SCIENTIFIC REPORTS

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ERRATA

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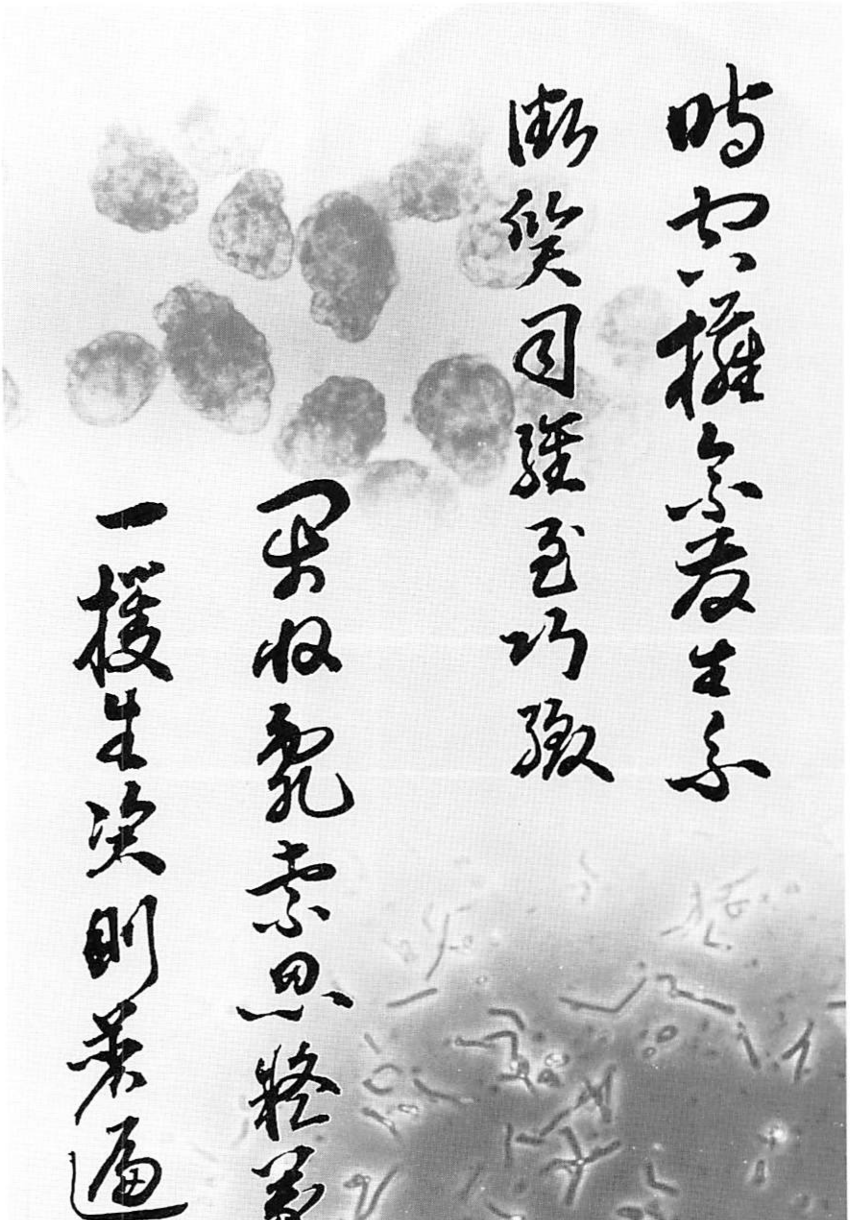
Molecular Biology



DEPARTMENT OF MOLECULAR BIOLOGY

GENERAL SUMMARY

The general aim in the research activities of this department has been concerned with molecular mechanisms of genetic expression and regulation as functions of bacterial spore germination, embryonic development and tumor virus induction. These projects should be naturally based on fundamental knowledge so far obtained in analysis of elementary processes for genetic expression, especially whenever either appropriate prediction to a clue or plausible elucidation for actually obtained results would be necessary. At the same time, much efforts in clarifying the specific expressions or reactions, which are truly responsible or may have some relevance to the events in question, must be made. On the aspects, reserving the actual results for latter Abstracts, we elect two main projects from them and will briefly describe their specific aims in dealing with such systems.

1. **Sequential gene activation**

In the first project, our interest has focussed on activation of the *Bacillus subtilis* spore resulting in probably a sequential genetic expression during germination. The process is very unique with respect to development of cellular functions, indispensable for the vegetative phase, from the dormant state. Therefor, it might be thought that the gemination process provides a key to open a new subject how an inert status of transcriptional machinery including DNA can be awoke at an early step of developmental processes in general. Our investigation is currently directing toward analysis of RNA synthesized in the germination step, since the activation of the machinery must be recognized primarily by its transcripts. The results shown in Abstract (1), as predicted in the previous issue, clearly demonstrate that the activation proceeds stepwise through distinct three phases, namely, Gm 1, Gm 2 and Gm 3, with respect to the rate of RNA synthesis, and further that such distinct phases are coincidently characterized as a reflection from successive expression of genes, judging from different two types of qualitative analyses of RNA.

