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## PREFERENTIAL SYNTHESIS OF MITOCHONDRIAL DNA DURING THE INITIAL STAGE OF TISSUE GROWTH IN POTATO EXPLANT CULTURES

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### Introduction

Excision of plant tissues and subsequent culture in a nutrient medium supplemented with phytohormones leads to a variety of physiological changes, which include increased respiratory metabolism, activation of macromolecule synthesis and replication of DNA prior to cell division. These changes have been shown to be a developmentally programmed process. Explants from Jerusalem artichoke tubers<sup>1)</sup>, potato tubers<sup>2)</sup> and carrot tap roots<sup>3)</sup> have been widely used for analyzing these events, since the cell cycle of these cells starts synchronously by incubation under appropriate conditions. Cytological investigation of the changes in DNA synthetic activity during the incubation of explants has demonstrated that the DNA syntheses require a relatively long lag phase after the preparation of explants and that the activity increases rapidly leading to the first maximum synthesis<sup>4)</sup>.

Satellite DNA is a molecular species separable from bulk or main-band DNA when equilibrated in CsCl density gradients, and has been found in various types of plant cell<sup>5)</sup>. The dense satellites include GC-rich nuclear DNA<sup>6)</sup> and organelle DNAs<sup>7)</sup>. Many reports have described evidence for compositional changes in whole-cell DNA, including an increase in the relative amount of satellite DNA, in developing plants or tissues cultured *in vitro*. These are considered attributable to selective replication of nuclear DNA or amplification of organelle DNAs. The seedlings of cucumber contain a high amount of heavy satellite<sup>8)</sup>, and the relative amount has been demonstrated to change in response to application of

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Abbreviations : IAA, indole-3-acetic acid; BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetra-acetic acid; SDS, sodium dodecyl sulfate; PB, phosphate buffer; SSC, 0.15 M NaCl, 15 mM sodium citrate, pH 7.5

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exogenous phytohormones to the seedlings; this change has been ascribed to selective replication of mitochondrial and chloroplast DNA<sup>10</sup>. A few investigations have also revealed the emergence of transient DNA satellite in cultured tobacco pith tissue<sup>11</sup> and carrot root explants<sup>9</sup>. The heavy fraction of nuclear DNA in carrot replicates differentially from bulk DNA during the early stage of callus formation<sup>12</sup>. However, there is only limited information on the selective synthesis of DNA in nuclei and the amplification of organelle DNA, and these species have not been characterized in detail.

Since the central core of potato tubers is composed mainly of parenchymatous tissue, the cells express physiological responses uniformly in culture. Culture of explants from potato tubers, therefore, appears to provide a good system for analyzing molecular events occurring during the early stage of tissue growth. Using this system, we present here data indicating that the preferential synthesis of a dense satellite takes place in explant cells following the initiation of tissue culture and that a major part of the DNA is involved with the synthesis of mitochondrial DNA. These results suggest that the mitochondrial DNA starts to replicate prior to the period of synthesis of major nuclear DNA. We also discuss the possibility that selective synthesis of the satellite includes the nuclear and plastid DNA.

## Materials and methods

### Culture of potato tuber derived explants

Tubers of potato (*Solanum tuberosum* L. cv. May Queen) were surface-sterilized in 10% sodium hypochlorite solution for 30 min and washed in sterile H<sub>2</sub>O. Cylindrical tissue samples were excised from the central core of tubers using a cork borer (6 mm diameter). The tissue was then chopped into disks 1 mm thick with a fine razor blade<sup>2</sup>. The explant disks were placed in Petri dishes containing a modified White medium supplemented with  $5.7 \times 10^{-7}$ M IAA,  $2.3 \times 10^{-6}$ M zeatin and 0.8% (w/v) agar and incubated at 25 °C under continuous illumination of  $8 \text{ W/m}^{-2} \cdot \text{s}^{-1}$  from cool white fluorescent tubes<sup>13</sup>.

