Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9279–9283, December 1989
Botany

Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts

(plant hormones/*Nicotiana tabacum*/cell cycle/dedifferentiation)

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Communicated by Georg Melchers, August 21, 1989 (received for review June 22, 1989)

ABSTRACT **A cDNA clone for an auxin-regulated gene was isolated from a tobacco mesophyll protoplast cDNA library by differential screening. Nucleotide sequence analysis showed that the deduced product of the gene, which we have designated *par*, is hydrophilic and is composed of 220 amino acids. No significant homology to other known proteins was detected. The mRNA of the *par* gene is ≈900 bases long and its accumulation was detected in cultured mesophyll protoplasts as early as 30 min after the addition of 2,4-dichlorophenoxyacetic acid to the culture medium. The *par* mRNA was not detected in leaves or freshly prepared protoplasts or in protoplasts in the absence of 2,4-dichlorophenoxyacetic acid. Expression of the *par* gene was detected at a low level in actively dividing BY-2 tobacco suspension culture cells. The conspicuous accumulation of *par* mRNA before the initiation of DNA synthesis in tobacco mesophyll protoplasts suggests that the *par* gene product could play a role in the initiation of meristematic activity in differentiated mesophyll cells.**

It has been generally accepted that one of the most conspicuous characteristics of plant cells is totipotency, in which differentiated and nondividing somatic cells of plant tissues regain the ability to divide and ultimately regenerate whole plants. In contrast, no such phenomenon exists in animal systems. A central feature of totipotency, often referred to as "dedifferentiation," is the reinitiation of cell division in differentiated and nondividing cells. This phenomenon, in which differentiated cells pass through the restriction point from the G_0 phase to the S phase of the cell cycle, remains unexplained in molecular terms.

Tobacco mesophyll protoplasts—which are highly homogeneous, typically differentiated, and nondividing—are able to initiate DNA synthesis, divide, and form colonies (1), a process that is dependent on the presence of both auxin and cytokinin. The regeneration of whole plants from these colonies has also been established (2, 3). Thus, tobacco mesophyll protoplasts appear to be suitable material for the analysis of the reinitiation of cell division activity in differentiated plant cells.

In this study, to elucidate the mechanism of initiation of meristematic activity in differentiated and nondividing tobacco mesophyll cells, we tried to identify genes that could be activated by plant hormones in the early stages of protoplast culture. Since two-dimensional gel electrophoresis has shown that auxin is more effective than cytokinin in inducing the appearance of proteins that are not found in freshly prepared mesophyll cells (4), we chose to examine genes regulated by auxin during the early stages of culture of tobacco mesophyll protoplasts. In doing so, we isolated the cDNA for a gene that could play a role in the initiation of cell division.§

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MATERIALS AND METHODS

Preparation of Protoplasts. Protoplasts were prepared from mesophyll tissues of tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) according to the two-step procedure described by Okada *et al.* (5) except that cellulase YC (Seishin Pharmaceutical, Tokyo) was replaced with cellulase Onozuka RS. The protoplasts (10^5 per ml) were cultured in the medium of Nagata and Takebe (1) with or without a supplement of 2,4-dichlorophenoxyacetic acid, (2,4-D, at 1 mg/liter) for 24 hr under continuous illumination of 2000 lux at 27°C. The protoplasts were harvested by centrifugation at 90 × *g* for 1 min, frozen in liquid nitrogen, and stored at −70°C until required for extraction of RNA.

Preparation of RNA. Total RNA was isolated as described (6) except that 4 M guanidinium thiocyanate was used instead of guanidinium chloride. Poly(A)$^+$ RNA was purified from total RNA by oligo(dT)-cellulose column chromatography.

Construction of cDNA Library. A cDNA library was constructed from poly(A)$^+$ RNA from protoplasts cultured for 24 hr in the presence of 2,4-D. Double-stranded cDNA was prepared according to Gubler and Hoffman (7) and then ligated to λgt10 with *Eco*RI linkers as described (8).