Thus far our survey now throws light on onset of t-RNA and r-RNA synthesis, by which the first two phases, Gm 1 and Gm 2, are well specified respectively. Further experiments by use of various inhibitors acting in different fashions reveal that the onset of r-RNA synthesis in Gm 2 absolutely depends upon protein synthesis and/or a structural releasing process of DNA from its frozen status in the preceding Gm 1. The fact that antibiotics acting on DNA-gyrase, nalidixic acid and novobiocin, inhibit phage promoter-dependent trascription of *trp* operon in *Φ*80*trp* was recently reported by Smith *et al.* (Nature, **275**, 420, 1978). Our finding on this subject might be the second and strongly suggests that an alteration of DNA conformation might be an essential prerequisite for expression of certain kind of gene(s). These results are summarized in Table to visualize more easily. Our further investigation to clarify the entities responsible for the conformational alteration and the regulating

Table. RNA synthesis during germination of *B. subtilis* spore.

Synth. of \ Phase		Gm 1 (0–15 min)	Gm 2 (15–40 min)	Gm 3 (40–60 min)
RNA	Rate:	gradually increases	reaches a definite level	explosively increases
	Species:	t-RNA (major) 5S RNA smaller RNA no r-RNA mRNA (a little?)	t-RNA 5S RNA 16S, 23S r-RNA starts mRNA starts	t-RNA 5S RNA 16S, 23S r-RNA continues mRNA increses
	CM: LM, SM, PM, EM:	no inhibition to t-RNA the same as above	40% inhibition no r-RNA&mRNA the same as above	remarkable inhibition no r-RNA & mRNA the same as above
	Nal, NB:	no inhibition to t-RNA	60% inhibition no r-RNA&mRNA	90% inhibition no r-RNA & mRNA
Protein		only a limited synthesis	starts & reaches a definite level	linearly increases
DNA		no synthesis	no synthesis	starts

CM: chloromycetin, LM: lincomycin, SM: streptomycin, PM: puromycin, EM: erythromycin, Nal: nalidixic acid and NB: novobiocin.

mechanisms for the stepwise transcription would be most comprehensive for future progress.

We must emphasize, with many thanks, that our success in device with a series of inhibitors acting on DNA synthesis, especially on DNA-gyrase, was exclusively owing to a quite pertinent advice from Prof. H. Yoshikawa of the Department of Biophysics in this Institute.

2. Distinction of embryonal and differentiated status

The second main project in this department deals with embryonic development. The problem of how uncommited cells become determined and finally specialized in embryonic development has been of central interest to biology for a long time. The experimental systems and concepts are now becoming available by introducing the teratocarcinoma cells derived from 129 mouse. Considering thus far obtained brilliant results on its cellular and genetic aspects, we believe that such noble material has a great advantage in clarifying certain relevances between embryonal potencies for differentiation and for tumor generation.

We previously reported that single cells of embryoid bodies (EB) are able to proliferate in a diffusion chamber and produce many spherical cells and a few spindle shaped cells on the surface of membrane. Such features of the

cells suggest that a great majority of spherical cells might be still remaining in an embryonal status, whereas that a few spindle shaped cells might be in a differentiated status. Thus, it could be said that some factors due to unique environmental conditions are operating in the transition from "embryonal" to "differentiated" status. On this aspect, we intended in the past years to survey the environmental requisites for the transition by controlling proliferation of cells derived from EB in a chamber.

The results in Abstract (3) and (4) as a whole could be interpreted as that the single cells derived from EB can not express any specified antigens, as far as they are maintained in the proliferating state, while as that they can express more or less specific antigens, when their proliferation reaches the nearly saturated state, hence entering into "differentiated" status. Now we can exclusively specify whether the cells in a chamber are in one or another status by controlling their proliferation.

Considering various factors for being possible to control, the chamber technique seems fortunately to have a great advantage. We can easily observe cells growing on the membrane surface whereon they may first anchor. The surface might be well pertinent as anchorage and also preferable for floating cells in supporting their proliferation somehow due to its nature, permeability and construction etc., differently from that of glass or plastic. In such circumstance "embryonal" cells extensively proliferate *in statu quo* at a constant rate. The membrane surface is then successively occupied by dividing cells forming a sheet. Reaching the nearly saturated state, since no rooms to anchor, the cells then encounter with entirely different microenvironment. Finally this effect might lead the "embryonal" to "differentiated" status by unknown mechanisms. Accepting such elucidation, it is expected "embryonal" status could be maintained forever by a large number of serial transfers. Results are so far well agreeing with this extraporation.

The results also confirm that the differentiation of EB-cells depends upon their attachment to glass surface even in the *in vivo* culture. In contrast, the cells grown in the second layer remain at "embryonal" status, nevertheless they are equally derived from the same origin as those in the first layer. In connection with this, much attentions must be paid to the fact that embryonal carcinoma cells could not differentiate *in vitro* on feeder cells.

It has been unexpected that the single EB-cells just after mild treatment for dissociation exhibit to some extent an absorption of cytotoxicity. This absorption might be explained alternatively by Ia antigen detectable or by host antigens contaminating in some EC-cell lines (unpublished data). Further qualifications of "embryonal" and "differentiated" status on their antigenicity, tumorigenicity and responsibility to various lectins are now actively in progress.

These studies were supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture , Japan.

ABSTRACT

(1) Transcription and regulation during germination of the *Bacillus subtilis* spore. I. Three phases of RNA synthesis.

