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Nerve growth factor-induced changes in neural cell adhesion molecule (N-CAM) in PC12 cells

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The effects of nerve growth factor (NGF) on the expression of neural cell adhesion molecule (N-CAM) in PC12 cells were determined. A quantitative immunoassay was used to show that NGF induces a 4- to 5-fold increase in relative N-CAM levels over a 3-day period. This increase could not be mimicked by cholera toxin suggesting that it is not a simple consequence of morphological differentiation. The changes in N-CAM levels induced by NGF were accompanied by changes in N-CAM molecular forms. The 140-kd N-CAM species is the major N-CAM expressed by naive PC12 cells, while NGF-treated cultures express N-CAM species of 180 kd and 140 kd. Northern analysis showed that naive cells express a 6.7-kd N-CAM mRNA species only, while NGF-treated cultures express both a 6.7-kb and a 7.2-kb transcript. As the 6.7-kb and 7.2-kb mRNAs are alternative spliced transcripts of a single gene, this result shows that NGF can activate a neuron-specific splicing mechanism. This is the first description of control of N-CAM expression by a growth factor.

Key words: nerve growth factor/N-CAM/alternative splicing/PC12 cells

Introduction

Neural cell adhesion molecule (N-CAM) is a cell surface glycoprotein expessed in a variety of cell types, including neurons, glia and skeletal muscle (Edelman, 1984; Rutishauser and Goridis, 1986). It has been implicated in a variety of events in the development of the nervous system, each involving cell–cell recognition processes (Edelman, 1984; Rutishauser, 1984). In the nervous system N-CAM exists as three heavily glycosylated polypeptide chains of 180 000, 140 000 and 120 000 (N-CAM-180, N-CAM-140, N-CAM-120) that are differentially expressed during development and also in specific cell types (Rutishauser and Goridis, 1986). It has been shown that the main difference in size between the different N-CAM isoforms resides in the size of the cytoplasmic C-terminal region (Cunningham *et al.*, 1983; This suggests that the extracellular portions of the molecule may be identical although this has not been formally proven. To approach these questions a number of N-CAM cDNA clones have been isolated and analysed from chick (Murray *et al.*, 1984, 1986a,b), mouse (Goridis *et al.*, 1985; Gennarini *et al.*, 1986; Nyugen *et al.*, 1986) and human (Walsh *et al.*, 1986) tissues. In all three species it seems likely that N-CAM is a single copy gene residing on chromosome 9 in the mouse (D'Eustachio *et al.*, 1985) and chromosome 11 in humans (Nguyen *et al.*, 1986; Walsh *et al.*, 1986). In the chick nervous system it has been shown that diversity of N-CAM species is generated by alternative splicing (Murray *et al.*, 1986a,b); a 7.2-kb mRNA transcript encodes N-CAM-180 while a 6.7-kb transcript encodes N-CAM-140. The 6.7-kb transcript is generated via splicing out of an exon that is present in the 3′ region of the 7.2-kb transcript (Murray *et al.*, 1986b). Interestingly, it is the 6.7-kb transcript that appears first during nervous system development, while the 7.2-kb transcript does not appear until later, suggesting that neural development is accompanied by a neuron-specific splicing mechanism and subsequently the appearance of the 7.2-kb transcript and its product N-CAM-180 (Murray *et al.*, 1986b). To further analyse these transitions in N-CAM expression in individual neural cells it would be advantageous to have available *in vitro* model systems for study. One possibility would be to study neuroblastoma cells such as N2A that express N-CAM-180 and N-CAM-140 (Goridis *et al.*, 1985; Gennarini *et al.*, 1986; Pollerberg *et al.*, 1985, 1986). An alternative system is PC12 cells (Greene and Tischler, 1976) that differentiate in response to nerve growth factor (NGF) and also express N-CAM (Friedlander *et al.*, 1986). NGF treatment of PC12 cells is accompanied by extensive neuritic outgrowth and extensive changes in gene expression which is dependent on new RNA synthesis (Burstein and Greene, 1978).

