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Synergism between temporally distinct growth factors: bFGF, insulin and lens cell differentiation

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Abstract

Fibroblast growth factors (FGFs) are the only known factors that can induce differentiation of the mammalian lens epithelial cell, while insulin acts only as a mitogen, not as a morphogen. We show here that insulin enhances expression of the αA-crystallin gene in lens epithelial cells and induces the synthesis of lens fibre cell specific β2- and γ-crystallins in early differentiated fibre cells. Different signal transduction pathways are required for bFGF or insulin maintained fibre cell differentiation. A 15 min preincubation with bFGF was sufficient for the lens epithelial cells to become competent to undergo insulin maintained differentiation. The phorbol ester TPA could replace bFGF. The bFGF instructed competence to differentiate decays with a half-life of about 30 h. Hence, bFGF and insulin can act in concert to produce a differentiated phenotype even when they are not present simultaneously. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

The classical example of embryonic induction is the induction of lens by factors emanating from the optic cup. In mammals the lens inducing factor is likely to be fibroblast growth factor (FGF) as purification of this inducing activity yielded basic fibroblast growth factor (Chamberlain and McAvoy, 1987, 1989; Schulz et al., 1993; for recent review see Chamberlain and McAvoy, 1997). Furthermore, perturbation of the FGF levels or those of its transmembrane receptor in transgenic mice causes aberrant lens development and differentiation (Chow et al., 1995; Robinson et al., 1995a,b). Finally, in an in vitro differentiating system based on explanted rat lens epithelial cells, the addition of bFGF (or aFGF) suffices to induce fibre cell differentiation and FGF is the only known factor to be able to do so (Chamberlain and McAvoy, 1989).

The lens epithelial cell can be considered as the stem cell of the lens in that it is a self-renewing population of cells that also gives rise to terminally differentiated progeny, the lens fibre cell. The lens epithelial cell faces the classical dilemma of a stem cell as to when to divide and when to differentiate (see also Morrison et al., 1997). The problem is compounded by the fact that bFGF acts not only as a morphogen but also as a mitogen for lens epithelial cells. The choice between cell division and differentiation is apparently dependent upon the concentration of bFGF. At low doses (1 ng/ml) bFGF induces mitosis, at intermediate doses cell migration and at high doses (100 ng/ml) differentiation (McAvoy and Chamberlain, 1989). It is not clear how the lens epithelial cell can distinguish between a mitotic and a morphogenic signal from the same factor. Literature data suggest several not necessarily mutually exclusive possibilities, such as the activation of different splice variants of one of the members of the FGF receptor family or the strength of the signal emanating from the activated receptor (see for example Shi et al., 1993; Mason, 1994; Wang et al., 1994; Shaoul et al., 1995). The intracellular routing of FGF could also play a role as it has been shown that a nuclear action of acidic FGF is required for the stimulation of DNA synthesis (Imamura et al., 1990; Wiedlocha et al., 1994, 1996). A similar action of bFGF in the nucleus is likely but as yet not rigorously proven (Bikfalvi et al., 1995).

In a previous study we have shown that the presence of a
nuclear localization signal (NLS) in bFGF inhibits the differentiation, but not the mitotic, response of lens epithelial cells (Leenders et al., 1997). As the presence of an NLS enhanced nuclear uptake of exogenous bFGF and thus could increase the putative nuclear signal of bFGF, we suggested that the balance between the nuclear signal and that emanating from the FGFRs may steer lens epithelial cells toward mitosis or differentiation. Possible support for this hypothesis is the finding that stimulation of another transmembrane tyrosine kinase receptor, the IGF-I receptor, enhances the differentiation response of lens cells to bFGF (Chamberlain et al., 1991; Liu et al., 1996). By itself, IGF-I (or insulin at high concentration) cannot induce differentiation of rat lens epithelial cells but only cell division. Unlike chicken lens cells (Beebe et al., 1987), rat lens cells retain their epithelial phenotype during incubation with insulin or IGF-I (Chamberlain et al., 1991; unpublished data). However, when bFGF and IGF-I or insulin are added together, less bFGF is required for the differentiation response. To determine whether the suggested synergy between bFGF and IGF or insulin in promoting lens differentiation is indeed due to enhanced membrane receptor signalling, we have taken a closer look at the effect of insulin on lens differentiation. We show here that insulin can maintain lens differentiation once it is initiated by bFGF. We have made use of this property of insulin to determine the minimal exposure time to bFGF required to initiate differentiation. Our results show that initiation of differentiation is an early response to bFGF, possibly mediated by protein kinase C as the phorbol ester TPA can replace bFGF in the initiation of differentiation. We further show that the FGF-induced competence to differentiate decays only slowly. Hence, growth factors can act synergistically to induce a certain phenotype even when they are not simultaneously present.

