Modulation of NCAM Expression by Transforming Growth Factor-Beta, Serum, and Autocrine Factors

Regine Roubin, Hermine Deagostini-Bazin, Marie-Rose Hirsch, and Christo Goridis

Centre d'Immunologie Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique de Marseille-Luminy, Case 906, F-13288 Marseille Cedex 9, France

Abstract. The expression of NCAM (neural cell adhesion molecule) is precisely regulated in terms of cell type specificity and developmental control. We searched for extracellular factors that may be involved in this regulation using N2A neuroblastoma and NIH 3T3 fibroblastic cells. Factors contained in FBS promoted a two- to threefold increase in NCAM protein and mRNA abundance in both cell lines. This increase in NCAM expression in high serum could be entirely attributed to enhanced levels of the NCAM-140 message. Modulation of NCAM synthesis via an autocrine mechanism is suggested by the observation that medium conditioned by N2A cells stimulated NCAM mRNA expression by 3T3 and N2A cells. Among the pure factors tested, transforming growth factor-beta (TGFβ) was found to act as an inducer of NCAM expression in 3T3 but not in N2A cells. 3T3 cells responded to exposure to TGFβ with a two- to threefold increase in NCAM protein and mRNA. Exposure of early-passage embryonic cells to TGFβ resulted in four- and twofold increases in NCAM protein and mRNA abundance, respectively, suggesting a role for TGFβ in modulating NCAM expression in the embryo. TGFβ seems to act by stimulating the transcriptional activity of the NCAM gene because it did not affect transcript stability and stimulated transcription from a proximal promoter element of the NCAM gene.

THE neural cell adhesion molecule (NCAM)[1] is the name given to a group of closely related cell surface glycoproteins that function as ligands in the formation of cell–cell contacts. NCAM, together with the members of the cadherin family (Takeichi, 1988), belongs to the primary or general cell adhesion molecules that are expressed early in development and on a variety of cell types. These molecules are believed to play critical roles in specifying cell patterning, movement, and differentiation in the embryo and in maintaining tissue integrity in the adult organism (Edelman, 1985; 1988; Rutishauser and Jessel, 1988; Takeichi, 1988; Thiéry, 1989). NCAM expression is precisely regulated in terms of tissue distribution and developmental control. The total amounts of NCAM and the relative proportions of its isoforms, which arise from a single gene by differential processing of the pre-mRNA (Cunningham et al., 1987; Barbas et al., 1988; Santoni et al., 1989), change during development and differentiation (Pollerberg et al., 1985; Murray et al., 1986). In the embryo, NCAM is expressed transiently on derivatives of all three germ layers, often in morphogenetically active regions such as the notochord, placodes, somites, and the neural crest (Thiéry et al., 1982; Crossin et al., 1985; Balak et al., 1987; Levi et al., 1987). The tissue distribution of NCAM becomes more limited during further development. In the perinatal period, it still includes most, if not all, neurones and glial cells and skeletal and cardiac muscle fibers (Langley et al., 1983; Chuong et al., 1984; Rieger et al., 1985; Covault et al., 1986; Prediger et al., 1988). NCAM persists on most neurons and astrocytes in the central nervous system, and on neuronal cell bodies, their unmyelinated axons and nonmyelinating Schwann cells in the peripheral nervous system in the adult (Langley et al., 1983; Chuong et al., 1984; Nieke and Schachner, 1985). It can, however, be reinduced in the denervated muscle and at sites of nerve injury and repair (Covault et al., 1986; Daniloff et al., 1986; Martini and Schachner, 1988).

The three main NCAM isoforms in the mouse, which we have called NCAM-180, -140, and -120 according to their apparent relative molecular masses in SDS gels (Rutishauser and Goridis, 1986), are encoded by four size classes of mRNAs: transcripts of 6.9 and 6.1 kb code for NCAM-180 and -140, respectively; two smaller transcripts of 4.8 and 2.7 kb code for NCAM-120. These messages are generated from the single NCAM gene by a combination of alternative splicing and the choice among one of three poly(A) addition signals (Barbas et al., 1988). Their expression changes during development and differentiation as has been particularly well documented during muscle development (Covault et al., 1986; Moore et al., 1987).

Despite a wealth of information on the expression se-

1. *Abbreviations used in this paper*: EGF, epidermal growth factor; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; TGF, transforming growth factor.

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quences of NCAM during embryonic development, we know very little as to how its expression is regulated. In particular, the extracellular signals involved in the control of NCAM levels in the embryo and its reinduction after nerve injury have remained unknown, the only growth or differentiation factor reported to influence NCAM expression being nerve growth factor (NGF) (Prentice et al., 1987; Doherty et al., 1988).

We have used the N2A mouse neuroblastoma and the NIH 3T3 fibroblastic cell lines to search for extracellular factors that might influence NCAM expression by these cells. N2A cells are known to express constitutively high amounts of NCAM which has been studied with respect to its protein and mRNA forms and its mobility at the cell surface (Pollerberg et al., 1985; 1986; Gennarini et al., 1986). Most fibroblastic cells are NCAM negative. However, NCAM has been found on some fibroblasts from peripheral nerve and denervated muscle (Martini and Schachner, 1988; Gatchalian et al., 1989) and its expression in embryonic mesenchyme (Crossin et al., 1985) suggests that it can be expressed on fibroblast precursors. In the course of this study, we found that NCAM is indeed expressed in primary and secondary cultures of fibroblastic cells from the mouse embryo commonly referred to as embryonic fibroblasts.

In this study, we demonstrate that NCAM protein and mRNA levels are regulated in N2A and 3T3 cells by (a) factor(s) contained in serum and in N2A cells in addition by an autocrine mechanism. When testing a variety of growth and differentiation factors, we found that transforming growth factor-beta (TGFβ) promoted a net increase in NCAM protein and mRNA levels in fibroblastic cells, not only in an established cell line, but also in early-passage embryo-derived cultures. These findings suggest that TGFβ is involved in the regulation of NCAM expression in the embryo, possibly also at sites of nerve injury where NCAM has been found to be induced on fibroblastic cells (Nieke and Schachner, 1985; Gatchalian et al., 1989).

Materials and Methods

Cell Culture

The C1300 mouse neuroblastoma-derived N2A cell, the mouse L cell (subclone LM tk$^-$) and the NIH 3T3 fibroblastic cell line were grown in DME containing 10% FBS (complete growth medium) at 37°C in an atmosphere of 7.5% CO_2 in air. Identical results were obtained when newborn bovine serum was used in place of FBS. Routinely, the cells were grown to 80% confluency before serum starvation or analysis of the effects of different agents. Serum-starved cultures were prepared by switching to DME with 0.2% FBS for N2A and to DME without serum for 3T3 cells. In some experiments, DME/F12 (1:1) medium supplemented with 10 μg/ml transferrin and 30 nM selenium was used. The following growth and differentiation factors were added in the concentrations and for the time periods specified in the legends: TGFβ (ultrapure TGFβ from human platelets, Calbiochem-Behring Corp., La Jolla, CA), purified β_1^- and β_2^- subtypes of TGFβ from porcine platelets (British Biotechnology Ltd., Oxford, UK), human recombinant PDGF (Genzyme Corp., Boston, MA), epidermal growth factor (EGF) from mouse submaxillary glands, and basic fibroblast growth factor (bFGF) from bovine pituitary and nerve growth factor (NGF) (7S NGF from mouse submaxillary glands). EGF, bFGF, and NGF were purchased from Sigma Chemical Co. (St. Louis, MO) as well as retinoic acid, phorbol myristate acetate, and actinomycin D.

Embryo-derived fibroblastic cells were prepared from 16-d-old embryos of Swiss mice as described by Arnheiter and Staeheli (1983) with slight modifications. After removal of head, limbs, and bowels, the trunks were minced and incubated in 0.125% crude trypsin (Gibco Laboratories, Grand Island, NY) in PBS at 37°C. Every hour, the released single cells were collected, fresh trypsin was added and the incubation continued for a total of 4 h. The cells were pooled, collected by centrifugation and allowed to adhere to tissue culture plastic dishes for 1 h in DME with 10% FBS. After 1 h, the nonadherent cells were discarded and the cultures grown in DME with 10% FBS. They were subcultured by trypsinization. First and second passage cells were used in all experiments.