In the present study we have determined whether there are qualitative and quantitative changes in N-CAM protein and mRNA species in response to NGF treatment. We show that NGF treatment leads to increases in N-CAM protein levels, the appearance of a 7.2-kb N-CAM mRNA and the concomitant appearance of N-CAM-180. This is the first report of a growth factor controlling expression of N-CAM and these positive results contrast with a previous study (Friedlander *et al.*, 1986) that did not show

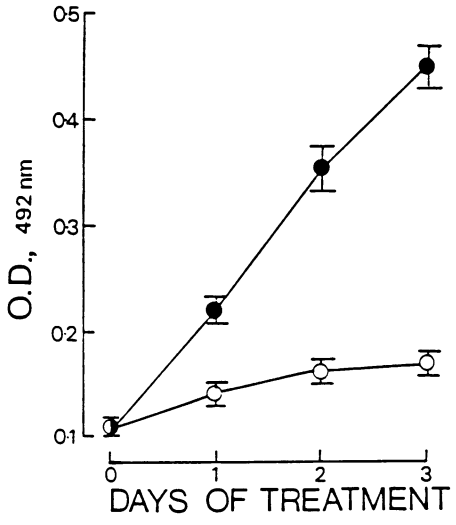

Fig. 1. PC12 cells were grown in 50 ng/ml NGF for up to 3 days. The graph shows the absolute binding of anti-N-CAM to the cultures of NGF-treated (●) and also naive (○) cells. Anti-N-CAM binding was as described in Materials and methods and each value is the mean ± SE of four independent observations with conjugate control subtracted. The latter was determined by omitting anti-N-CAM from the reaction sequence.

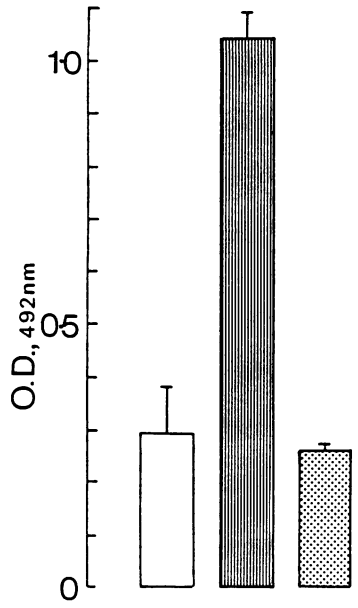

Fig. 2. PC12 cells were grown in SATO media (□). SATO media supplemented with 50 ng/ml NGF (▥) or, alternatively, SATO media supplemented with 100 ng/ml cholera toxin (▨). After 48 h the cultures were fixed and N-CAM levels determined as in Figure 1. Each value is the mean + SE of six independent determinations.

any changes in N-CAM in another commonly available line of PC12 cells.

Results

NGF-induced changes in N-CAM expression in PC12 cells

PC12 cells were grown in the presence of 50 ng/ml NGF for up to 5 days. As found previously (Dickson *et al.*, 1986b; Doherty and Walsh, 1987), NGF induces extensive neurite outgrowth in the majority of PC12 cells over this time course. Enzyme-linked immunosorbent assay (ELISA) was used to quantitate relative N-CAM levels after NGF treatment. Figure 1 shows that there is a rapid increase in relative N-CAM levels upon NGF treatment. Significant increases can be measured after 24 h of culture and by 3 days this has risen to a 4- to 5-fold increase. NGF is anti-mitotic for PC12 cells (Greene and Tischler, 1976) and over the initial 3-day culture period we found no significant increase in cell numbers in NGF-treated cultures. Thus the increased accumulation of N-CAM can be considered to be an increase in N-CAM per cell rather than an increase in cell number. Several agents that either directly or indirectly increase cAMP levels can also promote morphological differentiation of naive PC12 cells (Gunning *et al.*, 1981; Richter-Landsberg and Jastorff, 1986; Doherty *et al.*, 1987; Doherty and Walsh, 1987). We therefore grew naive cells for 48 h in media supplemented with NGF (50 ng/ml) or cholera toxin (100 ng/ml). Over this time period both agents stimulate neuritic outgrowth to a similar