M. Matsuda and T. Kameyama

It has been well known that RNA synthesis starts at an early phase prior to onset of protein and DNA synthesis during germination of the *B. subtilis* spore (Fig. 1). In this report the rate of RNA synthesis as a primary index of transcription is analyzed. Using pulse labeling with ^{3}H-uridine, the rate of RNA synthesis during germination showed three distinct phases. The result in Fig. 2 demonstrates such a typical profile; RNA synthesis starts after about 5 min, the rate increases linearly up to 15–20 min (Gm 2), and finally the rate increases explosively (Gm 3). In accordance with this profile, the rate of protein sythesis also remains constant in the Gm 2 phase (Fig. 2).

In the next attempt further qualification of each phase was carried out. When ^{3}H-RNA probes labeled in Gm 1 (10–20 min), Gm 3 (55–60 min) and log phase (for 1 min) were hybridized and saturated to a constant amount of *B. subtilis* DNA, they attained saturation at 2.5%, 7.5% and 20% of DNA added respectively. The result indicates only small region of DNA are transcribed in Gm 1, while the regions increase successively through Gm 2 and Gm 3. Neither RNA probes obtained at 20 min and 60 min con-

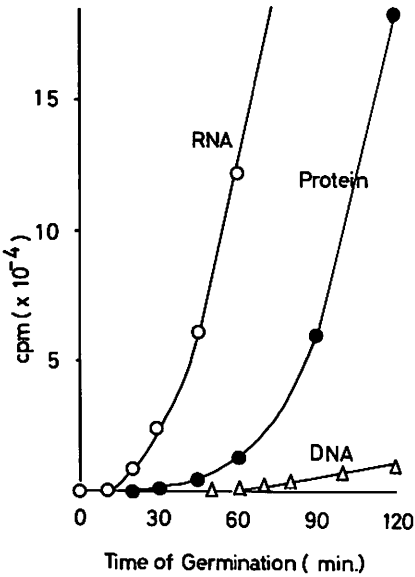


Fig. 1. Sequential synthesis of RNA, protein and DNA during germination. *B. sublitis* spore in LP-medium supplemented with L-alanine was used. ^{3}H-uridine, ^{3}H -amino acid mixtures and ^{3}H-thymidine were added at 0 min.

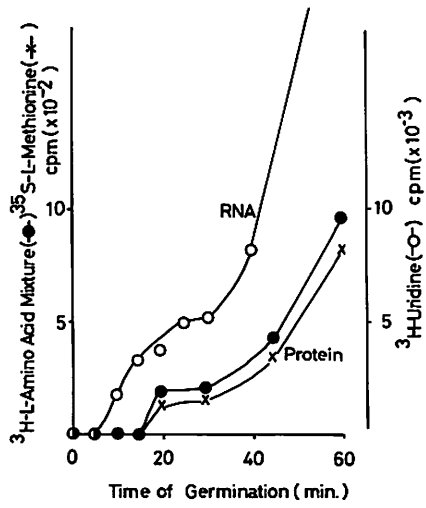


Fig. 2. The rate of RNA and protein synthesis during germination. Isotopes were added at times indicated and a pulse incorporation for 1 min was counted.

tain any specific RNA species for each phase, since they were almost completely in competition with cold RNA prepared from log phase cells.

These results led us to analyze RNA species synthesized in each phase in order to determine which gene(s) are activated at first and how gene order to be activated proceeds successively during germination. The pulse labeled ^{3}H-RNA obtained at times indicated were subjected to gel-electrophoresis (Fig. 3a and b). The result from 0.5% agarose-2% acrylamide gel revealed very clearly that the major products were 4-5S RNA after 15 min (Gm 1) but 23S and 16S r-RNA at Gm 3 as well as in the log phase. These ribosomal RNA were never synthesized at Gm 1 and then started to be expressed when the phase proceeded into Gm 2, where the smaller RNA species were also synthesized. For closer detection, 10% acrylamide gel was used (Fig. 3b). The result indicated that the smaller species at Gm 1 were 4S, 5S and other minors, that 6S RNA disappeared at Gm 2, and finally that larger RNA, probably mRNA, were detected as heterogeneous products at Gm 3 (45–60 min).

Consequently, it can be concluded that the three phases of germination, for which the rate of RNA synthesis was determined, coincide astonishingly with regulated change of major RNA synthesis, and that Gm 1 is definitely characterized by onset of 4-5S RNA genes activation. Gm 2 is specified only by switching on ribosomal RNA genes. The pattern of RNA species at Gm 3, however, is similar to that for log phase cells and more complicated in relation to DNA replication.