2. Results

2.1. The effect of insulin on lens fibre cell differentiation

Lens fibre cell differentiation is accompanied by complex phenotypic and biochemical changes, amongst which is the copious synthesis of the water-soluble structural lens proteins, the crystallins. In rat, as in most mammals, the crystallins are encoded by three gene families, i.e. the α-, β- and γ-crystallin genes. The accumulation of the products from these three crystallin gene families serves as a convenient marker for lens cell differentiation; α-crystallin is already present in the lens epithelial cells but its synthesis rate increases markedly during early fibre cell differentiation, synthesis of β-crystallin commences in the intermediate stages of fibre cell differentiation, while γ-crystallin synthesis is found only in late differentiated fibre cells (McAvoy and Chamberlain, 1989; Peek et al., 1992).

The influence of the bFGF concentration on the accumulation of αA-, βB2- and γ-crystallin during in vitro differentiation is illustrated in Fig. 1A. A high concentration of bFGF is required to promote differentiation. In agreement with the results of Chamberlain et al. (1991) the addition of insulin significantly lowers the requirement for bFGF: βB2-crystallin is now detectable upon culture with as little as 1 ng/ml bFGF. In control experiments, in which only insulin is added, no accumulation of βB2- or γ-crystallin was seen, but unexpectedly, αA-crystallin did accumulate in response to insulin treatment to the same extent as it accumulated in response to bFGF (Fig. 1B, compare lanes 1 and 2, note that the upper αA-crystallin band on the blot corresponds to αAIns). Apparently, the accumulation of this crystallin is not strictly coupled to fibre cell differentiation.

The increase in crystallin accumulation in the presence of bFGF and insulin could be due to faster differentiation, to an
increased rate of transcription of the crystallin genes or to an increased rate of translation of the crystallin mRNAs. To distinguish between these possibilities, the change in crystallin mRNA levels was followed in time. As shown in Fig. 2, the addition of insulin has some effect on the level of αA-crystallin mRNA but more notable is the marked increase in the levels of βB2- and γ-crystallin mRNAs. The addition of insulin also speeds up the differentiation process as indicated by the earlier appearance of the βB2- and γ-crystallin mRNAs. Insulin thus affects both the rate of differentiation of the fibre cells and the rate of transcription of the crystallin genes.

2.2. A short pulse of bFGF suffices to initiate differentiation

The finding that insulin alone stimulates the accumulation of αA-crystallin suggested to us that insulin might also suffice to stimulate accumulation of the βB2- and γ-crystallins once fibre cell differentiation has been initiated. In an initial test of this possibility, explants were exposed to bFGF for various lengths of time, washed with PBS to remove bFGF and then cultured for an additional 8 days with insulin. Without added growth factors, no further accumulation of βB2-crystallin mRNA was seen and γ-crystallin mRNA did not appear (see also Fig. 3), in agreement with our previous finding that withdrawal of bFGF stops the differentiation process (Peek et al., 1992). To our surprise we found that addition of insulin after a preincubation of only 15 min with bFGF allowed differentiation to continue as evidenced by the copious accumulation of both βB2- and γ-crystallin. To insure that this result was not due to remaining traces of bFGF bound to the extracellular matrix, we repeated the experiment but washed the explants with 2 M NaCl to remove extracellular matrix bound bFGF (Springer et al., 1994). As shown in Fig. 3, even when using a high-salt wash, 15 min of exposure to bFGF was sufficient for the lens epithelial cell to acquire competence to differentiate. Preincubation with bFGF for up to 2 h did not significantly increase the differentiation response over that seen after a 15 min preincubation. However, after 12 h of preexposure to...
FGF the differentiation response, as assessed from the accumulation of βB2-crystallin, showed an increase between two- and five-fold over that seen after a 30 min preincubation with bFGF (data not shown). The concentration of bFGF required to obtain a half-maximal initiation of differentiation response after a 30 min incubation is about the same (5 ng/ml) as found (under our conditions, using the same batch of bFGF) for the half-maximal differentiation response after continuous incubation with bFGF (Leenders et al., 1997). Whether bFGF concentration required for the initiation of differentiation is dependent upon the time of incubation with bFGF has not been tested.

