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Induction by growth factors of polysaccharide synthases in bean cell suspension cultures

G. Paul BOLWELL and Don H. NORTHCOTE
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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Suspension cells of bean subcultured into medium that maintains the culture and stimulates cell division but not differentiation brings about an increase in arabinan synthase activity. Subculture into a medium that induces both cell division and xylogenesis brings about in addition an increase in xylan synthase. Both synthases are membrane-bound and are concerned with the formation of neutral pectin or hemicellulose of the cell wall respectively. During the rising phase of the induction of these activities in the appropriate culture medium, the increases in activities were inhibited by either actinomycin D (an inhibitor of transcription) or D-2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide (an inhibitor of translation). Thus the control for the induction of the enzyme activities involves transcription and possibly translation. Subculture of the cells brought about an increase, probably non-specific, in total membrane-bound translation, as indicated by increased amounts of bound polysomes and incorporation of [^{35}S]methionine into membrane proteins. If the control of the appearance of specific mRNA molecules is partially effected by growth factors then these are probably operative during the period of the cell cycle that is stimulated by subculture and it is probably at this time that the growth factors act to bring about the changes necessary for differentiation.

Jones & Northcote (1981), investigating the effect of a number of inhibitors on total translation and transcription and on the activity of PAL during the induction of differentiation of suspension cultures of *Phaseolus vulgaris*, found that actinomycin D and MDMP produced a response, but no effect was observed over a range of concentrations with α-amanitin, cycloheximide and emetine. The effects of the inhibitors of transcription (actinomycin D) and of translation (MDMP) were studied under conditions of controlled cell density and optimized inhibitor dose. The rising phase of PAL activity was inhibited by both inhibitors, indicating that both transcription and translation are required for the hormonal induction of PAL during differentiation.

Bolwell & Northcote (1981) have demonstrated that the cessation of neutral pectin synthesis and the onset of xylan synthesis in both hypocotyl or callus of *Phaseolus vulgaris* may be regulated in part at the synthase stage. This has now been shown for suspension cultures in the present study. As an initial approach to the investigation of the nature of this control step in the qualitative regulation of polysaccharide synthesis, the effect of actinomycin D and MDMP on the induction of the arabinan synthase and xylan synthase has been studied. The results indicate that, as for the induction of PAL, both transcription and possibly translation are processes at which control of the syntheses of the enzymes can be exerted.

Abbreviations used: PAL, phenylalanine ammonia-lyase; MDMP, D-2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide.

Materials and methods

The cell line was isolated from hypocotyl explants of French bean (*Phaseolus vulgaris* L., cv. Canadian Wonder) (Haddon & Northcote, 1975) by vigorous shaking in CMD medium [B5 medium of Gamborg *et al.* (1968) supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/litre), deproteinized coconut milk (20%, v/v) and sucrose (2%, w/v)], at 25°C in darkness. Suspension cultures were maintained on CMD medium. Induction medium (IM) to induce xylogenesis was B5 medium supplemented with 1-naphthylacetic acid (1 mg/litre), kinetin (0.5 mg/litre) and sucrose (3%, w/v).

Actinomycin D was obtained from Sigma.

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MDMP was a gift from Dr. R. Baxter, Shell Research, Sittingbourne, Kent, U.K. UDP-α-D-[U-^{14}C]xylose (5.7 GBq · mmol^{-1}) was obtained from New England Nuclear; UDP-D-xylose was from Sigma. UDP-β-L-[1-^{3}H]arabinose and UDP-β-L-arabinose were synthesized as described previously (Bolwell & Northcote, 1981).

Treatment of cultures

Suspension cultures were maintained in CMD medium and subcultured every 8 days. Time courses were followed after subculture into either CMD medium or IM. For incubations with inhibitors, 10 ml samples were placed in pre-sterilized plastic pots; controls without inhibitors were treated similarly. Cells were harvested by filtration.

Enzyme assays

PAL (EC 4.3.1.5) was assayed by the method of Bevan & Northcote (1979*a*,*b*) and UDP-D-xylose 4-epimerase (EC 5.1.3.2) by the method of Dalessando & Northcote (1977*a*,*b*). Total membrane preparations were made and xylan synthase and arabinan synthase were assayed as described previously (Bolwell & Northcote, 1981), with either UDP-α-D-[U-^{14}C]xylose or UDP-β-L-[1-^{3}H]-arabinose as substrates.