Differential Screening. Replica filters were obtained from the cDNA library and were screened with single-stranded ^{32}P-labeled cDNA probes prepared from poly(A)$^+$ RNA of protoplasts cultured for 24 hr in either the presence or the absence of 2,4-D. Hybridization was at 65°C for 72 hr in 6× SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0) containing 1% sodium dodecyl sulfate ($NaDodSO_4$). Filters were washed with 2× SSC/1% $NaDodSO_4$ at 65°C and were autoradiographed for 50 hr with an intensifying screen at −70°C. Clones that hybridized with the 2,4-D(+) probe, but not with the 2,4-D(−) probe, were plaque-purified, and the cDNA inserts were characterized further by Northern blotting.

Northern Hybridization. RNA was subjected to electrophoresis in 1% agarose gel containing 2.2 M formaldehyde (9) and blotted onto GeneScreen*Plus* filters (DuPont). The filters were hybridized with random-primed probes (specific activity, 4–8 × 10^8 cpm/μg).

Primer Extension Analysis. A 19-nucleotide-long DNA (see Fig. 2) was synthesized as a primer and its 5′ end was labeled with the use of polynucleotide kinase and [γ-^{32}P]ATP. After

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthalene-1-acetic acid; IAA, indole-3-acetic acid; BAP, 6-benzylaminopurine.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29274).

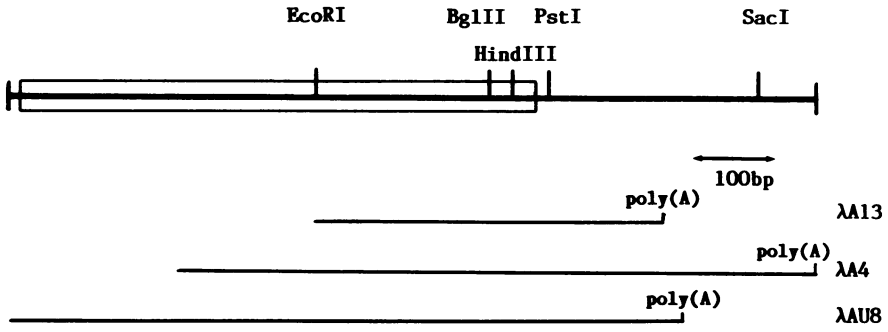

FIG. 1. Restriction map of *par* cDNA clones. Cleavage sites for some restriction enzymes are shown. Boxed area represents the proposed amino acid coding region. bp, Base pairs.

heating at 65°C for 5 min, hybridization was carried out in a 30-μl reaction mixture containing 40 mM Tris (pH 7.5), 0.4 M NaCl, 1 mM EDTA, 0.2% $NaDodSO_4$, 5 μg of poly(A)$^+$ RNA, and 0.5 pmol of 5′-end-labeled primer at 37°C for 5 hr. This was followed by reverse transcription and the product was analyzed by electrophoresis in a 6% polyacrylamide/8 M urea gel along with appropriate size markers.

RESULTS

Differential Screening. The cDNA library from protoplasts cultured in the presence of 2,4-D contained 2×10^6 independent clones with an average insert length of 1 kilobase (kb). Screening of ≈15,000 plaques by differential hybridization resulted in the selection of 15 clones that were found to hybridize preferentially to the cDNA probe prepared from poly(A)$^+$ RNA of protoplasts cultured in the presence of 2,4-D. Further analysis by Northern hybridization revealed that 4 of the 15 clones were cDNAs for an auxin-induced gene. As assessed by Southern hybridization, these 4 clones carried virtually identical sequences. Since neither of them covered the entire coding region, we rescreened the cDNA library with one of these clones, λA4 (Fig. 1), as a probe to obtain a longer insert. The clone λAU8, which carried the longest insert, about 900 bp, was chosen for subsequent analysis.

DNA Sequence and Primer Extension Analysis. The cDNA clone represents an auxin-regulated gene that we call *par* (protoplast auxin-regulated). A restriction map of the *par* cDNA is shown in Fig. 1. We sequenced the *par* cDNA by the dideoxy chain-termination procedure. The DNA sequence analysis and the deduced amino acid sequence are shown in Fig. 2.