2.3. TPA can substitute for bFGF in the initiation of differentiation

Our assay for the initiation of differentiation, a short exposure to bFGF, followed by incubation with insulin, provides the experimental means to test the effect of inhibitors or agonists of the signal transduction pathways emanating from the activated membrane receptor. In a first attempt to unravel the signalling pathway, we tested whether different agonists can replace bFGF as an initiator of differentiation. Arachidonic acid and bradykinin had no effect (data not shown), but the phorbol ester tetradecanoyl phorbol acetate (TPA) did have an effect (Fig. 4A). Exposure of the lens epithelial cells to TPA for 30 min, followed by insulin, induced a full differentiation response, showing that TPA can substitute for bFGF in this assay (Fig. 4A).

The effect of TPA is generally considered to be due to activation of protein kinase C (PKC). If so, the TPA effect should be blocked by staurosporine, a general serine/threonine kinase inhibitor. As shown in Fig. 4B, the initiation of differentiation by either bFGF or by TPA was not blocked by staurosporine, even when staurosporine was present during the first 4.5 h of the subsequent incubation with insulin. To ensure that staurosporine is effective in this cell system, the effect of staurosporine on the stimulation of DNA synthesis by either bFGF or TPA was also determined. In this assay, staurosporine inhibited the effect of either bFGF or TPA by 50%, showing that staurosporine is indeed effective.

An alternative way of demonstrating the involvement of PKC is by showing inhibition of the inductive effect after downregulation of PKC by prolonged incubation with TPA. Explanted lens epithelial cells were therefore incubated with TPA for up to 24 h and then tested for their capacity to differentiate, either in response to insulin alone, or in response to bFGF → insulin (Fig. 5A). No significant inhibition of differentiation was found, showing that prolonged incubation with TPA does not downregulate the initiation of differentiation response.

One possible explanation for the lack of effect of staurosporine as well as for the failure to downregulate by prolonged exposure to TPA is that the activation of PKC or of its target is not transient but stable. To measure the stability of the initiation of differentiation directly, explanted lens epithelial cells were incubated with bFGF or TPA for 30 min but the subsequent addition of insulin was delayed for up to 48 h. No decay was found in the first 7 h, after 24 h the response was about 60% of the control and after 48 h it was about 20% of the control (Fig. 5B). Hence, the ability to initiate differentiation decays only slowly, suggesting per-
initiated by bFGF are distinct from those initiated by insulin. To test whether the signal transduction pathways used in the maintenance of differentiation by these two factors also differ, the effect of cdc on explant differentiation was tested. In our hands, cdc did partially block the mitotic response to bFGF but did not affect the differentiation response to bFGF significantly (Fig. 6A). However, in the bFGF → insulin

Fig. 5. (A) The effect of prolonged incubation with TPA on the initiation of differentiation. Explants were incubated with TPA for the time indicated (in hours), washed, then further incubated with insulin or treated with bFGF for 30 min before insulin was added. Factors were used at the concentrations given in the legend to B. The differentiation response, measured as indicated in the legend to B, is expressed relative to that obtained after 2 h of exposure to bFGF followed by incubation with insulin, which was set at 100%. (B) The stability of the competence to differentiate. Explants were preincubated for 30 min with bFGF (25 ng/ml), washed with 2 M NaCl and cultured in medium without added factors for the time indicated. Then insulin was added and the culture was continued for 8 days. Differentiation is expressed relative to that obtained when insulin was added directly after preincubation with bFGF. The error bars show the standard deviation.

consistent activation of a component of the signal transduction pathway.

2.4. Insulin and bFGF use different pathways to maintain differentiation

The stimulation of DNA synthesis in rat lens epithelial cells by insulin has been reported to be sensitive to inhibition by cinnamyl 3,4 dihydroxy-α-cyanocinnamate (cdc), an inhibitor of 12-lipoxygenase, while the effect of bFGF on DNA synthesis was not blocked (Lysz et al., 1996). Hence, the signal transduction pathways leading to cell division as

