Expression of the Unique NCAM VASE Exon Is Independently Regulated in Distinct Tissues during Development

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Abstract. During development of the rat central nervous system, neural cell adhesion molecule (NCAM) mRNAs containing in the extracellular domain a 30-bp alternative exon, here named VASE, replace RNAs that lack this exon. The presence of this alternative exon between previously described exons 7 and 8 changes the predicted loop structure of the derived polypeptide from one resembling an immunoglobulin constant region domain to one resembling an immunoglobulin variable domain. This change could have significant effects on NCAM polypeptide function and cell–cell interaction. In this report we test multiple rat tissues for the presence of additional alternative exons at this position and also examine the regulation of splicing of the previously described exon. To sensitively examine alternative splicing, polymerase chain reactions (PCRs) with primers flanking the exon 7/exon 8 alternative splicing site were performed. Four categories of RNA samples were tested for new exons: whole brain from embryonic day 11 to adult, specific brain regions dissected from adult brain, clonal lines of neural cells in vitro, and muscle cells and tissues cultured in vitro and obtained by dissection. Within the limits of the PCR methodology, no evidence for any alternative exon other than the previously identified VASE was obtained. The regulation of expression of this exon was found to be complex and tissue specific. Expression of the 30-bp exon in the heart and nervous system was found to be regulated independently; a significant proportion of embryonic day 15 heart NCAM mRNAs contain VASE while only a very small amount of day 15 nervous system mRNAs contain VASE. Some adult central nervous system regions, notably the olfactory bulb and the peripheral nervous system structures adrenal gland and dorsal root ganglia, express NCAM which contains very little VASE. VASE is undetectable in NCAM PCR products from the olfactory epithelium. Other nervous system regions express significant quantities of NCAM both with and without VASE. Clonal cell lines in culture generally expressed very little VASE. These results indicate that a single alternative exon, VASE, is found in NCAM immunoglobulin-like loop 4 and that distinct tissues and nervous system regions regulate expression of VASE independently both during development and in adult animals.

THE primary strategy for developmental regulation of the expression of most individual gene products is at the level of abundance. Most genes give rise to a single polypeptide product whose abundance is controlled through mechanisms regulating transcriptional and translational events and polypeptide turnover. The evolution of multigene families has allowed a second strategy for regulation of gene expression: selective expression of single members of the family in different tissues or distinct times in development. A third strategy for selective polypeptide expression is via alternative splicing. In genes that generate multiple polypeptide products through alternative splicing, both the relative abundance and the precise splicing patterns of all gene products must be regulated. A large and rapidly increasing number of genes are known to be alternatively spliced (Breitbart et al., 1987). While some genes have splicing patterns that are apparently stochastic, others are clearly regulated in tissue-specific or developmental patterns.

Neural cell adhesion molecule (NCAM)[1] is a group of cell surface glycoproteins derived from a single gene. NCAMs are found in high levels in neural tissues in adult animals and are expressed more widely during early development (Edelman, 1988; Linneman and Bock, 1989). A number of studies have demonstrated the role of NCAM in cell–cell adhesion including the interaction of cells of the

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While this manuscript was under review we learned of an additional alternatively spliced exon between exons 7 and 8 of rat NCAM which is present at ~3% the abundance of the VASE exon (Barbara Bowen and Walter Gilbert, personal communication). This abundance is similar to that estimated above for the maximum abundance of any exons in additions to VASE.

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1. *Abbreviations used in this paper:* NCAM, neural cell adhesion molecule; PCR, polymerase chain reaction.

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neural and muscle lineages (Brackenbury et al., 1977; Grumet et al., 1982; Rutishauser et al., 1983). The deduced polypeptide sequence indicates NCAM is a member of the immunoglobulin gene superfamily which also contains a number of other cell–cell adhesion molecules (Rutishauser and Jessell, 1988; Williams and Barclay, 1988). The importance of NCAM to the neural phenotype is suggested by the observation that it is one of the first genes expressed after induction of the neural phenotype (Kinter, 1987). In *Xenopus*, NCAM transcripts can be detected 2 h after neural induction of the ectoderm by the dorsal lip. Alternative splicing of large RNA segments results in four major size classes of NCAM polypeptides, 180, 140, 120, and 105 kD (Barbas et al., 1988; Santoni et al., 1989; Gower et al., 1988). The abundances of these forms vary during development in part due to cell type–specific expression and also other factors (Small et al., 1987; Chuong and Edelman, 1984). The three largest polypeptide forms are cell surface membrane associated and capable of mediating cell–cell adhesion through their common amino terminal extracellular domains (Rougon and Marshak, 1986). The extent of differences in biologic functions among these NCAM forms is unknown although it is attractive to speculate that the two largest forms which have transmembrane segments and cytoplasmic domains could be involved in intracellular signaling (Schuch et al., 1989).

