The effects of the *neuN* and *neuT* genes on differentiation and transformation of mammary epithelial cells

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**SUMMARY**

Overexpression of the proto-oncogene product, p185<sup>neuN</sup>, in a non-tumorigenic mammary epithelial line (31E) facilitates aspects of lactogenic differentiation. Formation of branching cords and induction of β-casein synthesis by 31E cells normally require co-culture of these cells with fibroblasts, or the presence of collagen or fibronectin. In contrast, 31E cells expressing p185<sup>neuN</sup> spontaneously form branching cords when grown on tissue culture plastic and can synthesize β-casein in the absence of exogenous substrates or feeder layers. Under these conditions, the cells deposit laminin and fibronectin, indicating a possible role for p185<sup>neuN</sup> in the deposition of extracellular matrix proteins. Overexpression of the corresponding oncogene product, p185<sup>neuT</sup>, has markedly different effects. Expression of p185<sup>neuT</sup> does not facilitate the formation of branching cords or the synthesis of β-casein when grown on tissue culture plastic, although these cells do deposit laminin and fibronectin. Confocal microscopy indicates a significant difference in the distribution of laminin and fibronectin in 31E cells expressing p185<sup>neuT</sup> compared to those expressing p185<sup>neuN</sup>

The effects of p185<sup>neuN</sup> and p185<sup>neuT</sup> expression on cell transformation depend on cell type. Expression of both p185<sup>neuN</sup> and p185<sup>neuT</sup> increases anchorage-independent growth of 31E cells, but only p185<sup>neuT</sup> induces anchorage-independent growth of NIH 3T3 fibroblasts. This lineage specificity in the action of p185<sup>neuN</sup> may be related to observations that overexpression of p185<sup>c-erbB-2</sup> (the human homologue of p185<sup>neuN</sup>) is only associated with the development of human epithelial cancers. The effects of p185<sup>neuN</sup> on laminin deposition by 31E cells may be relevant to the transforming ability of p185<sup>neuN</sup>, since laminin can induce anchorage-independent growth of mouse mammary cells. These results suggest that p185<sup>neuN</sup> and p185<sup>neuT</sup> could exert their effects on differentiation and transformation of mammary epithelial cells in part by promoting the deposition of extracellular matrix proteins.

**Key words:** *neu*, mammary, differentiation, transformation, laminin, fibronectin

**INTRODUCTION**

Identification of proteins capable of modulating pathways of cell division and differentiation has been greatly facilitated by the realization that the normal cellular counterparts of oncogenes often play important roles in the function of untransformed cells. Many of these cellular genes (proto-oncogenes) have been found to encode proteins that are involved in the cascade of events that occur in the cell following stimulation with hormones or growth factors (reviewed by Bishop, 1991). Of these genes, a limited number have been associated with particular aspects of developmental regulatory mechanisms (Bishop, 1991). However, in most instances, the role of proto-oncogenes in normal development remains unknown.

One protein of considerable interest in both developmental biology and studies on neoplasia is the *neu* (*c-erbB-2*) receptor tyrosine kinase, the first described receptor for ligands of the *neu* differentiation factor/heregulin/glial growth factor/ARIA family (Wen et al., 1992; Holmes et al., 1992; Marchionni et al., 1993; Falls et al., 1993), henceforth termed neueregulin. *neu* was first isolated as an oncogene (*neuT*) from a rat neuroblastoma cell line (Schechter et al., 1984) and found to encode a 185 kDa transmembrane glycoprotein, p185<sup>neuT</sup>, which possesses intrinsic protein tyrosine kinase activity (Stern et al., 1986; Bargmann et al., 1986a), *neuT* arose from the proto-oncogene *neuN* by a single point mutation that resulted in an amino acid substitution (Val to Glu) in the transmembrane domain of the gene product (Bargmann et al., 1986b). This substitution increases protein tyrosine kinase activity (Bargmann and Weinberg, 1988a,b), increases the rate of p185<sup>neu</sup> degradation (Stern et al., 1988) and alters the aggregation state of...
the p185 neu protein, favouring dimer formation (Weiner et al., 1989). Recent studies have suggested that p185 neu can be a receptor for at least one member of the neuregulin family, although the increasing complexity in this family for both ligands and relevant receptors makes it difficult at this point to assign ligands to specific receptors.

The pattern of p185 neu expression during normal differentiation has been the subject of a number of investigations. During rat embryonic development, p185 neu expression has been detected in the nervous system, connective tissue and secretory epithelium, whereas in adult rats, p185 neu expression is limited to the secretory epithelial tissues and basal cells of the skin (Kokai et al., 1987; Cohen et al., 1989). It has also been shown that neuregulin can induce differentiation of human breast cells in vitro (Wen et al., 1992).