After an appropriate culture time, the disks were labeled with [methyl-<sup>14</sup>C] thymidine (143 kBq/ml, 2.0 GBq/mmol, Amersham, Buckinghamshire, UK) for 2 h or 4 h as indicated in Results. After being labeled, the disks were blotted dry on filter paper, frozen and stored until the extraction of DNA. Otherwise, the change in incorporation was followed; after labeling for 1 h the disks were harvested and acid-insoluble radioactivity was determined using a scintillation spectrometer, as described previously<sup>2</sup>.

### Extraction of DNA

The extraction of DNA was done according to the procedure of Laulhere and Rozier<sup>14</sup> with modifications. Frozen disks were ground to powder in a mortar

and pestle, and blended with an extraction buffer comprising 100 mM tris (hydroxymethyl) aminomethane (Tris)-Cl (pH 8.0), 20 mM EDTA, 50 mM NaCl, 0.1% DEPC, 10% ethanol and 2% SDS. After incubation of the mixture at 37 °C for 10 min, the insoluble materials were removed by centrifugation at 10000 × g for 10 min. The pooled supernatant was adjusted to 0.5 M NaCl and kept at 0 °C for 5 min. Following the removal of precipitates by centrifugation at 18000 × g for 10 min, the nucleic acids were precipitated by the addition of two vols. of ethanol and subsequent centrifugation. The pellet was dissolved in 20 mM Tris-Cl (pH 8.0), 4 mM EDTA and 1% SDS and deproteinized once with an equal volume of chloroform-isoamylalcohol (24 : 1). The nucleic acids in the aqueous phase were ethanol-precipitated, washed in absolute ethanol and dried under vacuum. The DNA was finally purified by enzymatic digestion of RNA, followed by deproteinization with a chloroform-phenol mixture and ethanol precipitation.

### Isopycnic centrifugation of DNA in CsCl

DNA in 1xSSC containing 0.1% Sarkosyl was adjusted to 1.700 g·cm<sup>-3</sup> with solid CsCl and centrifuged at 40000 rpm for 48 h in a Hitachi 65TA rotor. The gradients were fractionated from the bottom of the tubes, and then absorbance at 260 nm was determined after dilution with aliquots of H<sub>2</sub>O. The gradients of CsCl were estimated from the refractive indices. Radioactivity of fractions was determined for TCA-insoluble precipitates collected on GF/C paper (Whatman, Madison, UK) using a liquid scintillation spectrometer.

### Extraction of mitochondrial DNA

Peeled tubers of potato were ground with a grater (kitchen tool) in 0.44 M sucrose, 50 mM Tris-Cl (pH 7.4), 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.2% BSA and then homogenized in a mortar and pestle. After filtration through two layers of gauze, starch grains and nuclei were removed by centrifugation at 800 × g for 10 min. The mitochondria were collected by centrifugation at 12000 × g for 10 min, incubated in 50 μg/ml DNase I for 30 min at 25 °C and finally purified by sedimentation in a linear 30% to 60% sucrose gradient<sup>15)</sup>. The DNA was extracted from the mitochondrial fraction using essentially the same procedure as that for disks from tubers.

### DNA reassociation kinetics

DNA reassociation experiments were performed according to the method of Dillon *et al.*<sup>16)</sup> with slight modifications. DNA extracted from labeled tissue was sonicated in 0.12 M PB at 0 °C, to give a mean fragment length of 600 base pairs. The sheared DNA was denatured at 100 °C for 5 min and incubated for reassociation at a concentration of 10 μg/ml and 700 μg/ml DNA in 0.12 M PB or 0.24 M PB. Reassociation was carried out at 25 °C below the  $T_m$ . DNA of *E. coli* was

reassociated at 40  $\mu\text{g/ml}$  and 500  $\mu\text{g/ml}$  in 0.12 M PB at 60 °C. The reaction was terminated by diluting the DNA with cold  $\text{H}_2\text{O}$ , and then the single- and double-stranded DNA was separated by hydroxyapatite chromatography. Equivalent *Cot* values (mole  $\times$  s/l) were calculated for each sample after correction to standard salt concentration (0.12 M PB)<sup>17</sup>.

