3′ and for *Pax*-3 (a) 5′-GGAATACAAAGAGAGAACCCG-3′, (b) 5′-CTTCATCTCACTGAGGTGCG-3′.

2.5. Cell proliferation studies

ND7 cells were plated onto 9 cm dishes in medium containing 10% serum at a density of 5×10^5 cells per dish. The medium was replaced with 0.1% serum/1 mM cyclic AMP for 1, 2, 6, 24, 48 and 72 h. 1 μCi of [^{3}H]methylthymidine (1 mCi/ml, Amersham) was added to each of the dishes for the last hour of incubation. The cells were then washed twice with 1 ml phosphate buffered saline (PBS) and harvested by scraping into 62.5 mM Tris-HCl pH 7.5. Triplicate aliquots were then precipitated with ice-cold 10% trichloroacetic acid (TCA). After 30 min at 0°C, the precipitates were diluted with 0.5 ml of TCA and collected onto G/C filters (Whatman). The filters were then washed with 10% TCA, dried and counted in optiphase Hi-safe 3 scintillant (Fisons). Values shown represent the average relative amounts of tritiated thymidine incorporation in triplicate cultures of 0.1% serum/1 mM cyclic AMP-treated cells compared to triplicate control cultures of proliferating ND7 cells.

2.6. Neurite outgrowth

Neurite outgrowth was determined by counting the number of processes greater than 20 μm in length. The number of cells exhibiting outgrowths is expressed as a percentage of the total number of cells.

3. Results and discussion

3.1. Pax-3 DNA binding activity in proliferating neuronal cells

To determine the role that *Pax*-3 plays in neuronal cell development, we have studied the DNA binding activity of this transcription factor in a series of proliferating neuronal cell lines. The DNA binding activity of P*ax*-3 was analysed by electrophoretic mobility shift assay (EMSA), in which nuclear extracts from the different cell lines were incubated with a radiolabelled oligonucleotide containing the e5 sequence. The e5 sequence contains a high affinity binding site for P*ax*-3, which is derived from the *Drosophila even skipped* promoter [16]. As shown in Fig. 1A, one major e5 binding protein was observed in extracts made from proliferating ND7 cells. The binding of this protein was sequence-specific, since binding was abolished by competition with an excess of unlabelled e5 oligonucleotide but not by competition with an excess of unlabelled oligonucleotide containing the binding site for the general transcription factor Sp1. Furthermore, the identity of this e5 binding protein as P*ax*-3 was confirmed by the addi-