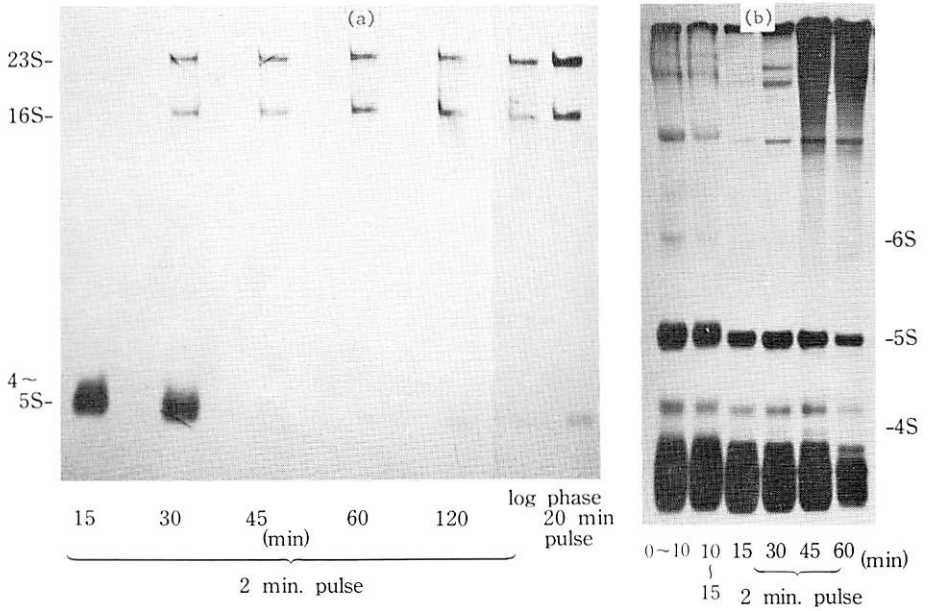


Fig. 3. Gel-electrophoresis of RNA synthesized during germination. a) 0.5% agarose -2% acrylamide gel. b) 10% acrylamide gel. ^{3}H-RNA prepared at times indicated. For detection the method of fluorography was used.

(2) Transcription and regulation during germination of the *Bacillus subtilis* spore. II. Effects of various inhibitors on RNA synthesis.

M. Matsuda and T. Kameyama

Based on the preceding report, further studies on the three phases of RNA synthesis were carried out. The most important problem is to determine unknown factors or molecular entities responsible for the specific transcription in each phase. In order to obtain a clue to opening this problem, the effects of various metabolic inhibitors on the rate of RNA synthesis and RNA species specifying each phase were surveyed.

As shown in Fig. 1, chloromycetin (100 μg/ml) had no inhibitory effect on the start of RNA synthesis after 5 min and on the linear increase of the rate up to 15 min if added at time 0, while the drug completely inhibited the gradual increase of RNA synthesizing rate at Gm 2 phase if added at either time 0 or 20 min. The inhibition increased further as the phase proceeded, and furthermore the rate of RNA synthesis declined slowly and attained the level of that at 15 or 20 min. This characteristic feature was common to other drugs tested, inhibitors of protein synthesis, since similar results were observed for lincomycin (100 μg/ml), streptomycin (300 μg/ml), puromycin (200 μg/ml) and erythromycin (10 μg/ml). Considering the fact that Gm 1 phase was insensitive but Gm 2 was sensitive to chloromycetin, a complete deletion of r-RNA synthesis might be expected by addition of the drug.

RNA species synthesized at the indicated times corresponding to three phases respectively in the presence of the drug from time 0 were analyzed

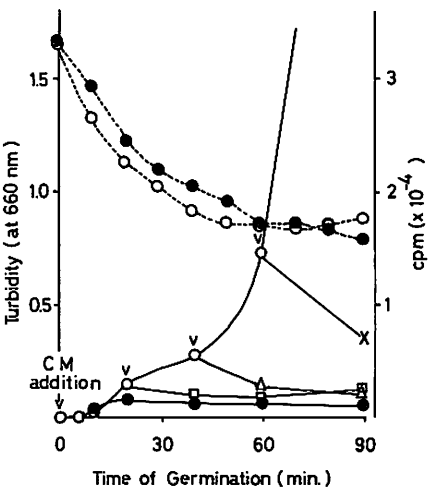


Fig. 1. Effect of chloromycetin (CM) on the rate of RNA synthesis during germination. Turbidity changes are shown by broken lines. Chloromycetin (100 μg/ml) was added at 0, 20, 40 and 60 min as indicated. ^{3}H-uridine pulse incorporation into RNA for 1 min was counted (solid lines). ○: without CM, ●: CM was added at 0 min, □: at 20 min, △: at 40 min, ×: at 60 min.

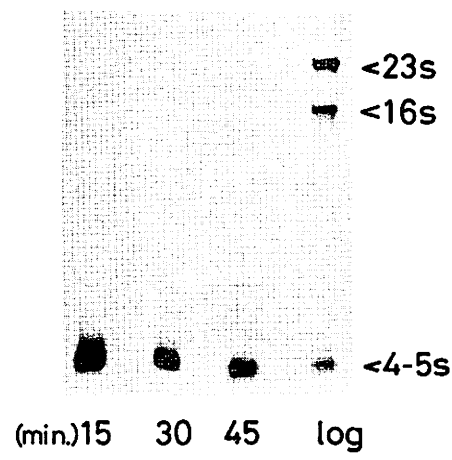


Fig. 2. Gel-electrophoretic patterns of pulse-labeled ^{3}H-RNA in the three phases and in the log phase. In the presence of CM two min-pulse labeled ^{3}H-RNA prepared at the indicated times was subjected to 0.5% agarose-2% acrylamide gel-electorophoresis. The detection was the same as in Fig. 3 of Abstract (1).

by 10% acrylamide gel-electrophoresis. The result in Fig. 2 clearly demonstrates that smaller RNA (4S, 5S, 6S and minors) were synthesized even if the drug was added, in accordance with the control, and that ribosomal RNA specific for Gm 2 and mRNA specific for Gm 3 were completely deleted. These results suggest that for the transition from Gm 1 to Gm 2 *de novo* synthesis of protein(s) should be required.