### Restriction analysis of DNA

DNA samples were incubated for 3 h with *Eco* RI, *Hind* III or *Bam* HI (Nippon Gene, Tokyo) at 4 units/ $\mu\text{g}$  DNA under the conditions directed by the supplier. The digests were electrophoresed on 0.8% agarose gels, stained with ethidium bromide and photographed under UV-illumination. Radioactivity on the agarose gels was visualized by fluorography; the dried gels impregnated with scintillant were exposed to preflashed screened X-ray film (RP-50, Kodak) at -80 °C for 24 to 120 h<sup>18</sup>.

### Hybridization

A radioactive 25S rRNA probe was prepared as described previously<sup>19</sup>. rRNA/DNA hybridization was carried out according to the procedure of Jaworski and Key<sup>20</sup>.

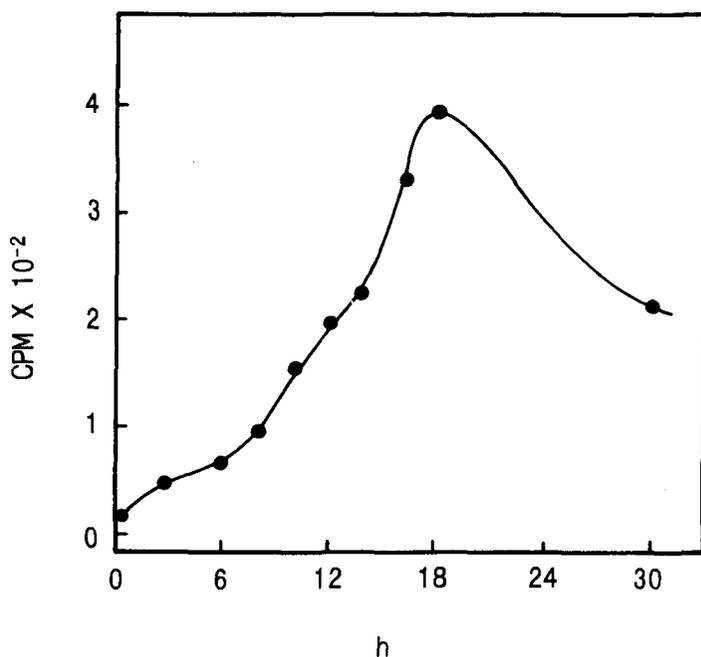
## Results

### Time course changes in thymidine incorporation

Changes in DNA synthetic activity following the start of culture of explants from potato tubers was assessed from the radioactivity in the acid-insoluble fraction. When explants were exposed to radioactive thymidine for 1 h during the first 30 h of culture, appreciable incorporation into the disks was detected within 4 h of culture, and a moderate increase followed until 7 h (Fig. 1). Incorporation then started to rise sharply, showing the first maximum at 18 h of culture and decreasing thereafter. Although the incubation time required to produce the maximum incorporation varied slightly depending on the period of storage after harvest of the tubers, the profile which represents the early phase showing first a moderate increase and a subsequent rapid increase was always observed.