To test for cell viability and changes in cell number under different culture conditions the MTT assay (Mosmann, 1983) as modified by Doherty et al. (1988) was used. Dimethylthiazol-2-yl-2,5 diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) was added at 0.5 mg/ml to cells in 96-well-microtiter plates for 2 h at 37°C. After stopping the reaction with 0.08 N HCl in isopropanol, color formation due to production of MTT formazin was measured at 540 nm with a microelisa reader (Multiscan; Titertek, Flow Laboratories S.A., Puteaux, France).

Immunochemical Detection and Quantification of NCAM and L1 Protein

For immune blot analysis, NP-40 extracts were prepared and NCAM proteins revealed after electrophoresis and transfer to nitrocellulose by rabbit anti-NCAM antibodies and ^{125}I-protein A as described (Gennarini et al., 1986).

The NCAM and L1 antigen content of the cultures was determined by a dot blot assay. Cells were lysed in 0.1% deoxycholate, 20 mM Tris HCl pH 8.5, 20 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT, 0.1 mM PMSF. Serial dilutions of the cleared (12,000 g, 30 min) lysates were spotted on nitrocellulose paper (0.2 μm pore size, Schleicher & Schüll, Dassel, FRG) with the help of a dot blot apparatus. To block nonspecific binding, the nitrocellulose sheets were incubated for 1 h at 37°C in PBS containing 5% defatted dry milk. Specific antibody appropriately diluted in the same solution was then added for 16–20 h at 4°C. The filters were washed four times in PBS containing 5% dry milk and bound antibody revealed by incubation for 1 h at room temperature with protein A (0.5 $\times$ 10^6 cpm/ml) iodinated with [^{125}I] to a specific activity of 20–30 $\times$ 10^6 cpm/μg. The radioactive spots were cut out and the radioactivity determined in a gamma counter. The amounts of antigen in the lysates were calculated from the linear region of the dilution curves and expressed in cpm per μg protein contained in the undiluted extracts.

The preparation and specificity of the rabbit anti-NCAM (anti-NCAM-120, -140, and -180) antiserum (Gennarini et al., 1986) and of the rabbit anti-L1 antiserum (Rathjen and Schachner, 1984; a kind gift of M. Schachner, ETH Zürich) have been described. Rabbit antibodies specific for NCAM-180 were prepared against a bacterial fusion protein composed of a genomic fragment encoding the NCAM-180-specific exon (Barbas et al., 1988) coupled to β-galactosidase, as will be described in detail elsewhere. In immune blots of brain extracts or of affinity-purified NCAM, this antiserum recognized only an 180-kD band. As shown by immunofluorescence experiments, the antibodies did not stain NCAM-negative cells and reacted only with permeabilized NCAM-180-expressing cells due to the intracellular localization of the epitope (results not shown). All rabbit sera were used at a 1/500 dilution.

Northern Blot Analysis and Quantification of mRNA

Total RNA was extracted directly from the cell monolayer in culture flasks in 4 M guanidinium thiocyanate followed by phenol/chloroform extraction according to Chomczynski and Sacchi (1987). RNA was quantified by measuring the OD at 260 nm. For Northern blot analysis, samples containing 10–15 μg of RNA were fractionated on 0.8% formaldehyde-containing agarose gels as previously described (Gennarini et al., 1986). The RNA was transferred on Hybond-C (Amersham Chemical Co.) and hybridized with 2–3 $\times$ 10^6 cpm/ml of the radioactive probe. Probe labelling and conditions for hybridization, washing and autoradiography were as described (Gennarini et al., 1986). The NCAM cDNA probe used was the 5' part of clone DW3 (DW3LE; Barthels et al., 1987); clone K13 (Moos et al., 1988; a kind gift of R. Tacke, Centre d'Immunologie de Marseille-Luminy) was used as a probe to reveal the L1 mRNA. Depending on the size markers used, different sizes have been assigned to the four size classes of NCAM mRNAs detected in the mouse. To arrive at a consistent nomenclature and in keeping with a previous publication (Santoni et al., 1989), we will call the transcripts according to their calculated sizes (minus the poly[A] tail) deduced from the cDNA sequence of the corresponding brain transcripts. In all Northern blots, RNA from mouse brain was run alongside and the comigrating bands in the different cell types were assigned accordingly.

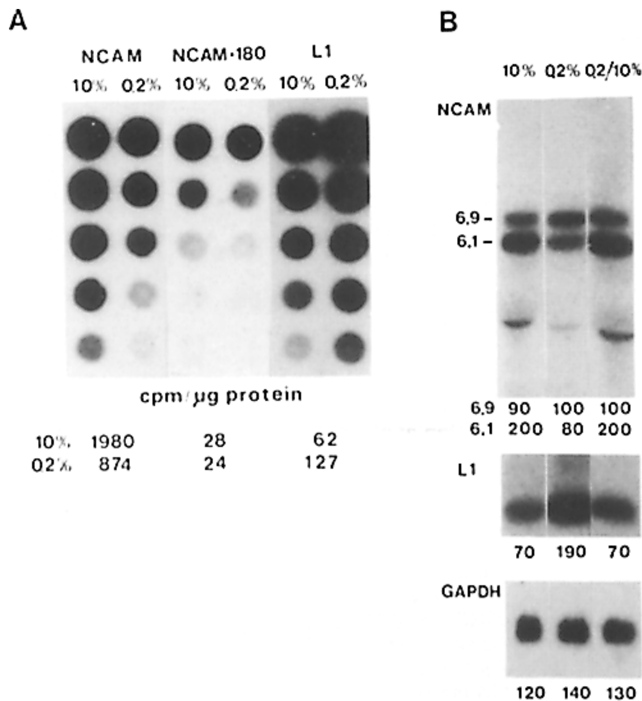


Figure 1. Changes in NCAM and L1 expression in N2A cells cultured in high and low serum. N2A cells were cultured in 10 or 0.2% FBS for 24 h before harvesting and lysis in 0.1% deoxycholate. (*A*) Autoradiograph of dot blots containing serial twofold dilutions of N2A cell lysates developed with rabbit anti-total NCAM (*NCAM-180, -140,* and *-120*) serum (*NCAM*), NCAM-180-specific rabbit serum (*NCAM-180*), and rabbit anti-L1 serum (*L1*). The dot blot was first incubated with the relevant antiserum diluted 1:500 and bound antibody was revealed with [^{125}I]protein A. The radioactive spots were then cut out and counted. The total NCAM, NCAM-180 and L1 immunoreactivities expressed as counts per minute/microgram protein are given below. The dilution series developed with anti-L1 serum started at a twofold higher protein concentration and was exposed four times longer. In each case, the counts from three to four dilutions that fell within the linear range of the response were averaged. Very similar results were obtained in more than six independent experiments. No detectable NCAM immunoreactivity was found in extracts of L cells that do not express NCAM (not shown). (*B*) Northern blot done with total RNA extracted from N2A cells and probed for NCAM, L1, and GAPDH mRNA. The cells, seeded at 0.5 × 10^6 cells per 25-cm^2 flask, were grown for 48 h in 10% FBS, then switched to 0.2% FBS and harvested 24 h later (0.2%), or after 24 h in 0.2% FBS, they were switched back to complete growth medium for another 24 h (0.2/10%). Cells grown in 10% FBS for 48 h were taken as a control (10%). The sizes of the NCAM-180- (6.9) and NCAM-140- (6.1) specific bands are given in kilobases. They comigrated precisely with the corresponding mRNA species from mouse brain, for which sizes of 6.9 and 6.1 kb (minus the poly[A] tail) have been determined by sequence analysis. The fainter band of smaller size runs just ahead of the 28S rRNA and probably represents hybridizing material that is being pushed ahead by the large amounts of rRNA. The intensity of the bands was quantified by densitometric scanning of the autoradiographs. The values are given below the autoradiographs in arbitrary units.

