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Sequence of a cDNA clone encoding the polysialic acid-rich and cytoplasmic domains of the neural cell adhesion molecule N-CAM

(amino acid sequence/nucleic acid/hydrophobicity/modulation/transmembrane glycoprotein)

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ABSTRACT **Purified fractions of the neural cell-adhesion molecule N-CAM from embryonic chicken brain contain two similar polypeptides (M_r, 160,000 and 130,000), each containing an amino-terminal external binding region, a carbohydrate-rich central region, and a carboxyl-terminal region that is associated with the cell. Previous studies indicate that the two polypeptides arise by alternative splicing of mRNAs transcribed from a single gene. We report here the 3556-nucleotide sequence of a cDNA clone (pEC208) that encodes 964 amino acids from the carbohydrate and cell-associated domains of the larger N-CAM polypeptide followed by 664 nucleotides of 3′ untranslated sequence. The predicted protein sequence contains attachment sites for polysialic acid-containing oligosaccharides, four tandem homologous regions of polypeptide resembling those seen in the immunoglobulin superfamily, and a single hydrophobic sequence that appears to be the membrane-spanning segment. The cytoplasmic domain carboxyl terminal to this segment includes a block of ≈250 amino acids present in the larger but not in the smaller N-CAM polypeptide. We designate these the ld (large domain) polypeptide and the sd (small domain) polypeptide. The intracellular domains of the ld and sd polypeptides are likely to be critical for cell-surface modulation of N-CAM by interacting in a differential fashion with other intrinsic proteins or with the cytoskeleton.**

The neural cell-adhesion molecule N-CAM is the first cell–cell adhesion molecule (CAM) to be structurally and functionally well-defined (1–4), and its known properties provide strong evidence that cell-surface modulation (5) is a fundamental mechanism in cell recognition and morphogenesis. N-CAM is expressed at key sites of embryonic induction and is ubiquitous in the embryonic and adult nervous system (6, 7). It is a large integral membrane glycoprotein (3), some forms of which are mobile in the plane of the membrane (8). Its homophilic binding (9) is mediated by its amino-terminal domain (10) and is strongly conserved during evolution (11). Major changes in N-CAM binding behavior can occur dynamically by changes in N-CAM prevalence or distribution at the cell surface or by embryonic to adult conversion, the alteration of polysialic acid at one or more asparagine-linked oligosaccharides near the binding region (9, 12, 13). The observations that such surface modulation events strongly alter CAM binding focus attention on the structure of N-CAM domains mediating the various types of modulation—i.e., the polysialic acid-rich and cell-associated domains.

Previous analyses using cDNA clones indicated that there is one gene for N-CAM in the chicken (14) and in the mouse (on mouse chromosome 9) (15) but that at least two forms of N-CAM mRNA exist in both species (14–16). In the chicken, these mRNAs give rise to two forms of N-CAM that differ in their COOH-terminal regions (17). Together these results suggested that the two N-CAM polypeptide chains arise by translation of different mRNAs generated by alternative splicing of transcripts from a single N-CAM gene.

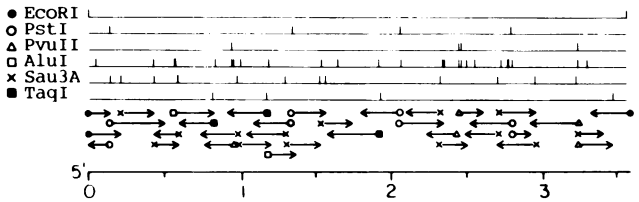

FIG. 1. Restriction enzyme map and sequencing strategy for the cDNA insert from pEC208. The restriction sites used to generate fragments for subcloning into bacteriophage M13 are shown at the top; the direction and extent of sequencing of M13 subclones beginning at the indicated restriction sites are shown at the bottom. The scale is calibrated in kilobases and shows the 5′ to 3′ orientation of the cDNA relative to the N-CAM mRNA.

In the present study, we describe the nucleotide sequence of a 3.6-kilobase (kb) chicken N-CAM cDNA clone (pEC208) (17) and some structural features of the encoded polypeptide. The predicted protein sequence contains attachment sites for polysialic acid-containing oligosaccharides, four tandem homologous regions that have elements in common with members of the immunoglobulin superfamily, and a single hydrophobic stretch, which is a candidate membrane-spanning segment. The sequence indicates that a major difference between the two N-CAM polypeptides comprises a large hydrophilic segment in the larger polypeptide carboxyl terminal to the proposed membrane spanning sequence that is likely to be intracytoplasmic. These results suggest that the two N-CAM polypeptides with different cytoplasmic domains may interact differentially with cortical cytoskeletal elements and thus may be subject to different forms of cell-surface modulation.