Isolation of rough endoplasmic reticulum

Precautions against RNA degradation were carried out as described by Martin & Northcote (1981). Suspension cultured cells (5 g) harvested by filtration were ground together with a little coarse sand in a pestle and mortar with 5 ml of extraction buffer [50 mM-Tris/HCl, pH 9.0, containing 400 mM-KCl, 25 mM-$MgCl_2$ and 13% (w/w) sucrose] at 4°C. The slurry was filtered through muslin and centrifuged at 1000 ***g*** for 25 min and the supernatant was centrifuged at 15 000 ***g*** for 10 min at 4°C to sediment most of the larger organelles. The supernatant was centrifuged on to a discontinuous sucrose gradient composed of 1.25 ml each of 34% (w/w) sucrose and 55% (w/w) sucrose in gradient buffer (50 mM-Tris/HCl, pH 8.0, containing 20 mM-KCl and 10 mM-$MgCl_2$). The gradients were centrifuged at 250 000 ***g*** for 4 h in a Beckman SW50.1 rotor. The interface at 55% (w/w) sucrose was harvested and pelleted by centrifugation in homogenization medium at 100 000 ***g*** for 30 min. The pellet was resuspended in 200 μl of gradient buffer containing 8% (w/w) sucrose for further analysis or in 0.1 M-cacodylate buffer, pH 7.2, containing 5% (v/v) glutaraldehyde and 13% (w/w) sucrose for electron microscopy.

Membranes prepared for electron microscopy were thin-sectioned, stained with uranyl acetate and alkaline lead citrate and examined in an AEI EM6B electron microscope at 60 kV (Brett & Northcote, 1975).

Protein was determined by a modification of the Coomassie Blue assay (Read & Northcote, 1981), RNA at 260 nm after phenol/chloroform extraction and phospholipid by the method of Ames (1966) after lipid extraction.

Polysome profiles were obtained by treatment of membranes with 200 μl of 10 mM-EGTA or 1% Triton in gradient buffer containing 8% (w/w) sucrose and analysed in 4 ml continuous gradients of 10–40% (w/w) sucrose on a cushion of 55% (w/w) sucrose by centrifugation at 250 000 ***g*** for 45 min in a Beckman SW50.1 rotor. Sucrose solutions were made in gradient buffer. The contents of the gradients were pumped through a Uvicord (LKB) set at 254 nm and recorded by a 'linear-log' amplifier coupled to a chart recorder. Relative levels of polysomes were estimated by comparing the integrated area under the curves.

[^{35}S]Methionine incorporation *in vivo* was determined by incubating 5 g of cells (estimated from the growth curves) with 1.85×10^{5} Bq of [^{35}S]-methionine in presterile plastic pots until incorporation into the membrane fraction reached saturation (3–4 h; Jones & Northcote, 1981). The cells were then harvested and the rough endoplasmic reticulum prepared as described above.

Results

Growth of cultures

Suspension cultures were routinely maintained on CMD medium to avoid rapid loss in morphogenic potential (Bevan & Northcote, 1979*b*), and subcultured every 8 days ensuring that the cells did not enter stationary phase (Fig. 1). Subculture into IM to bring about differentiation slowed growth considerably and the cells remained in exponential growth phase over the period of investigation. Cultures retain the ability to produce vascular nodules after transfer to IM over many maintenance subcultures on CMD medium although this ability diminishes with age (Dudley & Northcote, 1978; Bevan & Northcote, 1979*b*; Jones & Northcote, 1981) and is not as great as that observed in solid culture (Haddon & Northcote, 1975; Bolwell & Northcote, 1981).

Timing of cell division

It has not yet proved possible to synchronize these cultures by methods such as those used by Roberts & Northcote (1970), which do not involve major perturbations encountered, for example, with use of DNA synthesis and mitotic inhibitors. Bevan & Northcote (1981*b*) report that *Phaseolus* cultures divide asynchronously over the period of 24–36 h after subculture. Observation of the suspension