A cDNA coding for glyceraldehydephosphate dehydrogenase (GAPDH) (Hanauer and Mandel, 1984; a kind gift of J. L. Mandel, Laboratoire de Génétique Moléculaire des Eucaryotes, Strasbourg) was used as reference probe. The levels of GAPDH protein and mRNA are known not to change during the G_0 to G_1 transition and not to be influenced by various growth factors including TGFβ (Edwards et al., 1985; 1987). The NCAM- and L1-specific bands in the autoradiographs were quantified by scanning using a Vernon densitometer. Each blot was exposed for different times, and the values were taken from exposure times that fell in the linear range of the film and densitometer response. The crude values obtained for NCAM and L1 mRNA abundance were normalized by dividing them by the values obtained for GAPDH mRNA.

In the actinomycin D chase experiments, NCAM mRNA levels were determined by a dot-blot procedure. 5 μg of total RNA were dissolved in 50 μl H_2O, 30 μl 20× SSC (1× SSC is 15 mM Na citrate, 0.15 M NaCl, pH 7.0) and 20 μl 37% formaldehyde and denatured for 15 min at 60°C. Serial dilutions in 20× SSC were dotted on Hybond-C. The blots were first hybridized with the NCAM probe and, after total dehybridization, with the GAPDH probe. After autoradiography, the intensity of the spots was determined by densitometric scanning. Mean values for different dilutions were determined by regression analysis of the linear portion of the dilution curves.

Transfections and Determination of Chloramphenicol Acetyltransferase Activity

The recombinant plasmid used contains a 608-bp fragment (position -645 to -37 relative to the start site of translation) from the 5′ end of the mouse NCAM gene coupled to the chloramphenicol acetyltransferase (CAT) gene from pconaCAT (Kimura et al., 1986). In a previous study (Hirsch et al., 1990), this fragment has been shown to contain the transcriptional start sites and to exert high promoter activity in NCAM-expressing cells.

NIH 3T3 cells were plated at a density of 5 × 10^5 cells per 250-ml flask. 20 h later, they were transfected with 20 μg plasmid DNA by the calcium phosphate coprecipitation method (Gorman, 1985). After 16 h, a 10% DMSO shock was applied for 10 min. For each experiment, four sister cultures were transfected. After the DMSO shock, the cells from the four flasks were recovered by trypsinization, pooled, and redistributed into four flasks at a ratio of 1:1:3:3. The two sparser cultures were maintained in DME with 10% FBS throughout; in the two denser ones, the serum concentration was lowered to 1% 6 h and to 0.2% 16 h after replating. 6 h after subculturing, one of each of the high- and low-serum cultures received 200 pM TGFβ. All cultures were harvested between 40 and 48 h after subculturing. Approximately the same numbers of cells were recovered from high- and low-serum cultures in the presence or absence of TGFβ. CAT activity was determined on aliquots of cell lysates containing equal amounts of protein according to Gorman (1985). The percent conversion of [^{14}C]chloramphenicol into its acetylated derivatives was determined by cutting out the corresponding spots from the thin layer plates followed by scintillation counting.

Results

Modulation of NCAM Protein and mRNA Levels in N2A Cells by Serum and Autocrine Factors

The N2A mouse neuroblastoma cell line was chosen as an example of a neural cell line to analyze the effects of culture conditions and extracellular factors on NCAM expression. A quantitative dot-blot immunoassay, an example of which is shown in Fig. 1 *A*, was used to measure the NCAM protein content. N2A cells expressed around two- to threefold higher amounts of NCAM protein per μg total protein when grown in 10% (complete growth medium) than in 0.2% (low-serum medium) FBS (Fig. 1 *A*) or in serum-free medium supplemented with selenium and transferrin (synthetic medium) (not shown). By contrast, the levels of the L1 adhesion molecule (Rathjen and Schachner, 1984; Moos et al., 1988) were higher in low-serum than in complete (Fig. 1) or in synthetic medium (not shown). Hence, serum starvation caused opposite changes in the levels of two structurally related (Moos et al., 1988) adhesive proteins. In contrast to the decrease in total NCAM protein abundance in low serum, NCAM-180 levels measured with a specific antibody were not modified in the presence of low serum (Fig. 1 *A*), showing that an increase in the relative proportions of NCAM-180 to NCAM-140 expressed by N2A cells is induced by serum starvation

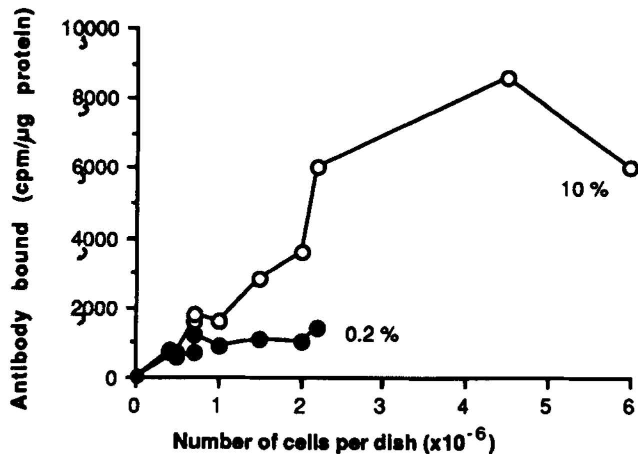


Figure 2. Effect of cell density on NCAM levels in N2A cells. N2A cells were plated at various cell densities (0.1-1.5 × 10^6 cells per 25-cm^2 culture dish) in 10 or 0.2% serum. After 48 h, the cells were harvested and counted with a hemocytometer. Cell lysates (25-50 μg protein) were dot blotted and assayed for total NCAM immunoreactivity as described in the legend to Figure 1. The results expressed as counts per minute/microgram protein are plotted as a function of cell density at the time of harvest.

alone and does not require the presence of additional factors such as DMSO and laminin (Pollerberg et al., 1985; 1986). Similar changes occurred in the levels of the corresponding mRNA species. As shown in Fig. 1 *B*, serum starvation resulted in an over twofold decrease in the level of the 6.1-kb message that codes for NCAM-140 (Barbas et al., 1988), whereas the 6.9-kb species, which is specific for NCAM-180, was not affected. The effect was reversible since the values were restored to control levels when serum was added back to the cultures. As already observed at the protein level, L1 mRNA expression increased in low-serum conditions. Rehybridization with a cDNA probe for GAPDH, a key enzyme of the glycolytic pathway, which is not affected by the G_0 to G_1 transition of the cell cycle (Edwards et al., 1985), showed that equivalent amounts of total mRNA has been applied to the different lanes. Serum factors appear thus to regulate NCAM and L1 expression at the level of synthesis, processing or stability of the corresponding mRNAs.

In addition to the serum content of the medium, cell density had a pronounced effect on NCAM protein levels of N2A cells grown in 10% serum with a steep increase between 1 and 2 × 10^6 cells per dish (Fig. 2). Serum starvation still caused a clear down-regulation of NCAM levels at identical cell densities, making it unlikely that an increase in cell density was in fact responsible for the observed effect of serum factors. The cell-density dependence of NCAM expression suggested to us that an autocrine factor might be involved in the regulation of NCAM expression by N2A cells. Indeed, medium conditioned by N2A cells promoted a threefold increase in the level of total NCAM transcripts over that measured in N2A cultures exposed to 3T3 cell-conditioned medium (Fig. 3, lanes *a* and *b*). There was no difference in NCAM transcript expression between cultures grown in fresh or 3T3 cell-conditioned medium (results not shown). In contrast to serum, conditioned medium affected both the 6.9- and the 6.1-kb species; therefore, the putative autocrine and the serum factors must be different molecules.

