Alternatively Spliced mRNAs Code for Different Polypeptide Chains of the Chicken Neural Cell Adhesion Molecule (N-CAM)

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Abstract. Rabbit polyclonal antibodies directed against the chicken neural cell adhesion molecule (N-CAM) were used to isolate four overlapping cDNA clones from a chicken cDNA expression library in bacteriophage λgt11. These clones collectively accounted for 3.8 kilobases of N-CAM mRNA sequence and hybridized specifically to two 6–7-kilobase brain polyadenylated RNA species that co-migrated with previously identified N-CAM mRNAs. DNA fragments derived from an internal region of the cloned cDNA sequences hybridized to the larger but not to the smaller N-CAM mRNA species, while fragments on either side of this region hybridized to both mRNAs. A cDNA fragment that recognized only the larger mRNA was subcloned into λgt11, and the expressed fusion protein was used to affinity-purify rabbit polyclonal antibodies; the antibodies recognized only the larger of the two structurally related N-CAM polypeptides. In contrast, when several cDNA clones that recognized both mRNAs were used to purify antibodies, the antibodies recognized both polypeptides. The results, in conjunction with other data indicating that there is one gene specifying N-CAM, suggest that different N-CAM polypeptides are synthesized from multiple N-CAM messages generated by alternative splicing of transcripts from a single N-CAM gene.

CELL adhesion molecules are likely to be of critical importance in embryogenesis (11, 12). The neural cell adhesion molecule (N-CAM)[1] and the liver cell adhesion molecule, for example, are expressed in specific, dynamically regulated patterns in many tissues during all stages of vertebrate development, from the pregastrulation embryo through the adult animal (4, 5, 7, 10, 13, 34, 35). In many cases, dramatic shifts in the levels of expression of these molecules are coincident with important inductive events, for example, at neurulation (34, 35), during the development of the kidney (13, 35), and in the formation of the feather (4, 5), implying that control of cell adhesion molecule gene expression may be a key process in the generation of embryonic form.

Two glycopolypeptides with apparent molecular weights of 170,000 and 140,000 are detected in purified embryonic chicken N-CAM from neural sources after removal of the large amounts of sialic acid present on the molecule. The polypeptide components (M_r 160,000 and 130,000) are very similar in structure, having identical amino-terminal amino acid sequences and giving nearly identical peptide maps (8). To determine the molecular basis for this similarity, we have isolated N-CAM cDNA clones and used them to analyze the structure of the N-CAM mRNAs and polypeptides.

The cDNA clones obtained in our initial study hybridized to two large (6–7 kilobase [kb]) presumptive N-CAM mRNAs in embryonic chick brain (26). Blotting experiments using chicken genomic DNA suggested that both of these messages are derived from a single gene. Here we report the isolation of four additional N-CAM cDNA clones, covering 3.8 kb of N-CAM mRNA sequence. RNA blot hybridization analyses using fragments of these cDNA clones as probes indicated that the larger N-CAM RNA species contains internal sequences that are missing from the smaller species, flanked on either side by sequences that are common to both RNAs. Furthermore, these unique RNA sequences code for a polypeptide segment that shares antigenic reactivity only with the larger N-CAM polypeptide. These results provide strong support for the hypothesis that different N-CAM polypeptides are translated from different N-CAM mRNAs that arise from the alternative splicing of transcripts from a single N-CAM gene.

[1] *Abbreviation used in this paper:* N-CAM, neural cell adhesion molecule.

Materials and Methods

Preparation and Screening of cDNA Libraries

RNA was isolated from 9-d embryonic chicken brains by guanidine thiocyanate extraction (3). Polyadenylated RNA from this material was used to prepare a cDNA library in bacteriophage λgt11 (14, 38, 39). The unamplified library consisted of 16 independent sections of 3×10^4 clones each. After amplification, the library was screened using rabbit polyclonal antibodies to chicken N-CAM. Purification of positive clones, generation of lysogens in *Escherichia coli* strain Y1089, preparation of DNA from induced lysogens, and subcloning of cDNA inserts into plasmid pBR328 were all as previously described (14).