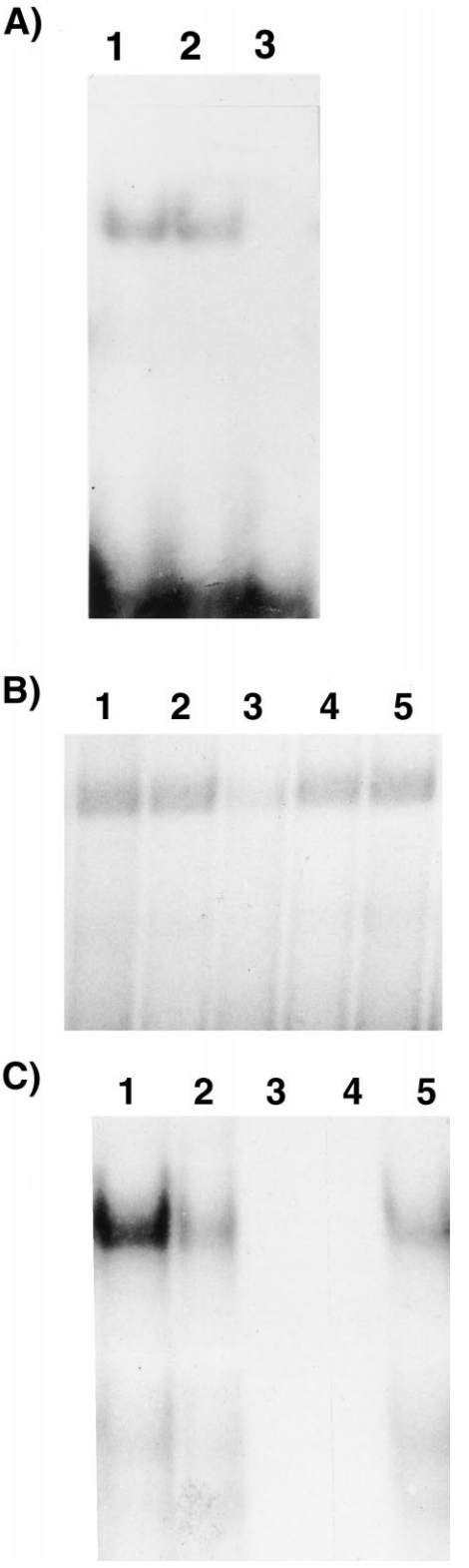

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Fig. 1. A: EMSA using the e5 oligonucleotide with nuclear extracts from proliferating ND7 cells alone (track 1), in the presence of one hundred fold excess of unlabelled Sp1 oligonucleotide (track 2), and in the presence of 100-fold excess of unlabelled e5 oligonucleotide (track 3). B: EMSA with the e5 oligonucleotide with nuclear extracts from proliferating ND7 cells alone (track 1), or in the presence of preimmune serum (track 2), anti-P*ax*-3 antiserum (track 3), P*ax*-5 antibody (track 4) and B*rn3b* antibody (track 5). C: EMSA with the e5 oligonucleotide with nuclear extracts from proliferating ND7 cells (track 1), PC12 cells (track 2), ND7 cells grown in 0.1% serum/1 mM cyclic AMP (track 3), PC12 cells in the presence of 5 ng/ml NGF (track 4) and N18 cells (track 5).

tion of P*ax*-3-specific antibody, which abolished binding (Fig. 1B) [19]. No effect on binding was observed by preincubation with antibodies raised either against P*ax*-5, another member of this developmental gene family, or against the homeodomain transcription factor B*rn3b* (Fig. 1B).

High levels of P*ax*-3 DNA binding activity were found not only in proliferating ND7 cells, but also in nuclear extracts made from dividing N18 cells, a mouse neuroblastoma cell line, and from mitotically active PC12 cells, an adrenal medulla phaeochromocytoma cell line (Fig. 1C). In contrast, P*ax*-3 binding activity could not be detected in ND7 cells grown in the presence of 1 mM cyclic AMP/0.1% serum, which induces both a cessation in cell proliferation and morphological differentiation. The DNA binding activity of P*ax*-3 was also downregulated upon PC12 differentiation. Within 72 h of the addition of 5 ng/ml nerve growth factor (NGF), which induces both the morphological differentiation of the PC12 cells into a mature sympathetic neurone-like phenotype and a cessation in cell proliferation, we found that the DNA binding activity of P*ax*-3 had declined to undetectable levels (Fig. 1C). Thus P*ax*-3 binding activity in vitro appears to be restricted to mitotically active immature neuronal cells of both sensory and sympathetic lineages.

3.2. The DNA binding activity of Pax-3 is rapidly downregulated upon ND7 differentiation

To analyse what role *Pax*-3 may play in the differentiation of neuronal cells, we set up a time course experiment to investigate the kinetics of the decrease in P*ax*-3 DNA binding activity. To do this, ND7 cells were grown either in 10% serum or transferred to medium containing 1 mM cyclic AMP/0.1% serum for 1, 6 and 72 h. At each of these time points, nuclear extracts were made and incubated with the e5 oligonucleotide. As shown in Fig. 2, upon the addition of differentiation medium, the DNA binding activity of P*ax*-3 fell rapidly. Within 1 h of the addition of cyclic AMP/0.1% serum, the DNA binding activity of P*ax*-3 had fallen to undetectable levels despite long exposures of the EMSA to X-ray film.

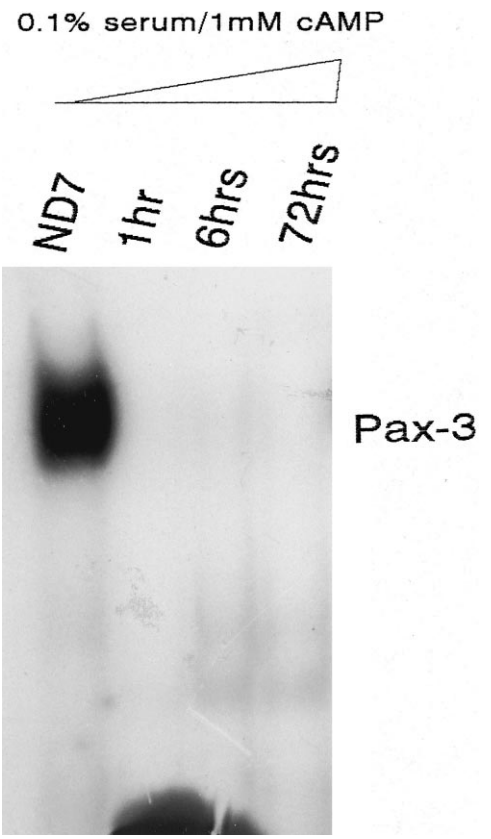


Fig. 2. EMSA showing the decrease in P*ax*-3 binding activity upon ND7 cell differentiation. The e5 oligonucleotide was incubated with nuclear extracts from ND7 cells grown in 10% serum, or with extracts made from ND7 cells incubated in 0.1% serum/1 mM cyclic AMP for 1, 6 or 72 h.