NCAM Expression by NIH 3T3 Cells

Most normal fibroblasts do not express NCAM, but in the embryo, NCAM is present in undifferentiated mesoderm (Crossin et al., 1985) which should include fibroblast precursors. We chose the NIH 3T3 cell line as an example of embryo-derived, fibroblastic cells. As shown in Fig. 4, 3T3 cells expressed readily detectable levels of NCAM-140 but not of NCAM-180; a faint band in the NCAM-120 region became visible after prolonged exposure. The NCAM-140 band from these cells consistently run with a slightly lower mobility than the corresponding isoform from N2A cultures or whole mouse brain. This slight mobility shift may be due to glycosylation differences or to the presence of a short additional stretch of amino acids as has been reported for muscle NCAM (Dickson et al., 1987). The material analyzed on lanes *2* and *3* was derived from identical numbers of cells showing that on a per cell basis, 3T3 cells contained approxi-

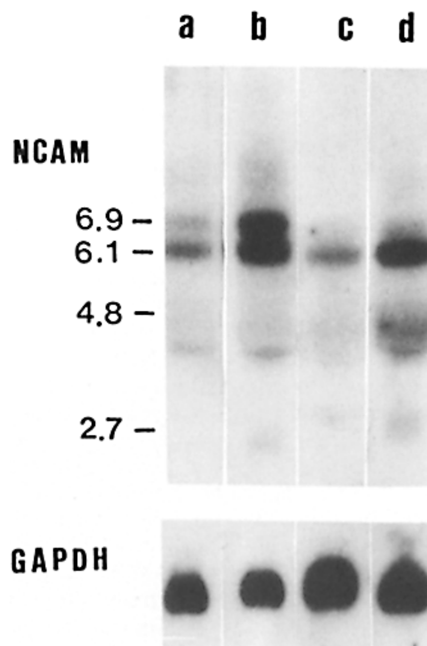


Figure 3. Effect of N2A-cell conditioned medium on NCAM mRNA expression by N2A and 3T3 cells. A Northern blot probed with a mixture of NCAM and GAPDH cDNAs is shown. Lanes *a* and *b*, N2A cells, lanes *c* and *d*, 3T3 cells; lanes *a* and *c*, cultures exposed to 3T3-conditioned medium; lanes *b* and *d*, cultures exposed to N2A cell-conditioned medium. The sizes of the bands are given in kilobases. In this experiment, small amounts of the 4.8- and 2.7-kb transcripts were also revealed. The band that runs just in front of the 4.8-kb species is an artifact due to hybridizing material pushed ahead by the 28S rRNA. The smaller-size bands represent at most 20% of the total hybridizing material and were not included in the quantitative analysis of the conditioned medium effect. Fresh complete growth medium was added to near confluent cultures of N2A or 3T3 cells. The medium was harvested 24 h later, cleaned by centrifugation, and stored at −20°C. N2A and 3T3 cells (5 × 10^5 cells per 25-cm^2 dish) were grown in complete medium for 24 h, then medium consisting of 50% conditioned medium and 50% fresh complete growth medium was added. After 24 h in the presence of conditioned medium, the cells were harvested and processed for RNA analysis.

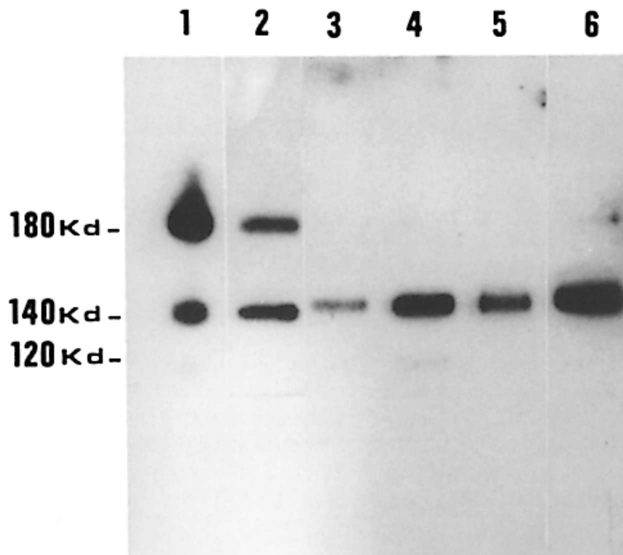


Figure 4. Immune blot analysis of NCAM protein in 3T3 and early-passage embryo cells in the presence and absence of TGFβ. Extracts of postnatal-d 20 mouse brain (lane *1*), of N2A cells grown in complete medium (lane *2*), of 3T3 cells grown in complete medium in the absence (lane *3*) or presence (lane *4*) of 200 pM TGFβ and of first passage cells from 16-d-old embryos grown in complete medium in the absence (lane *5*) and presence (lane *6*) of 200 pM TGFβ were fractionated by SDS-PAGE and transferred to nitrocellulose. NCAM-immunoreactive bands were revealed by rabbit anti-NCAM serum followed by [^{125}I]protein A. In lanes *2–4*, material from 4×10^5 cells was analyzed, in lanes *5* and *6*, 2×10^5 cell equivalents were loaded on each lane. TGFβ treatment was for 24 and 48 h for 3T3 and embryo cells, respectively.

mately one third of the NCAM protein expressed by N2A cells. Northern blot analysis showed that 3T3 cells expressed nearly exclusively the 6.1-kb mRNA species as could be anticipated from the immune blot data (see Fig. 8).

The levels of NCAM protein and mRNA in 3T3 cells were also affected by serum factors: serum-starved 3T3 cells contained around two- to threefold less NCAM protein per μg of total protein and two- to threefold lower levels of NCAM transcripts than cultures grown in 10% FBS (see Figs. 6, *A* and *B* and 8). By contrast, the pronounced dependence of NCAM expression on cell density observed in N2A cells was not seen in 3T3 cultures. In fact, the relative abundance of NCAM protein and mRNA was very similar whether cells seeded at the same density were grown for 1, 2, or 3 d in 10% serum (data not shown). This result fits in with the fact that we could not detect an autocrine effect on NCAM expression mediated by 3T3 cell-conditioned medium. 3T3 cells did, however, respond to N2A cell-conditioned medium with a very similar increase in NCAM transcript expression as did N2A cells themselves (Fig. 3, lanes *c* and *d*). Hence, the factor(s) released into the medium by N2A cultures also affect(s) NCAM expression by a different cell type.

Modulation of NCAM Expression in NIH 3T3 Cells by TGFβ

We tested a variety of pure substances known to affect growth and differentiation for their ability to restore NCAM protein levels in serum-starved N2A and 3T3 cells. The cells were cultured up to 80% confluency in standard growth medium, then switched to medium containing either low serum (0.2 or 0% for N2A and 3T3 cells, respectively) or 10% serum, which was supplemented with various agents. We tested a variety of growth factors (PDGF, bFGF, EGF, insulin, and TGFβ) and agents with effects on the state of cellular activity or differentiation (retinoic acid and PMA). In N2A cells, none of these agents added for up to 48 h changed the steady-state levels of NCAM protein. In addition, NGF in concentrations from 2 to 8 nM was without effect on NCAM expression (data not shown). In serum-starved NIH 3T3 cells by contrast, TGFβ restored the NCAM levels to those measured in complete medium. All other agents tested had no or marginal effects (Table I).

The response to TGFβ was half-maximal at 18 pM, in good agreement with a dissociation constant of 26 pM determined for the interaction of TGFβ with its receptor on 3T3 cells (Massagué and Like, 1985) and reached a plateau at ~100 pM (Fig. 5). The stimulation of NCAM protein expression by TGFβ was not yet apparent after 8 h; after 15 h in the presence of the agent, the increase in anti-NCAM antibody binding averaged 94% over that measured in its absence (Fig. 6 *A*). TGFβ was also capable of stimulating NCAM expression in cultures grown in complete medium on top of the increase elicited by serum alone. A similar increase in NCAM protein content in the presence of TGFβ could also be demonstrated by immune blot analysis (Fig. 4, lanes *3* and *4*). Since in this experiment the same number of cells from treated and untreated cultures was analyzed, TGFβ augments NCAM protein levels not only relative to total protein content but also on a per cell basis.

In the experiments shown in Fig. 6, 3T3 cells in complete growth medium were switched to TGFβ-containing medium with or without serum. Very similar results were obtained when quiescent cultures, serum-starved for 48 h, were stimulated with TGFβ alone or with TGFβ plus 10% FBS (Fig. 7). In this type of experiment, TGFβ increased NCAM protein levels already after 8 h. Again, TGFβ or 10% FBS alone stimulated NCAM protein expression to a very similar ex-

Table I. Effect of Various Agents on NCAM Protein Levels in 3T3 Cells

	Antibody bound		
Culture condition	Exp. 1	Exp. 2	Exp. 3
		cpm/μg protein	
10% FBS	360	640	470
0% FBS	171	120	140
0.3 nM PDGF	200	230	260
20 nM EGF	210	150	180
2 nM bFGF	154	ND	190
100 nM insulin	230	ND	ND
0.2 nM TGFβ	343	730	305
20 nM PMA	ND	90	180
20 nM retinoic acid	ND	130	170

3T3 cells were grown up to 80% confluency and the medium replaced for 24 h with fresh 10% FBS- or 0% FBS-containing medium supplemented or not in the latter case with the various agents at the concentration indicated. Cell lysates were dot blotted and assayed for total NCAM immunoreactivity as described in the legend to Figure 1.