Fig. 6. (A) Effect of cdc on [3H]thymidine incorporation and differentiation. Explants were cultured as indicated. Cinnamyl 3,4 dihydroxy-α-cyanocinnamate (cdc) was used at 10 μM and was present either during the whole time of culture or only during the incubation with bFGF (25 ng/ml) or insulin (5 μg/ml). [3H]Thymidine incorporation (open bars) was measured after 3 days and the ratio of βB2- to αA-crystallin (solid bars) was measured after 7 days. Both are shown relative to the response to bFGF which was set at 100%. The error bars show the standard deviation. (B) Effect of hydroxyurea on [3H]thymidine incorporation and differentiation. Explants were cultured in the presence of 2 mM hydroxyurea (hu) as indicated. In those cases where hydroxyurea was present at the beginning of the culture period (bFGF + hu), explants were preincubated for 30 min with hydroxyurea before addition of bFGF. bFGF was used at 25 ng/ml and insulin was used at 5 μg/ml. [3H]Thymidine incorporation (open bars) was measured after 2 days and the ratio of βB2- to αA-crystallin (solid bars) was measured after 10 days. The error bars show the standard deviation. Note that the higher background in this experiment is due to the fact that explants were harvested after 10 days. Cells at the periphery of the explants were preexposed to FGF in vivo and accumulated some β-crystallin during prolonged culture (see also Chamberlain et al., 1991).
protocol both the mitotic response and the differentiation response were strongly inhibited providing cdc was also present during the preincubation with bFGF. These results show that the signal transduction pathways used by bFGF or by insulin in maintaining differentiation differ. They also raise the question whether perhaps cell division is a prerequisite for cell differentiation in the bFGF → insulin protocol. To test this possibility, we used hydroxyurea to block DNA synthesis. In the presence of this compound, [3H]thymidine incorporation was completely blocked, yet differentiation proceeded normally (Fig. 6B). Hence, cell division is not required for the induction of differentiation in agreement with previous results (McAvoy and Chamberlain, 1989).

3. Discussion

There has always been a curious discrepancy between mammals and birds in the nature of the factor that causes lens differentiation. The purification of the lens differentiation factor or eye-derived growth factor from rat or calf eyes has led to the identification of αFGF or bFGF (Courty et al., 1986; Chamberlain and McAvoy, 1987), while lentropin in chicken has the characteristics of insulin-like growth factor (Beebe et al., 1987; for recent review see Schoen and Chader, 1997). Our results show that the action of insulin (or IGF-I, note that insulin at the concentration used in our studies will also activate the IGF-I receptor) in the rat has more in common with the IGF-like activity in chicken than originally thought. Insulin in the rat lens is not merely an enhancer of bFGF action as it also plays its own distinct role. Insulin activates the transcription of a crystallin gene already active in epithelial cells, αA-crystallin, just as the chicken IGF-like activity causes the accumulation of δ-crystallin, also a crystallin gene active in epithelial cells (Alemany et al., 1989). Insulin can also activate the transcription of the β- and γ-crystallin genes once initial fibre cell differentiation has occurred.

The experiments reported here dissect the process of lens differentiation into two parts, i.e., initiation of differentiation and maintenance of differentiation. Differentiation can be initiated by bFGF but not by insulin, while maintenance can be performed by either insulin or bFGF. However, our data suggest that the signal transduction pathways used by these two growth factors in maintaining differentiation differ, as the action of insulin, but not of bFGF, is blocked by cdc.

We have previously shown that a continuous signal from bFGF is required for the progression of lens fibre cells through the various stages of differentiation (Peek et al., 1992). We show here that this signal can also be provided by insulin. As the growth factor signal required for the maintenance of differentiation is apparently not unique to bFGF and as many parts of the signal transduction cascade are common to various growth factors, other growth factors besides insulin and bFGF might be able to maintain differentiation. We have not tested this possibility directly but there is suggestive evidence in the literature that PDGF-A could have such an effect as overexpression of PDGF-A in the lens leads not only to aberrant cell division but also to (partial) differentiation of the epithelial cells (Reneker and Overbeek, 1996). An in-depth discussion of the in vivo role of the various ocular growth factors can be found in two recent reviews (Chamberlain and McAvoy, 1997; Schoen and Chader, 1997).

Unexpectedly, adding both insulin and bFGF during the maintenance phase yielded higher levels of β- or γ-crystallin mRNA than achieved by either factor alone. The fact that neither growth factor can saturate the signal transduction pathways that impinge on the transcriptional activators of these genes indicates that at least two separate pathways must be operative. The simplest model would be that the transcription factors responsible for a basal transcription level are the common targets of both insulin and bFGF signal transduction pathways. Additional responsive elements (and their cognate factors) dedicated to either insulin or bFGF would then be responsible for the enhanced rate of transcription observed when both bFGF and insulin are present.

The division of lens epithelial differentiation in an induction and a differentiation phase provides us with an experimental system to study the signal transduction pathways involved in the initiation of differentiation. An initial screen of a number of antagonists showed them to have either no effect (genistein, wortmannin) or to be lethal (okadaic acid). Of the three agonists tested, namely arachidonic acid, bradykinin and phorbol ester, only the TPA could replace bFGF in the induction of differentiation. TPA has been shown to induce differentiation in several human tumour cell lines, such as promyelocytic and erythroleukemic cell lines. In those cells, differentiation was shown to be correlated with the activation of a particular isozyme of protein kinase C, PKC-α (Hocevar et al., 1992; Mischak et al., 1993; Murray et al., 1993). Whether the activation of an isozyme of protein kinase C is a crucial step in the initiation of differentiation of lens epithelial cells as well is not yet clear from our data. At first glance, the lack of inhibition by staurosporine as well as the lack of downregulation by prolonged exposure to TPA would argue against the involvement of PKC. However, the finding that the competence to differentiate is a stable state within the time scale of these experiments makes the interpretation of these data less straightforward. For example, if the target of PKC is stably activated as an initial effect of TPA, no effect of downregulation of PKC after prolonged incubation with TPA would be seen. Similarly, if the inhibition by staurosporine is only 50%, as seen in the stimulation of DNA synthesis, sufficient activated PKC target could accumulate to allow the acquisition of the competence to differentiate.

TPA promotes not only differentiation, it also promotes DNA synthesis and presumably mitosis. Apparently, the activation of PKC is involved in both processes. It has
I always seen even under conditions that optimally promote in vivo conditions exactly in vitro, as some cell division is be a valuable tool in elucidating these pathways.

In vivo the onset of fibre cell differentiation is coupled with cessation of cell division. As yet, we cannot mimic the in vivo conditions exactly in vitro, as some cell division is always seen even under conditions that optimally promote differentiation (see also McAvoy and Chamberlain, 1989; Liu et al., 1996). We have considered the possibility that the mitotic response derived from cells which went into mitotic arrest upon culturing in vitro. However, the time course of incorporation of \( ^{3}H \)thymidine, which reaches a maximum between 24 and 72 h (data not shown), does not support this possibility. We could be dealing with a non-homogeneous population of cells in which some divide and some differentiate. The alternative is that, in this culture system, cells that acquire fibre cell characteristics, namely the expression of fibre cell specific crystallins, are still capable of cell division. Such a situation is seen in vivo when PDGF-A is overexpressed in the lens (Reneker and Overbeek, 1996) or in lenses deficient in the retinoblastoma protein or in p57\textsuperscript{KIP2}. The lack of pRB or of p57\textsuperscript{KIP2} prevents exit from the cell cycle, yet \( \beta \) - and \( \gamma \)-crystallin expression is found (Fromm et al., 1994; Maandag et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Fromm and Overbeek, 1996; Zhang et al., 1997). These data suggest that proper fibre cell differentiation is not the result of interlocking pathways but rather of parallel pathways that are normally regulated to be coincident. In the normal mouse lens, cyclin D and cdk4 are still present in early differentiated fibre cells (Fromm and Overbeek, 1996), while in the chicken lens cyclin B and cdk2 are still present in the post-mitotic fibre cells (Gao et al., 1995). Hence, an important target for the regulation of the exit from the cell cycle is likely to be p57\textsuperscript{KIP2}, a cdk inhibitor also present in early differentiated lens fibre cells (Matsuoka et al., 1995; Harper and Elledge, 1996; Zhang et al., 1997). How the synthesis of p57\textsuperscript{KIP2} is regulated during in vitro culture with bFGF or insulin is not known. In vivo, contact with the posterior capsule could play a role; fibrillar collagen has been shown to inhibit smooth muscle proliferation through upregulation of p27\textsuperscript{KIP1} and p21\textsuperscript{Cip1/Waf1} (Koyama et al., 1996).