Immunoblot Analysis of Fusion Proteins

Chicken N-CAM and rabbit polyclonal anti-N-CAM antibodies were purified as described (17). Detergent extracts of induced lysogens (38) were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (19), transferred to nitrocellulose (36), and probed with anti-N-CAM antibodies that had been absorbed with autoclaved *E. coli* strain Y1089 in order to remove possible antibodies against *E. coli* antigens (39). For neutralization of antibody binding to immunoblots, 200 μg of undenatured affinity-purified chicken N-CAM in 200 μl of phosphate-buffered saline (PBS) and 200 μg of N-CAM that had been boiled for 3 min in 200 μl PBS + 0.1% sodium dodecyl sulfate were added to 500 μl of blocking buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.2 mg/ml NaN_3, 30 mg/ml crude ovalbumin, 0.1% Triton X-100) on ice. 4 μl of absorbed, filtered anti-N-CAM antiserum was added, and the mixture was incubated on ice for 2 h before being used in immunoblot analysis.

Nucleic Acid Analysis

Restriction enzyme analysis, preparation of restriction maps, preparation of nick-translated probes, and RNA and DNA blot hybridization analyses have been described (22, 26). DNA fragments were electrophoresed in low-melting-point agarose, extracted (22), and labeled by nick-translation for use in blot hybridization analyses.

Determination of mRNA Orientation

The 750-base pair (bp) *PstI-EcoRI* fragment from pEC208 (at the right end of the pEC208 insert as diagrammed in Fig. 2) was cloned in opposite orientations into each of the single-stranded bacteriophages M13mp8 and M13mp9 (40), and hybridization probes were prepared (24) for RNA blot analysis. Only the probe cloned in M13mp8 hybridized with N-CAM mRNA. Because the polylinker in the bacteriophage DNA strand of M13mp8 is in the orientation 5′-*EcoRI* site-*PstI* site-3′, the complementary strand in the mRNA must have the orientation 5′-*PstI* site-*EcoRI* site-3′, establishing the orientation of the mRNA as shown in Fig. 2.

Preparation of λN252

Plasmid pEC201 was digested to completion with *PvuII*, then *EcoRI* linkers were added (22), and the DNA was digested with *EcoRI*. The mixture was phenol-chloroform extracted, precipitated, and cloned directly into alkaline phosphatase–treated λgt11 arms. By this procedure, the *EcoRI* site at the 5′ end of the pEC201 insert, which is known to be in an appropriate reading frame for expression of a β-galactosidase-N-CAM fusion protein, was preserved. A clone that reacted with anti-N-CAM antibodies was purified and its structure was verified by nucleic acid hybridization with cloned cDNA fragments (probes *B–E* of Fig. 2).

Analyses of Affinity-Purified Anti-N-CAM Antibodies

About 1.5×10^4 plaque-forming units of a stock of recombinant bacteriophage were used to infect *E. coli* Y1090 (38). After 4 h at 42°C, a nitrocellulose filter (previously soaked with 10 mM isopropyl-β-D-thiogalactopyranoside and dried) was laid onto the plate and incubation was continued for 12 h at 37°C (14, 39). The filters then were peeled off and soaked at room temperature for at least 1 h in blocking buffer plus 0.5% (vol/vol) Tween 20. Approximately 2-cm squares cut from each filter were incubated with 1 ml of the same buffer containing 25 μg of *E. coli* Y1089–absorbed rabbit polyclonal anti-N-CAM antibodies. After 6–16 h at room temperature, the filters were washed five times with PBS plus 0.5% Tween 20, and antibodies were eluted as described (31). These antibodies were used to probe immunoblots of extracts prepared from 7-d embryonic chick retinal cells that had been infected with Rous sarcoma virus strain tsNY68 and cultured for 9 d (1). These cells expressed the 170- and 140-kD glycosylated adult forms of N-CAM; they were used because the embryonic form of N-CAM found in brain tissue migrates as a diffuse zone on sodium dodecyl sulfate polyacrylamide gel electrophoresis due to its high content of polymeric sialic acid, and the individual polypeptides cannot be detected.