In addition to alternative splicing which results in readily detectable polypeptide size differences, splicing that alters shorter segments of the NCAM polypeptide chains has been identified (Dickson et al., 1987; Thompson et al., 1989; Small et al., 1988; Santoni et al., 1989; Prediger et al., 1989). Between previously identified exons 12 and 13 (Owens et al., 1987), several new small exons have been recently identified. These are expressed in a significant proportion of muscle NCAM mRNAs but some combinations of them also appear in brain NCAM mRNAs (Walsh and Dickson, 1989; Prediger et al., 1989). NCAM and other neural and non-neural adhesion molecules have immunoglobulin-like domains whose primary sequences are somewhat distinct from those of true immunoglobulin variable and constant domain regions. These domains have been termed C2 (Williams and Barclay, 1988) or H (Hunkapiller and Hood, 1989) domains to indicate this difference. Although NCAM C2/H domains are somewhat distinct, they still have many features consistent with the basic tertiary structure of an immunoglobulin domain: a compact group of antiparallel beta-pleated sheets linked by a conserved disulfide bond and adjacent tryptophan which form a "pin" structure (Lesk and Chothia, 1982; Edmundson et al., 1975). True immunoglobulin variable domains differ from constant domains in several features, most notably the presence of two extra beta-pleated sheets (named C' and C") which form the surface of one edge of the domain and comprise a portion of the hypervariable region involved in antigen binding. We have previously identified within the fourth immunoglobulin-like loop of NCAM an amino acid sequence encoded by a 30-bp alternative exon (Small et al., 1988). The position of this 10 amino acid insertion within the fourth NCAM Ig-like loop is coincident with the position of amino acids which make up hypervariable regions of true immunoglobulin polypeptides. Thus, the inclusion of this 10 amino acid insert changes the predicted structure of the fourth NCAM loop to one somewhat more similar to an Ig-

like variable domain. In the absence of structural data it is not known if these 10 amino acids form a beta-pleated sheet, an extra loop on the surface of the fourth domain, or another configuration. However, as similar sequence alterations affect the structure and function of immunoglobulin domains, this alternative exon could substantially alter the ability of NCAM to mediate adhesion during development. For convenience we propose the provisional name VASE for this exon to signify its properties of mimicking the formation of a domain more like an immunoglobulin *variable* domain, and being an *a*lternatively *spliced exon*. This acronym has the advantage of both convenience and translatability into German.

The identification of the VASE exon in Ig-like loop 4 raises the question of whether additional alternative exons are found at this position. Some neurons in primary cultures express VASE, while others do not, raising the additional question of what cells and nervous system regions express VASE in vivo. Here we describe the use of the polymerase chain reaction (PCR; Saiki et al., 1988) to test RNA populations both for additional alternative NCAM exons and also for differential VASE expression during development and in distinct nervous system regions.

Materials and Methods

Tissues and Cell Lines

All rat tissues were taken from Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN). For embryonic tissues the day after the night the animals were mated is defined as day 0. Sources of neural and muscle cell lines have been previously described (Williams et al., 1985). Liver cell lines H4TG and H411E were obtained from the American Type Culture Collection (Rockville, MD).

RNA Extraction and cDNA Production

Total cellular RNA was purified from dissected rat tissues and cultured cell lines by the guanidine isothiocyanate-CsCl method (adapted from Maniatis et al., 1982). The total RNA was then used as a template for specific NCAM first strand cDNA synthesis using the 30 base oligonucleotide 17-2: 5'-AGGACACACGAGCATGCCTGCGTACCACCA-3'. This oligonucleotide is complementary to the coding strand of the rat NCAM cDNA pRl8 (bases 1,317–1,346 of Small et al., 1987) except for a single base mismatch: at base 17 the complementary base G is replaced by a C to create an Sph I restriction site (see Fig. 1). This oligo hybridizes to NCAM RNA just downstream of the splice junction between exons 7 and 8. In most cases, 30 μg of total cellular RNA was used in the first strand cDNA synthesis reaction, but in several cases where tissue mass was limiting, significantly less RNA was used. As little as 0.5 μg of RNA was sufficient to give a detectable product after PCR amplification of the first strand cDNA. The cDNA synthesis reactions were performed with minor modifications of a published method (Geliebter et al., 1986). Briefly, the RNA was coprecipitated with 10 ng of the 30mer 17.2, dried down, and dissolved in 10 μl of annealing buffer (250 mM KCl, 10 mM Tris, pH 8.3, at 50°C). The mixture was then denatured at 80°C for 3' and allowed to anneal for 90' at 55°C. Then 16.5 μl of reverse transcriptase mix (24 mM Tris 8.3 at 50°C, 16 mM MgCl₂ 8 mM DTT, 2 mM each dNTP, 20 U AMV reverse transcriptase; Life Sciences Inc., St. Petersburg, FL) was added to each annealing reaction, and incubated at 50°C for 45'. 1–4 μl of this reaction mix was then used directly as a template for amplification by the PCR.