### Density gradient centrifugation of early synthesized DNA

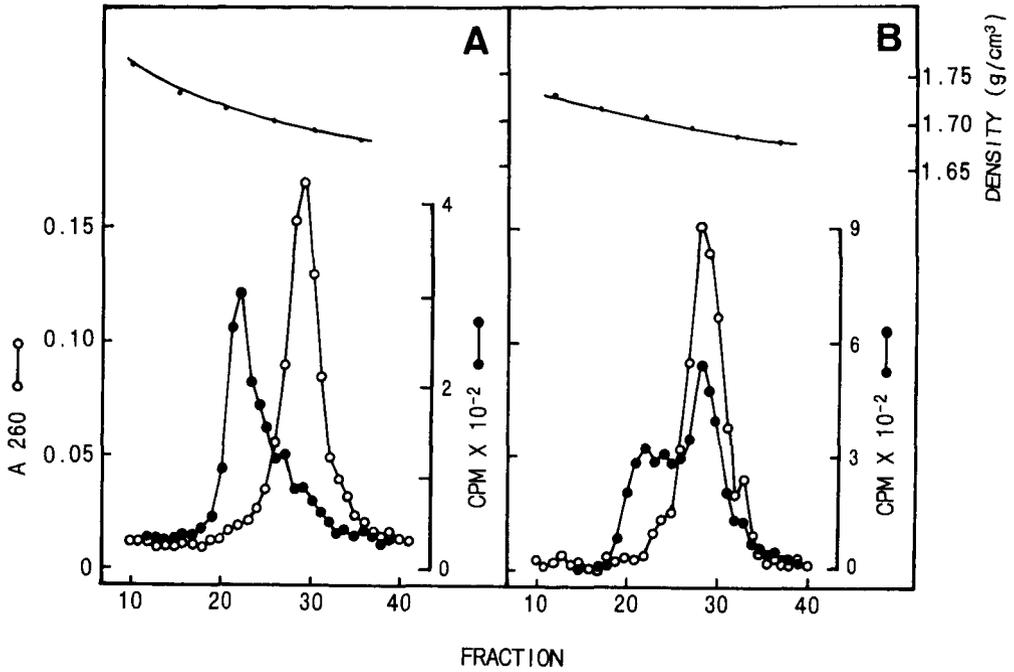
To obtain preliminary information on the nature of the DNA synthesized before and after 7 h of culture, the explants were labeled with radioactive thymidine for 2 h after 5 or 8 h of culture. The isopycnic distribution in CsCl gradients of labeled DNA is shown in Fig. 2. The whole DNA determined by absorbance at 260 nm showed bulk DNA banding at 1.694  $\text{g}\cdot\text{cm}^{-3}$  with small satellite components on the dense side of the main band. Almost all the radio-



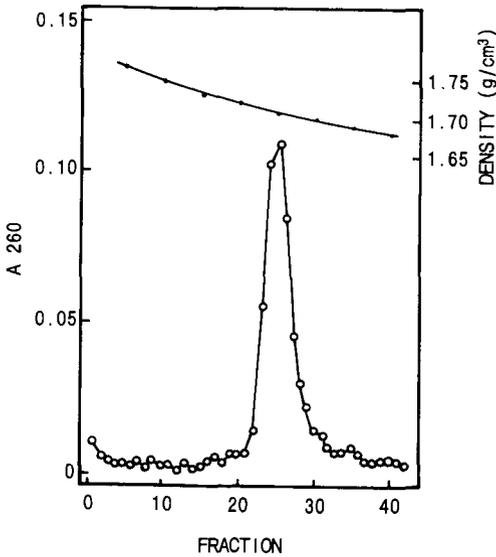
**Figure 1.** Time course changes in thymidine incorporation into acid-insoluble fraction of potato explants following the start of culture. Radioactive thymidine was applied to the explants at the time indicated in the Figure. After labeling for 1 h, the explants were processed to determine the acid-insoluble radioactivity.

activity (more than 70%) in DNA labeled from 5 to 7 h of culture was distributed in the density range  $1.700$  to  $1.712 \text{ g}\cdot\text{cm}^{-3}$  (Fig. 2A). While the distribution of the radioactivity was somewhat asymmetrical and significant incorporation into less dense fractions was detected, the radioactivity did not form the distinct peak at the main band until 7 h after the start of culture. The DNA sample extracted from disks labeled from 2 to 4 h of culture showed lower radioactivity, whereas it also gave a profile displaying pronounced incorporation into the satellite components (data not shown). Significant incorporation into the main band was detected by labeling from 8 h to 10 h and produced a distinct peak at a density of  $1.694 \text{ g}\cdot\text{cm}^{-3}$  (Fig. 2B).