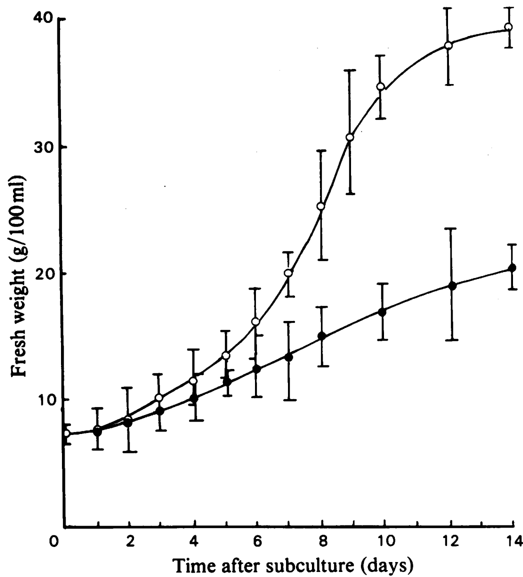

Fig. 1. *Growth curve for suspension cultured cells after subculture into CMD medium* (○) *or IM* (●)
Cells were subcultured in a standard inoculum (approx. 8 g fresh wt./100 ml of medium).

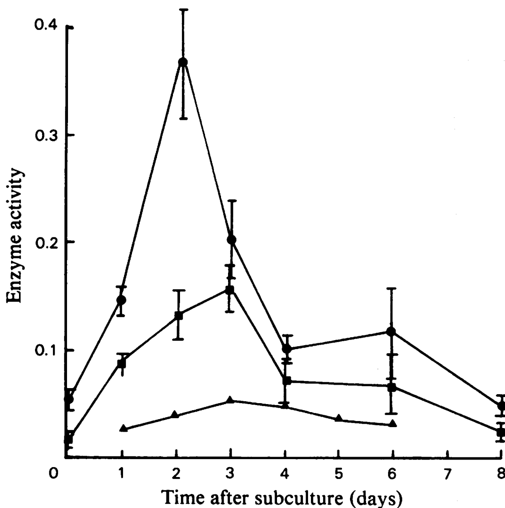

Fig. 2. *Changes in levels of enzyme activity during growth of cells subcultured into CMD medium*
●, Arabinan synthase [nmol of arabinose incorporated·min^{-1}·(mg of protein)$^{-1}$]; ■, xylan synthase [nmol of xylose incorporated·min^{-1}·(mg of protein)$^{-1}$]; ▲, PAL [nmol of cinnamate produced·min^{-1}·(mg of protein)$^{-1}$ × 10^{-1}]

cultures studied here showed that they divided over the same period, though an accurate mitotic index was not measured.

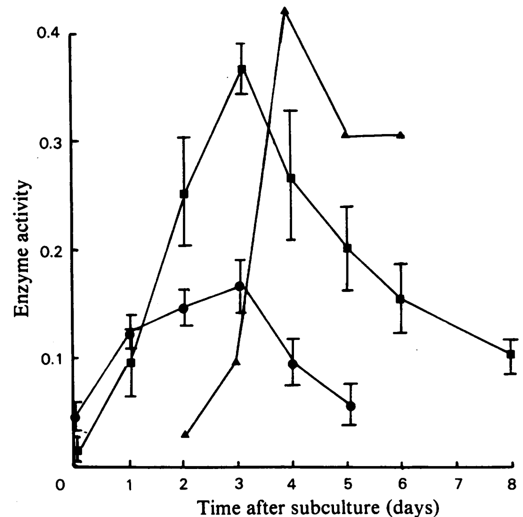

Fig. 3. *Changes in levels of enzyme activity during growth and differentiation of cells subcultured into IM*
●, Arabinan synthase [nmol of arabinose incorporated·min^{-1}·(mg of protein)$^{-1}$]; ■, xylan synthase [nmol of xylose incorporated·min^{-1}·(mg of protein)$^{-1}$]; ▲, PAL [nmol of cinnamate produced·min^{-1}·(mg of protein)$^{-1}$ × 10^{-1}].

Changes in enzyme levels after subculture into CMD medium

Fig. 2 shows the changes in enzyme levels associated with subculture into CMD medium. After subculture there was a rapid 6-fold increase in arabinan synthase activity, reaching a peak at 48 h. After this time there was a rapid decline in activity so that the peak is sharp. The enzyme activity at 48 h has been characterized and labelling *in vivo* with [^{3}H]arabinose has shown that most of the incorporation is into water-soluble polysaccharides containing more than 90% of the radioactivity in arabinose in both cell wall and endomembrane system (Bolwell & Northcote, 1983). In contrast xylan synthase activity remained considerably lower, but is probably distinct, as epimerization by membranes is less than 6%. Incorporation of [^{3}H]-arabinose into xylose-containing polysaccharides was low (Bolwell & Northcote, 1982). No induction of PAL activity was observed during the period of culture between 24 and 144 h.