PCR

Two basic protocols were used: "cold" and "hot" (radioactive) PCRs. Oligo 17.2 (see above) and oligo 17.1: 5'-ACCTGCAGAACGTCCACCCGAAA-CATCA-3', were used as primers for PCR amplification in both protocols. Oligo 17.1 contains 28 bases of rat NCAM pRl8 cDNA coding sequence (bases 1,229–1,256 of Small et al., 1987) except for a single mismatch: at

Figure 1. VASE Position within NCAM. The position of VASE between exons 7 and 8 which comprise the fourth immunoglobulin-like domain of NCAM is shown. Oligo nucleotide 17.1 is identical to the cDNA sequence shown except at the position noted. Oligonucleotide 17.2 is complementary to the cDNA sequence shown except at the position noted.

base 6 of the oligo the coding strand G is replaced by a C creating a Pst I restriction site. This sequence lies just 5' of the splice site between exons 7 and 8 (Fig. 1).

Cold PCRs were carried out on various cDNA templates under the following conditions in 25–100 μl volume: 50 mM KCl, 10 mM Tris, pH 8.0, 0.01% gelatin, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 3 mg/ml each primer and 20 U/ml Taq I polymerase (Cetus Corp., Emeryville, CA). The reactions were subjected to an initial cycle of 3' at 93°C, 1' at 55°C, and 2' at 72°C, followed by 29 cycles of 1' at 92°C, 1' at 55°C, and 2' at 72°C. The last 72°C step was extended for 8' to maximize full extension of the final product. Using these conditions ∿1 μg of double-stranded fragment was routinely produced in a 100-μl reaction. 10-μl aliquots were directly loaded on 6% nondenaturing polyacrylamide gels and visualized by poststaining for 30' with 0.75 μg/ml ethidium bromide.

Hot reactions (10 μl final volume) were performed under exactly the same conditions described above except that only 1 ng of 32P 5' end-labeled primer (oligo 17.1) was used with an excess of the other primer (oligo 17.2), which was unlabeled. We found that a 25:1 excess of unlabeled 17.2

Figure 2. Developmental expression of VASE in brain. RNA samples and cDNAs were prepared from the indicated tissues and "cold" PCRs performed (Materials and Methods). The tissue samples were taken from whole brain of the indicated embryonic (E) or postnatal (P) age or adult animal. The product of ∿250 bases is indicated with an arrow. This experiment was repeated four times with similar results. The top of the gel is to the right in Figs. 2, 3, 5, 6, and 7.

primer/labeled 17.1 primer gave the best results. After the reaction was complete, 40 μl of loading dye (95% formamide, 0.5× TBE, 0.5 mg/ml bromophenol blue, 1 mg/ml xylene cyanol) was added to the reaction, which was boiled for 3' and then transferred immediately to an ice bath. 1–3 μl of each reaction was then loaded onto a 6% urea-containing sequencing gel. The gel was run at 1,200 V, dried, and exposed to film for 3–12 h.

Results

The PCR was chosen to test for the presence of alternatively spliced exons in the immunoglobulin-like loop 4 region due to both its sensitivity and its ability to detect multiple products in a single reaction. Classical Northern analysis or nuclease S1 protection assays can sensitively analyze expression but do not lead directly to isolation and cloning of new sequences. Standard cDNA library production methods can be used to clone and sequence new DNA products but are very laborious. Thus, the PCR combines both sensitivity and efficiency in detection and identification of new DNA products. The complementary oligonucleotides used for PCRs give 118-bp products on NCAM cDNAs containing the VASE exon and 88-bp products on NCAM cDNAs lacking the VASE exon (Fig. 1).