MATERIALS AND METHODS

Plasmids pEC201, pEC202, pEC204, and pEC208 (17) were digested with restriction enzymes and the resulting fragments were subcloned into bacteriophage M13 mp8 or M13 mp9 (18); in some cases, individual fragments were purified by gel electrophoresis before subcloning. DNA nucleotide sequence analysis was performed by the dideoxynucleotide chain-termination method (19, 20). Sequence data were compiled by using the computer programs of Staden (21). Restriction sites and reading frames were analyzed with the ANALYSEQ program (22).

Purified embryonic chicken N-CAM (3) was digested with CNBr or *Staphylococcus aureus* V8 protease. Peptides were

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Abbreviations: CAM, cell-adhesion molecule; kb, kilobase(s) or kilobase pair(s); N-CAM, neural CAM.

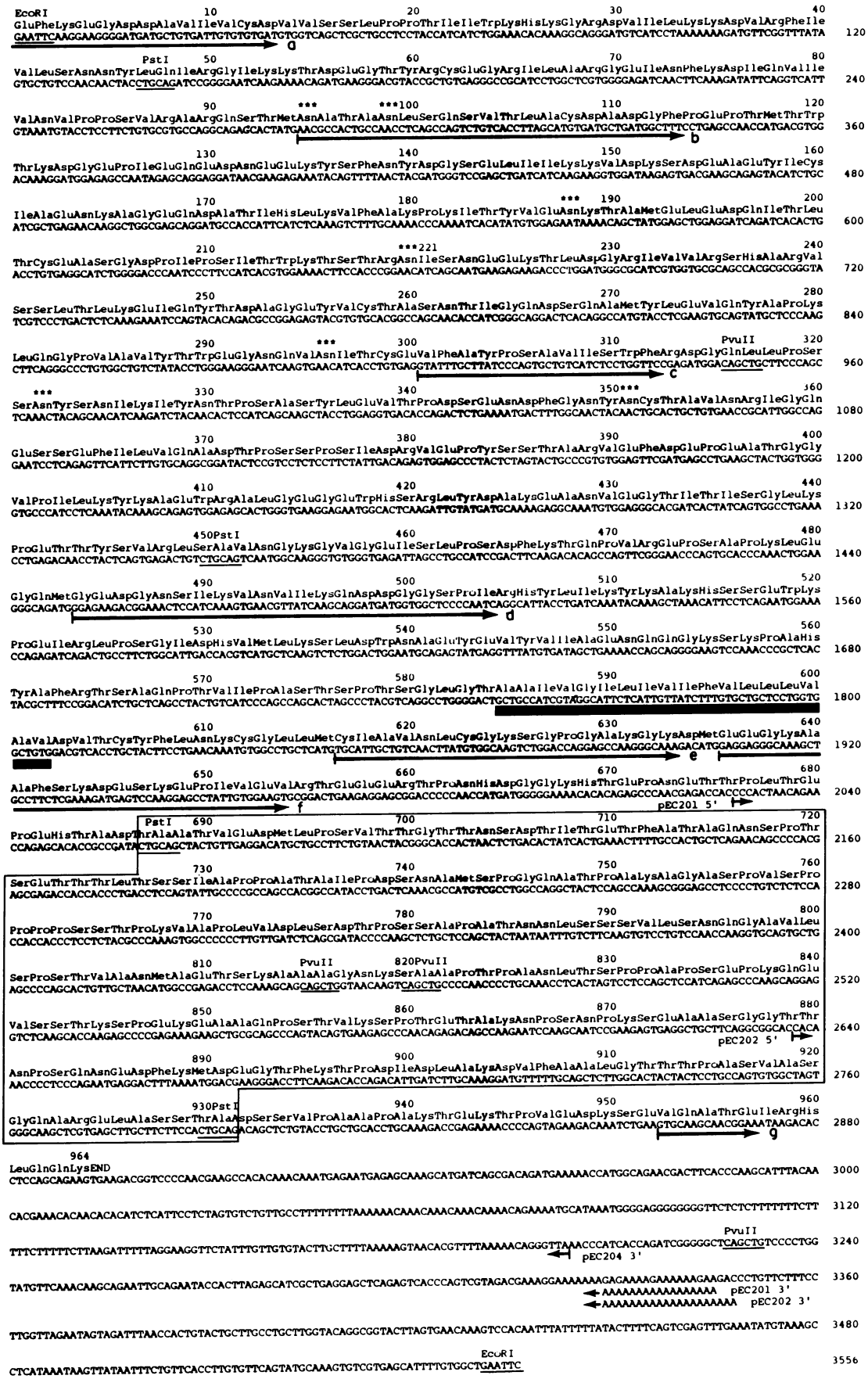

FIG. 2. (*Legend appears at the bottom of the opposite page.*)

purified by gel filtration chromatography or preparative sodium dodecyl sulfate gel electrophoresis (23) and were subjected to Edman degradation in a spinning cup sequenator (24) or in an Applied Biosystems (Foster City, CA) gas-phase sequencer (25).

Hydrophobicity was calculated by using amino acid hydrophobicity values (26) averaged over a sliding window of 17 residues. The Dayhoff protein sequence database (National Biomedical Research Foundation, Washington, DC) was searched by using the rapid homology search program FASTP (27). This program was also used in the pairwise alignment of the internal tandem repeats in the N-CAM sequence that were combined to give the overall alignment shown in Fig. 5.

RESULTS

Nucleotide Sequence of the pEC208 Insert. The strategy used to determine the nucleotide sequence of the pEC208 cDNA insert is shown in Fig. 1, and the complete sequence of 3556 nucleotide pairs is shown in Fig. 2. The *Eco*RI, *Pst* I, and *Pvu* II sites predicted from this sequence agree with our previous restriction maps (17) except that the two closely spaced *Pvu* II sites at positions 2444 and 2459 were not resolved earlier. The *Eco*RI sites at both the 5′ and 3′ ends of pEC208 differ in sequence from the synthetic linkers (CGGAATTCCG) used to construct the library, implying that both ends arose from natural *Eco*RI sites in the N-CAM mRNA sequences. This is substantiated for the 5′ site inasmuch as the sequence recognized by *Eco*RI codes for amino acids present in known N-CAM protein sequences (Table 1 and Fig. 2).