Materials

Materials were obtained from the following sources: guanidine thiocyanate from Fluka (Hauppauge, NY); Tris base, Tris HCl, Triton X-100, Tween 20, and crude ovalbumin (grade II) from Sigma Chemical Co. (St. Louis, MO); low-melting-point agarose, M13 cloning kits, and oligonucleotide primers from Bethesda Research Laboratories (Gaithersburg, MD); restriction enzymes and other molecular biological reagents from Bethesda Research Laboratories, New England Biolabs (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN); nitrocellulose from Schleicher & Schuell, Inc. (Keene, NH); and ^{32}P-deoxynucleotides for nick-translation from New England Nuclear (Boston, MA) or Amersham Corp. (Arlington Heights, IL). Other chemicals were reagent grade.

Results

Isolation of N-CAM cDNA clones

Polyadenylated RNA was isolated from 9-d embryonic chick brain and was used to construct a cDNA library in the bacteriophage expression vector λgt11. Four independent bacteriophages that reacted with rabbit polyclonal anti-N-CAM antibodies were isolated. All four produced plaques that reacted specifically with anti-N-CAM antibodies but not with preimmune IgG. These recombinant bacteriophages were designated λN201, λN202, λN204, and λN208.

Sequences in cDNA cloned in bacteriophage λgt11 are expressed under the control of the *E. coli lac* operon as fusion proteins with *E. coli* β-galactosidase (38, 39). To verify that these fusion proteins reacted with anti-N-CAM antibodies, lysogens were constructed for each N-CAM recombinant bacteriophage (39). Anti-N-CAM antibodies specifically bound to single, high molecular weight species in immunoblots of extracts from induced lysogens of λN201 and λN202 (Fig. 1), and this binding was abolished by preincubation of the antibodies with authentic chicken N-CAM that had been purified by two cycles of immuno-affinity chromatography using a highly specific anti-N-CAM monoclonal antibody. Discrete high molecular weight immunoreactive material was not detected in blots of lysates from λN204 or λN208, although a series of components that migrated at lower molecular weights reacted faintly with anti-N-CAM antibodies in some experiments. However, the strong and specific reactivity of plaques of these bacteriophages with anti-N-CAM antibodies, and the fact that these clones hybridized extensively with and shared restriction maps with λN201 and λN202 (see below), indicate that λN204 and λN208, like λN201 and

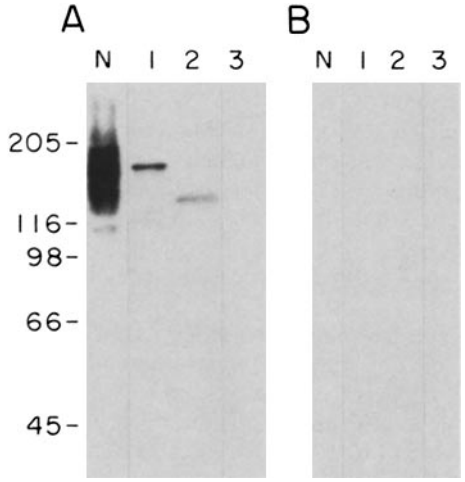

Figure 1. Immunoblot analysis of N-CAM fusion proteins. Affinity-purified embryonic chick N-CAM (1 μg, lanes *N*) or extracts prepared from induced lysogens of λN201 (lanes *1*), λN202 (lanes *2*), or λL301 (lanes *3*) were electrophoresed in a 7.5% polyacrylamide gel and transferred to nitrocellulose. *A* was probed with rabbit polyclonal anti-N-CAM antibodies, whereas *B* was probed with the same antibodies that had been preincubated with purified N-CAM. Molecular masses (kilodaltons) of marker proteins are indicated at the left.

λN202, were derived from N-CAM mRNA. Anti-N-CAM antibodies did not react with the fusion protein from λL301, a λgt11 cDNA clone derived from mRNA for the structurally unrelated liver cell adhesion molecule (14).

Restriction Mapping and Alignment of Clones

To facilitate further analysis, the cDNA inserts were excised by digestion with *EcoRI* and were cloned into the *EcoRI* site of plasmid pBR328, yielding the corresponding plasmids pEC201, pEC202, pEC204, and pEC208. Restriction maps of the cDNA inserts were deduced and aligned from fragment patterns obtained after single and multiple digestions with restriction endonucleases *EcoRI*, *PstI*, and *PvuII*, and the orientation of the clones with respect to the mRNA was determined by RNA blot analysis using single-stranded probes (Fig. 2). To verify the maps, the DNA fragments designated *A–E* in Fig. 2 were prepared, labeled with ^{32}P by nick-translation, and used to probe DNA blots of single and multiple restriction digests of all four cDNA clones. In all cases, the pattern of hybridization was consistent with Fig. 2. These experiments also demonstrated directly that all four cDNA clones share nucleic acid sequence homology. None of the four cDNA clones hybridized with either of the two previously described chicken N-CAM cDNA clones, pEC001 and pEC020 (22), indicating that the two sets of cDNA clones arose from different regions of the N-CAM mRNAs.

Detection of cDNA Fragments Hybridizing Only to the Larger N-CAM mRNA Species

In initial RNA blot transfer experiments using the insert from pEC201 as a probe, two high molecular weight RNA species were seen that co-migrated with the species detected by the previously characterized N-CAM cDNA clones pEC001 and pEC020 (26). However, the pEC201 probe hybridized much more strongly to the larger than to the smaller mRNA species. To test the possibility that pEC201 contains sequences unique to the larger RNA species, we used the cDNA fragments indicated in Fig. 2 to probe RNA blots (Fig. 3). Probes A, D, and E hybridized to both of the N-CAM RNA species, while probes B and C hybridized only to the larger component.

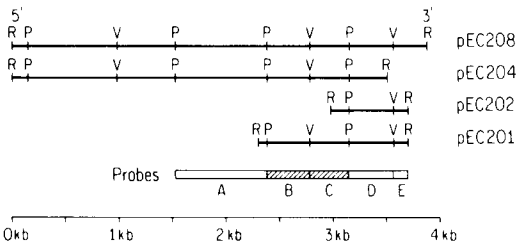

Figure 2. Restriction maps of N-CAM cDNA clones, indicating the sizes and alignments of the cDNA inserts from the indicated plasmids. Lengths of the cDNA inserts are given in kilobase pairs (*kb*) by the scale at the bottom of the figure. Restriction endonuclease cleavage sites are shown for *EcoRI* (*R*), *PvuII* (*V*), and *PstI* (*P*). The 5′ to 3′ (left to right) orientation of the mRNA from which the cDNA was copied is indicated at the top of the map. Purified DNA fragments that were used to verify the map and to probe the RNA blots of Fig. 3 are indicated. Probe A was obtained from pEC204; the remaining probes were obtained from pEC201. The shaded boxes represent fragments that reacted only with the larger N-CAM RNA band, and the open boxes represent fragments that reacted with both RNA bands.

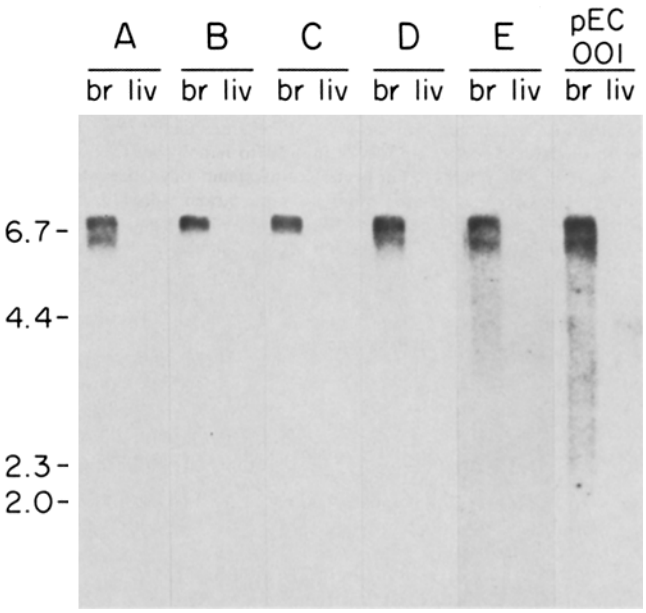

Figure 3. RNA blot hybridization analysis using N-CAM cDNA probes. Polyadenylated RNA (1 μg per lane) isolated from 10-d embryonic chick brain (*br*) or liver (*liv*) was electrophoresed in a 0.8% agarose gel in the presence of formamide and formaldehyde, transferred to nitrocellulose, and hybridized to nick-translated probes A–E (Fig .2), or to pEC001 plasmid DNA. Migrations of the indicated molecular weight markers (kb) are indicated at the left.

The Larger N-CAM Polypeptide Is Translated from the Larger N-CAM mRNA

To determine whether the nucleic acid sequences unique to the larger N-CAM RNA code for a protein sequence found only in one N-CAM polypeptide chain, the 450-bp 5′ *EcoRI-PvuII* fragment of pEC201 (corresponding to probe *B* and about 40 bp of probe *A*, Fig. 2) was excised, converted to *EcoRI* ends, and subcloned in λgt11, giving λN252. To confirm that this fragment recognized only one of the N-CAM mRNAs, the cDNA insert in λN252 was excised and subcloned into pBR328, giving pEC252; this probe hybridized only to the larger N-CAM mRNA (data not shown).

Fusion proteins synthesized from λN252 and the four original N-CAM recombinant bacteriophages were used to affinity-purify subpopulations of rabbit polyclonal anti-N-CAM antibodies. These subpopulations were then used to stain immunoblots of extracts of cultured chick retinal cells that express the 170- and 140-kD glycoproteins found in the adult form of N-CAM (1) (Fig. 4). Antibodies purified using the λN208 or λN204 bacteriophages, which contain a substantial amount of sequence shared by both N-CAM RNAs upstream (5′) from probes B and C, recognized both the 170- and 140-kD N-CAM polypeptides. In contrast, antibodies purified using the λN252 bacteriophage, which has a cDNA insert derived from sequences unique to the larger N-CAM RNA, recognized only the 170-kD N-CAM polypeptide. Both the λN201 and λN202 bacteriophages contain sequences unique to the larger N-CAM RNA, as well as downstream (3′) sequences present in both RNAs. Antibodies purified using these bacteriophages also recognized only the 170-kD N-CAM protein species.

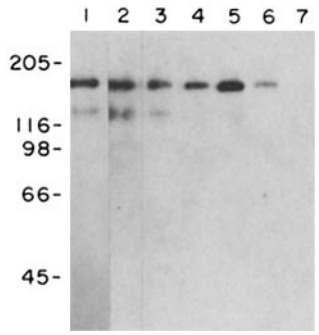

Figure 4. Immunoblots of antibodies purified using cDNA fusion proteins. Extracts of cultured chick retinal cells were electrophoresed in a 7.5% polyacrylamide gel and were transferred to nitrocellulose. Duplicate strips were probed with rabbit polyclonal anti-N-CAM antibodies (lane *1*) or with anti-N-CAM antibodies eluted from filters prepared from cultures of λN208 (lane *2*), λN204 (lane *3*), λN252 (lane *4*), λN201 (lane *5*), λN202 (lane *6*), or λgt11 (lane *7*). Molecular masses (kilodaltons) of marker proteins are indicated at the left.

Discussion

Our previous study (26) suggested that a single N-CAM gene gives rise to multiple N-CAM mRNAs which, in turn, are translated into different N-CAM polypeptides. This hypothesis is consistent with the mapping of the mouse N-CAM gene to a single chromosomal location (9) and with RNA blot analyses showing multiple N-CAM RNAs in the mouse (15). Strong additional evidence for a single gene for chicken N-CAM has been obtained in recent genomic cloning studies (Owens, G. C., G. M. Edelman, and B. A. Cunningham, unpublished data) that demonstrated that all of the chicken genomic *EcoRI* fragments that hybridize to pEC201 or pEC204 are derived from a single locus. The studies of new N-CAM cDNA clones reported here, in conjunction with the genomic cloning results, strengthen the hypothesis that N-CAM mRNAs arise by alternative splicing, a process that is known to occur during the expression of many eukaryotic genes (reviewed in reference 2).

The strong cross-hybridization observed between the four cDNA inserts examined here and their ability to affinity purify anti-N-CAM antibodies supports the conclusion that they all contain N-CAM protein coding sequences. Even more compelling, recent DNA sequencing work (Hemperly, J. J., B. A. Murray, G. M. Edelman, and B. A. Cunningham, unpublished data) has demonstrated regions of DNA sequence in pEC208 corresponding to N-CAM amino acid sequences obtained by direct protein sequencing. Both λN201 and λN202 gave rise to stable fusion proteins that could be detected specifically in immunoblots of extracts from induced lysogens. Although the λN204 and λN208 bacteriophages reacted strongly and specifically with anti-N-CAM antibodies when tested as plaque cultures, discrete high molecular weight fusion proteins were not detected in immunoblots, perhaps reflecting the relative susceptibility of large segments of foreign proteins to degradation in *E. coli* (38). Similar results have been seen for fusion proteins in other systems. For example, some cDNA clones for terminal transferase react with antibodies in plaques but fail to be recognized in immunoblots of induced lysates (20).