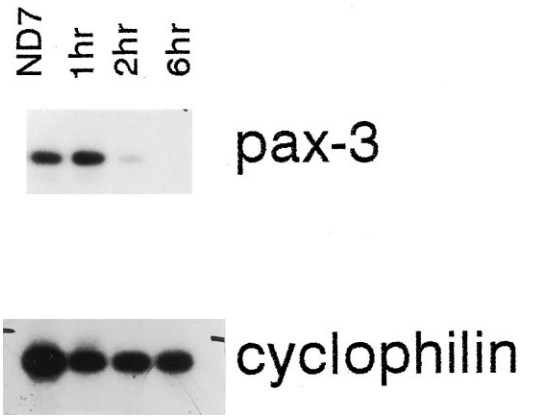


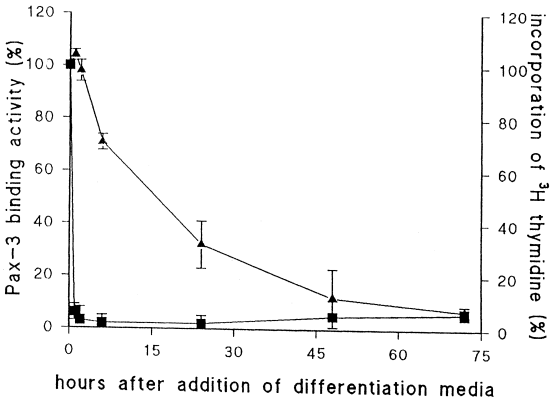
Fig. 3. RT-PCR showing a decrease in the level of P*ax*-3 mRNA upon differentiation. cDNA was prepared from ND7 cells grown in 10% serum or grown in 0.1% serum/1 mM cyclic AMP for 1, 2 or 6 h.

Having shown that the DNA binding activity of P*ax*-3 is rapidly downregulated upon differentiation, we also wished to establish whether the fall in P*ax*-3 binding activity was mirrored by a similar decrease in the level of P*ax*-3 mRNA expression. To achieve this, RNA was isolated from both proliferating ND7 cells and from cells grown for 1, 2, 6 or 24 h in 1 mM cyclic AMP/0.1% serum. The RNA from each of these time points was then used as a template for cDNA synthesis and amplified using quantitative PCR with primers specific for *Pax*-3 and the housekeeping gene cyclophilin. We found, as shown in Fig. 3, high levels of *Pax*-3 mRNA in proliferating ND7 cells. Interestingly, unlike the DNA binding activity of P*ax*-3 which was downregulated within 1 h, no change in P*ax*-3 mRNA levels was initially observed within 1 h of the addition of differentiation medium. However, 2–6 h after the induction of differentiation, P*ax*-3 mRNA levels did fall dramatically to very low levels. The levels of cyclophilin remained unchanged. These results show that at least in part the downregulation in P*ax*-3 binding activity is mediated by a decrease in P*ax*-3 mRNA expression, although the initial fall in P*ax*-3 binding activity may be mediated by post-transcriptional changes in either P*ax*-3 expression or binding activity.

3.3. The role of Pax-3 in ND7 differentiation

To analyse what role the rapid downregulation in *Pax*-3 activity plays in the differentiation of neuronal cells, we wished to determine how the decline in the DNA binding activity of P*ax*-3 correlated with the appearance of overt signs of morphological differentiation and/or the onset of cell cycle arrest, which accompanies morphological differentiation. The degree of morphological differentiation, judged by counting the percentage of cells bearing neurite outgrowths, and the

rate of cell proliferation, measured by incorporation of tritiated thymidine, was analysed and compared with changes observed in the binding activity of P*ax*-3 upon addition of differentiation medium. We found, as shown in Fig. 4, that the fall in the DNA binding activity of P*ax*-3 occurs prior to any overt signs of morphological differentiation, and prior to any detectable changes in the rate of cell proliferation. This argues that the downregulation of this transcription factor is not merely a consequence of differentiation but may instead be a necessary requirement for either the production of a morphologically mature neurone or for the onset of cell cycle arrest which accompanies morphological differentiation. Therefore, to determine whether the fall in P*ax*-3 binding activity is involved in the morphological differentiation of the cells rather than in the onset of cell cycle arrest, we treated ND7 cells with 1 mM cyclic AMP in the presence of 10% serum, which induces morphological differentiation without a cessation in cell proliferation [21]. Under these conditions, the DNA binding activity of P*ax*-3 fell within 1 h of the addition of 1 mM cyclic AMP (Fig. 5). This suggests that the decrease in the activity of this transcription factor is closely associated with the production of a morphologically mature neurone, and raises the possibility that the downregulation in P*ax*-3 activity may be an important regulatory event in the morphological differentiation of neuronal cells.