Sequences and positions of the ends of the cDNA inserts from pEC201, pEC202, and pEC204 are indicated in Fig. 2. The 5′ end of pEC204 was identical with that of pEC208; the remaining ends contained synthetic *Eco*RI linker sequences that presumably were added during cDNA cloning. pEC201 and pEC202, but not pEC204, contained short poly(A) tracts at their 3′ termini, suggesting that their synthesis was primed by oligo(dT) that hybridized to the internal A-rich region at this position in pEC208 (Fig. 2). Translation of the pEC208 insert yielded a single open reading frame of 2892 nucleotides, encoding 964 amino acids with a predicted M_r of 103,566. The other two reading frames contained multiple stop codons throughout the sequence. The predicted COOH-terminal amino acid is lysine, consistent with the results of carboxypeptidase analysis of N-CAM (3). The presence of multiple stop codons downstream from this point corroborates this lysine as the carboxyl-terminal amino acid and suggests that the remaining 664 nucleotides in pEC208 are 3′ untranslated sequences.

pEC208 codes for the larger (M_r, 160,000) N-CAM polypeptide (17). From its length and from the predicted molecular weight of the protein sequence encoded by pEC208, we infer that 50–60 kDa of amino-terminal N-CAM protein sequences are not represented in pEC208. Other cDNA clones and the size of the N-CAM mRNA suggest that N-CAM message sequences extend 3′ at least 3 kb from the stop codon in pEC208; consistent with this, the sequence of pEC208 does not contain the consensus poly(A) addition signal AATAAA.

Table 1. Amino acid sequences of peptides used to corroborate the nucleotide sequence of pEC208

Peptide*	Origin†	Sequence‡
a (1–13)	CNBr	. . .EFKEGDDAVIV(C)D
b (94–113)	CNBr	[M](N)ATANLSQ(S)VTLA(C)DA(D)GF
c (301–312)	V8	[E]VFAYPSAVISWF
d (483–504)	CNBr	[M]GEDGNSIKVNVIKQDDGG(S)PI
e (616–633)	CNBr	[M](C)IAVNL(C)GK(S)GPGAKGK
f (635–654)	CNBr	[M]EEGKAAF(S)KDE(S)KEPIVEV
g (952–957)	V8	[E]VQATE

*Peptides are labeled and aligned with the sequences shown in Fig. 2 at the positions indicated in parentheses.