In the first test for additional Ig-like loop 4 exons, PCR products from a variety of brain ages were examined on nondenaturing gels. Three products were observed corresponding to 88, 118, and ∿250 bases (Fig. 2). The 118 base product steadily increased in abundance during perinatal development in these PCR reactions consistent with the previously described appearance of the VASE exon using RNA hybridization methods (Small et al., 1989). The 250 base product represented a candidate alternative exon. However, we noted that its developmental appearance and abundance seemed to be directly proportional to the abundance of the 118 base product. To test the identity of the 250 base product, individual bands were cut out, melted, rerun, and also tested for single-stranded character with mung bean nuclease. These experiments indicated the 250 base product was a heteroduplex between the 118 and 88 base strands which migrates anomalously on nondenaturing gels, presumably

R5.6 (+)

R8.2 (-)

E15

P1
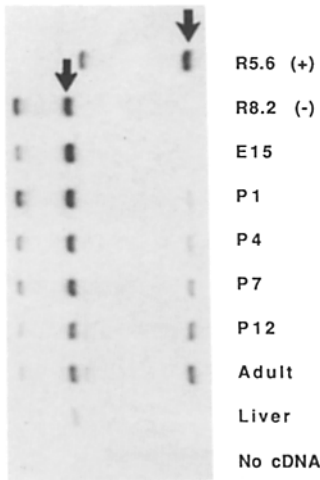
P4

P7

P12

Adult

Liver

No cDNA

Figure 3. PCR analysis of VASE expression during brain development. RNA samples and cDNAs were prepared from the indicated tissues and "hot" PCRs were performed and run on denaturing gels. Samples R5.6 and R8.2 are mouse L cell lines transfected with 140 kD rat NCAM containing and lacking the VASE exon, respectively. The 118-bp product is denoted by the arrow in the R5.6 lane and the 88-bp product is denoted by the arrow in the R8.2 lanes. Each of these lanes also contains a faster migrating artifactual band. These bands appear to be due to partial hybridization of the PCR oligonucleotide(s) with the extension product based on the following evidence: (*a*) their abundance is decreased by increasing the formamide concentration of the sample buffer used to load the gel; and (*b*) their abundance diminishes when the ratio of unlabeled to labeled oligonucleotide in the PCR is decreased. Unfortunately, in order to achieve a significant yield of labeled PCR product from the small amounts of RNA and cDNA available from some samples, we were forced to use a higher ratio of unlabeled to labeled oligonucleotide in order to drive the reaction. Other samples are whole brain from embryonic day 15 (*E15*), the indicated postnatal (*P*) day, adult brain, adult liver, and a control to which no RNA was added. A very weak band is observed here with adult liver. Kinetic analysis of the appearance of the PCR products suggests that the abundance of NCAM RNA in liver is ~1,000-fold less than that in brain (data not shown). This experiment was performed at least twice with independent preparations of all samples.

due to its partial single-stranded character (data not shown, Small, S. J., and R. Akeson, manuscript in preparation). As an additional test of the identity of the products obtained, PCRs were performed with 32-P–labeled oligonucleotide and analyzed on urea containing denaturing gels. As controls, fibroblasts transfected with cDNAs for a single NCAM isoform were included. These experiments demonstrated 118 base products (Fig. 3, *right arrow*) using the recombinant cell line R5.6, which expresses 140 kD NCAM+VASE, and 88 base products (Fig. 3, *left arrow*) with the recombinant cell line R8.2, which expresses 140 kD NCAM–VASE. The additional minor bands seen in these lanes are mobility artifacts of the major bands (Fig. 3 legend). Only products of these same sizes, 88 and 118 bases, were seen with brain samples from all the ages tested, suggesting that no additional alternative exons are found in brain. To confirm the identity of these samples, PCR products from adult brain were sequenced and found to be identical to the previously reported rat NCAM sequence (data not shown). The perinatal developmental appearance of the VASE exon which was previously observed by Northern analysis (Small et al., 1988) is reproduced almost exactly by the PCR products, indicating that the PCR can be used to quantitatively estimate the relative proportion of NCAM RNAs with and without VASE in a tissue.

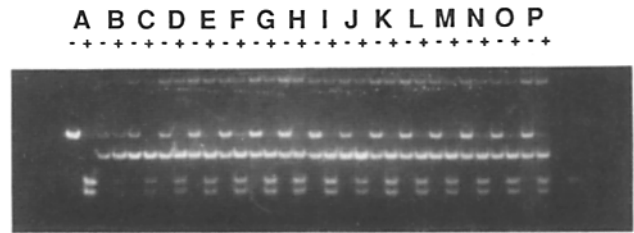These combined experiments indicate that within the limits of sensitivity of the methods, if any additional alternative exons between exons 7 and 8 exist, they must be 30 bp

A B C D E F G H I J K L M N O P
- + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +

Figure 4. Taq I Digestion of PCR products. "Cold" PCR reactions were performed with cDNAs from the cell lines and brain regions indicated below. Half of each reaction was digested with the restriction enzyme Taq I, the samples were run in adjacent lanes on a nondenaturing gel, and visualized by staining with ethidium bromide. The control sample in lane A is from recombinant R5.6 cells which express only 140 kD NCAM+ VASE. Total digestion of the upper 118-bp band to two smaller products is seen. In the other samples the 118-bp band is also digested to completion but the 88-bp band (from NCAM-VASE) is not digested as predicted. The samples are *A*, R5.6 cells; *B*, E15 heart; *C*, P1 brain; *D*, P4 brain; *E*, P7 brain; *F*, P12 brain; *G*, adult brain; *H*, cerebellum; *I*, cerebrum; *J*, olfactory bulb; *K*, thalamus; *L*, olfactory tubercule; *M*, basal Ganglia; *N*, Midbrain; *O*, brain stem; *P*, adult heart. All brain regions were dissected from adult rats. This experiment was performed twice with similar results.

in size. As an initial test of the existence of such additional 30-bp alternative exons, PCR products were digested with Taq I a restriction enzyme which cuts within the VASE sequence but not elsewhere within the amplified sequence. When PCR-amplified products produced from cDNAs from rat brain ages embryonic day 11 through adult were cut with Taq I, complete digestion was observed in all cases (Fig. 4). Thus, there is no evidence for alternative exon(s) which do not have a Taq I site.

The Taq I digestion experiment indicates that all alternative exons at the 7/8 junction must be 30 bp in length and must contain a Taq I site. To determine whether any alternative exons in addition to the one previously observed are present in the rat, the 118-base PCR product was labeled and used to probe genomic clones spanning the entire region between exons 7 and 8. This probe should hybridize to both the previously identified exon and also any possible additional exons amplified by PCR. In Southern blot experiments a single 1,285-bp Ava I–Hind III genomic fragment hybridized to this probe. Thus, any alternative exons must be within this fragment. This genomic fragment was completely sequenced. Five Taq I sites were found (data not shown). The only Taq I site that was properly positioned within a 30-bp segment surrounded by the consensus splice donor and acceptor sequences was contained within the previously identified alternative VASE exon. Thus, within the limits of sensitivity of the methods employed, VASE is the only alternative exon at the 7/8 junction expressed in rat brain throughout development.

In these experiments it is possible that additional exons present at the 7/8 junction were not detected due to their low abundance. Exons that represent a minor portion of total brain NCAM RNA could be expressed by cells with an anatomically restricted distribution and represent a major proportion of the NCAM RNA of these cells. To test this pos-
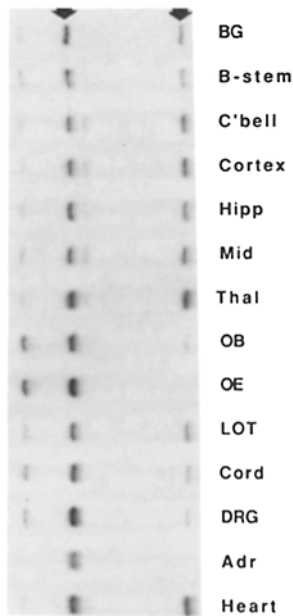
Figure 5. VASE expression in distinct adult brain regions. RNA preparations from the indicated adult nervous system regions were prepared and PCRs performed as in Fig. 3. *BG*, basal ganglia; *B-stem*, brainstem; *C'bell*, cerebellum; *cortex*, cerebral cortex; *Hipp*, hippocampus; *Mid*, midbrain; *Thal*, thalamus; *OB*, olfactory bulb; *OE*, olfactory epithelium; *LOT*, lateral olfactory tract; *DRG*, dorsal root ganglia; *Adr*, adrenal; *Heart*, adult heart. Results from a representative experiment are shown. All regions were analyzed at least twice using independent dissections and RNA preparations.

Figure 6. VASE expression in the rat embryo. RNA preparations from embryonic day 15 (*E15*) whole embryos or the regions indicated were prepared and PCRs performed. Adult heart is shown for comparison with the 118- and 88-bp PCR products denoted by the *right* and *left* arrows, respectively.