The survey using metabolic inhibitors was further continued and extended to drugs having inhibitory effect on DNA synthesis, since the phases Gm 1 and Gm 2 prior to DNA sythesis might be involved in certain processes from a frozen state of DNA as dormancy to a somehow activated state. When nalidixic acid (40 and 100 μg/ml) was added at time 0, the rate of RNA synthesis in Gm 2 was exclusively repressed (Fig. 3). Novobiocin (5–10 μg/ml) also exhibited exactly the same effect. Since these two drugs are known as potent inhibitors to DNA-gyrase, activation of frozen DNA should be a prereqiuisite for attaining Gm 2 and could be accomplished by DNA-gyrase. The above hypothesis was further strengthened by different inhibitors such as hydroxyurea (0.05–0.2 M) and 6-hydroxyphenylazouracil (0.08 mM) acting on other steps of DNA synthesis. These drugs inhibited only the phase Gm 3 without any effect on Gm 2.

Finally, the pulse labeled ^{3}H-RNA synthesized at each phase in the presence of nalidixic acid was analyzed coincidently by two types of gel -electrophoresis. As shown in Fig. 4, the synthesis of ribosomal RNA was completely inhibited at Gm 2 but that of smaller RNA species was consistently detected at Gm 1 and Gm 2.

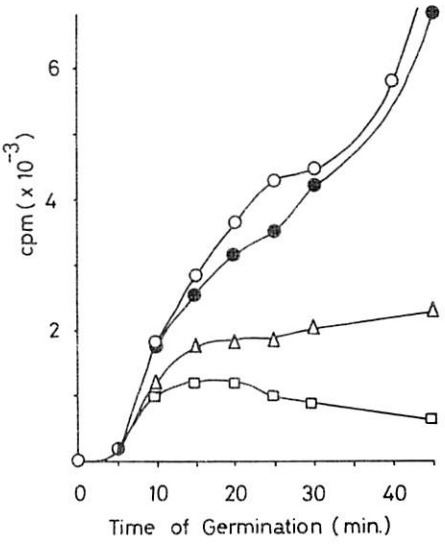


Fig. 3. Effect of nalidixic acid on the rate of RNA synthesis during germination. Nalidixic acid (no addition: ○, 10 μg/ml: ●, 40 μg/ml: △ and 100 μg/ml: □) was added at 0 min. One min-pulse labelling by ^{3}H-uridine was carried out.

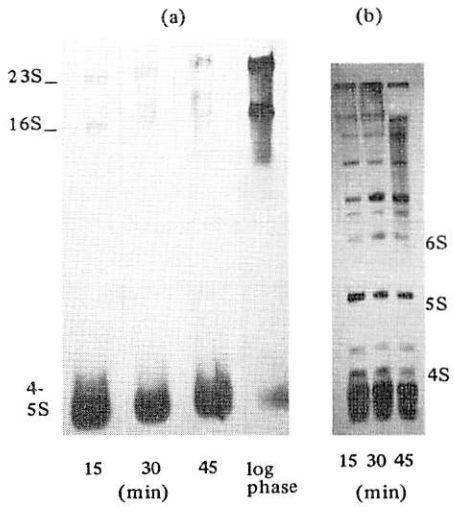


Fig. 4. Gel-electrophoretic patterns of pulse labeled ^{3}H-RNA in the presence of nalidixic acid (40 μg/ml). Two min-pulse labeled ^{3}H -RNA prepared at the indicated times was subjected to (a) 0.5% agarose-2% acrylamide gel -electrophoresis and to (b) 10% acrylamide gel -electrophoresis. The detection was the same as in Fig. 2.

(3) Diffusion chamber culture of a single cell from the embryoid body of teratoma of the strain 129 mouse.

T. Nomura, N. Satoh and T. Kameyama

Teratocarcinoma OTT6050 is the transplantable testicular teratoma of the strain 129 mouse having an embryonal carcinoma cell as a stem cell. The ascitic form of this tumor is called an embryoid body because of its resemblance to a 6 day mouse embryo in both morphology and debelopmental potency[1]. The solid tumors contain various tissues originating from all three germ layers and stem cells. Embryonal carcinoma cells not only have tumorigenicity but also developmental capacity and are able to develop normally under some conditions which can nominate their character[2].

In the previous report of this issue, we reported the cultivation of a single embryoid body by the *in vivo* diffusion chamber technique. The details of the experiments were reported elsewhere[3]. To summarize them, embryoid body cells grow very well in a diffusion chamber without maintenance of the specific structure, and the cell masses become so sensitive to trypsin that it is easy to measure the cell number according to their growth. This report describes briefly the culture of a single cell from the embryoid body in a diffusion chamber, and an instance that appears to imply a change in cell morphology and surface antigenicity.