†Peptides were generated by cleavage with CNBr or with *S. aureus* V8 protease (V8).

‡Sequences show agreement between the observed and predicted protein sequences. Residues consistent with the cleavage specificities of CNBr and *S. aureus* V8 protease are enclosed in brackets, and residues not unambiguously assigned by protein sequence analysis are enclosed in parentheses. In peptide a, residues encoded by the *Eco*RI site at the 5′ end of pEC208 are underlined; . . . represents additional peptide preceding pEC208. Amino acids are designated by the single-letter code.

Comparison with Protein Sequences from N-CAM. The pEC208 cDNA sequence was corroborated by limited protein sequencing of peptides generated by cleavage of N-CAM with CNBr or *S. aureus* V8 protease (Table 1). The peptide sequences agreed with the sequences predicted from pEC208 for all seven fragments, totaling ≈100 residues and spaced throughout the coding region of pEC208 (Fig. 2). Moreover, the residue predicted to precede each polypeptide fragment agrees with the known specificities of CNBr and *S. aureus* V8 protease cleavages (after methionine or glutamic acid, respectively). These results confirm that the cDNA clones represent N-CAM mRNAs and verify the reading frame indicated in Fig. 2.

Comparison with Other Protein Sequences. The predicted amino acid sequence from pEC208 was compared to the Dayhoff protein sequence database. Similarities to immunoglobulin domains are discussed below. No other clearly significant similarities were detected, although exact matches of seven amino acids were observed with the human and bovine fibronectin sequences (N-CAM position 435, human fibronectin protein position 1473; sequence TISGLKP) and of five amino acids with the feline sarcoma virus gag–pol polyprotein sequence (N-CAM position 202, gag–pol position 987; sequence CEASG).

DISCUSSION

Differential cell-surface modulation of N-CAM and other CAMs is likely to be critically important throughout embryogenesis (5). The cDNA insert in pEC208 codes for two regions involved in such modulation: a large intracellular domain that differs between the two N-CAM polypeptides and a major portion of the extracellular region, including the sites where polysialic acid is attached.

A schematic representation of these features of N-CAM and their alignment with the sequence of pEC208 is presented in Fig. 3. The amino-terminal 108 kDa of chicken N-CAM

FIG. 2. Sequence of the cDNA insert from pEC208 and the deduced amino acid sequence. Nucleotides are numbered at the right; amino acid residues are numbered above the sequence. *Eco*RI, *Pst* I, and *Pvu* II sites are labeled and underlined. Positions corresponding to the peptides sequenced in Table 1 are marked (a–g). The 18-residue hydrophobic (presumably transmembrane) segment is indicated by a heavy bar. Potential asparagine-linked glycosylation sites on the amino-terminal side of this region are indicated by asterisks. Sequences corresponding to the *Pst* I fragment present in the larger but not in the smaller N-CAM mRNA (17) are boxed. The 5′ and 3′ ends of pEC201, pEC202, and pEC204 are indicated below the nucleotide sequence; the As below the sequence upstream of position 3355 indicate oligo(A) tracts at the 3′ ends of pEC201 and pEC202.