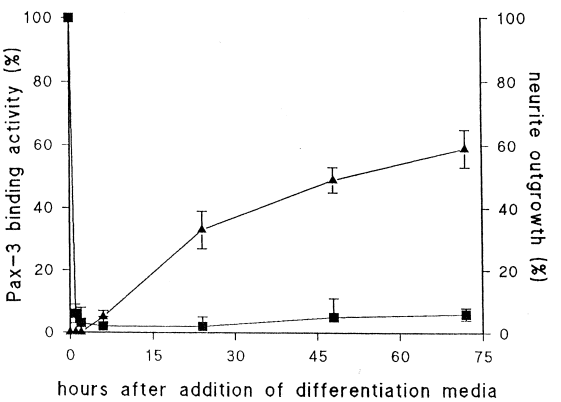


Fig. 4. A: Time course of the decline in P*ax*-3 DNA binding activity (■), measured by EMSA, and the decline in cellular DNA replication (▲), measured by incorporation of tritiated thymidine upon addition of 0.1% serum/1 mM cyclic AMP. The DNA binding activity and incorporation of thymidine are expressed as a percentage of the value observed in proliferating cells. B: Time course of the decline in P*ax*-3 DNA binding activity (■) and the increase in neurite outgrowth (▲) upon addition of 0.1% serum/1 mM cyclic AMP. Neurite outgrowth is expressed as the number of cells exhibiting neurites longer than 20 μm as a percentage of the total number of cells.

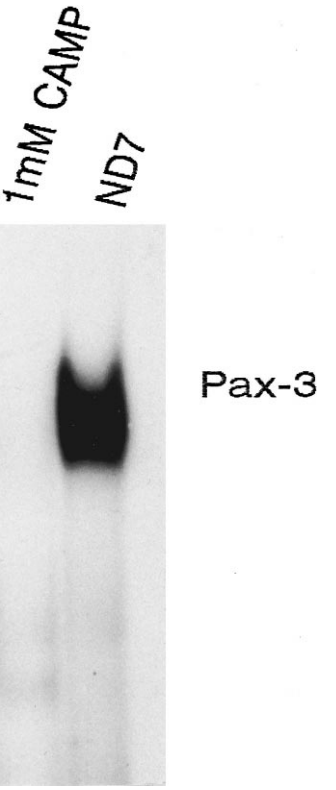


Fig. 5. EMSA with nuclear extracts from proliferating ND7 cells (ND7) and from ND7 cells incubated for 1 h in growth medium containing 1 mM cyclic AMP.

The data in this report therefore suggest that one function of P*ax*-3 in immature neuronal cells is to maintain the undifferentiated phenotype of these progenitor cells until appropriate environmental cues signal a downregulation in P*ax*-3 activity and the production of a morphologically mature neurone. Cyclic AMP may itself play an important neurotrophic role in the development of the peripheral nervous system. Rydel and Green have shown that cyclic AMP analogues promote neurite outgrowth and cell survival in primary cultures of both sensory and sympathetic neurones [22]. This cyclic AMP neurotrophic pathway is mediated by the activation of cyclic AMP-dependent protein kinases and is independent of NGF. One possible downstream target for this cyclic AMP pathway may therefore be the transcriptional regulator P*ax*-3 whose downregulation may be important for either the activation of genes involved in the structure and function of the mature neurone or the repression of genes important in the maintenance of the immature phenotype. Interestingly, Kioussi et al. [14] have also shown that P*ax*-3 expression is controlled by intracellular cyclic AMP levels. Treatment of Schwann cells in culture with forskolin, an agonist of cyclic AMP, leads to the repression of P*ax*-3 RNA expression and the activation of myelin basic protein, a gene whose expression is inhibited in the presence of P*ax*-3. It will now be important in order to gain further insights into the

role of P*ax*-3 in neuronal cell development to begin to identify some of the target genes regulated by P*ax*-3 during neuronal cell differentiation.

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