sibility, adult rat brain was dissected into discrete regions. RNA preparations from these regions were tested using the PCR methodology. In addition two components of the peripheral nervous system (PNS), adrenal gland and dorsal root ganglia, and also adult heart were examined. All central nervous system (CNS) and PNS regions contained significant amounts of NCAM lacking any 7/8 exon as indicated by the presence of the 88 base PCR product (Fig. 5, *left arrow*). The VASE exon was also observed (*right arrow*) but no additional size classes of alternative exons were seen in these experiments. Thus, there is no support for the hypothesis of anatomically restricted expression of additional 7/8 exons. However, examination of the ratios of the upper band (with VASE) to the lower band in these experiments did reveal significant region-specific regulation of splicing of VASE. Most CNS regions expressed mixtures of NCAM with and without VASE. Some CNS regions, particularly olfactory bulb and spinal cord, predominantly expressed NCAM without VASE. The VASE exon could not be detected at all in the olfactory epithelium sample. The PNS tissues had much lower (dorsal root ganglia) or undetectable (adrenal) levels of VASE. Overall VASE expression in the PNS was generally less than that in the CNS. In film exposures 20 times longer than those shown, VASE expression could be detected at low levels in all the tissues examined with the lowest levels observed in olfactory epithelium followed by adrenal. Assuming linearity of film response, NCAM RNAs in these tissues are ~99.9% VASE negative. These combined results demonstrate that alternative splicing at the 7/8 junction is independently regulated in distinct regions of the nervous system.

As a further test of alternative NCAM splicing at the exon 7/8 junction, nonneural tissue from embryonic day 15 (E15) whole embryos and also E15 rat brain, body, and heart cDNA samples were examined. Heart was the muscle tissue chosen for emphasis because it can be relatively cleanly dissected free of neural or glial elements. It is very difficult to obtain samples of skeletal muscle which are free of these ele-
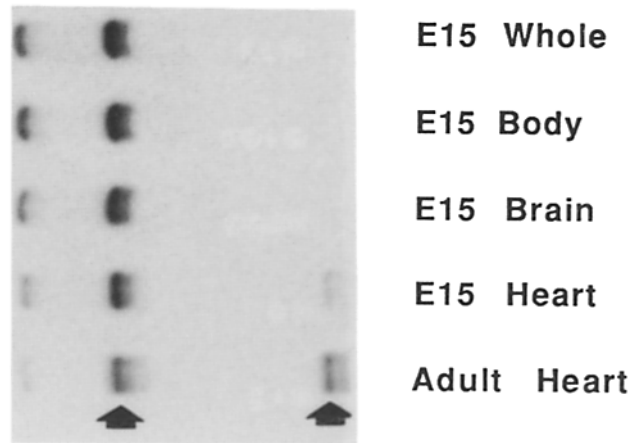
ments, particularly the Schwann cells of the ingrowing nerves. At E15, very little VASE is observed in whole embryo, body, or brain (Fig. 6, the 118-base band is the *right arrow*) but the embryonic heart sample shows significant VASE levels. These results with brain samples are consistent with those obtained using the unlabeled PCR reaction (Fig. 2). It is interesting to note that when the whole body sample (including heart) from an E15 rat is assayed, little VASE is observed indicating the effects of RNA dilution on the PCR assay. VASE could be detected in these E15 body samples in longer film exposures which grossly overexposed the 88 base band. Adult heart has large amounts of NCAM with VASE. Again, no additional exons were observed in these nonneural tissues at the exon 7/8 junction. These experiments do illustrate that NCAM splicing at the 7/8 junction is independently regulated in different tissues during development.

The experiments testing NCAM splicing in nervous system regions and embryos are limited by the fact that each of these samples contains multiple cell types. As a final test for the presence of additional 7/8 exons, individual cell types were tested with the PCR. By using clonal cell lines in culture, cells of known phenotype could be examined. The muscle cell line L6; the pheochromocytoma cell line PC12 and additional neuronal lines B35 and B104; the Schwannoma cell line RN22 and the additional glial cell lines C6 and B12; and also the control liver cell lines H4TG and H411E and control fibroblast L cells were examined. In all cases, no additional size classes of 7/8 exon were detected (Fig. 7). The positive control 118 and 88-base bands are denoted by the arrows in the lanes for recombinant cell lines R5.6 and R8.2, respectively. No NCAM expression was detected in the hepatocyte or fibroblast lines. All the neural and muscle cells expressed very little VASE. The regulation of splicing of VASE was also examined using two model systems for cell differentiation in vitro: the fusion of L6 myoblasts to form myotubes and the morphologic differentiation of PC12 cells after nerve growth factor treatment. Myoblast fusion does not increase the abundance of the 30-bp exon in L6 cells (lanes L6 vs L6-fused). Similar results were found with the muscle cell line H9c2 (data not shown). PC12 cells do ex-
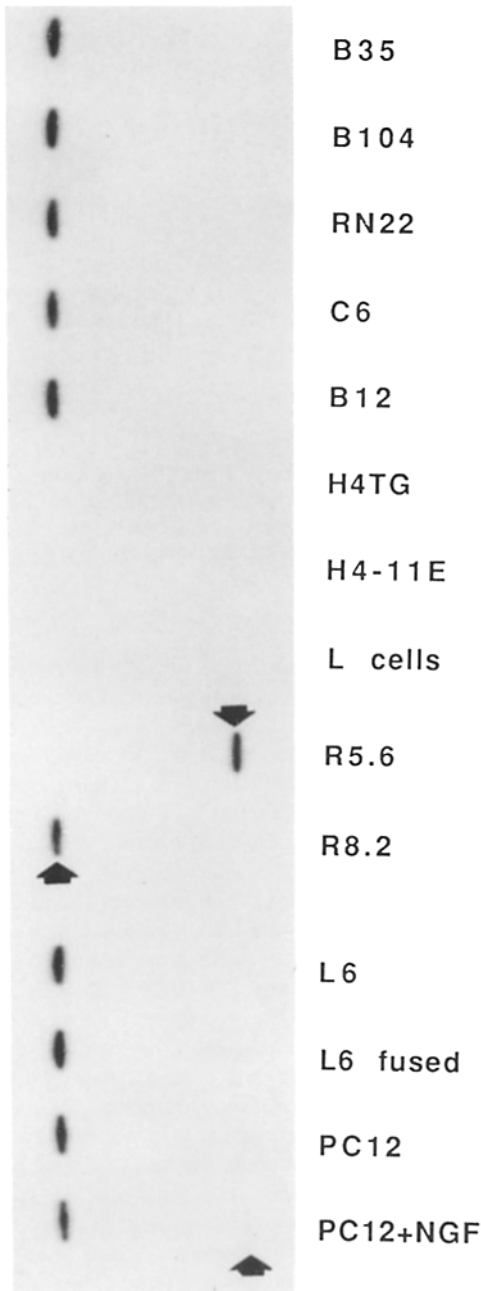
Figure 7. VASE expression in cell lines. RNA preparations from cells grown in culture were prepared and PCRs performed as in Fig. 3. L6 myoblasts were induced to fuse to form myotubes by growing myoblasts to high density in DME with 10% serum and then reducing the serum concentration to 2.5% for 6 d. Fusion was visually estimated at 40–50% at the time of cell harvest. PC12 cells were treated with nerve growth factor (Collaborative Research, 20 ng/ml) for 8 d before harvest.

press low levels of VASE under normal culture conditions but this amount does not increase in relative abundance after NGF treatment (*arrow*, lane *PC12+NGF*). In summary, these experiments also did not detect additional exons at the 7/8 junction, and further indicated that splicing at this junction is not regulated by mechanisms which play a role in two cell culture model systems.

Discussion

Using all four of the strategies for testing different tissues, cell types, and stages of development, no exon other than the previously described VASE was identified at the exon 7/8 junction in rat. The quantitative limits on this statement that no additional exons can be found can be estimated from data within the experiments. By overexposing films we are readily able to detect 118-base bands at abundances that are 1.0% of the 88-base bands. For example, the 118-base band is not readily detected in samples of whole embryonic day 15 rats but can be easily detected in samples of E15 heart. However, on exposures 5–20 times those shown, the 118-base band is present in the whole embryo samples as would be expected. Thus, these results indicate both that the PCR technique is extremely useful for examining developmental regulation of alternative splicing and also that its extreme sensitivity necessitates careful analysis of the reproducibility of the products obtained. We conclude that additional exons at the 7/8 junction, if any, must be expressed at abundances at or below the 1.0% level, in cell types that represent minor populations within the tissues examined, or in specialized tissues or cell types not examined here.

This conclusion highlights a striking contrast between two regions of alternative splicing in NCAM. The genomic organization of NCAM as described in the chicken (Owens et al., 1987) and confirmed by our laboratory in the rat (Chen et al., 1990 and Reyes, A. A., and R. Akeson, unpublished data) is a 5' small cluster of seven exons contained in ~8 kb of genomic sequence which encode the first three and one half immunoglobulin-like domains. These are followed by a 10.5-kb segment which contains only the VASE exon. This is followed by exons 8–12 which are clustered in ~6 kb and then another large gap of 15 kb between exons 12 and 13. Exons 12 and 13 encode a fibronectin-like region of NCAM. Recently, this latter large gap has been shown to contain several small alternatively spliced exons (Santoni et al., 1987; Barbas et al., 1988; Prediger et al., 1989). This result is in contrast to our finding of a single exon in the 10.5-kb genomic gap between exons 7 and 8.