To define the 5′ end of the *par* mRNA, primer extension analysis was performed by using a synthetic 19-nucleotide DNA (Fig. 2) as a primer on mRNA templates prepared from the protoplasts cultured for 24 hr in the presence of 2,4-D. The results revealed extension products of 78 and 110 nucleotides (Fig. 3). The size of the longer extension indicates that the cloned sequences lack no more than 54 nucleotides from the 5′ ends of the mRNAs. In the genomic clone (unpublished data), an in-frame nonsense codon, TAA, was found 19 bp upstream from the 5′ end of cDNA. Thus the translational initiation site is tentatively assigned to the ATG codon at positions 15–17, to which the first ATG triplet within the cDNA sequence corresponds. This ATG begins an open reading frame that encodes 220 amino acid residues. In the 3′ untranslated region, the *par* cDNA clones λA4, λA13, and λAU8 are polyadenylylated at different positions and do not contain the canonical polyadenylylation signal AATAAA (10).

***par* Gene Expression.** We examined the expression of the *par* gene in tobacco leaves, freshly prepared mesophyll protoplasts, protoplasts cultured in the presence or absence of 2,4-D for 24 hr, and BY-2 cells (11) 2 days after transfer to fresh medium, when they were most actively dividing (12). The *par* mRNA was abundantly expressed in protoplasts cultured in the presence of 2,4-D for 24 hr and at reduced levels in BY-2 cells, whereas significant expression was not detected in leaves, freshly prepared protoplasts, or protoplasts cultured in the absence of 2,4-D (Fig. 4). Although hybridization signals were sporadically detected in freshly prepared protoplasts, mostly from younger leaves, this signal, which could be due to the effect of endogenous auxins, was completely nullified during culture in the medium of Nagata and Takebe (1) without 2,4-D (data not shown). The tobacco *par* mRNA is ≈900 nucleotides long, indicating that λAU8 contains an almost full-length clone.

To assess the state of DNA synthesis in the same material, the same RNA blots were probed with the coding region of the wheat histone H3 gene (13), whose expression is closely coupled with DNA synthesis.[¶] The histone H3 gene was expressed only in actively dividing tobacco BY-2 cells (Fig. 4). This result indicates that even protoplasts cultured in the presence of 2,4-D for 24 hr had yet to enter S phase and strongly suggests that the initiation of *par* gene expression by auxin precedes the initiation of DNA synthesis. Labeling with [^{3}H]thymidine revealed that the S phase of protoplasts started more than 24 hr after the start of culture and that DNA synthesis increased until 48 hr, as described by Zelcer and Galun (14).

To assess "dedifferentiation" of the cultured cells, a pea ribulose-1,5-bisphosphate carboxylase small-subunit cDNA probe (15) was used as a leaf-specific differentiation marker, since expression of this gene has been shown to decrease during the culture of tobacco mesophyll protoplasts (16). The amount of ribulose-1,5-bisphosphate carboxylase small-subunit mRNA decreased at 24 hr of culture, and the presence of 2,4-D accelerated this decrease (Fig. 4).

Time-Course Experiment and Effect of Other Hormones. To investigate the induction kinetics of the *par* mRNA, total RNAs were isolated at various times after the addition of 2,4-D. To avoid the sporadically detected signals in freshly prepared protoplasts, 2,4-D was added to protoplasts that had been cultured in the medium of Nagata and Takebe (1) without 2,4-D for 24 hr. Northern hybridization showed that *par* gene expression began as early as 30 min after the addition of 2,4-D (Fig. 5*A*). Control hybridization with the λA1 probe, which is not affected by 2,4-D, demonstrated that equivalent quantities of intact RNA were present in each lane.

It has been shown (1) that NAA (1 mg/liter), but not IAA (1 mg/liter), can replace 2,4-D in inducing the initiation of cell division in tobacco mesophyll protoplasts. Northern hybrid-

[¶]Nakatsuka, A., Tabata, T., Okada, K., Nagata, T., Takebe, I. & Iwabuchi, M. (1988) Fifty-Third Annual Meeting of the Botanical Society of Japan, October 13–15, 1988, Okayama, p. 316 (abstr.).