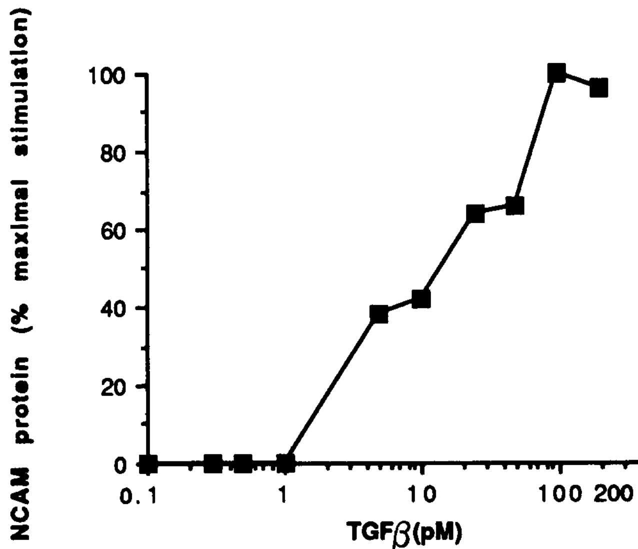


Figure 5. Dose response of the TGFβ effect on NCAM protein levels in NIH 3T3 cells. NIH 3T3 cells grown in 10% serum up to 80% confluency were switched to serum-free medium supplemented with 0.1 to 200 pM TGFβ. The cells were harvested 15 h later and the NCAM immunoreactivity measured by the dot-blot procedure. The results, in cpm/μg protein, are expressed as percent of maximum stimulation in the presence of 100 pM TGFβ. The 100% value corresponds to 850 cpm/μg protein.

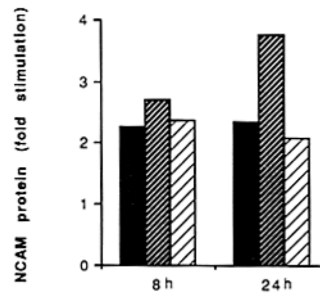


Figure 7. Stimulation of NCAM protein abundance in quiescent NIH 3T3 cultures by TGFβ and serum factors. Confluent 3T3 cultures made quiescent by serum starvation for 48 h were switched to medium containing 10% FBS with (▨) or without (■) 100 pM TGFβ or to serum-free medium supplemented with 100 pM TGFβ (▨). The cells were harvested after 8 or 24 h and NCAM immunoreactivity determined by the dot blot procedure. The results, in counts per minute/microgram protein, are the mean of determinations done on two parallel cultures (maximum variation between individual values was ±5%) and are expressed as fold stimulation of control values (225 cpm/μg protein) from untreated quiescent cultures. The control value did not vary over the time period studied.

tent, and the largest effect, a 3.8-fold stimulation of the control value in serum-free medium after 24 h, was produced by the combined action of serum and TGFβ.

The changes in NCAM protein content elicited by TGFβ were accompanied by changes of similar magnitude in NCAM mRNA levels. In the experiment shown in Fig. 8 *B*, treatment with 100 pM TGFβ for 8 h produced an approximate twofold increase in the relative abundance of NCAM transcripts in cells cultured in 10% serum and an approximate fourfold increase in serum-deprived cells. However, the kinetics of the effects on protein and mRNA levels differed. Stimulation of mRNA expression was detectable already after a 4 h exposure to TGFβ, peaked at around 8 h and was negligible after 24 h (Fig. 8 *A*). Together, serum factors and TGFβ were capable of modulating NCAM mRNA levels by a factor of five, the lowest values being recorded in cells serum-starved for 15 h, the highest ones after 8 h in the presence of serum and TGFβ (Fig. 6 *B*).

TGFβ appeared neither to affect cell viability nor to stimulate proliferation within the dose range and the times of exposure tested. This was confirmed by measuring the conversion of MTT to its formazan product (Mosmann, 1983) in parallel cultures. As shown in Fig. 6 *C* for a 15-h time point, formazin production was only slightly affected by the different culture conditions making it unlikely that changes in growth rate or metabolic state of the cells were responsible for the effects on NCAM expression.

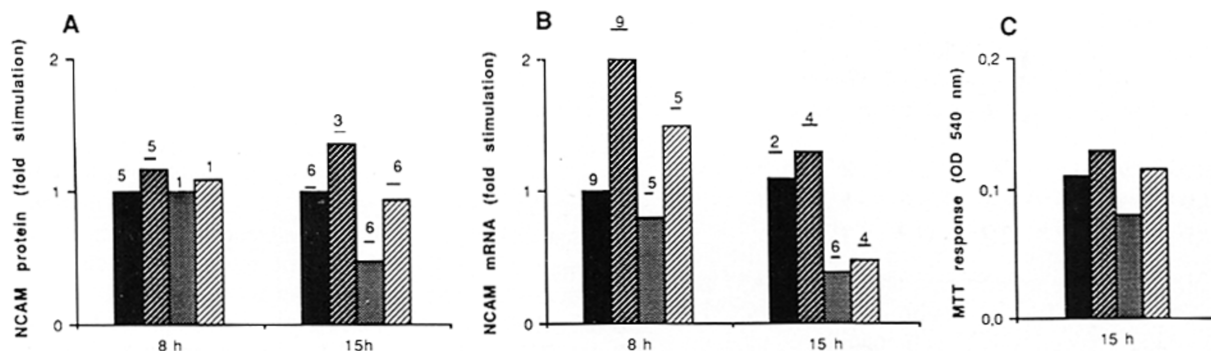
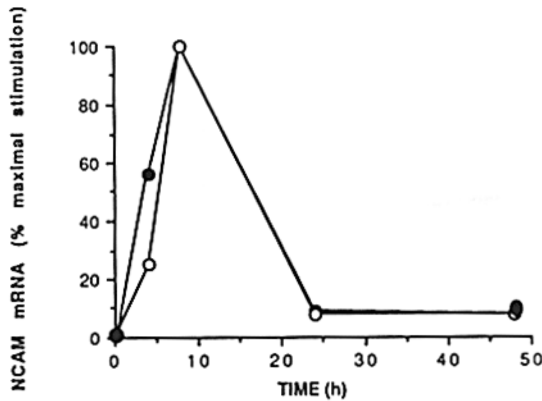


Figure 6. Stimulation of NCAM expression in NIH 3T3 cells by TGFβ. 3T3 cells were grown in 10% serum, then fresh 10% serum-containing (■, ■) or serum free medium (■, □) without (■, ■) or with (■, □) 100 pM TGFβ was added and the cells were harvested after 8 or 15 h as indicated. (*A*) Quantification of NCAM protein levels as measured by the dot blot procedure. The results, in counts per minute/microgram protein, are expressed as fold stimulation of the value measured in control cultures with 10% serum and without TGFβ, which was 515 ± 62 cpm/μg protein. (*B*) Quantification of NCAM mRNA levels as measured by densitometric scanning of autoradiographs of Northern blots. The results, in relative NCAM mRNA abundance after normalization relative to GAPDH mRNA, are expressed as fold stimulation of the value measured in the control cultures with 10% serum and without TGFβ. In *A* and *B*, the results are expressed as the mean + SEM (indicated by the bar) for the number of independent experiments given above. (*C*) Sister cultures maintained for 15 h in the various conditions were treated with MTT and the formazin production determined by measuring the absorbance at 540 nm as an indication of metabolic activity and cell density.



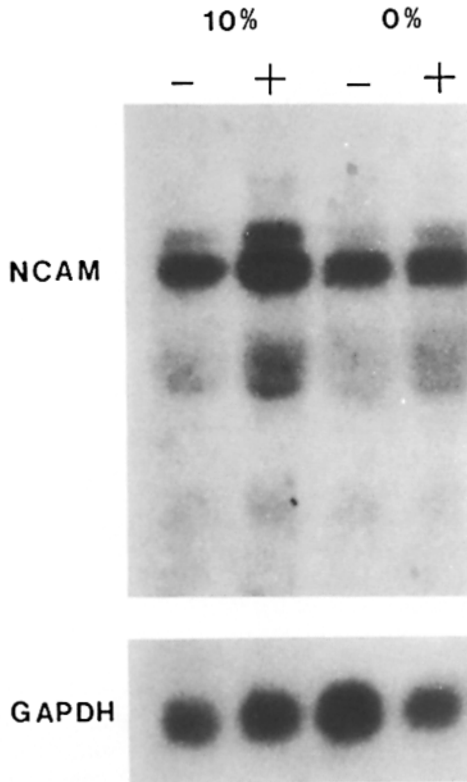


Figure 8. Time course of TGFβ-induced changes in NCAM mRNA abundance. (*A*) NIH 3T3 cells were cultured for various time intervals in 10% FBS-containing (●) or serum-free (○) medium with or without 100 pM TGFβ. Northern blots of total RNA were hybridized with NCAM and GAPDH probes. The autoradiographs were scanned and the densitometric values given by the 6.1-kb NCAM mRNA band normalized relative to the intensity of the GAPDH band revealed on the same blot. The results are expressed as the percentage of the maximum stimulation obtained after 8 h. The mean value from three independent experiments is given; the individual values differed by <±5%. (*B*) The autoradiograph of a typical Northern blot is presented for the 8-h time point. The crude and normalized values of the densitometric scanning are given below.

Table II. Actinomycin D Blocks the TGFβ Effect on NCAM Expression

Culture condition	Relative levels of NCAM mRNA − Actinomycin D	Relative levels of NCAM mRNA + Actinomycin D
10% FBS	0.11 ± 0.05	0.09 ± 0.04
10% FBS + 100 pM TGFβ	0.21 ± 0.09	0.08 ± 0.02

3T3 cells were grown to 80% confluency in 10% FBS. Then, new medium was added with or without 100 pM TGFβ and 2.5 μg/ml actinomycin D. The cells were harvested 6 h later. Results are the mean ± SD of three experiments.

The Effect of TGFβ on NCAM Expression Is Exerted at Least in Part at the Transcriptional Level

As a first step towards characterizing the mechanism whereby TGFβ increases NCAM mRNA levels, we sought to determine if transcription was required for the effect to occur and whether the stimulation of NCAM mRNA accumulation resulted from increased transcription of the gene or from increased stabilization of the mRNA. As shown in Table II, actinomycin D added together with TGFβ completely abrogated the response measured 6 h later without affecting the relative abundance of NCAM transcripts in the absence of TGFβ. Over this time period and at the dose used, actinomycin D did not appear to have any detrimental effect on cell viability (data not shown). Hence, the response to TGFβ requires the synthesis of new mRNA, either of transcripts that code for proteins which are intermediates in the chain of events leading to the increases in NCAM mRNA, or of NCAM mRNA itself (or both). We then measured the decay of NCAM mRNA in actinomycin D-treated cells in the presence and absence of TGFβ. Cultures in complete and serum-free medium were pretreated for 8 h with 100 pM TGFβ or not. Then, actinomycin D (5 μg/ml) was added to block transcription and the NCAM mRNA content determined by a dot-blot procedure. As shown in Fig. 9, NCAM mRNA levels declined with a very similar time course whether TGFβ was present or not. As a control, the GAPDH mRNA content was measured in the same blots after stripping of the NCAM probe; in accord with previous studies (Dani et al., 1984), the GAPDH transcripts decayed with a half-life of ∼8 h. We thus conclude that the increase in NCAM mRNA elicited by TGFβ does not result from stabilization of the message implying that the agent increases the transcriptional activity of the NCAM gene.

We turned to transfection assays using recombinant plasmids containing a previously identified transcriptional control region (Hirsch et al., 1990) from the 5′ end of the NCAM gene coupled to the CAT gene as indicator gene to demonstrate that TGFβ was indeed capable of stimulating transcription from the NCAM gene promoter. The constructs were transfected into 3T3 cells that were then either maintained in 10% FBS or shifted to low serum with or without 200 pM TGFβ and processed for determination of CAT activity 40 to 48 h later. A typical example is shown in Fig. 10. In three independent experiments, TGFβ stimulated CAT activity 1.95 ± 0.35- and 1.63 ± 0.09- (mean ± SEM) fold in the presence of low and high serum, respectively. The CAT expression directed by the NCAM promoter was also stimulated by serum, 2.90 ± 0.10 times as much enzyme activity being expressed in the presence than in the absence of 10% FBS. The combined action of serum and TGFβ produced a near fivefold change in CAT activity. The increases

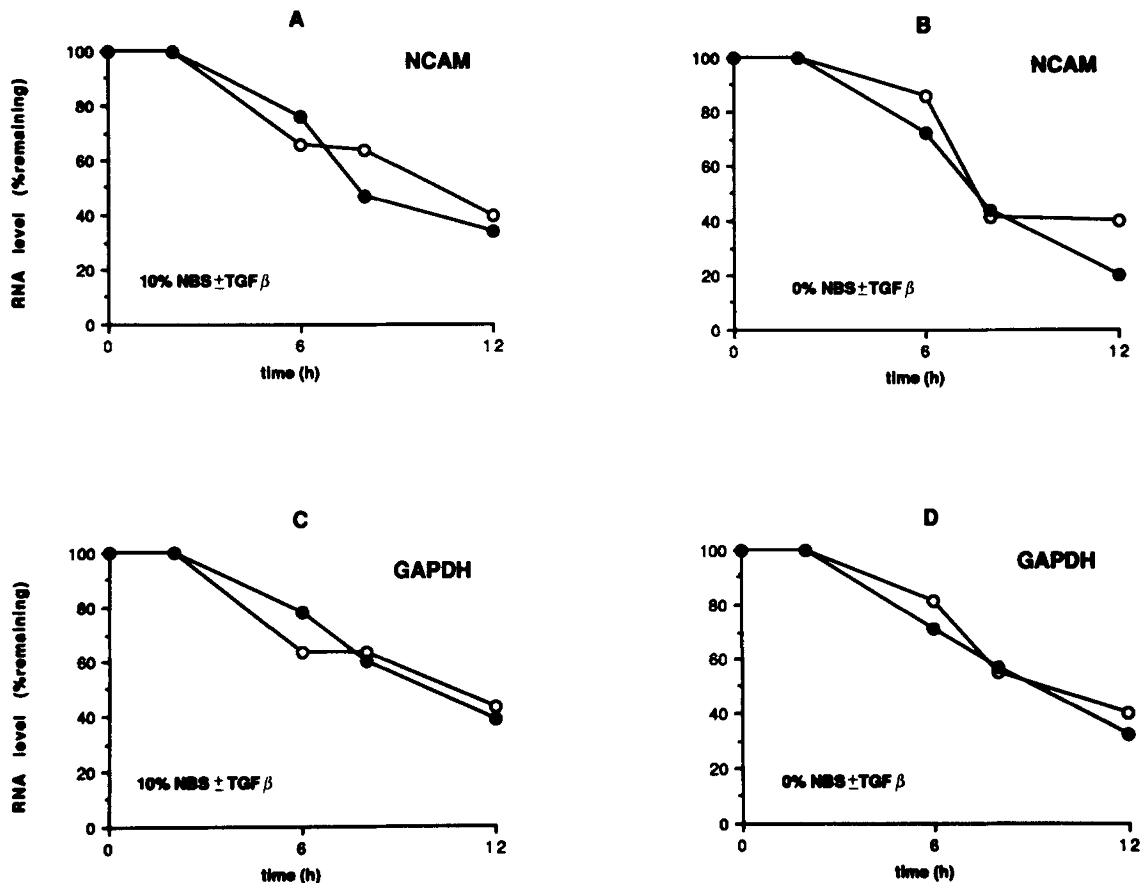


Figure 9. Effect of TGFβ on NCAM transcript stability in NIH 3T3 cultures. NIH 3T3 cells were cultured up to 80% confluency in 10% serum. Then, the cells were switched to 10% serum-containing (*A* and *C*) or serum-free (*B* and *D*) medium supplemented with 100 pM TGFβ (●) or not (○). 8 h later, 5 μg/ml actinomycin D was added and the cells harvested after the time intervals indicated. RNA was extracted and NCAM mRNA levels determined by dot-blot hybridization. As a control, the same blots were rehybridized with the GAPDH probe after stripping of the NCAM probe. The decay of NCAM and GAPDH mRNA levels was plotted as the percentage of specific mRNA remaining at the different times relative to the zero time sample, which was set at 100%.

in promoter activity elicited by TGFβ or serum were thus very similar to the magnitude of their effects on the steady-state levels of the mRNA suggesting that most if not all of the response to TGFβ and serum factors can be attributed to increased transcriptional activity of the NCAM gene.