All five NCAM Ig-like domains are each encoded by two exons consistent with the proposal of a primordial half-domain–encoding exon giving rise to the immunoglobulin supergene family (Bourgois, 1975; Hunkapiller and Hood, 1989). No alternatively spliced exons have been detected in NCAM between Ig-like domains within domains other than domain 4. The position of the VASE exon within an Ig-like domain as determined by this genomic organization is coincident with the major position of difference between true immunoglobulin constant and variable domains. Further experiments are clearly necessary to determine the nature of the NCAM structural and functional changes caused by VASE exon insertion. Thus, the acronym VASE is intended provisionally and should not be taken to indicate that the true structure of the VASE containing fourth NCAM domain is identical to that of a true immunoglobulin variable domain. NCAM has homology to a number of other neural adhesion molecules including L1, TAG-1, contactin, and DCC (Moos et al., 1988; Furley et al., 1990; Ranscht 1988; Fearon et al., 1990). It will be interesting to determine if the domains of these molecules are also encoded by split exons and also whether they exhibit alternative splicing within the domains.

Using an antibody to a synthetic peptide predicted by the VASE exon, we had previously demonstrated that individual neuronal cells in culture independently regulated expression of VASE; some cells had readily detectable VASE expression while others did not. The results obtained here with the PCR technique extend the observation that this splicing event is independently regulated in different cells by demonstrating that distinct brain regions show clear differential regulation of VASE expression. A portion of this regulation correlates with embryologic origins of the tissues tested. Both of the neural crest–derived elements which contribute to the peripheral nervous system, adrenal gland and dorsal root ganglia, showed low levels of VASE expression. The olfactory epithelium, which is derived from the olfactory placode, had essentially no VASE expression. In contrast, CNS regions which are derived from the neural tube, although varying in expression from low (olfactory bulb and spinal cord) to high (thalamus), all had readily detectable levels of VASE expression. Further tests of the correlation between VASE expression and embryologic origin would include analyses of additional placode and neural crest–derived tissues.

An alternative hypothesis consistent with much of the data presented is that expression of VASE is inversely correlated with the degree of synaptic plasticity of a tissue. In the developing nervous system, very little VASE is expressed during early development when there is significant cellular growth and synaptic connections are first being formed and are relatively labile. In adult rats in which brain growth has ceased and synaptic connections are less labile, higher levels of VASE are observed. In adult vertebrates, the olfactory system is unique in that continuous cellular turnover of mature neurons occurs in the epithelium which must be accompanied by continuous synaptic rearrangement within the olfactory bulb. Both components of this system are also uniquely low in their levels of VASE expression. Clearly this hypothesis is not sufficient to explain all aspects of regulation of VASE expression as adrenal, a seemingly static tissue, also has low levels of expression. Previous workers have shown a rough correlation between periods of synaptic development and plasticity and the presence of polysialic acid groups on NCAM. These groups are thought to decrease the ability of NCAM to mediate cell–cell adhesion (Hoffman and Edelman, 1983; Rutishauser et al., 1988). The relationship between VASE content and polysialylation is not known. The role of both VASE expression and polysialylation in the modulation of the strength of NCAM-mediated cell adhesion during development requires further analysis.

How is VASE expression regulated? These results clearly indicate that VASE regulation is not stochastic. However, the mechanisms which do regulate VASE expression are not clear. No changes in VASE expression were observed after treatment of PC12 cells with NGF. Likewise, L6 muscle cell fusion to form myotubes was not accompanied by VASE expression even though significant alterations in NCAM splicing at the exon 12/exon 13 junction were observed during this change (Reyes, A. A., S. J. Small, and R. Akeson, unpublished data). The experiments described here do eliminate the hypothesis that a simple biological clock such as a systemic hormonal factor induces the simultaneous developmental appearance of VASE expression in all cells of all tissues. Such a simple clock cannot regulate VASE splicing because this exon appears earlier in heart than in the nervous

system and some neural regions such as the olfactory epithelium do not express significant VASE at any time. However, a biologic clock in the form of a circulating factor could still regulate expression if it is further proposed that some cells (such as cardiac tissue) develop receptor for the factor earlier than others and some cells (such as olfactory epithelium) never express the receptor. Thus, there could still be a systemic component involved in regulation of VASE expression. The results described here indicate individual brain regions vary dramatically in VASE expression. The previous studies with antibody to the VASE-encoded peptide demonstrated that individual neurons in embryonic rat brain primary cultures regulated VASE expression independently. These combined results suggest that regulation of VASE expression occurs at the level of single cells. Thus, the simplest hypothesis consistent with all these data is that regulation of VASE expression is cell intrinsic. Regardless of the nature of regulation of VASE splicing and other splicing events that determine which NCAM forms are expressed, these results do emphasize that future studies of NCAM functions during development must consider the precise alternatively spliced form of NCAM being expressed. Conclusions based on reagents that identify most or all NCAM forms may not be appropriate to the potentially diverse biologic capabilities of the increasingly large number of individual distinct NCAM forms.

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