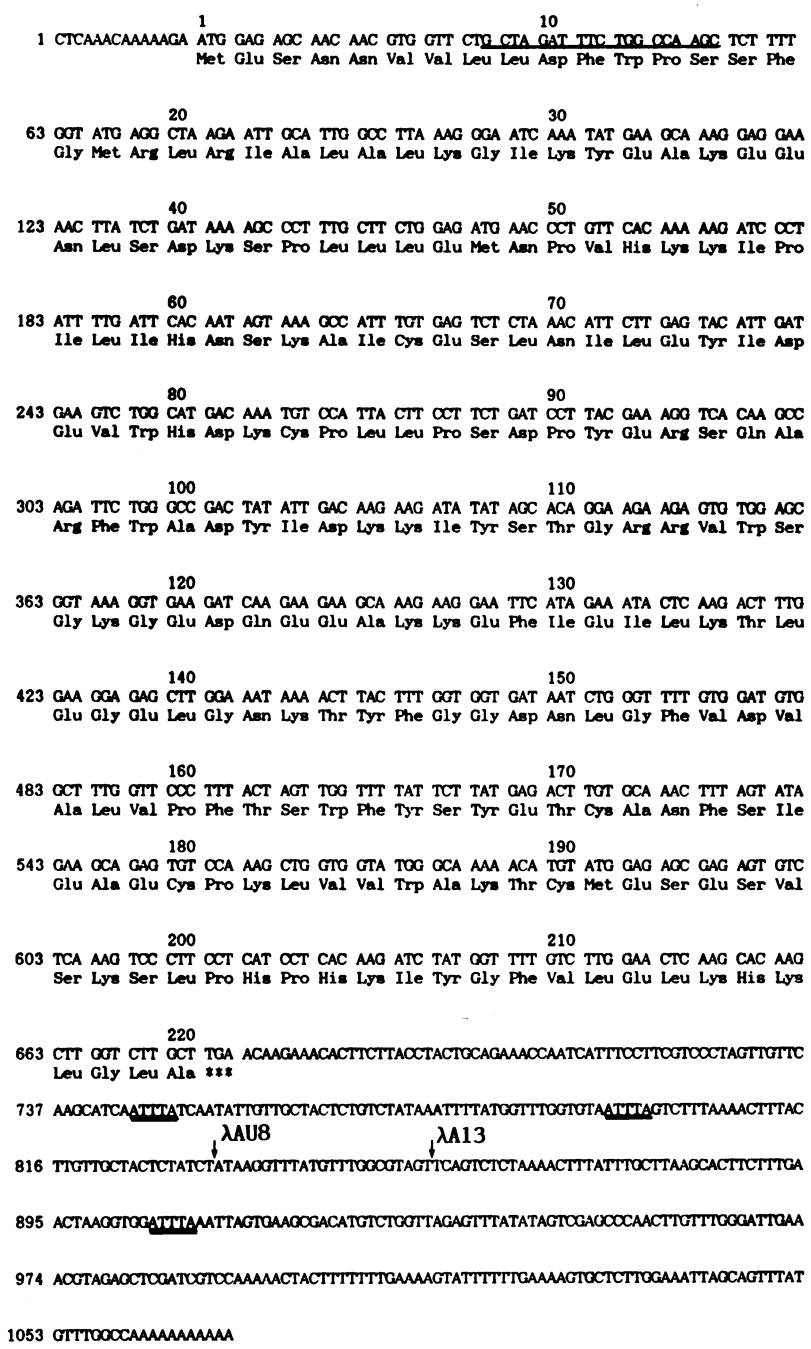

FIG. 2. Nucleotide sequence of *par* cDNA and the deduced primary structure of the protein. The 3′ termini of λA13 and λAU8-derived cDNAs are marked by arrows. The DNA sequence that corresponds to the synthetic 19-nucleotide primer used for primer extension analysis is underlined. The ATTTA sequences, which possibly confer mRNA instability (see *Discussion*), are heavily underlined.

ization showed that NAA was only slightly less effective than 2,4-D in accumulation of *par* mRNA, whereas *par* gene expression induced by IAA, which was comparable to that induced by 2,4-D at 1 hr (data not shown), decreased to a low level after 24 hr of culture (Fig. 5*B*). However, subtraction of the cytokinin BAP from the culture medium did not affect the expression of the *par* gene induced by 2,4-D (Fig. 5*C*).

DISCUSSION

We have isolated an auxin-regulated gene, designated *par*, from cultured tobacco mesophyll protoplasts by differential screening. Expression of the *par* gene was detectable as early as 30 min after the addition of auxin, while no such expression was detected in leaves and in protoplasts cultured in the absence of 2,4-D. Since the expression of the *par* gene preceded the commencement of DNA synthesis in the cultured mesophyll protoplasts, it appears that the product of this gene could play a role in the initiation of meristematic activity in differentiated mesophyll cells. This view is supported by the observation that *par* expression was not detectable in shoot tips including leaf primordia (data not shown). There is a strong correlation between the relative effects of 2,4-D and NAA on both the initiation of cell division and the accumulation of the *par* mRNA. The low level of accumulation induced by IAA at 24 hr could be due to biological metabolization of IAA during culture, as a significant level of *par* gene expression was detected at 1 hr of culture. That subtraction of BAP from the medium did not suppress *par* gene expression and yet did not result in cell

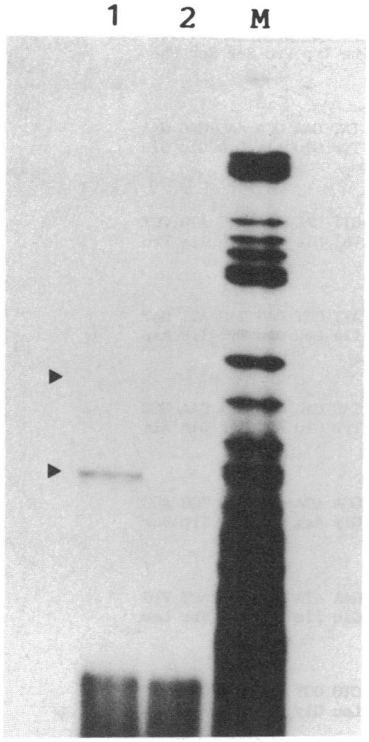

FIG. 3. Primer extension for defining transcription start point of *par* mRNA. The ^{32}P-labeled synthetic 19-nucleotide DNA (Fig. 2) was hybridized to poly(A)$^+$ RNA and extended with reverse transcriptase as described in *Materials and Methods*. Poly(A)$^+$ RNA samples (5 μg) were from mesophyll protoplasts cultured for 24 hr in the presence of 2,4-D (lane 1) or from leaf tissue (lane 2). Lane M, size markers (pBR322 *Hae* III digest). Extension products of 110 and 78 nucleotides are indicated by arrowheads at left.

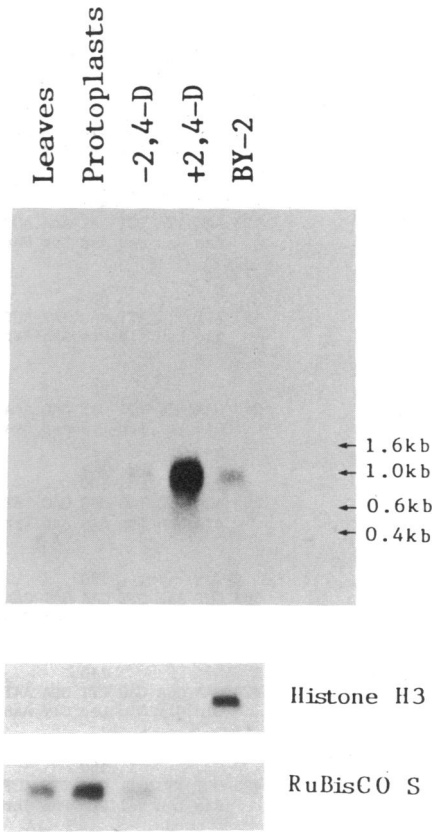

FIG. 4. Northern analysis of *par*, histone H3, and ribulose-bisphosphate carboxylase small-subunit (RuBisCO S) gene expression in tobacco leaves, freshly prepared protoplasts, protoplasts cultured for 24 hr in the absence of 2,4-D (−2,4-D), protoplasts cultured for 24 hr in the presence of 2,4-D (+2,4-D), and actively dividing BY-2 cells. Each lane received 1 μg of poly(A)$^+$ RNA. Leaves were from *N. tabacum* L. cv. Bright Yellow 2; the BY-2 suspension culture cells were derived from this cultivar as well. Protoplasts were obtained from *N. tabacum* L. cv. Xanthi nc.

division (1) suggests that the *par* gene alone is not sufficient for the induction of cell division and that cytokinin is required for some other activity (17).