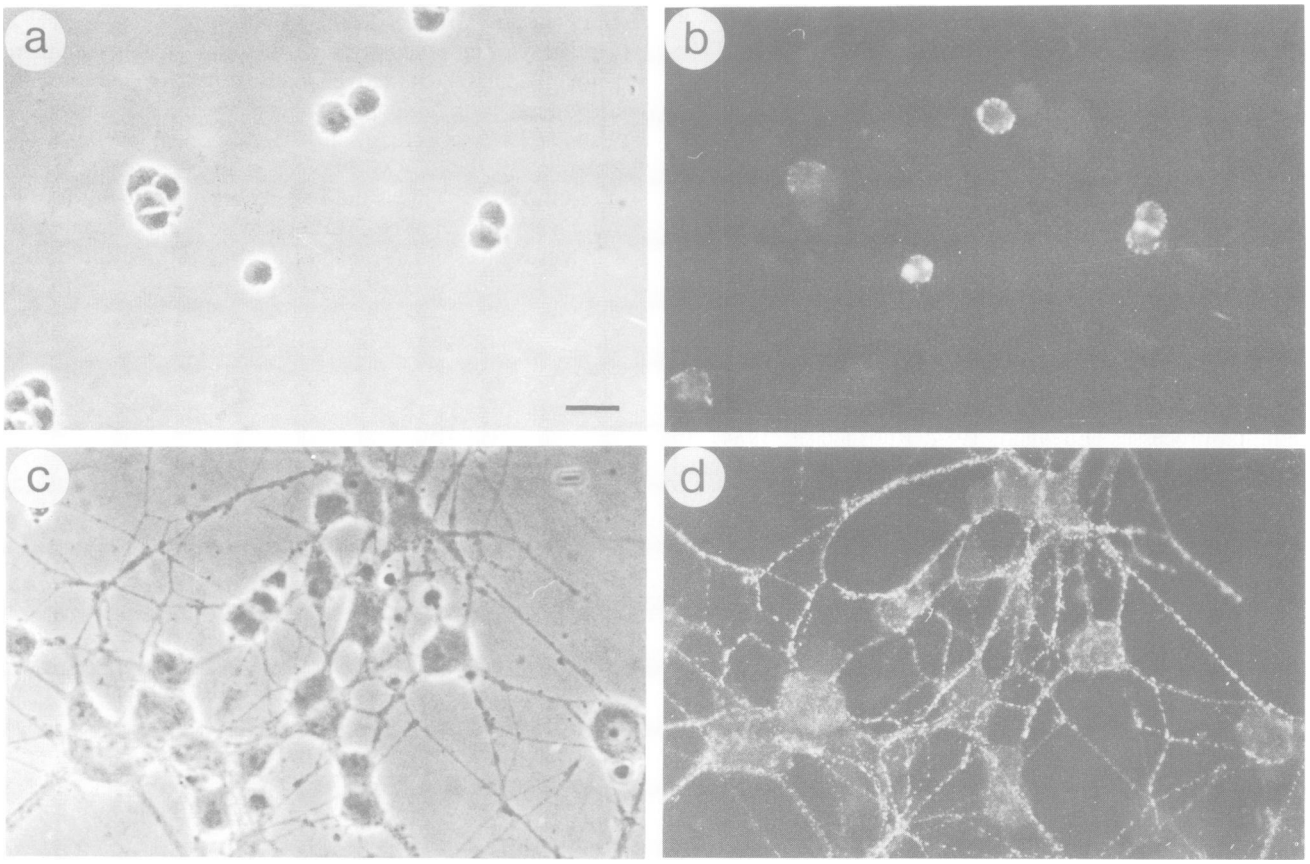

Fig.3. Expression of N-CAM in cell cultures of PC12 cells after NGF treatment. Indirect immunofluorescence was used to show the distribution of N-CAM in culture. Samples were **(a,c)** naive cells and **(b,d)** NGF-treated cells after 5 days of culture. Scale bar = 50 μm.

extent (Gunning *et al.*, 1981); however, as shown in Figure 2, increased expression of N-CAM is apparent only in NGF-treated cultures under conditions where cell numbers are identical.

Indirect immunofluorescence was used to localise N-CAM at the surface of PC12 cells (Figure 3). NGF induces extensive neurite formation in PC12 cells over a 5-day culture period. Naive PC12 cells express N-CAM all over the cell body. In NGF-treated cultures N-CAM was associated with neurites, and the cell bodies also remained strongly stained (Figure 3).