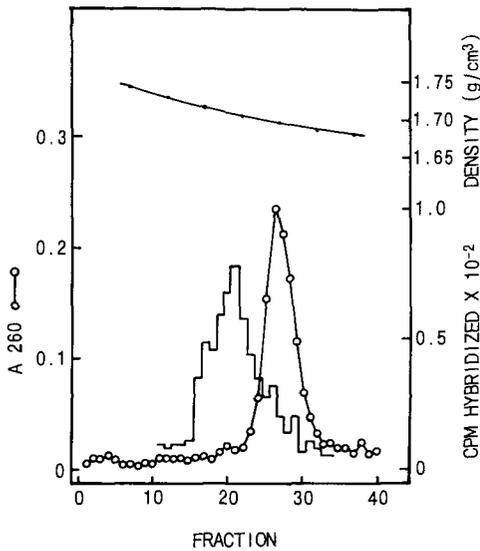
The distribution of potato mitochondrial DNA and rDNA in the CsCl gradient is shown in Fig. 3 and Fig. 4, respectively. The mitochondrial DNA showed a nearly symmetrical distribution, having a density of  $1.708 \text{ g}\cdot\text{cm}^{-3}$ . On the other hand, the density of rDNA was estimated at  $1.710 \text{ g}\cdot\text{cm}^{-3}$  by hybridization experiments of DNA fractions from the CsCl gradient and the 25S rRNA probe.



**Figure 2.** Distribution of DNA synthesized during the initial stage of tissue culture of potato explants in CsCl density gradients. Extracted DNA from explants labeled for 2 h after 5 h (A) or 8 h (B) of culture was banded in CsCl gradients. The gradients were fractionated after centrifugation, and refractive indices, absorbance at 260 nm and radioactivity were determined for each fraction. The density gradients were estimated from the refractive indices.



**Figure 3.** Density gradient centrifugation of mitochondrial DNA of potato. DNA extracted from mitochondria of tubers was banded in a CsCl density gradient.

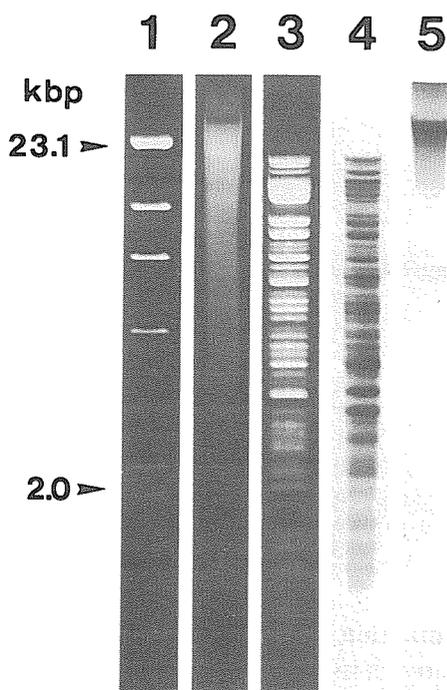


**Figure 4.** The distribution and buoyant density of potato rDNA in a CsCl gradient. The whole DNA was separated in CsCl gradients, and the fractions were collected from the bottom of the tubes. After determination of absorbance at 260 nm, DNA fractions were immobilized on nitrocellulose membranes and then reacted with a 25S rRNA probe.

### Restriction analysis and rRNA/DNA hybridization

Whether or not the DNA synthesized before 7 h included mitochondrial DNA was examined by comparing the electrophoretograms of restriction fragments from mitochondrial DNA and the fluorogram of fragments of DNA labeled *in vivo* (Fig. 5). Labeled DNA was extracted from disks which had been cultured for 3 h and labeled for an additional 4 h. The radioactive fractions from CsCl gradients were pooled and subjected to restriction analysis. We tested *Eco* RI, *Hind* III and *Bam* HI for restriction digestion, and the fragments generated by *Bam* HI gave finely comparable bands, which showed a discrete distribution upon agarose gel electrophoresis. The fluorography of DNA fragments from labeled disks indicated radioactive bands distributed from 20 to less than 2.0 kbp (Fig. 5, lanes 4). The electrophoretogram of *Bam* HI fragments of potato mitochondrial DNA showed a discrete distribution of the bands (Fig. 5, lane 3) which demonstrates a high similarity with the results of fluorography; on the basis of relative migration upon electrophoresis, more than 70% of DNA fragments from labeled disks were identified as derived from mitochondrial DNA. These results indicate that a considerable fraction of the DNA labeled before 7 h of culture included mitochondrial DNA.