Changes in enzyme levels after subculture into IM

Fig. 3 shows that there was about an 8-fold induction in xylan synthase activity and a peak was reached at about 84 h. The peak was much broader than that observed for arabinan synthase activity in CMD medium and this may be related to the relative rates of growth of the cells (Fig. 1), and possibly

lower turnover rate and consequently a much longer apparent half-life for the xylan synthase. The xylan synthase has been characterized and at the peak activity most of the incorporation of [^{3}H]arabinose is into hemicellulose polysaccharides, with an increased ratio of radioactive xylose to arabinose compared with the incorporation observed into cells cultured in CMD medium at 48h (Bolwell & Northcote, 1983). In cells grown in IM the arabinan synthase was induced to a much lower extent and also showed a broad peak, which indicated that there was possibly a lower turnover in these cells also. The PAL activity exhibited a rapid rise, peaking at about 96h, as described previously (Bevan & Northcote, 1979*a*,*b*; Jones & Northcote, 1981). The changes in enzyme levels (either xylan synthase or arabinan synthase) between cultures grown on CMD medium or IM and the observed incorporations into poly-

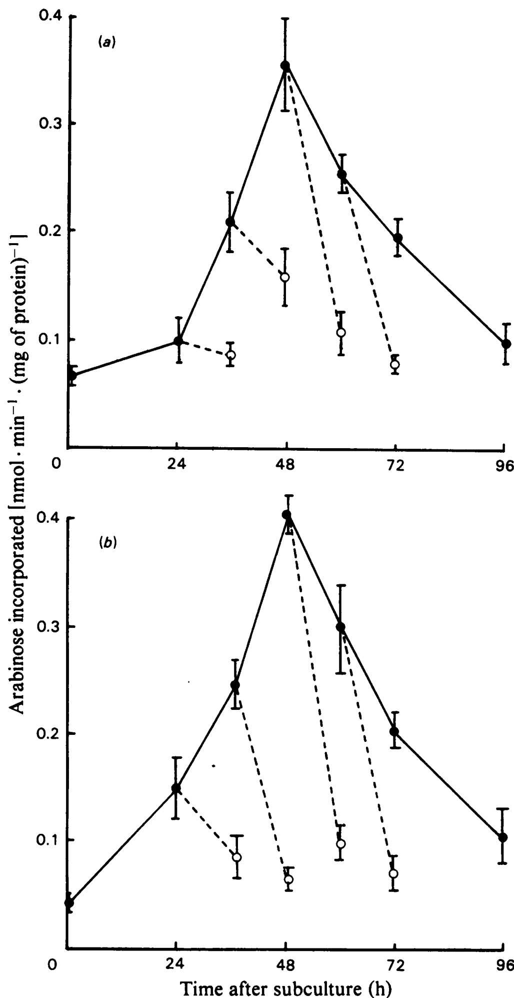

Fig. 4. *Inhibition of arabinan synthase activity by* (*a*) *actinomycin D* (*20μg/ml*) *and* (*b*) D-*2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide* (*15 μM*)

The time course of enzyme activity after subculture of cells into CMD medium in the absence (●——●) or presence (●----○) of inhibitor is shown. Inhibitor was added and enzyme activity was measured 12h later. Three replicates/time point per treatment were assayed and mean and standard error bars are shown.