Induction of N-CAM-180 upon NGF treatment

To determine whether the NGF-induced changes in N-CAM levels were accompanied by changes in N-CAM isoforms, a Western blotting analysis was carried out. Figure 4a shows native and desialo N-CAMs in PC12 cells grown in culture in the presence and absence of NGF. Naive PC12 cells grown for 1 or 5 days in culture (Figure 4, tracks A and B) express only N-CAM-140 and after NGF treatment there is a specific induction of N-CAM-180 (Figure 4, tracks C–F) which is very prominent after 5 days. To show that the N-CAM protein band was authentic N-CAM-180 and not an abnormally sialylated N-CAM-140, we neuraminidase-treated cell extracts from cells treated with NGF for 5 days. Figure 4 track G shows that neuraminidase digestion generates desialo N-CAM and leads to the formation of N-CAM bands of lower mol. wt.

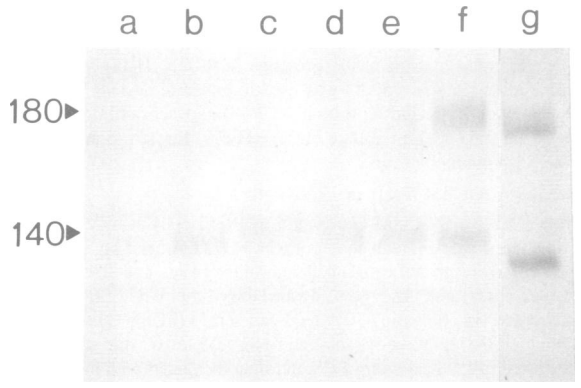

Fig. 4. Immunoblot analysis of N-CAM in cell cultures of PC12 cells. The figure shows PC12 cells before and after NGF treatment and also neuraminidase treatment. **Track A**, naive PC12 cells; **track B**, naive cells grown for 5 days. **Tracks C–F** show NGF-treated cultures grown for **(C)** 1 day, **(D)** 2 days, **(E)** 3 days or **(F)** 5 days. **Track G** shows the effect of neuraminidase digestion on a 5-day NGF-treated culture. Mol. wt markers are given in kilodaltons (kd).

Fig. 5. Northern blot analysis of N-CAM mRNA in naive and NGF-treated PC12 cells. Naive PC12 cells **(track 1)** or after 5 days of treatment with NGF **(track 2)** were harvested and mRNA isolated. mRNA samples were processed for Northern blot analysis [2 μg poly(A)$^+$ RNA per track] and hybridized to the N-CAM cDNA probe pM1.3. Filters were autoradiographed at −80°C in the presence of intensifying screens.

NGF specifically induces a 7.2-kb N-CAM mRNA species

The previous experiments showed specific changes in N-CAM isoforms and N-CAM levels upon NGF treatment. To determine whether the induction of N-CAM-180 was accompanied by changes in N-CAM mRNA species, a Northern blot analysis was carried out. The N-CAM cDNA probe used in the present experiments was the mouse probe pM1.3 which reacts with bands of 6.7, 5.2 and 2.9 kb in rodent muscle (Covault *et al.*, 1986; Moore *et al.*, 1987) and additionally with a band of >7. kb in brain (Covault *et al.*, 1986; Gennarini *et al.*, 1986; H.Prentice, unpublished observations). Figure 5 shows that naive PC12 cells express a single 6.7-kb N-CAM mRNA band only, whereas NGF-treated cultures exhibit two bands of 6.7 and 7.2 kb. As equal amounts of poly(A) RNA were loaded in each track, then we conclude that NGF treatment of PC12 cells activates the expression of a 7.2-kb mRNA species. As the 6.7- and 7.2-kb transcripts are alternative spliced transcripts of a common gene, this result shows that NGF can regulate the splicing of the N-CAM gene and, in particular, can activate a neuron-specific splicing mechanism. Similar results were found using a human N-CAM cDNA probe (Walsh *et al.*, 1986; data not shown).