Amplification of the human homologue of neuN (c-erbB-2) occurs with high frequency in many human adenocarcinomas, which are tumours derived from secretory epithelial cells (Slamon et al., 1987, 1989; Berger et al., 1988; Van de Vijver et al., 1987; Yokota et al., 1986; Venter et al., 1987; Varley et al., 1987). There is a significant correlation between overexpression of p185 neu in human breast and ovarian adenocarcinomas and poor prognosis (Slamon et al., 1989). Studies using cells in culture have shown that overexpression of either p185 neu or p185 neu-2 in NIH 3T3 fibroblasts is associated with promotion of anchorage-independent growth, again suggesting a role for these proteins in cellular transformation (Bargmann et al., 1986b; Di Fiore et al., 1987; Hudziak et al., 1987; Kokai et al., 1989).

Despite the increasingly documented evidence for a role of overexpression of p185 neu in human tumours, attempts to demonstrate that the rodent counterpart of this gene product (p185 neuN) can contribute to neoplastic transformation have been largely unsuccessful. Overexpression of p185 neuN was without effect in the studies using NIH 3T3 fibroblasts mentioned above, whereas overexpression of either p185 neu or p185 neu-2 was associated with promotion of anchorage-independent growth (Bargmann et al., 1986b; Di Fiore et al., 1987; Hudziak et al., 1987; Kokai et al., 1989). Thus, it is not clear at present whether p185 neu and p185 neu-2 have fundamentally different effects from p185 neuN on cellular properties, or whether there is simply a difference in the thresholds that cells have for transformation by these proteins.

In an attempt to understand the possible role of p185 neuN and p185 neuT on differentiation and transformation of secretory epithelial cells, we introduced the neuN and neuT genes into the mouse mammary epithelial cell line 31E (Reichmann et al., 1989). This is a spontaneously immortalized cell line derived from the mammary gland of mid-pregnant mice, and has retained many features corresponding to the organization and differentiated functions of mammary epithelial cells in vivo. Thus, when 31E cells are stimulated with lactogenic hormones, they synthesize the differentiation-specific product, β-casein (Reichmann et al., 1989). A prerequisite for this differentiation is the presence of fibroblasts or collagen type I gels. Coculture of 31E cells with fibroblasts and the culture of 31E cells on a collagen substrate both promote the formation of branching cords in which the deposition of laminin, the reorganization of intermediate filaments and the competence to produce β-casein coincide (Reichmann et al., 1989).

Overexpression of p185 neuN and p185 neuT had profound, but different, effects on the differentiation competence and growth characteristics of the 31E cells. We were particularly interested in the fact that expression of p185 neuN in 31E cells resulted in a different phenotype that had previously been observed only in the presence of fibroblasts or collagen type I gels, and this led us to investigate further the phenotype of these cells. The expression of p185 neuN by 31E cells induced the deposition of the two ECM proteins, laminin and fibronectin. These results are likely to shed significant light on the roles p185 neuN and p185 neuT in differentiation and transformation of mammary epithelial cells.

MATERIALS AND METHODS

Constructs

To facilitate conditional expression of p185 neuN and p185 neuT, the respective neu cDNAs were placed under the transcriptional control of the long terminal repeat (LTR) of mouse mammary tumour virus as shown in Fig. 1a. To construct the LTR neu plasmids, a 1.7 kb HindIII-Sal fragment of the LTR of mouse mammary tumour virus was first cloned into pPolyIII (Lathe et al., 1987). Subsequently, HindIII/ecoRI fragments from the pSV2neuT and pSV2neuN plasmids (Bargmann et al., 1986a,b), consisting of neuT neuN cDNAs and splice and polyadenylation signals from SV40, were cloned into pPolyIII-LTR. The resulting plasmids were amplified and screened for correctly sized inserts.

Cell culture

Cell culture reagents were purchased from Gibco/BRL, UK. Cell lines were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum, penicillin (60 i.u./ml), streptomycin (100 µg/ml), EGF (10 ng/ml, Collaborative Research Inc, MA) and insulin (5 µg/ml, Sigma Chem. Co. UK).

Transfections

The LTRneu constructs were transfected into both 31E epithelial cells and NIH 3T3 fibroblasts. DNA transfection was carried out by the calcium phosphate precipitation technique (Wigler et al., 1977). Stably transfected cell lines were isolated by co-transfection of pSV2neo (Southern and Berg, 1982) or pY3 (Blockiger and Diggelmann, 1987) with the LTRneu constructs, and selection with either 1 mg/ml G418 sulphate (Gibco/BRL, UK) or 200 ng/ml hygromycin B (Calbiochem, USA). Resistant colonies were isolated after 2 weeks of selection, and tested for the presence and expression of the introduced plasmids.

β-Casein induction

Rat tail collagen type I (Sigma Chemical Co. UK) was dissolved at 2 mg/ml in 0.1% acetic acid. Collagen gel was prepared by addition of 1 part setting solution (7x RPMI, 50 mM NaOH, and 0.2 % NaHCO3) and 1 part DMEM to 8 parts 2 mg/ml collagen solution. The mixture was poured quickly into tissue culture dishes to form a 4 mm thick gel and left to solidify at 37°C for 30 minutes. Cells were plated on the collagen gels and induced with lactogenic hormones when they reached confluence. The induction medium consisted of DMEM supplemented with 5% FCS, 1 µg/ml hydrocortisone (Sigma Chemical Co. UK), 5 µg/ml bovine insulin, and 5 µg/ml ovine prolactin (Sigma Chemical Co. UK). The cultures were incubated for 3 days at 37°C, and lysed by addition of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 1 mg/ml aprotinin, 3 mM PMSF). All experiments were performed in triplicate.

Western blot analysis

The protein concentration of the cleared lysates was determined by
the method of Bradford (1976). The levels of p185\(^{\text{neoN}}\) and p185\(^{\text{neoT}}\) were determined after electrophoretically separating the proteins by 7% SDS-PAGE (Laemmli et al., 1970) and transferring the proteins to nitrocellulose (Schleicher & Schuell) using a semi-dry blotting apparatus. Non-specific binding was blocked with 0.5% gelatin in PBS and all subsequent steps were performed at 37°C. The antibody α21N was used to examine p185\(^{\text{neoN}}\) and p185\(^{\text{neoT}}\) expression and has been described in detail elsewhere (Gullick et al., 1987). The nitrocellulose blots were incubated for 1 hour in α21N antibody, diluted 1:1,000 in PTG (PBS containing 0.02% gelatin and 0.02% Triton X-100) and then washed five times for 10 minutes in PTG. Blots were then incubated for 1 hour with 1 μCi 125I-Protein A (30 mCi/mg Amersham, UK) in 10 ml PTG, and washed five times for 10 minutes with PTG. Blots were air dried and then subjected to autoradiography. The intensity of the bands was quantified by densitometric scanning using an LKB Filmscanner to determine peak heights.

The same method was used in the β-casein western blots, except that 11% SDS-PAGE gels were used, and the nitrocellulose was exposed to anti-β-casein antiserum (Reichmann et al., 1989), also diluted 1:1,000.

**Immunofluorescence**

Cells were cultured on sterile glass coverslips either uncoated or coated with collagen by soaking for at least one week in collagen (2 mg/ml). The coverslips were placed in 24-well tissue culture dishes (Nunc) and the trypsinized cell suspension was plated on top of the coverslips. The buffer used for washes and staining (Buffer I) was composed of Hanks’ balanced salts (Imperial Laboratories, UK), 5% donor calf serum (Gibco/BRL, UK), and 40 mM HEPES, pH 7.5. Cells were washed 5 times with buffer I and then incubated with 50 μl of antibody for 30 minutes in a humid chamber at room temperature. The antibodies used were rabbit anti-mouse laminin (Gibco/BRL, UK) diluted 1:50 and rabbit anti-mouse fibronectin (UCB bioproducts, Belgium) diluted 1:16. The cells were fixed using 1:1 acetone/methanol at −20°C and then kept at −20°C for at least 10 minutes. The cells were rehydrated and washed in buffer I and then incubated with 50 μl of FITC-conjugated donkey anti-rabbit (Amersham) diluted 1:100. The coverslips were washed in buffer I, and mounted in glycerol containing 2.5% 1,4-diazabicyclo-2.2.2 octane (DABCO, Sigma Chemical Co. UK) to prevent fading (Johnson et al., 1982). The coverslips were sealed to glass slides using nail varnish and the cells were visualized using a Zeiss Axioskop microscope fitted with a Bio-Rad confocal imaging system.

Throughout the study, the microscope (Axioskop, Zeiss, W. Germany) fitted with an infinity corrected, ×63, NA 1.4, planapochromatic objective and a 10× eyepiece was placed under the scanning, beam folding and confocal optics of an MRC 500 (Bio-Rad, UK). The images collected contained 393216 picture elements (pixels) in a matrix of 768×512 elements. The microscope and confocal visualization system were aligned so that >96% of all pixels had grey scale levels within 10% of the mean and >76% within 5% of the mean when a 2 mm fluorescein film was examined at a laser input of 0.05–0.1 mW and the image averaged over ten scans. The means of regulating the laser input and other details of the system have been described elsewhere (Entwistle et al., 1990; Entwistle and Noble, 1992a). When collecting data, the level of the laser illumination was set so that the level of fluorescent emission was not detectably saturated (Entwistle and Noble, 1992b) and the detector was set so that the relationship between the level of the incident illumination and the pixel grey level value recorded was both linear and continuous (Entwistle and Noble, 1990, 1992a). For the collection of images the diameter of the pinhole in front of the detector was set to approximately the same diameter as the Airy disk of the image (Entwistle and Noble, 1992a). The Z0.5 value for the vertical point spread function (an estimate of the thickness of the confocal section defined as the distance between points of half-maximal intensity in the image along the vertical axis when an infinitely thin object is examined), measured by examining scattered light, was 0.6–0.9 μm throughout the field and 0.7–0.8 μm over >80% of the field. In all image files the images represent the average of 30–120 passes of the laser and the background was measured and subtracted prior to processing. FITC images were collected using a fluorescein bandpass filter (filter set C: Entwistle and Noble, 1992b). Reflectance images were collected using a 488±5 nm bandpass filter and rotatable dichroic polarization selective filter (Melles Griot).