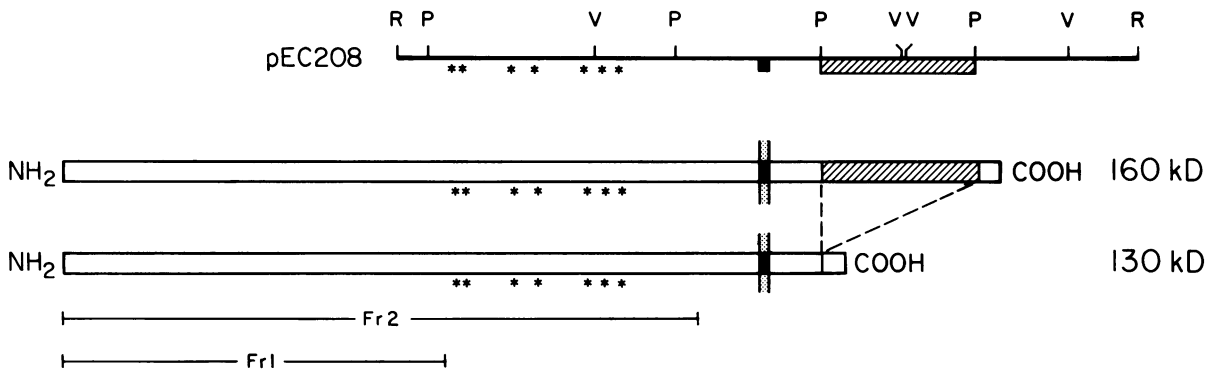

FIG. 3. Schematic representation of the polypeptide chains of N-CAM aligned with pEC208. Restriction sites for *Eco*RI (R), *Pst* I (P), and *Pvu* II (V) in pEC208 are indicated. The locations and estimated extents of fragments Frl and Fr2 (10) are indicated. Black regions represent the membrane-spanning hydrophobic region. The *Pst* I fragment defining the approximate region of sequence difference between the ld polypeptide (160 kDa) and the sd polypeptide (130 kDa) is indicated by cross-hatching. Potential sites for the attachment of asparagine-linked carbohydrates are indicated by asterisks.

(Fr2) are exterior to the cell membrane (10); similar results have been reported for mouse N-CAM (28). The remaining 52 kDa of the 160-kDa N-CAM polypeptide are contained within the sequence encoded by pEC208. This sequence contains a single hydrophobic segment (Fig. 4) of 18 amino acids (residues 585–602 in Fig. 2), similar to those presumed to span the lipid bilayer in other membrane proteins (29). The molecule is thus divided into an amino-terminal extracellular portion of ≈121 kDa (≈1100 amino acids) and a cytoplasmic portion of 37 kDa (362 amino acids).

The mRNA for the larger N-CAM polypeptide contains sequences corresponding to a 735-base-pair *Pst* I fragment (boxed in Fig. 2) that are absent in the smaller mRNA (17). Assuming that the *Pst* I sites (amino acid positions 688 and 931) are close to its boundaries, the difference region corresponds to ≈250 amino acids and the smaller chain should have an intracellular domain of ≈110 amino acids (M_r, 12,000). This difference is sufficient to account for the difference in size between the polypeptides (17). The possibility that differential splicing might also occur in other regions of the mRNAs cannot be excluded; moreover, the continuity and validity of the entire sequence of pEC208 will have to be confirmed by further studies of cDNA and genomic clones. Because the two N-CAM polypeptides differ in the size of their cytoplasmic domains, we designate the 160-kDa chain the large intracellular domain (ld) polypeptide and the 130-kDa chain the small intracellular domain (sd) polypeptide. A third polypeptide is seen in small and variable amounts in chicken N-CAM (3, 10) and more consistently in mouse N-CAM (4, 30). From its size (M_r, 120,000), the position of the hydrophobic segment in pEC208, and its properties (28, 31), it should contain little if any intracellular region and may even lack a membrane-spanning region; we therefore propose the designation ssd (smallest domain) polypeptide.

The finding that the difference between the ld and sd

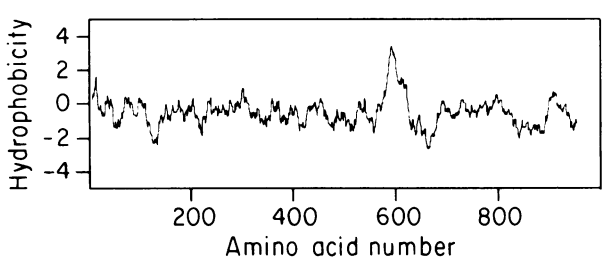

FIG. 4. Hydrophobicity plot of the predicted amino acid sequence from pEC208.

polypeptides appears to be in a cytoplasmic domain provides a sound structural basis for a variety of earlier observations. The polypeptides differ in the extent and sites of phosphorylation on serine and threonine residues (32); the additional segment in the ld polypeptide has an unusual amino acid composition (rich in threonine, serine, alanine, and proline) that could include these additional phosphorylation sites. The finding that a monoclonal antibody recognizing only the larger polypeptide of mouse N-CAM stains cultured cells only after they are permeabilized (33) and recent *in vitro* translation studies (31) both suggest that the corresponding polypeptides of rodent N-CAM also differ in their cytoplasmic domains.