Discussion

PC12 cells grown in the absence of NGF are mitotic, do not express neurites or differentiated neural characteristics and resemble adrenal medulla chromaffin cells (Greene and Tischler, 1976). When exposed to NGF their mitotic index drops, neuritogenesis is initiated and a number of differentiated neural characteristics are expressed. NGF has been shown to increase the level of specific membrane glycoproteins in PC12 cells, such as the NGF-inducible large external glycoprotein (NILE, Salton *et al.*, 1983), which has recently been shown to be identical to neuronal glia adhesion molecule (Friedlander *et al.*, 1986) and L1 adhesion molecule (Bock *et al.*, 1985). Changes have also been found in Thy-1 antigen (Richter-Landsberg *et al.*, 1985; Doherty and Walsh, 1987) and its mRNA (Dickson *et al.*, 1986b). Only one previous study has analysed N-CAM expression in PC12 cells (Friedlander *et al.*, 1986). That study reported no change in N-CAM levels upon NGF treatment and, in addition, there was no shift in molecular forms; naive and NGF-treated PC12 cells expressed N-CAM-180 and N-CAM-140. The present study produced dramatically different results. We showed that NGF treatment was associated with a 4- to 5-fold increase in N-CAM surface protein levels, and an activation of the expression of N-CAM-180. This activation was specific to NGF and not simply a consequence of morphological differentiation since increasing cAMP levels by cholera toxin did not activate N-CAM-180. The activation of N-CAM-180 was also associated with the appearance of a 7.2-kb N-CAM mRNA which has been shown previously to encode N-CAM-180 (Murray *et al.*, 1986b). How could these two studies produce such different results? One possibility is the

nature of the PC12 cells used in each study, alternatively, culture conditions may influence the basal expression of N-CAM. The neuritic response of the PC12 cells in the study of Friedlander *et al.* (1986) bears little resemblance to the cells used in the present study. The cells used in the present study respond rapidly to NGF treatment and produce an extensive neuritic network (Doherty and Walsh, 1987; Doherty *et al.*, 1987). In addition, there is a dramatic 30-fold increase in the neurofilament protein levels (Doherty *et al.*, 1987) and a doubling of the level of Thy-1 antigen (Doherty and Walsh, 1987). In addition, we showed changes in the transcriptional level of the mRNA encoding the 68-kd neurofilament protein and Thy-1 antigen (Dickson *et al.*, 1986b). These studies clearly demonstrate that the line of PC12 cells used in the present study differentiate morphologically to the same extent as the parent line described by Greene and Tischler (1976).

Neural development *in vivo* is accompanied by the expression of specific N-CAM isoforms. N-CAM-140 appears to be first expressed at a variety of locations in the developing nervous system (Murray *et al.*, 1986b; Pollerberg *et al.*, 1985). It is only as neurons become more differentiated or post-mitotic that they acquire N-CAM-180 which also suggests non-coordinate regulation of these isoforms. Neural development is, therefore, accompanied by the sequential replacement of the molecular forms of N-CAMs and there are likely to be parallels with other well-defined systems such as muscle (Caplan *et al.*, 1983). Although the molecular transitions of the N-CAM family have been found *in vivo* there has been no evidence that similar transitions could occur in single neurons. N2A cells have been of value in analysing the mobility of N-CAM-180 in the lipid bilayer but these cells do not replicate the N-CAM transitions seen *in vivo* as they constitutively express N-CAM-180 and N-CAM-140 (Pollerberg *et al.*, 1986). Treatment with dimethyl sulphoxide however leads to down-regulation of N-CAM-140 and expression of only N-CAM-180 (Pollerberg *et al.*, 1986). These cells may therefore represent a later stage of neural maturation. The activation of N-CAM-180 in PC12 cells upon treatment with NGF and the continued expression of N-CAM-140 shows for the first time that this molecular transition can occur in single cells and implicates target-derived growth factors in the controlling mechanism. The effect is also specific to NGF treatment as increasing cytoplasmic cAMP levels did not increase the relative level of N-CAM expression. It is also likely that the 6.7- and 7.2-kb transcripts have different translational efficiencies, since a smaller amount of the 7.2-kb mRNA produces similar amounts of N-CAM-140 and N-CAM-180 and this should be studied further. Priming of PC12 cells involves the transcriptional activation of a set of gene products that subsequently allow a more rapid neuritic response (Burstein and Greene, 1978; Greene *et al.*, 1982). N-CAM-180 has been suggested to be involved in neurite extension (Pollerberg *et al.*, 1985) and the activation found in the present study is consistent with this proposal.

In the genome N-CAM is present at single copy level and diversity appears to be generated at the protein level by a process of differential splicing (Murray *et al.*, 1986a,b). N-CAM-140 is encoded by a 6.7-kb mRNA which is homologous to the 7.2-kb transcript that encodes N-CAM-180. The 7.2-kb transcript however contains an additional 3′ exon (Murray *et al.*, 1986b). Thus the acquisition of a differentiated neuronal phenotype is accompanied by the operation of this neuron-specific splicing mechanism. PC12 cells appear to be an ideal model system for analysing this splicing mechanism and its products further.