The relative amount of rDNA was determined by quantitative hybridization of the 25S rRNA probe and DNA from cultured disks (Table 1). No significant change in rDNA redundancy was detected at the end of 7 h of culture, irrespective of the application of phytohormones.



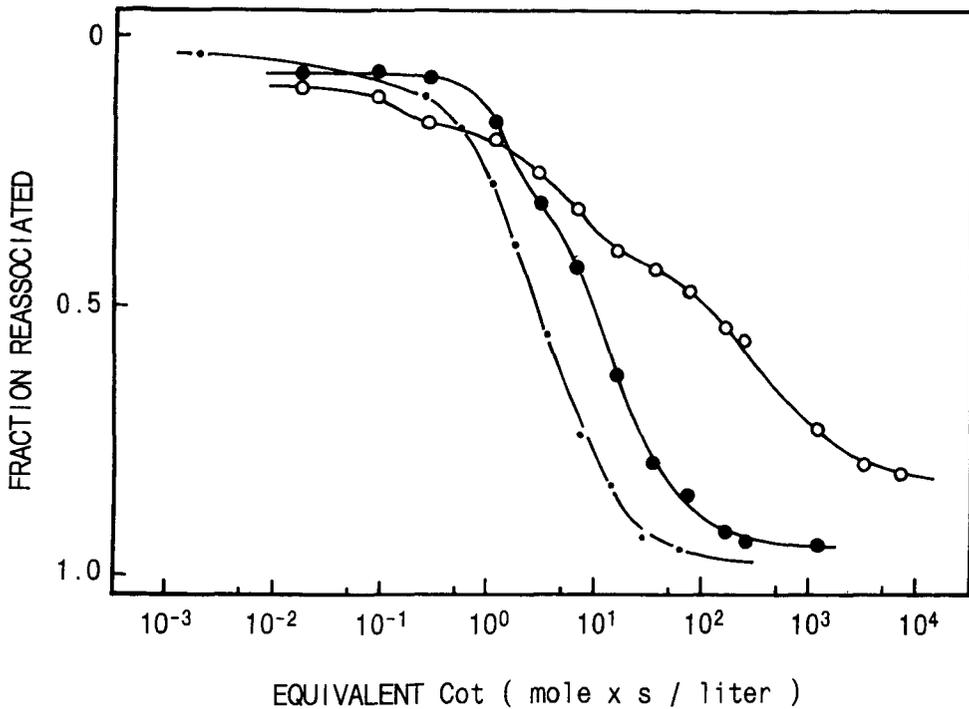
**Figure 5.** Fluorogram of *Bam* HI fragments of DNA synthesized during the initial stage of tissue culture of potato, compared with the electrophoretogram of mitochondrial DNA digested with the endonuclease. Explants from potato tubers were cultured for 3 h and labeled subsequently for 4 h with [ $^{14}\text{C}$ ]thymidine. The extracted DNA was purified by banding in CsCl gradients and used for subsequent analysis with endonuclease. The *Bam* HI fragments separated on agarose gel were stained with ethidium bromide (*lane 2*), and the radioactivity was visualized by fluorography (*lane 4*). Otherwise, the *Bam* HI fragments of potato mitochondrial DNA were separated on agarose gel and stained with ethidium bromide (*lane 3*). The fluorogram of uncut DNA from labeled explants is shown in *lane 5*. *Lane 1* indicates molecular size markers.