Basal staining was visualized by locating the position of the coverslip using the strong reflections of light from the glass/ mountant interface with the laser scanning confocal microscope in reflectance mode. Apical staining was visualized at a distance of 8.1 μm from the coverslip. This distance was selected because it gave a typical representation of the tops of the cells without showing the middles of the neighbouring cells. In all cases, different heights were examined to ensure that these images were representative of the population as a whole regardless of density.

**Anchorage-independent growth assays**

Anchorage-independent growth was determined by assessing the colony-forming efficiency of cells suspended in soft agar. All experiments were conducted using 60 mm tissue culture dishes containing a 6 ml cell-free feeder layer (0.5% agarose in DMEM supplemented with 10% FCS) and a 1 ml top layer (0.33% agarose in DMEM supplemented with 10% FCS) in which 10⁵ cells were suspended. The top layer also contained 50 nM dexamethasone (Collaborative Research Inc, MA) where indicated. Colonies were counted after 10 days.

**RESULTS**

The neuN and neuT genes under the control of the mouse mammary tumour virus long terminal repeat (Fig. 1a) were introduced into the mouse mammary epithelial cell line 31E (Reichmann et al., 1989) and into NIH 3T3 cells. Successful introduction of the LTRneu constructs was shown by Southern blotting, and expression of p185\(^{\text{neo}}\) was analysed in the presence and absence of dexamethasone. For each transfection 20 clones with various levels of p185\(^{\text{neo}}\) expression were used for further study, and the data presented here represent only a selection of the results obtained for the clones showing the highest levels of p185\(^{\text{neo}}\) expression. These clones are 31E/neuT14, 31E/neuT3, 31E/neuN2, 31E/neuN11, NIH/neuT7, NIH/neuT19, NIH/neuN19 and NIH/neuN17. Southern analysis confirmed that these were all unique clones, and this, together with the fact that the effects reported here are observed only upon addition of dexamethasone, would indicate that the results are genuine effects of expression of p185\(^{\text{neoN}}\) and p185\(^{\text{neoT}}\) and not due to peculiarities of individual clones.

**Expression of p185\(^{\text{neoN}}\) and p185\(^{\text{neoT}}\)**

The data in Fig. 1b illustrate the levels of p185\(^{\text{neoN}}\) and p185\(^{\text{neoT}}\) found in the absence and presence of dexamethasone in a sample of the transected cell clones used for subsequent experiments (the levels of p185\(^{\text{neo}}\) in 31E/neuT14, 31E/neuN11, NIH/neuT19 and NIH/neuN19 are shown). We attribute the p185\(^{\text{neo}}\) observed in the absence of dexamethasone to the expression of endogenous mouse p185\(^{\text{neo}},\) since there was no detectable increase above the levels detected in non-transfected cells. Expression of p185\(^{\text{neo}}\) in the transected cells was compared with that of a well studied cell line, DHRF/G-8.
Fig. 1. Constructs used in transfections, and levels of p185\textsuperscript{neuN} and p185\textsuperscript{neuT} in the transfected cell lines. (a) Schematic representation of LTR\text{neu} constructs. LTR denotes the long terminal repeat of mouse mammary tumour virus; neu, the rat cDNAs neuN or neuT; SV40, the splice and polyadenylation sites of simian virus 40. (b) Expression of p185\textsuperscript{neuN} and p185\textsuperscript{neuT} in the transfected 31E and NIH 3T3 cell lines. Cells were lysed after overnight treatment with 50 nM dexamethasone as described in Materials and Methods. Total cell lysate (100 µg protein) was loaded on polyacrylamide gels and transferred to nitrocellulose filters. Lanes labelled (+) refer to cells treated with dexamethasone, whereas (-) lanes denote cells grown in the absence of dexamethasone. DHFR/G-8 cells (D) express high levels of p185\textsuperscript{neuN} and were included as a control.

which was established by Hung et al. (1986) and is thought to possess approximately 10\textsuperscript{6} p185\textsuperscript{neuN} molecules per cell (Stern et al., 1986). The levels of p185\textsuperscript{neuN} and p185\textsuperscript{neuT} detected in the transfectants was further studied by laser scanning densitometry, and all the cell lines used possessed approximately 10\textsuperscript{6} p185\textsuperscript{neuN} or p185\textsuperscript{neuT} molecules per cell, a level similar to that observed for p185\textsuperscript{c-erbB-2} expression in human tumour cell lines in which the c-erbB-2 gene has been reported to be amplified and overexpressed (Kraus et al., 1987). The cell lines used for the subsequent experiments reported here are 31E/neuT14, 31E/neuN11, NIH/3T3 T9 and NIH/3T3 N9 (levels of p185\textsuperscript{neu} shown in Fig. 1b), and 31E/3T3 ME, 31E/neuN2, NIH/3T3 T7 and NIH/3T3 N7 (which possessed similar levels of p185\textsuperscript{neuT} or p185\textsuperscript{neuN}).