Embryoid bodies were obtained from peritoneal fluid of the mouse which received embryoid bodies about a week before. They were washed ten times with Eagle's MEM medium until undetectable in contaminated cells microscopically. One week old embryoid bodies have looser structure and are less insensitive to trypsin in contrast to the older ones. Embryoid bodies were partially dissociated by treatment with 0.1% trypsin and 0.2% collagenase mixture. The cells thus obtained were not homogeneous in morphology and were roughly classified into three groups in diameter; that is, 6μm, 12μm and over 20μm cells. From the cell population, a single cell was isolated on a coverglass tip under the microsope and was transfered into a diffusion chamber with MEM medium. The chamber was implanted into the peritoneal cavity of a 129 mouse. After many days culture, the diffusion chamber was taken out and the proliferated cells were examined (Fig. 1). In some chambers cells had not proliferated but in many cases cells had proliferated; therefore, the cloning efficiency of this technique may be very high (probably over 50%). These proliferated cells could be observed on the coverglass tip put into a diffusion chamber in the begining, and on the surface of the membrane filters, or in the fluid of the diffusion chamber. We examined only the smallest class cells and the black cells, because the smallest cell may be an undifferentiated stem cell and the black one, which was found by chance in the dissociated embryoid body cells, may be differentiated.

After 120 days culture, a single smallest cell proliferated and many

heterogeneous progeny cells were observed in the fluid of the diffusion chamber. These progeny cells were spherical and included all the three groups of cells in diameter just corresponding to cells of the embryoid body. In this case, the progeny cells attached on the coverglass tip showed more complicated morphology that seemed to be differentiated (Fig. 2). By the cytotoxicity test with C3H anti-129 serum, 75% of these cells attached on the coverglass tip were affected, but only a few of embryoid body cells were affected. On the other hand, after 180 days culture, the black cell proliferated and many black progeny were observed in the diffusion chamber and on the coverglass tip (Fig. 3). The black progeny thus obtained were about 10 μm in diameter, corresponding well with the original black cell. When about 10^6 of these black progeny were injected intraperitoneally into 129 male mice, no tumors had developed within 120 days, while in contrast, heterogeneous cells from embryoid bodies produced tumors and killed the mice within 60 days.

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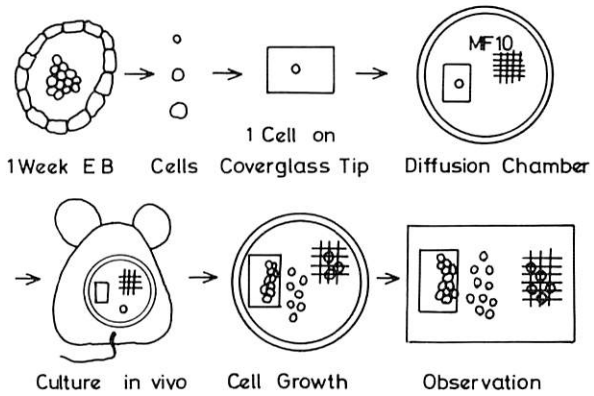
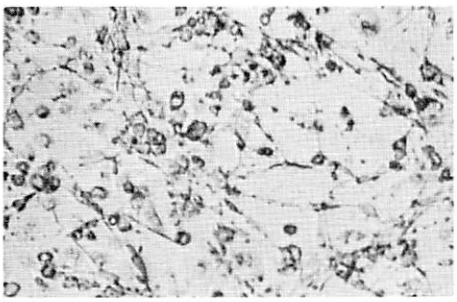


Fig. 1. Schema of experimental procedure.



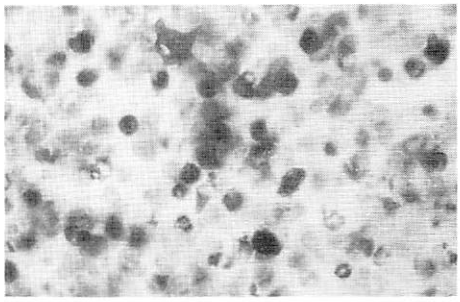


Fig. 2. Progeny of the smallest cell attached to the coverglass tip in the diffusion chamber.
Fig. 3. Black progeny cells on the surface of the membrane filter of the diffusion chamber.

(4) Distinction of the embryonal state from the differentiated state by the diffusion chamber culture of EB-cells from mouse teratoma OTT6050.

N. Satoh, T. Nomura and T. Kameyama

Recently, several embryonal carcinoma (EC) cell lines have been established *in vitro*. There are some nullipotent lines and a series of multipotent EC cell lines[1,2,3]. Among them, PCC3 maintained in the growing phase remain in the original EC state. In contrast, when such cells are allowed to reach confluent, foci of differentiated cells appear in the culture[4]. It suggests that the differentiation of EC cells are affected quite well by the cellular state whether they are proliferating or saturating. In connection with this, we intend to clarify the cellular requisites for transition from embryonal to differentiated state by controlling the growth of cells from the embryoid body (EB).

Cell culture: 10^4 EB-cells from OTT6050 were implanted into diffusion chambers and the *in vivo* culture was started. Cells proliferated logarithmicaly for the first 40 days with a doubling time of 3 days. Then, cells grew at a slower rate reaching the 10^8 level by the 60th day. Between days 60-80, cells grew very slowly with a doubling time of about 10 days. Cells were now divided into two halves on the 60th day. Half of the cells were transfered to another diffusion chamber lowering the number from 10^8 to 10^6 cells. They showed again logarithmic growth up to the 80th day (the proliferating state). The other half, as control, without transfer was allowed to grow up to the 80th day (the nearly saturated state). This half was maintained by serial transfer in the nearly saturated state over a year.