Materials and methods

Cell culture

PC12 cells (Greene and Tischler, 1976) were obtained from Dr L.Greene and were grown on collagen-coated tissue culture dishes in serum-free media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with progesterone, 0.062 mg/l; putrescine, 16.1 mg/l; thyroxine, 0.4 mg/l; selenium, 0.039 mg/l; triiodothyronine, 0.337 mg/l; insulin, 10 mg/l; transferrin, 100 mg/l. The media were further supplemented with 1% (v/v) Path-O-cyte 4 (bovine serum albumin solution from Miles Chemical Company). The 2.5S subunit of mouse NGF was a generous gift from Dr A.Leon (Fidia Research Laboratories, Abano Terme, Italy) and was added to cultures at a level of 50 ng/ml. Cultures were given 50% media changes every 48 h. Cholera toxin was obtained from Sigma Chemical Company.

Immunoblotting

Immunoblotting of PC12 cells with anti-N-CAM was carried out as described previously with some modifications (Moore and Walsh, 1985). PC12 cells were extracted with 1% Nonidet P-40 and in general 50 μg of extracted cellular protein was added per gel track. After electrophoresis samples were transferred to nitrocellulose and non-specific binding sites blocked with phosphate-buffered saline (PBS) containing 2% casein. The anti-N-CAM used in the present study was rabbit anti-mouse N-CAM and the detecting antibody was sheep anti-rabbit Ig labelled with peroxidase. Reaction products were visualized with Biorad HRP colour development reagent in the presence of 0.02% H_2O_2.

Neuraminidase (Sigma type X) was used to generate desialo N-CAM. PC12 cell cultures were extracted with Nonidet P-40 and neuraminidase added (0.5 units/ml in 2.5 mM sodium acetate buffer pH 5.0) for 1 h. Samples were then treated as for immunoblotting.

Quantitation of N-CAM levels in PC12 cells

The relative level of N-CAM expression in PC12 cells at different periods of culture and after treatment with cholera toxin was determined by ELISA. Cultures of PC12 cells were grown in 96-well microtitre plates and fixed by 4% paraformaldehyde for 60 min. They were washed three times with PBS and then further incubated with PBS containing 10% fetal calf serum (FCS). Cultures were incubated with a 1:9000 dilution of rabbit anti-N-CAM for 60 min at 20°C, washed three times with PBS containing FCS and then incubated with a 1:1000 dilution of peroxidase conjugated sheep anti-rabbit Ig for 60 min. Cultures were washed four times with PBS, twice with distilled water and finally incubated with 50 μl of 0.2% (w/v) *o*-phenylenediamine and 0.02% (v/v) H_2O_2 in citrate phosphate buffer. The reaction was stopped after 15 min by the addition of 50 μl of 4.5 M H_2SO_4 and reaction products measured at 492 nm using a Flow Titertek Multiscan apparatus.

Indirect immunofluorescence

Indirect immunofluorescence staining of PC12 cultures was carried out as described previously (Walsh *et al.*, 1983). A 1:1000 dilution of rabbit anti-N-CAM was used and, as detecting antibody, a 1:100 dilution of rhodamine labelled anti-rabbit Ig was used.

Northern blot analysis

Isolation of poly(A)$^+$ RNA, glyoxal−agarose gel electrophoresis and capillary blotting to Genescreen transfer membrane (New England Nuclear) was performed as previously described (Dickson *et al.*, 1986a). Filter hybridizations were performed in the presence of formamide (50%) and dextran sulphate (10%) using N-CAM cDNA probe pM1.3 (Goridis *et al.*, 1985). The 0.6-kb insert was excised by *Eco*RI, purified on a 0.7% agarose gel and labelled with [^{32}P]dCTP (3000 Ci/mmol) by the replacement synthesis method (Dickson *et al.*, 1986a) to a specific activity of $>5 \times 10^7$ c.p.m./μg. Filters were routinely washed for 60 min in 2 × saline sodium citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1% (w/v) SDS at 65°C followed by 0.1 × SSC, 0.1% SDS at 25°C for 60 min and then exposed to Fuji-RX film with intensifying screens at −80°C.

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