**Table 1.** Hybridization of 25S rRNA to DNA from freshly prepared and cultured explants of potato tubers. The DNA was extracted from freshly prepared explants and ones cultured for 8 h on medium containing  $5.7 \times 10^{-7}$  M IAA with or without  $2.3 \times 10^{-6}$  M zeatin. Each filter contained 13  $\mu\text{g}$  DNA. Specific radioactivity of RNA was  $7 \times 10^3$  cpm/ $\mu\text{g}$ . Blank filters incubated with RNA probe contained 35 to 40 cpm.

DNA source	cpm bound to filter	relative %
Explants	$209 \pm 4$	100
+ IAA	$212 \pm 6$	101
+ IAA + Zeatin	$207 \pm 3$	99

### Reassociation kinetics

The complexity of the early synthesized DNA was analyzed by reassociation kinetics experiments (Fig. 6). The DNA was labeled and purified by the same protocol as that employed for the restriction analysis. Following the density gradient centrifugation, the fractions with densities ranging from 1.680 to 1.720  $\text{g} \cdot \text{cm}^{-3}$ , which includes whole DNA, were processed for reassociation experiments. The reassociation of whole DNA, estimated by absorbance at 260 nm, demonstrated three distinct kinetic components. The *Cot* 1/2 values of highly repeated, intermediately repeated and rarely or unique repeated fractions were 0.1, 5 and 800, respectively. On the other hand, reassociation determined on the basis of



**Figure 6.** Reassociation kinetics of DNA synthesized during the initial stage of tissue culture of potato explants. The explants were labeled and purified as described in Fig. 5. DNA was sheared to 600 nucleotide fragment length, denatured and incubated for reassociation in phosphate buffer. The single and double stranded DNAs were separated by hydroxyapatite chromatography, and the fraction reacted was estimated based on the determination of absorbance at 260 nm (o—o) and radioactivity (●—●). The denatured fragments of *E. coli* DNA were reacted separately as a reference (—•—).

radioactivity revealed that approximately 60% of the labeled DNA showed a single kinetic component with the  $Cot\ 1/2$  of 10. The remaining minor fraction found in labeled DNA showed a slightly faster reassociation; synthesis of highly repeated DNA and single-copy DNA was not found. This implies that the intermediately repeated sequence was preferentially synthesized in potato explants during the first 7 h of culture. From the relationship between the  $Cot\ 1/2$  values based on the determination by radioactivities and that of the slowly reassociating fraction obtained by optical determination, the copy number of synthesized DNA was estimated; assuming that the slowly reassociating fraction with  $Cot\ 1/2$  of 800 is comprised of single-copy DNA, the  $Cot\ 1/2$  of 10 for a labeled fraction corresponds to 80 copies. In addition, on the basis of the proportional relationship in rate of reassociation and genome size, using *E. coli* DNA as a reference, a haploid genome size of  $1.28 \times 10^{-12}$  g was estimated for potato cells.

### Discussion

The present study demonstrated that a heavy satellite DNA was differentially synthesized from bulk DNA during the early stage of tissue growth in cultures of tuber-derived explants of potato. Restriction endonuclease analysis indicated that a major part of the early synthesized DNA consisted of mitochondrial genome. The determination of the buoyant density of purified mitochondrial DNA in the CsCl gradient supports this result.

Following the initiation of cell proliferation in explants from resting storage organs, respiratory metabolism is known to be activated very markedly. The activation is considered to be associated with various aspects of alteration in the structure and molecular constituents of the mitochondria. Changes in fine structure of the mitochondria, such as shape, size and the number of cristae, have been demonstrated for various tissues showing different types of proliferation activity<sup>21)</sup>. The supply of some tRNA species from plastids and nuclei and nuclei-directed polypeptides involved in the enzymatic complex of the respiratory chain appears to be essential for the building of functional mitochondria<sup>22,23)</sup>. In addition, the present experimental results suggest that an increase in the copy number of mitochondrial DNA is also responsible for activation of the respiratory metabolism in the cultured explants. On the other hand, synthesis of mitochondrial DNA may be required for replication of the organelle. However, the result indicating that onset of synthesis of DNA was one of the earliest responses elicited in the explants implies that the increase in copy number of the mitochondrial genome is closely linked with the early activation of respiratory metabolism in potato explants. The preferential synthesis of mitochondrial DNA thus appears to be a transient event during the change from a resting to proliferative state, independent of the phase of the cell cycle.

Analysis of labeled DNA by density gradient centrifugation demonstrates that some minor components, which overlapped with the dense fraction of the main band, are also preferentially synthesized. In addition, in the reassociation experiments, the labeled DNA was shown to include a minor fraction. These results indicate that some components differing from mitochondrial DNA are synthesized preferentially. In explants derived from carrot root, synthesis of a dense satellite component has been reported to start earlier than the bulk DNA, and the synthesis is due to the amplification or early, allocyclic replication of a portion of the nuclear DNA<sup>12,25)</sup>. These reports have also demonstrated that a slightly heavier fraction than the main band replicates at the early phase of the first synthetic period in explants from Jerusalem Artichoke tubers. In some vertebrates, a relative increase of heavy satellite DNA during oogenesis is well known, and the compositional changes in cellular DNA are attributed to the amplification of rRNA genes. In view of this, it was intriguing to explore the change in the relative amount of rDNA in potato explants; the synthesis of RNA

in tuber cells, which consists mainly of rRNA, is activated markedly following the initiation of tissue culture. In addition, our data indicate that the heavy satellite resolved from the main band DNA in CsCl gradients includes rDNA. However, there was no significant change in the relative amount of rDNA, suggesting that the machinery for activation of rRNA synthesis does not require an amplification of genes. In this regard, we have confirmed the conclusion by Siegel that the plant genome has a quite stable copy number of rDNA through growth and differentiation, since higher plant cells always contain sufficient copies of rDNA in their genome for urgent demands for rRNA<sup>26</sup>. Although the possibility of amplification of rDNA was almost excluded, it still remains to be resolved whether or not the early synthesized satellite in the potato system includes a dense fraction of nuclear DNA.

The reassociation kinetics revealed that the early synthesized DNA is lacking in highly repeated DNA as well as a single copy sequence. In addition, the minor fraction of labeled DNA consisted of an intermediately reassociating component. These results suggest that the labeled fraction includes the newly synthesized plastid DNA. The differential replication of plastid and nuclear DNA has been shown for tobacco cell suspension culture; DNA synthesis of plastids occurs exclusively during the first day of culture when the cells at stationary phase are transferred to fresh medium<sup>24</sup>.

Thus, the possibility that the minor fraction contains plastid DNA and/or intermediately repeated nuclear DNA remains to be explored. To define the origin of the early synthesized DNA and to characterize it would give a valuable clue for analyzing events which occur during the growth and development of explants in tissue culture.

### Summary

The DNA synthesized during the initial stage of tissue growth in culture of explants from potato tubers was characterized. An appreciable incorporation of radioactive thymidine into DNA was detected within 4 h of culture, and this increased moderately until 7 h. The incorporation then started to rise sharply, showing the first maximum at 18 h. Density gradient centrifugation of DNA in CsCl indicated that the label before 7 h was incorporated selectively into a heavy satellite DNA with a density ranging from  $1.700 \text{ g}\cdot\text{cm}^{-3}$  to  $1.712 \text{ g}\cdot\text{cm}^{-3}$ . This range encompasses the density of mitochondrial DNA and rDNA for the potato. The synthesis of bulk DNA displaying a main band at  $1.694 \text{ g}\cdot\text{cm}^{-3}$  was initiated at 7 h. The *Bam* HI digest from the early synthesized DNA was shown to contain fragments of mitochondrial DNA by comparative analysis upon agarose gel electrophoresis and fluorography. There was no significant change in the copy number for rDNA. Reassociation experiments demonstrated that the DNA labeled before 7h of culture represented at least two kinetic components compris-

ing repeated DNA sequences. These results suggest that preferential replication of mitochondrial DNA occurs during the initial stage of tissue growth in culture of explants from potato tubers.

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