Serum absorption test: C3H anti-129 serum was prepared by the serial injection of 129 spleen cells into a C3H mouse. This antiserum reacts against not only major histocompatibility antigens but also various other surface antigens. The terms "differentiated" or "embryonal" are used hereafter in the meanings of presence or absence of immunological reaction of the tested cells to the antiserum respectively. C3H anti-129 serum was absorbed by the two halves of cells as above at the concentration of 10^8/ml for 90 min at 37°C. Serum absorption was expressed by the cytotoxic activity to the thymus cells of a 129 mouse. The result of serum absorption of the two halves of cells is shown in the Figure. The result of other cellular states at 15, 60 and 290 days of culture and EB-cells are also plotted, comparing with the absorption curve of thymus cells of a 129 mouse. The cells in the proliferating state showed little absorption like the cells of 15 and 60 days of culture. The cells in the nearly saturated state showed a lot of absorption, and the cells of the extended culture of 290 days showed more increased. Contrary to our expectation, EB-cells showed absorption to some extent at the start of the culture.

Microscopic observation: To demonstrate the micro-environmental influence in relation to cell morphology, a tip of coverglass was put into a

diffusion chamber at the start of culture. The cells grown on the glass formed two layers by the 120th day. The first layer was composed of spindle shaped cells attached to the surface of glass. The second layer was composed of spherical cells loosely attaching to each other and to the cells of the first layer. They were treated with C3H anti-129 serum and rabbit complement for 60 min at 37°C, and then stained by Trypan Blue (Plate). Sixty five per cent of the cells were stainable in the first layer, whereas few cells were stainable in the second layer.

Summary: EB-cells were distinguishable into "embryonal" and "differentiated" state depending upon whether they were in the proliferating state or in the nearly saturated state respectively. As to differentiation, they were also distinguished by the two different states depending upon whether they were attached or not to the surface of the coverglass tip in the diffusion chamber.

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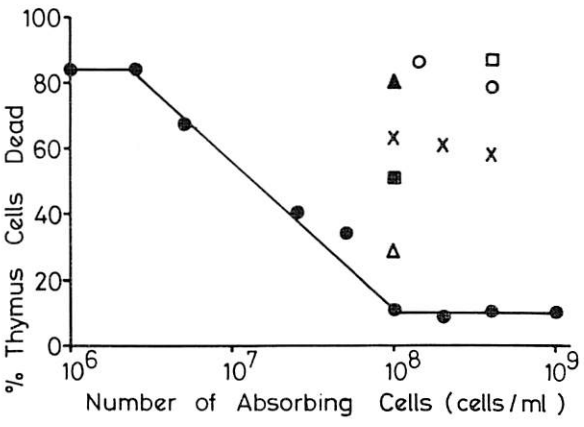

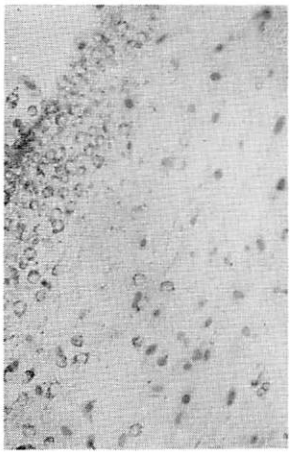

Figure. Quantitative absorption of C3H anti-129 serum by cultured cells.

C3H anti-129 serum was absorbed quantitatively by the thymus cells of 129 mouse and cultured cells. Cytotoxic activity of absorbed sera was tested with thymus cells of a 129 mouse as a target. Antiserum was absorbed by the cells of thymus(●), 15 days culture(□), 60 days culture(○), the proliferating state(▲), the nearly saturated state(▦), 290 days culture(△) and EB-cells(×).

Plate. Microscopic photograph of the cytotoxicity test on the cells attached to the coverglass.

Spindle shaped cells in the first layer are stained but spherical cells in the second layer are not stained.

(5) Induction of endogenous retrovirus from L-cell with 5-bromodeoxyuridine and cycloheximide.

K. Iida

Various mammalian cells, which are not infected with any viruses, produce retroviruses (RNA tumor viruses) spontaneously or with physical and chemical agents at a low frequency. It has been well known that the retroviruses could be induced more effectively from cultured mammalian cells of various species with halogenated derivatives of nucleoside such as 5-bromodeoxyuridine and 5-iododeoxyuridine and other inhibitors acting on protein synthesis[1].

From these results and other evidences, genomes of retrovirus were shown to be integrated in host chromosomes. It was also suggested that the expression of the endogenous viral genes are repressed in the normal state and derepressed with the halogenated nucleosides or inhibitors for protein synthesis. Moreover, the mechanisms for regulation of the expression of the viral genes are interesting not only in comparison with that of the expression of exogenous retroviruses but also in relation to that of the partial expression of gene(s) responsible for cellular transformation.