If both N-CAM mRNAs are transcribed from a single gene, they should have the same nucleotide sequences for much of their lengths. The results presented here confirm that the two N-CAM RNAs share considerable sequence homology and also identify an internal region of sequence where they differ. The simplest explanation of these results is that both RNA species are transcribed from a single gene and that probes B and C recognize an exon (or exons) that is present in the larger but has been spliced out of the smaller component. More complicated models involving multiple splice donor or acceptor sites (18, 30, 33, 37), splicing of alternative exons (2, 23), or additional regions of splicing in other areas of the mRNA, are also compatible with these data. The region of difference detected by probes B and C does not appear to arise from the use of alternative promoter or transcription termination sites for the two N-CAM RNA species, because probes A, D, and E, which surround this region in the cDNA, hybridized to both RNA species. All four cDNA clones reported here hybridized to probes B or C, and thus were derived from the larger RNA species.

The results in Fig. 4 indicate that the 170-kD N-CAM species contains polypeptide antigenic determinants unique to the translation product of the larger N-CAM RNA, and thus that the 170-kD polypeptide is coded for by the larger RNA. A similar immunological approach has been used recently to support the role of alternative splicing in the generation of multiple related polypeptides of fibronectin (29). We assume that the 140-kD N-CAM species is translated from the smaller N-CAM RNA species, although the data also are consistent with its translation from an alternatively spliced mRNA that co-migrates with the larger N-CAM mRNA; in either case, the 140-kD component would not react with the antibodies purified using λN252 because it would lack the protein sequences translated from the region unique to the larger N-CAM RNA. The 140-kD species is unlikely to result from posttranslational cleavage of the 170-kD species because cell-free translation of N-CAM mRNA in vitro (16, 26) and pulse-chase analysis of N-CAM synthesis in vivo (21) indicate that the two N-CAM polypeptides are synthesized from different mRNAs. The region of difference between the two RNAs that is defined by probes B and C is at least 760 bases long, which is sufficient to account for the 30 kD difference between the two polypeptides, and the location of these probes is consistent with the position of sites of phosphorylation that are known to differ between the two polypeptides (32).

In addition to DNA sequences unique to the larger N-CAM RNA, both λN201 and λN202 contain downstream sequences that are common to both RNAs (Fig. 2). The antibodies purified using either λN201 or λN202, however, bound only to the 170-kD N-CAM polypeptide. The amino acid sequences shared between the two polypeptides in this region may not be recognized by the rabbit polyclonal antibodies used in this study. Alternatively, the protein coding sequences may terminate at or close to the point of divergence of the RNAs, so that the carboxy termini of the polypeptides differ or share too few amino acids to be recognized immunologically. It is also possible that the smaller mRNA is spliced into a different reading frame, which would also give rise to different carboxy-terminal amino acid sequences in the two N-CAM polypeptides.

The cDNA probes and immunological reagents described in this paper make it possible to specifically detect the 170-kD N-CAM species in situ at both the protein and nucleic acid levels, and thus to address several important questions about N-CAM polypeptide heterogeneity and its develop-

mental regulation. For example, it will be possible to test whether developmentally regulated differences in RNA splicing are responsible for differences observed in the expression of N-CAM polypeptides between neural and nonneural tissues (4, 6, 25, 27, 28). The answers to these questions may have important implications for cell adhesion molecule function, if, as has been postulated, the regulation and modulation of expression of these molecules is intimately involved with the determination and differentiation of different cell and tissue types (11, 12).

We thank Patricia Ferrie, Colette Muenzen, and Kim Magloire for excellent technical assistance. J. J. Hemperly is a Lester Wolfe fellow of the Rockefeller University.

This work was supported by U.S. Public Health Service Grants HD-16550, HD-09635, AM-04256, and AI-11378.

Received for publication 12 August 1985, and in revised form 8 October 1985.

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