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

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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) 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 non-myelinating 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-
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 (TGF/β) 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 TGF/β 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% CO₂ 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 scratching 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: TGF/β (ultrapure TGF/β from human platelets, Calbiochem-Behring Corp., La Jolla, CA), purified β₁ and β₂ subtypes of TGF/β 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, 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 formazan was measured at 540 nm with a microelisa reader (Multiscan; Titertek, Flow Laboratories S.A., Puteaux, France).}

**Immunochromical Detection and Quantification of NCAM and L1 Protein**

For immune blot analysis, NP-40 extracts were prepared and NCAM protein and L1 antigen revealed after electrophoresis and transfer to nitrocellulose by rabbit anti-NCAM antibodies and 125I-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 HC1 pH 8.5, 20 mM NaCl, 1 mM MgCl₂, 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 & Schuell, Dassel, FROI) 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 × 10⁶ cpm/ml) iodinated with [125I] to a specific activity of 20–30 × 10⁶ 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) antisera (Gennarini et al., 1986) and of the rabbit anti-L1 antisera (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 antisera recognized only an 180-kD band. As shown by immunofluorescence experiments, the antibodies did not stain NCAM-negative cells and reacted only with perinuclear 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 × 10⁶ 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 (DWSLE; 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 the comigrating bands in the different cell types were assigned accordingly.
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 antisera diluted 1:500 and bound antibody was revealed with [125I]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 × 10^5 cells per 25-cm² 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 tRNA. The intensity of the bands was quantified by densitometric scanning of the autoradiographs. The values are given below the autoradiographs in arbitrary units.

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 pRSV-CAT (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 harvested and lysis in 0.1% deoxycholate. (B) 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 antisera diluted 1:500 and bound antibody was revealed with [125I]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 × 10^5 cells per 25-cm² 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 tRNA. The intensity of the bands was quantified by densitometric scanning of the autoradiographs. The values are given below the autoradiographs in arbitrary units.
**Figure 2.** Effect of cell density on NCAM levels in N2A cells. N2A cells were plated at various cell densities (0.1-1.5 $\times 10^6$ 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 $\mu$g 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.

**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-
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 were fractionated by SDS-PAGE and transferred to nitrocellulose. NCAM-immunoreactive bands were revealed by rabbit anti-NCAM serum followed by [32P]protein A. In lanes 2–4, material from $4 \times 10^5$ cells was analyzed, in lanes 5 and 6, $2 \times 10^5$ cell equivalents were loaded on each lane. TGFβ treatment was for 24 and 48 h for 3T3 and embryo cells, respectively.

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 [32P]protein A. In lanes 2–4, material from $4 \times 10^5$ cells was analyzed, in lanes 5 and 6, $2 \times 10^5$ cell equivalents were loaded on each lane. TGFβ treatment was for 24 and 48 h for 3T3 and embryo cells, respectively.

Approximately 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 $\mu$g 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 $\sim 100$ 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

| Culture condition | Exp. 1 | Exp. 2 | Exp. 3 |
|-------------------|-------|-------|-------|
| 10% FBS           | 360   | 640   | 470   |
| 0% FBS            | 171   | 120   | 140   |
| 2 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.

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

| Culture condition | Relative levels of NCAM mRNA |
|-------------------|-----------------------------|
| 10% FBS           | 0.11 ± 0.05                 |
| 10% FBS + 100 pM TGFβ | 0.21 ± 0.09                 |

3T3 cells were grown to 80% confluence 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
in promoter activity elicited by TGFB 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 TGFB and serum factors can be attributed to increased transcriptional activity of the NCAM gene.

**TGFB 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 TGFB 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 TGFB (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 TGFB 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 TGFB 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...
harvested 40-48 h after transfection and CAT activity determined by TGF-β (not shown).

Effect of serum and TGF-β 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 TGF-β (+) 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 [14C]chloramphenicol derivatives. As a control, sister cultures were transfected with RSVCAT (Gorman, 1985); the CAT activity directed by this plasmid was not affected by TGF-β (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. TGF-β 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 TGF-β, 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 TGF-β stimulated NCAM expression by 3T3 and normal mouse embryonic cells at both the mRNA and protein levels. In 3T3 cells, where this has been investigated, the TGF-β effect required de novo mRNA synthesis and did not appear to be due to changes in mRNA stability. The obvious implication that TGF-β 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), TGF-β 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 TGF-β.

TGF-β 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 TGF-β, 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 TGF-β 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, TGF-β is supposed to increase the adhesive inter-
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, but not TGFβ, has been shown to have mesoderm-inducing activity in amphibian embryos (Rosa et al., 1988). In our hands, pure β1 and β2 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 (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 immunocytochemistry (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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