In these respects, the induction of retrovirus from L-cell was carried out with 5-bromodeoxyuridine, cycloheximide, and cytochalasin B, which is an inhibitor of the cytokinesis. L-cells were grown in Eagle's minimum essential medium supplemented with 5% calf serum for 2 to 4 days. After pretreatment of L-cells with 5-bromodeoxyuridine overnight, the cells were cultured again in the fresh growth medium containing ^{3}H-uridine in the absence of the drug. The medium was changed daily or every other day during incubation with ^{3}H-uridine. The amount of the induced viruses was estimated by counting the radioactivities of the viral fraction following the sucrose gradient centrifugation of the cultured medium.

It was shown from these experiments that the endogenous retrovirus of L-cell was induced with 5-bromodeoxyuridine after 2 days of the treatment and its amount reached the maximum level after 4 to 6 days. A higher concentration of the drug (10^{-3} M) was required to induce the virus in L-cell compared to those in other murine cells (10^{-5} M).

Cycloheximide (1 to 10 μg/ml) and cytochalasin B (1 μg/ml) were also found to induce the endogenous retrovirus of L-cell. In these cases, the induction of the virus was maximum after 1 to 2 days.

Experiments on the virological properties of these induced viruses with three drugs are now in progress. The identification of the viruses induced with these different compounds also remain to be clarified, since murine cells involve more than one retrovirus in general[2].

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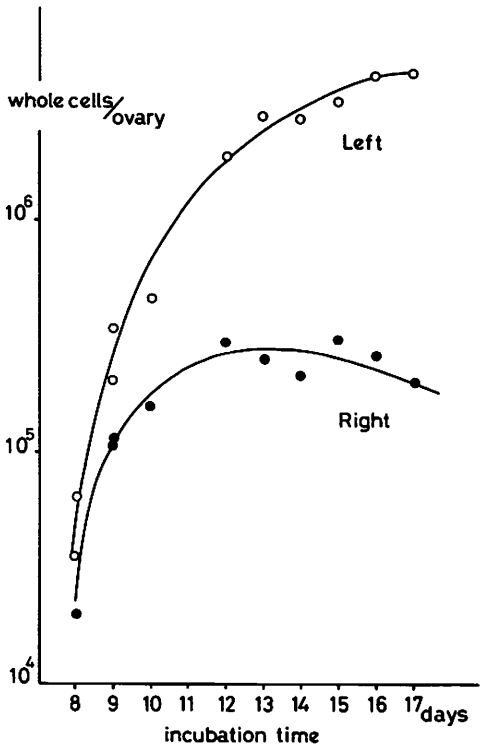
(6) Asymmetric degeneration of chick embryonic female gonads.

H. Kawakami

In birds, ovarian development progresses asymmetrically between the left and right sides, while in the male gonads it progresses equally on both sides[1]. Many investigators have shown that the left ovary is progressively growing and developing functionally, but the right one is smaller even at early embryonic stage and finally degenerates. At present, we have little knowledge as to this rudiment, resulting in a degeneration of the right side. There are cases where death of specific cells or a group of cells at a specific site is included in the embryonic development, and these deaths might be requisite[2].

I intend to investigate, what events are occurring in the cells of this rudiment, whether they are able to change their destiny, whether there are substantial differences between the functional cells of left side and the degenerating cells of right side, and how they connect with sex differentiation. I have just begun such studies.

First, a growing profile of the ovaries in both sides was studied by measuring the total cells of the whole. The gonads were dissected from the surrounding tissue, mesonephros, of female chick embryos incubated 8–17 days, and dispersed into single cells by 1% solution of Disparse II. The embryonic ovary was composed of several types of cells according to size. As shown in the figure, the cells of the left ovary were increasing in agreement with embryonic growth, while in the right it was less even after 8 days incubation and after 12–13 days no further increase, or had a tendency to decrease. Growing stopped only on the right side. Whether this rudiment in the right side will degenerate in the same way as observed *in vivo* when it is transfered to an organ culture, is now a question and in progress. The isolated single cells obtained from the right ovary of 10, 11 days embryos were actively growing as well as those from the left by *in vitro* cell culture containing 10% of calf serum. It may be that each cell is keeping its viability.



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(7) Postnatal developmental changes in microtubule protein of rat brain[1].

Y. Iida

In the external germinal layer of the rat cerebellum, precursors of granule cell neurons proliferate postnatally and migrate inward to the granular layer. During the migration, a differentiating granule cell extends a T -shaped axon which grows up a parallel fiber in the molecular layer[2]. As the parallel fiber contains many microtubules, the contents of the microtubule protein, tubulin, would increase during development of the granule cell. In this report, postnatal developmental changes in tubulin contents and its distribution between the supernatant and particulate fractions are described. Tubulin contents were determined by a colchicine-binding assay[3]. In the standard condition, that one molar tubulin binds one molar colchicine, was confirmed by analysis of the purified colchicine-binding tubulin.

In the cerebrum, the tubulin concentration was about 20% of total proteins at 0–10 days after birth, then decreased to 12% at 20 days and finally to 10% in the adult. In contrast, the tubulin concentration in the cerebellum was about 10% at 0–10 days, slightly increased until the 17th day, then decreased to 7% in the adult. The increase of tubulin content in the cerebellum in the second week seems to correspond to the remarkable development of the parallel fiber. