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Characterization of *N*-cadherin mRNA in chicken brain and heart by means of oligonucleotide probes

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It has previously been reported, that there is only one mRNA of 4.3 kb for chicken *N*-cadherin in brain and heart and one gene [(1988) J. Cell Biol. 106, 873–881]. Using three synthetic oligonucleotide probes derived from the published chicken *N*-cadherin cDNA sequence we found hybridization to at least four mRNAs in chicken brain and heart. The measured sizes were 8.0, 4.7, 3.8, and 3.3 kb. The 8.0 kb mRNA is only seen in brain, and only when a cytoplasmic probe is employed. The 3.8 kb mRNA is only observed in heart.

N-Cadherin; Gene expression; mRNA; Chicken

1. INTRODUCTION

N-cadherin is a calcium-dependent cell-cell adhesion molecule, which presumably plays an important part during embryonic development [1,2]. It is an integral membrane glycoprotein of M_r 127–135 kDa. It is probably identical to NcalCAM [3–5], ACAM [6,7] and a 130 kDa glycoprotein in lens cells [8,9]. *N*-cadherin has been demonstrated in several tissues including the nervous system, cardiac muscle, lens, retina, spleen, and bone marrow. It is also expressed transiently in skeletal and smooth muscle, lung and urogenital epithelium [7,10–12]. The nucleotide sequence of a chicken *N*-cadherin cDNA from brain has been reported [13]. Furthermore the amino acid sequence of mouse *N*-cadherin in brain has been published [14]. Mouse *N*-cadherin shows 92% similarity in amino acid sequence to the chicken homologue. Northern blotting, using a cDNA probe of about 350 bp covering the putative NH_2-terminal, displayed a single mRNA population of ca. 4.3 kb in chicken brain and heart [13]. In mouse two additional RNA species of 3.5 and 5.2 kb were observed using a cDNA probe of about 930 bp starting from the putative NH_2-terminal [14].

We here report the demonstration of four mRNA populations of 3.3, 3.8, 4.7, and 8.0 kb in chicken using three oligonucleotide probes covering the NH_2-terminal, a part of the extracellular domain called EC3 [13], and a part of the cytoplasmic domain. The main mRNA band of 4.7 kb was observed in both heart and brain using all probes. The 3.8 kb mRNA was only observed in heart using all probes, and the mRNA of 8.0 kb was only seen in brain and only when using the cytoplasmic probe. The mRNA of 3.3 kb was observed in both heart and brain with the cytoplasmic and the EC3 probe but not with the N-terminal probe. The existence of these mRNAs may be accounted for by alternative splicing of the *N*-cadherin gene or the existence of *N*-cadherin related mRNAs.

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Abbreviations: SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; E10, embryonal day 10; P0 and P10, postnatal day 0 and 10.

2. MATERIALS AND METHODS

2.1. *Northern blotting analysis*

Total RNA was extracted from chicken brain, heart, and liver by the phenol-chloroform method [15,16] and then passed through an oligo(dT)-cellulose column (Pharmacia, type 7) according to Aviv and Leder [17]. Poly(A)$^+$RNA was denatured in 50% formamide, 6% formaldehyde in water by heating for 15 min at 56°C followed by a few minutes on ice and fractionated by electrophoresis overnight through a 0.8% agarose gel. 5 μl RNA standard (BRL) was used to determine the size of RNA bands. The gel was capillary blotted overnight in 20×SSC (1×SSC = 0.15 M NaCl, 0.015 M sodium citrate) onto nitrocellulose filters (Schleicher & Schüll). Prehybridization (4 h) and hybridization (18–20 h) were performed at 60°C in 4×SSC, 0.1% SDS, 0.1% Denhardt's solution (2% Denhardt's solution = 2% w/v Ficoll, 2% w/v bovine serum albumin, 2% w/v polyvinylpyrrolidone), 0.2 mM EDTA, 200 μg/ml poly (A)$^+$ RNA, and 0.06% tetrasodium diphosphate-10-hydrate. After hybridization filters were washed twice for approximately 1 h in 1×SSC, 0.1% SDS at hybridization temperature. Autoradiography was performed at −80°C using Hyperfilm-MP (Amersham).