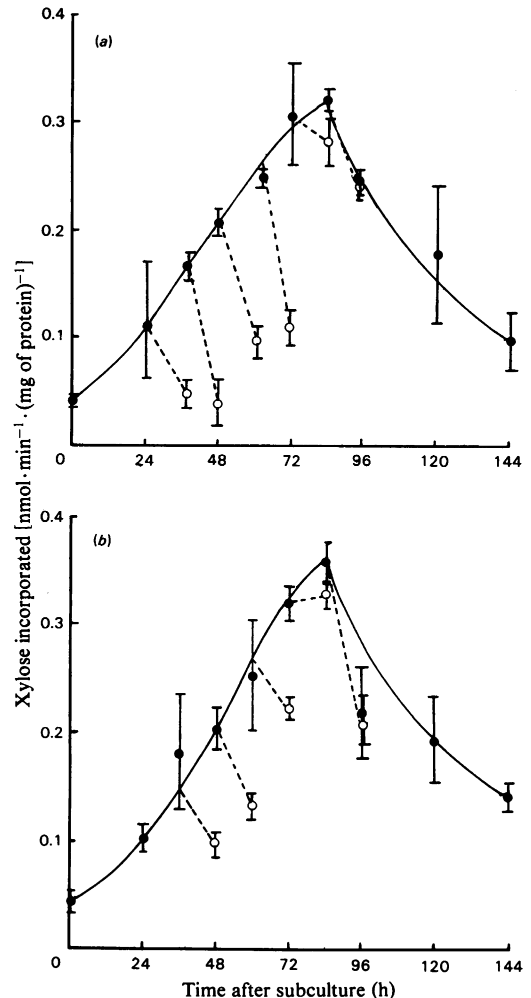

Fig. 5. *Inhibition of xylan synthase activity by* (*a*) *actinomycin D* (*20μg/ml*) *and* (*b*) D-*2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide* (*15 μM*)

The time course of enzyme activity after subculture of cells into IM in the absence (●——●) or presence (●----○) of inhibitor is shown. Inhibitor was added and enzyme activity measured 12h later. Two replicates per time point per treatment were assayed and means and ranges (indicated by the bars) are shown.

saccharide (either xylan or arabinan) (Bolwell & Northcote, 1983) therefore correlated very well with the different effects of these media on xylogenesis.

The effect of actinomycin D on the induction of enzymes after subculture

Jones & Northcote (1981) optimized the dose of actinomycin D at 20 μg/ml and the timing of exposure at 12h for inhibition of RNA synthesis. The antibiotic was added aseptically at intervals during the period of enzyme induction and the enzyme levels assayed 12h after treatment and compared with cells with no antibiotic. For both arabinan synthase in cells on CMD medium (Fig. 4*a*) and xylan synthase in cells on IM (Fig. 5*a*) the antibiotic inhibited the rise in activity throughout the period during which the enzyme activity normally increased. Actinomycin D inhibited the rise in PAL activity in cells on IM during this period (Jones & Northcote, 1981) but reverses the decline in enzyme activity from the peak onwards. This superinduction (Tomkins *et al.*, 1972) was not observed for the membrane-bound syntheses. Actinomycin D has little effect on cell viability under these conditions (Jones & Northcote, 1981).

The effect of MDMP on induction of enzymes after subculture

Jones & Northcote (1981) optimized the dose of MDMP at 15 μM for 12h for inhibition of protein synthesis without gross effects on cell viability. The inhibitor was added aseptically at points during the time course of enzyme induction and the enzyme levels were assayed 12h later and compared with those in cells not treated with the inhibitor. The inhibitor was effective throughout the rising phase of enzyme activity both for arabinan synthase in cells on CMD media (Fig. 4*b*) and xylan synthase from cells on IM (Fig. 5*b*). It was also effective on arabinan synthase during the period after the maximum induction of enzyme activity when the activity declined. In contrast, MDMP inhibited the rising phase but not falling phase for the induced peak of PAL activity in cells on IM (Jones & Northcote, 1981).

The effect of subculture on membrane-bound protein synthesis

The response of membrane-bound protein synthesis to subculture is indicated by the analysis of a rough endoplasmic reticulum fraction (Bolwell & Northcote, 1983) over a time course of 88h after subculture (Table 1). In addition to an increase in membrane protein, RNA and phospholipid (rough endoplasmic reticulum), there was also a stimulation in translation of membrane-bound proteins, as shown by incorporation of [^{35}S]methionine and the level of polysomes relative to that before subculture. It is very difficult to measure the rates of membrane bound protein synthesis accurately, since there is membrane flow into other compartments.

Discussion

The direction of polysaccharide synthesis is a major feature of the state of differentiation of plant tissue. Although the synthases for glycosylation may be translated on membrane-bound polysomes and inserted at the level of the endoplasmic reticulum, the bulk of glycosylation takes place in the Golgi apparatus (Ray *et al.*, 1976; Robinson *et al.*, 1976; Ray, 1980; Kawasaki, 1981; Bolwell & Northcote, 1983). The arabinan synthase and xylan synthase were active *in vitro* in both membrane compartments (Bolwell & Northcote, 1983) but *in vivo* are subject to controls, which may include transport and availability of substrate or some form of activation as they traverse the endomembrane system.

The requirement for transcription and translation on the qualitative regulation of polysaccharide synthesis in response to subculture and plant growth substances has been studied by using actinomycin D and MDMP treatments. Actinomycin D selectively prevents transcription by

Table 1. *Characterization of rough endoplasmic reticulum from suspension cultured cells*

Cells were harvested and rough endoplasmic reticulum was prepared. Protein, RNA and phospholipid determinations were made for cells grown both in the presence or absence of [^{35}S]methionine (1.85×10^5 Bq). Polysome determinations were made with non-radioactive cells. The recoveries from 5g fresh weight of tissue are shown. The number of replicates is shown in brackets. Abbreviation: n.d., not determined.