The structural differences between the ld and sd polypeptides raise a number of questions. Binding data suggest that N-CAM at the cell surface is polyvalent (9). Are the ld and sd chains separate on the cell surface, and if so, are they distributed at different locations? Do either or both interact with the cytoskeleton and is one more mobile in the membrane (8) than the other? What is the relationship between the differential expression of the ld and sd chains and N-CAM activity? It would appear that the polypeptides can be expressed independently, because some tissues (e.g., muscle) show a predominance of the sd chain (13).

It is significant that the cytoplasmic domains of the ld and sd polypeptides are both relatively large. Many membrane proteins have intracellular domains of only 30–80 amino acids, although the epidermal growth factor and insulin receptors have large intracellular domains (542 and 402 residues) that may have tyrosine kinase activity (34). N-CAM cytoplasmic domains may modulate binding by interacting with other intrinsic proteins either directly or indirectly, via the cytoskeleton, an event that might also influence the polarity modulation of N-CAM. A cluster of four tightly spaced cysteine residues (positions 606, 612, 617, and 623) near the inner face of the membrane may provide additional opportunities for such regulation.

In addition to the cytoplasmic domain, pEC208 codes for the region where polysialic acid is attached to the molecule. Although sialic acid does not participate directly in N-CAM binding (10), a decrease in this negatively charged sugar is responsible for embryonic to adult conversion of the molecule and an increase in binding rate (9). All of this sialic acid is attached to one or more asparagine-linked oligosaccharides within a single CNBr fragment (M_r 35,000) present in fragment Fr2 but not Frl (Fig. 3) (35). Estimates from the lengths of the N-CAM polypeptides and of Frl and Fr2 place this region in the center of the molecule; the sialic acid is thus presumably on one or more of the seven potential sites between residues 95 and 351 (Fig. 2). The only predicted

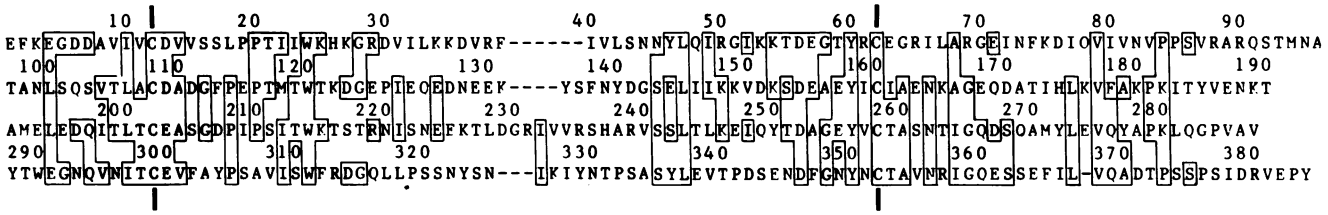

FIG. 5. Internal homologies of amino acid sequences from pEC208. The first 384 residues of the sequence predicted from pEC208 are aligned to show the four tandem regions of internal similarity. Residues identical in at least two of the aligned sequences are boxed. Vertical bars indicate cysteine residues conserved in all four sequences. Amino acids are designated by the single-letter code.

single CNBr fragment approaching this size is between Met-271 and Met-483; this region contains three potential glycosylation sites. The size of the predicted fragment (M_r 23,400), however, is smaller than that observed (35) and reconciliation with the previous results will require further study.

The existence of four tandem regions of intrachain similarity near the amino-terminal end of pEC208 (Fig. 5) suggests the occurrence of some partial gene duplication events during evolution. These regions of similarity are centered around four pairs of conserved cysteine residues spaced 50–56 residues apart and have some homology with immunoglobulin variable and constant domains (36). The seven residues that are identical in all four repeats in the N-CAM sequence (Fig. 5) correspond to highly conserved residues in the immunoglobulin variable region; many of these residues are also conserved in other members of the immunoglobulin superfamily (37). The significance of these similarities—i.e., whether they are homologous or analogous—remains to be established. No free sulfhydryl groups or interchain disulfide bonds have been detected in N-CAM (3), and we assume that these residues form intrachain bonds. Given the apparent extended structure of N-CAM (36), these cysteines may be linked to form a linear array of four homologous loops. Such loops might influence N-CAM binding by orienting the binding domain or by imposing a hinge structure (5, 36), or they might serve other as yet unidentified activities. Additional cDNA clones 5′ to pEC208 have been identified and should help clarify the relationship of these features to the complete binding domain (Fr1) of N-CAM.

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