4. Experimental procedures

4.1. Preparation and culture of lens explants

Explants of rat lens epithelial cells were prepared essentially as described (McAvoy, 1980). Two- to 3-day-old Wistar rats were decapitated and the eyes were removed. Lenses were isolated and explants were prepared by pinning down the capsule with the attached monolayer of epithelial cells in a culture dish, while carefully removing the bulk of lens fibre cells. Explants were cultured in serum free M199 medium (ICN), supplemented with 20 mM HEPES buffer (pH 7.4) and 0.1% BSA (Boehringer, Mannheim). One day after isolation, factors were added. bFGF (a kind gift from Scios, Mountain View, CA, USA) was used at the concentrations indicated while insulin (Sigma, St. Louis, MO, USA) was always used at a final concentration of 5 \( \mu \)g/ml. In some cases, explants were washed with phosphate buffered saline (PBS) or with high salt buffer (2M NaCl, 20 mM HEPES (pH 7.4)).

4.2. Crystallin accumulation

Explants were harvested in 50 \( \mu \)l SDS-PAGE PAGE buffer per explant and proteins were solubilized by boiling for 10 min. For \( \alpha A \)- and \( \beta B 2 \)-crystallin analysis, 5 \( \mu \)l of this sample was subjected to 12.5% SDS polyacrylamide gel electrophoresis according to standard protocols (Ausubel et al., 1994). For \( \gamma \)-crystallin measurements, 10 \( \mu \)l was used. Gels were electroblotted onto reinforced nitrocellulose filters (Schleicher and Schuell, Germany) and blots were preincubated with PBS containing 0.05% Tween-20 and 0.1% I-block (Tropix) to block aspecific binding sites. Subsequently, blots were incubated overnight at 4°C with appropriate dilutions of rabbit antisera against calf \( \alpha A \)-crystallin (1:1000, a kind gift of Dr W.W. de Jong, Department of Biochemistry, University of Nijmegen), calf \( \beta B 2 \)-crystallin (1:400) or recombinant \( \gamma C \)-crystallin (1:1000). The \( \alpha A \)-crystallin antibody and \( \beta B 2 \)-crystallin antibodies are specific and do not cross-react with other crystallins. The \( \gamma \)-crystallin antibody does cross-react with other \( \gamma \)-crystallins due to the high sequence similarity between these proteins. Blots were washed extensively with PBS/Tween-20 and then incubated for 2 h with alkaline phosphatase conjugated goat-anti-rabbit IgG. After washing with PBS/Tween-20 alkaline phosphatase activity was visualized with the NBT/BCIP chromogenic substrate. Staining was quantitated using a BioRad scanner. All experiments were repeated at least twice and each measurement of the level of \( \alpha A \)- and \( \beta B 2 \)-crystallin was performed in duplo. Differentiation was
quantitated as the ratio of the βB2-crystallin signal to the αA-crystallin signal. The amount of αA-crystallin is similar in explants incubated with bFGF, with insulin or with bFGF → insulin. The level of αA-crystallin thus serves as a measure of cell number used. In control experiments, where no growth factors are added, this ratio cannot be used (as synthesis of αA-crystallin is not stimulated). In those cases, the βB2-crystallin signal, if any, was used without correction.

4.3. Thymidine incorporation assays

Factors and [³H]thymidine were added to rat lens explants at day 1 after isolation in M199 medium, supplemented with 0.1% BSA and incorporation was measured 3 days later (when not indicated otherwise); explants were washed twice with 10% TCA containing 2 mM deoxythymidine triphosphate (dTTP, Sigma) and twice with 5% TCA. Subsequently, the explants were solubilized in 400 µl 0.3 M perchloric acid for 30 min at 90°C. After neutralization with 25 µl 10 N NaOH, scintillation fluid was added and samples were counted. In general four explants were used for each measurement, whereby the incorporation in each explant was determined separately. The typical incorporation per explant was 4 x 10⁴ cpm with a background (no growth factors added) of 2 x 10³. All experiments were done at least twice.

4.4. Northern blotting

RNA isolated from four explants using the method of Gough (1988) was electrophoresed in 1.2% formaldehyde gels under standard conditions (Ausubel et al., 1994). Gels were blotted onto reinforced nitro-cellulose filters (Schleicher and Schüll, Germany) in 20x SSC. Blots were prehybridized in 6x SSC, 5x Denhardt’s solution, 0.5% SDS, 50% formamide and 100 µg/ml sonicated herring sperm DNA and hybridized in the same solution overnight at 42°C. Blots were washed for 2 x 30 min in 2x SSC, 0.5% SDS and for 2 x 30 min in 0.1x SSC, 0.5% SDS at 42°C and autoradiographed for 1 day to 1 week. Probes were prepared using the random hexamer labelling technique (Ausubel et al., 1994). Autoradiographs were scanned using a Pharmacia Laser Densitometer.

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