2.2. *Oligonucleotides*

Three oligonucleotides were constructed using the published *N*-cadherin sequence of a 3.2 kb cDNA clone derived from a chicken

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brain and retina lambda gt11 library [13]. One 37-mer oligonucleotide, N-term, spanned the putative NH_2-terminus and the end of the pre-mRNA sequence (nucleotide 511–547 incl.) Another oligonucleotide, EC3, was a 34-mer and corresponded to the third extracellular segment (nucleotide 1264–1297). A 40-mer probe, cyt, was derived from the cytoplasmic part of the protein (nucleotide 2381–2420). The oligonucleotides were synthesized on a NUCSYN DNA synthesizer. Oligonucleotides were 3'-end labelled with [α-^{32}P]dATP (Du Pont), sp. act. >3000 Ci/mmol, using a DNA tailing kit (Boehringer Mannheim).

2.3. *Immunoblotting*

Tissue was homogenized on ice in sucrose-Tris/HCl buffer, pH 7.4, containing 2 mM $CaCl_2$, 15 mM NaN_3, 0.1 mM PMSF, 100 units/ml aprotinin and centrifuged at 1000 × *g* at 4°C for 10 min. The supernatant was centrifuged at 29 000 × *g* at 4°C for 30 min. The pellet was resuspended 1:1 in the above mentioned buffer leaving out sucrose and stored at –80°C until use. Samples were boiled for 5 min in sample buffer containing 2% v/v β-mercaptoethanol and 2% w/v SDS before SDS-PAGE. Alternatively, tissue samples were boiled directly in sample buffer for 5 min and centrifuged for 5 min at 12 000 rpm at 4°C before SDS-PAGE on a 7.5% w/v gel according to Laemmli [18]. The gel was electroblotted for 90 min onto nitrocellulose filters (Millipore) using a semi-dry electroblotter (Kem-En-Tec, Denmark). Nitrocellulose blots were handled essentially as described [19]. Monoclonal antibodies ID-7.2.3 [6] and NCD-2 [1] were generous gifts from Dr Benjamin Geiger, The Weizmann Institute, Rehovot, Israel and Dr Masatoshi Takeichi, Kyoto University, Kyoto, Japan.

3. RESULTS AND DISCUSSION

3.1. *Northern blotting*

With all probes a 4.7 kb mRNA band, probably corresponding to the 4.3 kb band described by Hatta et al. [13] appeared as the major mRNA species. Depending on the probes used and tissues examined three additional mRNA species of 3.3, 3.8, and 8.0 kb were also observed.

The N-term probe displayed only the 4.7 kb mRNA in brain, whereas in heart an additional mRNA of 3.8 kb was observed (fig. 1, lanes 1 and 2).

The EC3 probe (fig. 1, lanes 3–7) hybridized to both the 4.7 and 3.3 kb mRNAs in brain and heart. This mRNA species seemed to decrease with age, being clearly expressed in E10 brain (lane 3) and P0 heart (lane 5) and to a lesser degree in P0 brain (lane 4) and adult heart (lane 6). Using the EC3 probe the 3.8 kb mRNA was also observed in heart although as a very weak band in four out of five experiments (not shown).

With the cyt probe we observed an mRNA of ca. 8.0 kb in brain (fig. 1, lane 8). This mRNA species was not seen in heart (fig. 1, lane 9). In addition to the 4.7 kb mRNA in brain and heart, this probe also hybridized weakly to the 3.8 kb mRNA in heart and the 3.3 kb mRNA in brain and heart.

Broader more diffuse bands of ca 2.4 and 1.7–1.9 kb were seen in brain and heart in about one third of the experiments with all probes (see e.g. fig. 1, lanes 3 and 9). RNA from chicken liver (E10 to adult) was included in every experiment as a negative control, and no bands were observed in any experiment even with exposure times up to fourteen days. The results are summarized in table 1.