Effects of p185\textsuperscript{neuT} or p185\textsuperscript{neuN} expression on the morphological organization of 31E cells

Confluent cultures of untransfected 31E cells develop hemicysts or domes when cultured on plastic (Reichmann et al., 1989). When mixed with fibroblasts, they formed branching cords; formation of such branching cords has also been observed when 31E cells are grown in collagen (type I) gels (Reichmann et al., 1989). In contrast, 31E/neuN cells did not require the presence of fibroblasts or collagen to promote the formation of branching cords in vitro. When grown directly on tissue culture plastic, the 31E/neuN cells (31E/neuN2 and 31E/neuN11) formed branching cords that appeared to be very similar to those produced by untransfected 31E cells grown with fibroblasts (compare Fig. 2a with Fig. 2c). This effect was dependent upon the presence of dexamethasone. In contrast to 31E/neuN cells, 31E/neuT cells (31E/neuT14 and 31E/neuT3) grown on tissue culture plastic did not generate branching cords of cells. The 31E/neuT cells instead formed domes that were larger and less regular in shape than those observed in untransfected 31E cells (compare Fig. 2b with Fig. 2d).

In the absence of dexamethasone, both 31E/neuT and 31E/neuN cells resembled untransfected 31E cells, developing domes when cultured on plastic and forming branching cords of cells in the presence of fibroblasts, suggesting that the effects observed on addition of dexamethasone were due to overexpression of p185\textsuperscript{neuN} and p185\textsuperscript{neuT}.

Synthesis of β-casein

The caseins are well characterized markers of mammary epithelial differentiation, and β-casein is the predominant intracellular casein detected in extracts of mouse mammary cells (Durban et al., 1985). Untransfected 31E cells produce β-casein upon induction with lactogenic hormones only when cultured on collagen or when cultured in the presence of fibroblasts, but not when cultured directly on tissue culture plastic.

When cells were cultured on a collagen substratum, exposure to lactogenic hormones induced synthesis of β-casein in untransfected 31E cells, 31E/neuN and 31E/neuT cells (Fig. 3a). The levels of β-casein expression by different cell lines were compared using laser scanning densitometry, and the level of β-casein in 31E/neuT cells was 51% of that in control 31E cells, whereas the level of β-casein in 31E/neuN cells was 69% of that in control 31E cells.

When plated on tissue culture plastic, only 31E cells over-expressing p185\textsuperscript{neuN} were able to synthesize β-casein upon stimulation with lactogenic hormones. Neither control 31E cells nor 31E/neuT cells could be induced to synthesize β-casein on plastic (Fig. 3b).

Deposition of extracellular matrix proteins

In vivo, induction of mammary epithelial development by the mesenchyme is thought to be mediated through signals contained in the mesenchymal ECM that is deposited between the two tissues (Grobstein, 1954). At least one central component of the extracellular matrix appears to be laminin, since a laminin-rich basement membrane substratum facilitates production of β-casein by mammary epithelial cells under appropriate hormonal conditions in the absence of any additional extracellular matrix components and cell-cell contact (Streuli et al., 1991). The responsivity of 31E cells to lactogenic hormones in vitro requires the presence of mesenchymal cells or mesenchymal ECM proteins (Reichmann et al., 1989). The matrix molecules applied to 31E cells have been collagen and fibronectin, but it has been noted that the ability of 31E cells to synthesize β-casein occurs in association with the deposition of laminin by these cells (Reichmann et al., 1989).

The ability of 31E/neuN cells to synthesize β-casein when grown on tissue culture plastic suggested that these cells had either overcome the prerequisite for mesenchymal ECM or that the cells were depositing necessary ECM components themselves, and in this way had overcome requirements for the presence of exogenously produced matrix components. This
latter possibility was investigated by immunocytochemical staining of cells with antibodies against ECM proteins, and analysis of the stained cells by confocal microscopy. By using a confocal microscope it was possible to obtain accurate information about the localization of ECM components even when cells were growing on top of one another.

Laminin is the most abundant glycoprotein in basement membranes and has been shown to play a role in the differentiation of primary mouse mammary epithelial cells in culture (Li et al., 1987; Streuli et al., 1991), so the deposition of laminin by transfected and untransfected 31E cells was analysed. The results are summarized in Table 1 and the most significant results are shown in Fig. 4. In each case, laminin was detected as discrete fibrils between the cells. Untransfected 31E cells deposited laminin when maintained on a collagen substratum (Fig. 4a-c) whereas no laminin was detected when these cells were cultured on untreated coverslips (Table 1). In contrast, 31E/neuN and 31E/neuT cells showed a similar intensity of staining when grown on glass or on a collagen substratum, and the results are shown here on glass (Fig. 4e-l). The intensity of laminin staining was much stronger in 31E/neuN cells, and to a lesser extent in 31E/neuT cells, than was seen with untransfected 31E cells grown on collagen.

Examination of deposition of the ECM protein fibronectin, the predominant ECM component of mesenchymal tissues, also revealed striking effects of p185 neuN and p185 neuT overexpression on cellular phenotype, but now p185 neuN and p185 neuT had different effects on the 31E cells (Fig. 5 and 6).
Table 1. Analysis of extracellular matrix protein deposition transfected and untransfected 31E cell lines

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<th>Fibronectin staining</th>
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<td>Control 31E</td>
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Deposition of laminin and fibronectin was analysed as described in Materials and Methods, and the most important results are shown in Figs 4 and 5, respectively. The results are summarized here; ++, refers to strong staining; +, to clear staining; (+) indicates that a low level of staining was visible; and − indicates that there was no staining. +dex indicates the addition of 50 nM dexamethasone. Basal refers to staining at the coverslip; Middle to the staining 3.9 µm from the coverslip; and Apical to staining 8.1 µm from the coverslip. Collagen indicates collagen-coated coverslips, whereas glass indicates untreated coverslips.

Fig. 4. Analysis of laminin deposition in untransfected and transfected 31E cells. Control 31E cells (a-d), 31E/neuT (e-h) and 31E/neuN (i-l) cells were cultured on collagen-coated coverslips (a-d) or directly on glass coverslips (e-l). Cell culture medium was supplemented with dexamethasone (results without dexamethasone not shown). Immunofluorescence staining was performed after 5 days. Cells were incubated with rabbit antibody raised against laminin (diluted 1:50) for 30 minutes. After fixation, the cells were incubated with FITC-linked anti-rabbit antibody and the staining was visualized by confocal microscopy. (a, e and i) Basal staining; (b, f and j) staining in the middle of the cells, 3.9 µm from the coverslip; (c, g and k) apical staining 8.1 µm from the coverslip. (d, h and l) Reflectance images of the middle of the cells 3.9 µm from the coverslip. Bar, 25 µm.
Fibronectin was detected in untransfected 31E cells only when the cells were cultured on collagen-coated glass coverslips (Fig. 5a-c). The localization of fibronectin was basal, indicating the presence of fibronectin where the cells were in contact with collagen (Fig. 5a). No apical staining was detected (Fig. 5a). In contrast, 31E/neuN cells grown on untreated coverslips did show fibronectin staining (Fig. 5i-k). Moreover, fibronectin deposition was not concentrated at the basal layer (as it was in untransfected 31E cells grown on collagen) but was found distributed around the entire cell surface (Fig. 5i-k). The distribution of fibronectin in 31E/neuN cells was identical whether these cells were cultured on collagen or on glass.

When 31E/neuT cells were cultured on untreated coverslips, fibronectin was detected mainly on top of the cells and there was no staining on the basal surface of the cells (Fig. 5e-g). Thus the polarity of fibronectin deposition by these cells was the reverse of that expressed by untransfected 31E cells cultured on collagen. When 31E/neuT cells were cultured on collagen, fibronectin was detected mainly between the cells and some fibronectin was detected at both the apical and basal surfaces.

Although we were unable to detect synthesis of p185 neuN or p185 neuT in transfected cells in the absence of exposure to dexamethasone, we did observe differences between 31E cells and their transfected counterparts in the laminin and fibronectin staining seen in the absence of dexamethasone treatment (Table 1). There were two discrete patterns of staining observed in cultures grown in the absence of dexamethasone: labelling in most regions of a coverslip resembled the pattern observed in untransfected 31E cells, while in some regions the pattern of labelling resembled that observed in 31E/neuT cells grown in the presence of dexamethasone.

Studies on human neoplasias have previously indicated that over-expression of p185 neuN or p185 neuT in some cells even in the absence of exposure to dexamethasone.

**Anchorage-independent growth**

Studies on human neoplasias have previously indicated that over-expression of p185 neuN or p185 neuT is associated with neoplastic
progression (Slamon et al., 1989), and studies on experimental tumours have demonstrated the capacity of p185\textsuperscript{neuT} to function as a transforming gene (Bargmann et al., 1986b; Kokai et al., 1989). To extend these previous findings, we compared the effectiveness of p185\textsuperscript{neuN} and p185\textsuperscript{neuT} overexpression on promoting anchorage-independent growth of 31E mammary epithelial cells. The ability of transfected cells to display anchorage-independent growth was investigated by testing the cloning efficiency of the cell lines in semi-solid media. The results summarized in Table 2 show the percentage of seeded cells that gave rise to colonies in soft agar.

Expression of both p185\textsuperscript{neuN} and p185\textsuperscript{neuT} in 31E epithelial cells grown in the presence of dexamethasone resulted in anchorage-independent growth, although the efficiency of colony formation was lower in 31E/neuN cells than in 31E/neuT cells. The effects of p185\textsuperscript{neuN} on anchorage-independent growth were wholly dependent upon the presence of dexamethasone, while a small proportion of the p185\textsuperscript{neuT} cells were able to grow as anchorage-independent colonies even in the absence of this hormone.

In contrast to the promotion of anchorage-independent growth of 31E cells by both p185\textsuperscript{neuN} and p185\textsuperscript{neuT}, only p185\textsuperscript{neuT} was able to induce anchorage-independent growth of NIH 3T3 cells (Table 2). The lack of effect of p185\textsuperscript{neuN} on NIH 3T3 cells was not due to lack of expression, as both p185\textsuperscript{neuN} and p185\textsuperscript{neuT} were expressed at similarly high levels in both the 31E cells and the NIH 3T3 cells (see Fig. 1a).

The requirement for dexamethasone in promoting growth of transfected cells showed that the transfected cells had not been transformed spontaneously during the selection procedure, thus suggesting that the promotion of anchorage-independent growth was caused by the overexpression of p185\textsuperscript{neuN} and p185\textsuperscript{neuT}.

**DISCUSSION**

Our studies on the consequences of p185\textsuperscript{neuN} and p185\textsuperscript{neuT} overexpression in 31E mammary epithelial cells and in NIH 3T3 fibroblasts reveal striking differences between the effects of these two related proteins on cellular differentiation and have also demonstrated effects of these genes that appear to be relevant to both normal development and transformation. Overexpression of p185\textsuperscript{neuN} facilitated differentiation of 31E cells, in that formation of branching cords and induction of β-casein synthesis no longer required that cells be grown on ECM components. In contrast, such effects were not associated with overexpression of p185\textsuperscript{neuT}. Overexpression of either p185\textsuperscript{neuN} or p185\textsuperscript{neuT} promoted laminin and fibronectin deposition by 31E epithelial cells, but striking differences were observed between 31E/neuN and 31E/neuT cells in the localization of fibronectin. We also found that overexpression of either p185\textsuperscript{neuN} or p185\textsuperscript{neuT} promoted anchorage-independent growth of 31E cells, while only p185\textsuperscript{neuT} was able to promote anchorage-independent growth of NIH 3T3 fibroblasts.

It seems likely that one of the key factors involved in the effects of p185\textsuperscript{neuN} on differentiation of 31E cells was associated with induction of ECM production. 31E/neuN cells synthesized both laminin and fibronectin when grown on tissue culture plastic, unlike their untransfected counterparts. As untransfected 31E cells are only able to show these differentiation characteristics when grown on fibroblasts, collagen or laminin (Li et al., 1987; E. Lucassen and E. Reichmann, unpublished observations), the effects of p185\textsuperscript{neuN} on differentiation are likely to have resulted from the fact that 31E cells could now make these proteins themselves. One possible interpretation of these results is that p185\textsuperscript{neuN} plays a role in regulating the deposition of ECM during normal differentiation. It may be that overexpression of p185\textsuperscript{neuN} mimics ligand binding and in this way by-passes requirements for exposure to exogenously produced ECM components in the induction of secretory differentiation in vitro.

An alternative explanation for our results is that p185\textsuperscript{neuN} overexpression plays an indirect role in the differentiation of mammary epithelial cells by causing the transition of a subpopulation of epithelial cells to fibroblast-like cells, which could then trigger laminin deposition in the remaining epithelial cells. Such changes in phenotype have been reported in mammary epithelial cells in response to changes in hormonal status in the growth medium (Leppä et al., 1991). Moreover, it has recently been suggested that overexpression of c-fos in a mammary epithelial cell line can cause such a cell conversion (Reichmann et al., 1992). This explanation strikes us as unlikely, however, for several reasons. 31E cells overexpressing p185\textsuperscript{neuN} or p185\textsuperscript{neuT} were labelled by the ZO-1 antibody (which labels tight junctions; Anderson et al., 1988) and continued to express epithelial morphologies and uvomorulin protein (unpublished observations); in contrast, overexpression of c-fos was associated with the rapid loss of all such properties (Reichmann et al., 1992). In addition, we were unable to detect vimentin in the transfected 31E cells (unpublished observations).

If induction of laminin and fibronectin synthesis were responsible for the effects of p185\textsuperscript{neuN} on differentiation of 31E cells, then why was overexpression of p185\textsuperscript{neuT} also associated with the promotion of fibronectin and laminin deposition but...
not with the promotion of differentiation? This question is particularly intriguing in light of the observations that although 31E/neuT cells could not be induced to differentiate when grown on a substrate of tissue culture plastic (on which both fibronectin and laminin were produced), these cells could be induced to differentiate if they were grown on collagen. The failure of p185<sup> neuT </sup> to facilitate differentiation of 31E cells despite the presence of fibronectin and laminin may be related to the observation that, although fibronectin was detected on the apical surfaces of cells grown on tissue culture plastic, it was absent from the basal surface. 31E/neuT cells grown on collagen showed a partial distribution of fibronectin on the basal surfaces, suggesting that cultivation of these cells on collagen may restore a certain degree of polarity. It has previously been shown that competence to respond to lactogenic hormones is dependent on the development of a polarized cell morphology (Li et al., 1987; Streuli and Bissell, 1990). Studies utilizing permeable supports that allow access of nutrients, hormones and growth factors to the baso-lateral epithelial cell surface indicated that baso-lateral access is an important prerequisite for differentiated epithelial function (Strang et al., 1991; Simons and Fuller, 1985) and baso-lateral deposition of ECM proteins plays an important role in mammary epithelial differentiation (Parry et al., 1987). Thus the distribution of fibronectin may be significant in explaining the differing abilities of p185<sup> neuN </sup> and p185<sup> neuT </sup> in facilitating mammary differentiation.

Invoking polarized distribution of ECM components to explain our results may be less likely in the light of studies by Streuli et al. (1991) indicating that expression of milk proteins does not depend on morphological polarity. In this study, the authors demonstrated that E-cadherin and α6 integrin were distributed evenly around the surface of single cells that had been successfully induced to express β-casein, suggesting that the cells were not fully polarized. The results of Streuli et al. (1991) also indicate that the basement membrane directly regulates the synthesis of milk proteins in the mammary epithelium, and that integrins are involved in this process. Since the integrins analysed in this study (α6β1) are known to act as receptors for laminin and fibronectin, and tyrosine phosphorylation is known to play a role in the regulation of many extra-cellular matrix receptors (Buck and Horwitz, 1987), it will be interesting to determine the effects of p185<sup> neuN </sup> and p185<sup> neuT </sup> expression on this integrin-mediated signal transduction.

Along with their dissimilar behaviour in respect to facilitation of differentiation and localization of ECM protein, p185<sup> neuN </sup> and p185<sup> neuT </sup> also differed in their capacity to promote anchorage-independent growth of non-epithelial cells. Growth in semi-solid media was seen in 31E epithelial cells overexpressing p185<sup> neuN </sup>, but not in NIH 3T3 fibroblasts expressing similar amounts of this protein. In contrast, overexpression of p185<sup> neuT </sup> promoted anchorage-independent growth of both 31E cells and NIH 3T3 fibroblasts. There is a strong correlation between the ability of cells to grow in semi-solid medium and to form tumours in vivo (MacPherson and Montagnier, 1964), and our results suggest that p185<sup> neuN </sup> would only function as a transforming protein when expressed in appropriate lineages. Consistent with this view we have seen, following injection of p185<sup> neuN </sup>-expressing 31E cells and NIH 3T3 cells into a small number of nu/nu mice, that 31E cells overexpressing p185<sup> neuN </sup> gave rise to tumours after 35 days whereas no tumours were observed after injection of NIH 3T3 cells overexpressing p185<sup> neuN </sup> (unpublished observations). In contrast, overexpression of p185<sup> neuT </sup> was associated with tumour formation by both 31E cells and NIH 3T3 fibroblasts. The ability of p185<sup> neuN </sup> to elicit anchorage-independent growth of 31E cells is in contrast with previous suggestions, emerging from studies on fibroblasts, that only p185<sup> neuT </sup>, not p185<sup> neuN </sup>, has the capacity to cause cellular transformation (Bargmann et al., 1986b; Kokai et al., 1989). However, our results are consistent with previous indications that mammary epithelial cells and fibroblasts differ in their response to genes with transforming potential. For example, the mammary oncogene int-1 caused morphological transformation and tumorigenicity when expressed in mammary cell lines, but not when expressed in fibroblast cell lines (Rijswijk et al., 1987). In addition, when normal murine mammary epithelial cells were infected with a TGF-α retroviral vector, colony formation was induced in soft agar and the cells became tumorigenic in nude mice, whereas fibroblast cell lines such as Rat-1 and NIH 3T3 remained untransformed by the same procedure despite similar levels of TGF-α expression (McGeady et al., 1989). The p185<sup> neuN </sup>-induced deposition of laminin in 31E cells may be relevant to the transforming ability of p185<sup> neuN </sup> in 31E mammary epithelial cells, since other studies have shown that laminin can induce anchorage-independent growth of mouse mammary cells in semi-solid agar culture (Chou et al., 1989). Moreover, the cell-type specificity of action of p185<sup> neuN </sup> that we have observed is consistent with the many observations indicating that overexpression of p185<sup> neu </sup>-erbB-2 (the human homologue of p185<sup> neuN </sup>) is correlated with neoplastic transformation of human epithelial cells, but not other cell types, from a variety of different tissues (Slamon et al., 1989; Berger et al., 1988; Van de Vijver et al., 1987; Yokota et al., 1986; Varley et al., 1987).

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