		Total amount of:				
Time after subculture (h)	Medium	Protein (μg)	RNA (μg)	Phospholipid (μg)	[^{35}S]Methionine incorporated (c.p.m.)	Relative increase in polysomes (%)
0	—	205 ± 15 (8)	49 ± 7 (4)	12 (2)	6283 ± 1531 (4)	100 (4)
16	CMD	288 ± 89 (8)	108 ± 13 (4)	28 (2)	18116 ± 2360 (4)	154 ± 3 (4)
40	CMD	450 ± 42 (8)	89 ± 5 (4)	29 (2)	28966 ± 6863 (4)	160 ± 14 (4)
88	CMD	646 ± 170 (8)	98 ± 15 (4)	19 (2)	22040 ± 4928 (4)	173 ± 16 (4)
88	IM	518 ± 75 (4)	79 ± 11 (2)	22 (2)	22322 ± 5729 (2)	n.d.

complexing to deoxyguanosine residues (Goldberg & Friedman, 1971) and is extremely effective at 20 μg/ml in inhibiting mRNA synthesis in suspension cultures of bean, although the sensitivity of the PAL response was dependent on the timing and length of the exposure (Dudley & Northcote, 1979; Jones & Northcote, 1981). MDMP in contrast acts as an inhibitor of initiation of translation (Weeks & Baxter, 1972) and was effective in inhibiting incorporation of [^{35}S]methionine into total protein and the PAL response in this bean culture (Jones & Northcote, 1981).

The results from inhibitor studies on the rising phase of induction of arabinan synthase activity in CMD medium and xylan synthase activity in IM indicated that both transcription and translation are required for the rise in specific activity. During this period the incorporation of arabinose or xylose into the type of polysaccharide that was accumulated was a function of the state of differentiation of the cells, which in turn can be regulated by the application of different growth factors (Bolwell & Northcote, 1983). Specific gene products are therefore required either as direct products of the mRNA molecules for the synthases or systems involved in post-translational activation of pre-synthesized enzyme or to process the mRNA.

Unlike PAL, which is a cytosolic enzyme (Jones & Northcote, 1981), superinduction of the membrane-bound synthases was not observed in the presence of actinomycin D. Superinduction of PAL activity was interpreted as being due to decreased competition at the protein-synthesizing machinery of the mRNA coding for PAL compared with other messages, owing to its slower turnover compared with the other mRNA species, the production of which were inhibited by the actinomycin D. In general, membrane-bound proteins turn over faster than cytosolic proteins (Dean, 1978) and this may be due in part to faster message turnover.

After subculture of a suspension culture of plant cells there is probably an increase in protein synthesis. This is in part indicated for the cytosolic proteins by an increase in free polysomes (Bevan & Northcote, 1981*a*) and for membrane proteins by the increase in membrane-bound polysomes, which we have now shown also increase on subculture. The effect of subculture on both cytosolic and membrane-bound protein synthesis seems to be a rapid, perhaps non-specific, stimulation of the level of the translation apparatus as a prerequisite for cell division and growth. It was previously shown that the action of some auxins to alter qualitatively the pattern of mRNA production depends not only on the application of the growth factor but on subculture. The change in mRNA production was inferred from the appearance and disappearance of certain proteins detected in an '*in vitro*' synthesis system using the total mRNA extracted from the cells or from free polysomes to direct the protein synthesis (Bevan & Northcote, 1981*b*).

Subculture of the suspension culture stimulates the cells to grow and this is initially brought about by cell division, so that the action of the auxins may operate on actively dividing cells for the induction or repression of the transcription of certain mRNA species. At later stages of growth, suspension cultured cells that have been induced to differentiate by subculture into IM have a characteristic pattern of mRNA molecules that is different from that of cells subcultured into maintenance medium (Dudley & Northcote, 1978). In the intact plant the actively dividing cells are represented by the meristematic tissues such as the cambium. During differentiation of the cells to vascular tissue, we have shown that the appearance of polysaccharide synthase activity is controlled and this control is exerted at the stage of transcription and possibly translation. If the control of transcription is partially effected by auxins then this is probably operative during the period of the cell cycle to achieve the subsequent changes necessary for the differentiation.

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