The *par* cDNA sequence and the predicted amino acid sequence were examined for homologies with the sequences in the GenBank data base, but large-scale similarities with other genes, including auxin-regulated genes in the elongating tissues of soybean (18–20), were not detected. Hydropathicity plots (21) did not highlight any long hydrophobic regions, suggesting that the *par* gene product is not a secretory protein or an integral membrane protein. Furthermore, characteristic motifs for DNA-binding proteins, such as helix–turn–helix (22), zinc finger (23), and leucine zipper (24), were not detectable in the deduced amino acid sequence of the *par* gene product. Thus, at present, the *par* sequence data do not reveal any information about its function.

We have isolated three types of cDNA clones that are polyadenylylated at three different sites (Fig. 2). This may have some relation to the absence of the canonical polyadenylylation signal AATAAA within the 3′ untranslated region. Polyadenylylation at multiple sites has been reported for

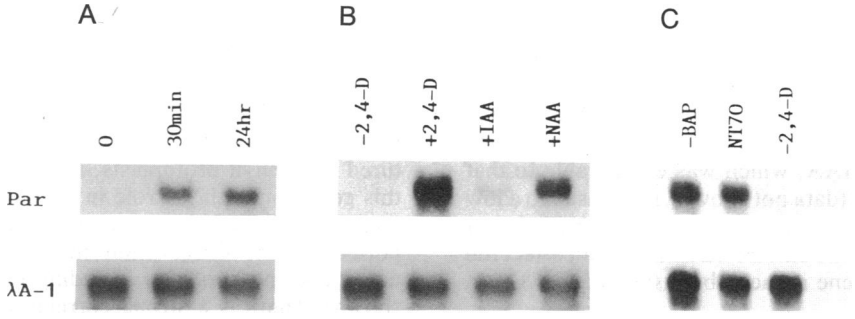

FIG. 5. (*A*) Induction of *par* mRNA accumulation by 2,4-D. Protoplasts were precultured in the medium of Nagata and Takebe (1) but without 2,4-D for 24 hr. RNA was extracted 0 min, 30 min, and 24 hr after the addition of 2,4-D (1 mg/liter). Each lane received 10 μg of total RNA. (*B*) Effect of various auxins on the accumulation of *par* mRNA. RNA was extracted after culture for 24 hr without 2,4-D or with 2,4-D, indole-3-acetic acid (IAA), or naphthalene-1-acetic acid (NAA). Each lane received 20 μg of total RNA. (*C*) Effect of 6-benzylaminopurine (BAP) on the accumulation of *par* mRNA. RNA was extracted after culture for 24 hr in the absence of BAP. (−BAP), in complete Nagata and Takebe medium containing BAP and 2,4-D (NT70), and in the absence of 2,4-D (−2,4-D). Each lane received 10 μg of total RNA. Blots were also probed with λA1, a clone isolated in the differential screening, whose expression is not affected by 2,4-D.

mouse α-amylase mRNA (25) and mouse dihydrofolate reductase mRNA (26). Three copies of the sequence ATTTA (27), found in many mammalian protooncogene (28) and growth factor response gene (29) mRNAs, are present within the 3′ nontranslated region of the *par* cDNA. This sequence is believed to play a role in the posttranscriptional regulation of gene expression by conferring instability on the mRNA (30). If the ATTTA sequence is also functional in plant cells, then the *par* mRNA might have a relatively short half-life.

In the differential screening of this study, four isolated cDNA clones carried the *par* cDNA. Whether other clones will be obtained with the same methods awaits further investigation. It will be of interest to determine whether the *par* gene product is related to two proteins that appeared in tobacco mesophyll protoplasts in medium containing 2,4-D (31). Our results are a step towards a molecular understanding of the role of auxin in the shift from G_0 to S phase of cell cycle in plant cells, and with further analysis it should be possible to elucidate the function of the *par* gene product.

Note Added in Proof. A recent data base search (GenBank) revealed that the amino acid sequence of the *par* gene has 34% homology to that of the auxin-regulated gene *Gmhsp* 26-A (32) from soybean, which is identical to the sequence G2-4 (18), and 23% homology to a stringent starvation protein of *Escherichia coli* that binds to RNA polymerase (33).

We wish to thank Dr. Callum Bell for his critical reading of the manuscript. This study was partly supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan to Y.T. and to T.N. The 19-mer oligonucleotide was synthesized at the Center for Gene Research, Nagoya University. This study was carried out under the Cooperative Research Program (89-101) and Program for Biomembrane Research of the National Institute for Basic Biology, Japan.

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