TGFβ Increases NCAM Protein and mRNA in Early Passage Embryo Cells

We used first- and second-passage embryo cells prepared from 16-d-old mouse embryos as a first approach to test whether the TGFβ effect could also be observed in cells, which can be considered as reflecting more closely the situation in the embryo than the 3T3 line. When analyzed by immune blotting, first passage embryo cells expressed easily detectable levels of the 140-kD form of NCAM, which were increased by exposure to TGFβ (Fig. 4, lanes *5* and *6*). An increase in NCAM protein levels was first seen after 24 h in the presence of the agent; after 48 h, an over fourfold stimulation of NCAM protein content was observed (Fig. 11 *A*).

In Northern blots, the embryo-derived cultures were found to express mainly the 6.1-kb RNA with smaller amounts of the two transcripts of 2.7 and 4.8 kb that code for NCAM-120 (Fig. 11 *B*). Treatment with TGFβ produced an increase in the steady-state levels of the 6.1-kb message, whereas this effect was less obvious for the smaller transcripts. The results from several experiments of this type were quantified by densitometry and expressed relative to the GAPDH levels. A near twofold increase in the relative abundance of the 6.1-kb band was recorded after an 8-h exposure, somewhat less after 48 h. Clearly, early passage embryo cells respond to TGFβ with very similar increases in NCAM protein and mRNA as do 3T3 cells.

Discussion

To fulfill its supposed role as a morphoregulatory molecule (Edelman, 1985; 1988), NCAM must not only regulate, but also be regulated by cellular interactions. However, the mechanisms and in particular the extracellular factors involved in the control of NCAM expression have remained largely unknown. Thus far, the only published examples of defined, naturally occurring factors that affect NCAM expression are NGF and laminin. NGF has been reported to upregulate NCAM in PC12 cells concomitant with a shift in the relative proportion of NCAM isoforms (Prentice et al., 1987; Doherty et al., 1988). Still, control of NCAM levels

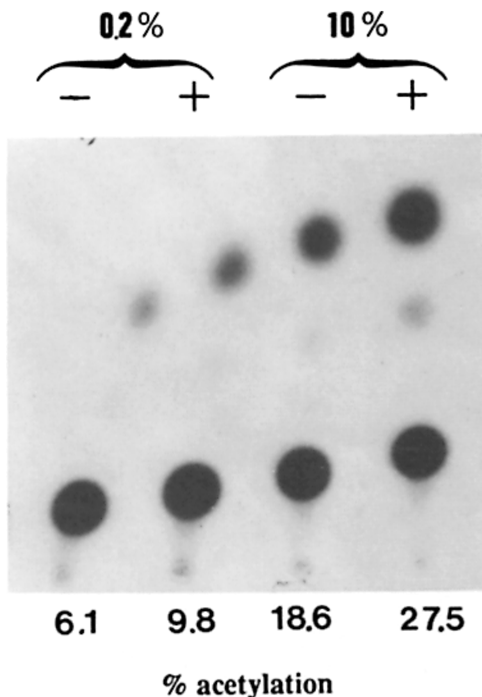


Figure 10. Effect of serum and TGFβ on NCAM promoter activity in 3T3 cells. The cells were transfected with 20 μg of a recombinant plasmid in which CAT gene expression is controlled by the sequence between positions −645 to −37 (relative to the initiation methionine) of the NCAM promoter (Hirsch et al., 1990). After transfection, the cells were pooled and redistributed into four flasks, one for each condition: medium containing 0.2 or 10% FBS and supplemented with 200 pM TGFβ (+) or not (−). The cells were harvested 40–48 h after transfection and CAT activity determined on aliquots containing equal amounts of protein. The autoradiograph of a thin layer plate is shown; below is given the percent conversion into acetylated [^{14}C]chloramphenicol derivatives. As a control, sister cultures were transfected with RSVCAT (Gorman, 1985); the CAT activity directed by this plasmid was not affected by TGFβ (not shown).

by NGF does not seem to be a general phenomenon; NGF had no effect on NCAM expression by PC12 cells in another study (Friedlander et al., 1986) and did also not affect NCAM levels in Schwann cells (Seilheimer and Schachner, 1987), although the adhesion molecule L1 was upregulated in both cell types. Laminin, when used as culture substrate, has been shown to change NCAM isoform expression by N2A cells (Pollerberg et al., 1986); however, the absolute levels of the NCAM forms have not been measured in this work. In this study, we have used two permanent cell lines to search for factors capable of modulating NCAM expression. In N2A and NIH 3T3 cells, NCAM expression was upregulated by serum and by a factor contained in N2A cell-conditioned medium. TGFβ was found to increase NCAM protein and mRNA levels in 3T3 and in early-passage fibroblastic cells from mouse embryos.

Serum increased the steady-state levels of NCAM protein and mRNA in N2A and 3T3 cells compared with total protein content and with the levels of GAPDH mRNA used as reference. The expression of the L1 adhesion molecule, by contrast, decreased in N2A cells grown in high serum, providing further evidence for the selective nature of the serum response. The increase in total NCAM message after addition of serum to serum-starved N2A cells can be entirely accounted for by increased levels of the 6.1-kb species that codes for NCAM-140 (Barbas et al., 1988). Since the different transcripts arise from a single gene by alternative splicing (Cunningham et al., 1987; Barbas et al., 1988), serum could act either by selectively increasing the stability of the 6.1-kb transcript, thus affecting both the ratio between the two mRNA species and the amount of total NCAM message, or by changing transcriptional activity and the mode of splicing via independent mechanisms. We favor the second possibility since serum factors are capable of stimulating NCAM promoter activity in 3T3 cells and since preliminary results suggest that this is also true for N2A cells.

The strong dependence of NCAM expression on cell density in N2A cultures suggested that the regulation of NCAM levels in these cells might also involve an autocrine mechanism. Indeed, exposure to medium conditioned over N2A cells resulted in increased steady-state levels of NCAM transcripts in both N2A and 3T3 cells. Previous studies have provided evidence that N2A cells produce and are responsive to PDGF (Van Zoelen et al., 1985). In our hands, however, PDGF was without effect on NCAM expression by N2A cells as were the other growth and differentiation factors tested. In any case, the putative autocrine factor cannot be TGFβ, which had no effect on N2A cells, and must be distinct from the serum factor, since it increased both the 6.9- and the 6.1-kb transcripts.

A major finding that emerges from our study is that TGFβ stimulated NCAM expression by 3T3 and normal mouse embryo cells at both the mRNA and protein levels. In 3T3 cells, where this has been investigated, the TGFβ effect required de novo mRNA synthesis and did not appear to be due to changes in mRNA stability. The obvious implication that TGFβ stimulates transcription is strongly supported by the finding that the factor increased the activity of the NCAM promoter in transfection assays. As shown recently (Rossi et al., 1988), TGFβ activates transcription from the collagen type I promoter, an effect mediated by a nuclear factor I-binding site. Interestingly, the NCAM promoter fragment used contains also a site to which this factor binds (Hirsch et al., 1990). We are currently investigating whether nuclear factor I is involved in the stimulation of the NCAM promoter by TGFβ.