As can be seen from our results, the 3.8 kb mRNA species was not observed in brain with any of the probes used. This indicates that it might be a heart-specific species, although several other tissues will have to be examined to confirm this. The lambda N1 cDNA probe of about 350 bp used in Northern blotting by Hatta et al. [13] also covered the putative NH_2-terminus as our N-term probe does. Therefore an identical picture should be expected, which was the case in brain but not in heart, where we observed the additional 3.8 kb mRNA species. The 3.3 kb mRNA was not observed in heart or

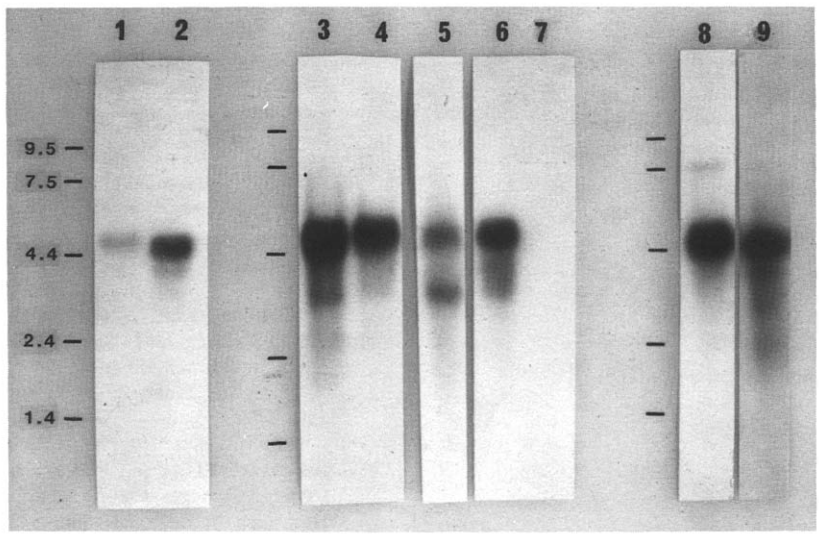

Fig.1. Northern blotting using 10 μg mRNA/lane. The N-term probe hybridized to P0 chicken brain (lane 1) and heart (lane 2). The EC3 probe hybridized to E10 (lane 3) and P0 (lane 4) brain and P0 (lane 5) and adult (lane 6) heart. Lane 7 shows P0 liver. The cyt probe hybridized to P0 brain (lane 8) and heart (lane 9). Numbers on the side refer to molecular weight standards (kb).

Table 1

mRNA species found in brain and heart using the probes N-term, EC3, and cyt described in section 2

	N-term		EC3		cyt	
	Brain	Heart	Brain	Heart	Brain	Heart
8.0	–	–	–	–	+	–
4.7	+	+	+	+	+	+
3.8	–	+	–	(+)	–	(+)
3.3	–	–	+	+	(+)	(+)

brain when using the N-term probe. This indicates, that if this mRNA is translated, the resulting protein does not contain the same NH_2-terminus as the proteins translated from the 4.7 kb mRNA.

The occurrence of more than one *N*-cadherin mRNA in chicken is in agreement with results obtained for mouse *N*-cadherin, where a 5.2 and 3.5 kb mRNA band were observed in heart and brain in addition to a main band of 4.3 kb [14].

3.2. *Immunoblotting*

Immunoblotting using the monoclonal antibody ID-7.2.3 and NCD-2 (fig. 2) displayed one major band in both brain and heart of M_r 135 000. We found that the ID-7.2.3 antibody weakly stained a band in liver (fig. 2, lane 3), whereas the NCD-2 antibody did not (not shown). More weakly stained additional bands with molecular weights lower than the main band were observed using both antibodies.

Immunoblots of *N*-cadherin published by different authors almost always exhibited weak bands with M_r's higher than the main band of ca 130 kDa [6,7,12,20,21]. These bands are generally regarded as degradation products. This is reasonable since the molecule is highly susceptible to proteolysis [21]. It is noteworthy that polyclonal antibodies to *N*-cadherin stain these bands much more intensively than monoclonal antibodies do

Fig.2. Immunoblotting using the monoclonal antibody ID-7.2.3 (lane 1–3) and NCD-2 (lane 4 and 5). P0 brain (lane 1 and 4), heart (lane 2 and 5), and liver (lane 3). Numbers on the side refer to molecular weight standards (kDa).

[20]. Differences in reaction between monoclonal and polyclonal antibodies are also observed in immunohistochemical staining of the developing retina [20], suggesting that *N*-cadherin molecules in the retina might be heterogenous.

Considering how often these weak bands are seen in spite of the precautions taken to avoid break-down and considering the demonstration of multiple mRNA species in this study, we suggest, that some of the weaker protein bands may be translation products of the less abundant mRNA species. These mRNA species could be the result of alternative splicing of the *N*-cadherin gene, or they may be transcribed from a gene closely related to the *N*-cadherin gene.

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