TGFβ was the only defined factor tested that restored the reduced NCAM content of serum-starved 3T3 cultures. In spite of this, the serum stimulation of NCAM expression is probably not due to TGFβ, which might have been present in the serum, since the increases in NCAM mRNA abundance elicited by optimum doses of the factor were very similar whether serum was present or not. The two- to threefold increase in the levels of NCAM protein (over fourfold in the embryo cells) we observed with optimum doses of TGFβ may seem modest. However, comparable changes in NCAM concentration have been reported to result in large effects on adhesion (Hoffman and Edelman, 1983) and on neurite outgrowth (Doherty et al., 1990), and an only twofold modulation in cell surface expression of NCAM may have important functional consequences.

Because of its stimulating effect on matrix deposition and stability and on the expression of the corresponding cellular receptors, TGFβ is supposed to increase the adhesive inter-

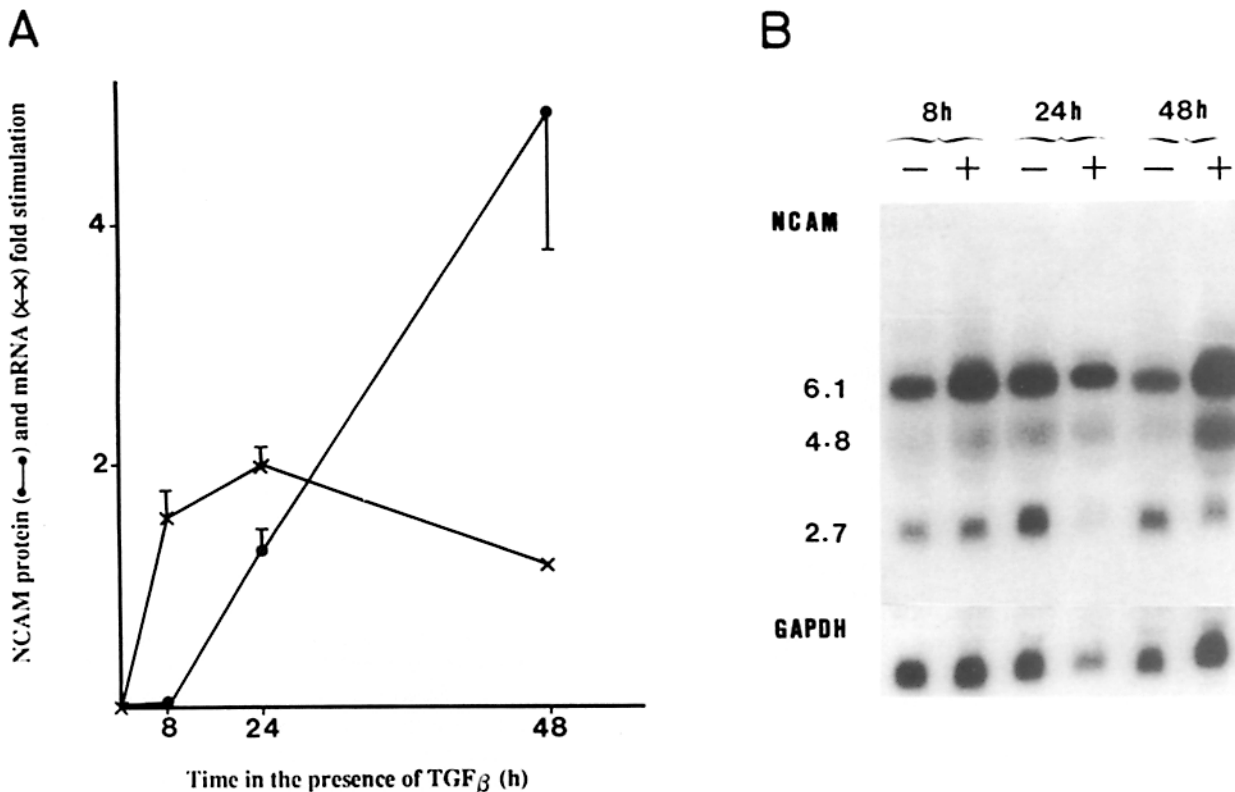


Figure 11. Effect of TGFβ on NCAM protein and mRNA abundance in first- and second-passage embryo-derived cells. (*A*) Time course of the changes in NCAM protein (●) and mRNA (×) levels. The cells were cultured for the time intervals indicated in the presence of 200 pM TGFβ. Control values in the absence of the agent did not vary over the time period considered. Protein levels were determined by scanning suitable exposures of immune blots like the one shown in Fig. 4; the results, in arbitrary units, were corrected with respect to total protein loaded on each lane and expressed as fold stimulation of the value measured in the absence of TGFβ. Autoradiographs of Northern blots like the one shown in Fig. 11 *B* were scanned and the densitometric values given by the 6.1-kb RNA band normalized relative to the intensity of the GAPDH band; the values are expressed as fold stimulation of the value measured in the absence of the agent. Except for the 48-h time point, where only two experiments have been performed and the mean value is given, the values shown are the mean ± SEM for three independent experiments. *B* shows a typical Northern blot for first passage cells. In addition to the major 6.1-kb species, minor bands at 4.8 and 2.7 kb are also revealed.

actions between cells and their extracellular environment (Ignotz and Massagué, 1987; Edwards et al., 1987; Ignotz et al., 1987; Heino et al., 1989). The stimulation of NCAM expression we observe implies that the same factor may also enhance adhesion between cells. Since the effect on NCAM seems to be less lasting than the one on matrix deposition, one may speculate that increased cell–cell adhesion is an initial response to TGFβ, to be supplanted later by enhanced interaction with extracellular substrates. However, the distinction between cell–cell and cell–matrix adhesion molecules is not always sharp, and NCAM may mediate cell–matrix adhesion via its interaction with heparin and heparan sulfate (Cole and Akeson, 1989) in addition to promoting adhesion between cells.

The striking association of TGFβ with morphogenetically active regions and with the mesenchyme in areas of embryonic induction has led to the suggestion that TGFβ participates in shaping the basic organization and architecture of the embryo (Heine et al., 1987; Akhurst et al., 1989). Our results suggest that TGFβ may do this in part by promoting the expression of NCAM. TGFβ_2 but not TGFβ_1 has been shown to have mesoderm-inducing activity in amphibian embryos (Rosa et al., 1988). In our hands, pure β_1 and β_2 types of TGF were about equally active in stimulating NCAM expression by 3T3 cells (results not shown), although we have done detailed dose-response and kinetic studies only with human platelet-derived TGFβ which should consist only of TGFβ_1 (Cheifetz et al., 1987).

Cellular and molecular mechanisms involved in embryogenesis may be reiterated during tissue repair in the adult. Indeed, a common theme underlying some of the diverse actions of TGFβ appears to be its function as a stimulator of repair and regeneration in response to injury (Sporn et al., 1987; Pierce et al., 1989; Rizzino, 1988). NCAM has been implicated in processes of regeneration in the peripheral nerve and the denervated muscle. Transecting or crushing the sciatic nerve stimulates NCAM protein expression in Schwann cells and fibroblasts as determined by immunocytology (Daniloff et al., 1980; Martini and Schachner, 1988) and denervation induces NCAM expression on the muscle fibers (Covault and Sanes, 1985; Rieger et al., 1985) and on interstitial fibroblasts (Gatchalian et al., 1989). These fibroblasts not only accumulate NCAM, but also fibronectin and tenascin, both of which have been shown to increase after exposure to TGFβ in other systems (Ignotz et al., 1987; Adams Pearson et al., 1988). TGFβ may thus function as one of the signals that mediate local stimulation of NCAM expression in muscle and nerve after injury.

Although we have no direct proof that TGFβ regulates NCAM expression in vivo, our results obtained with early-passage embryo cells strongly suggest that this factor participates in the control of NCAM expression in the embryo. TGFβ is certainly not the only factor involved in the regulation of NCAM expression as already indicated by the stimulatory effect of factors contained in serum and secreted by N2A cells. The identification of the extracellular signals that regulate NCAM levels in different cell types will be an important step towards unravelling the mechanisms by which cell-cell adhesion is regulated. Our evidence indicates that serum factors and TGFβ stimulate transcription from the NCAM promoter. It will now be possible to investigate the nuclear factors and their targets that are involved in this regulation.

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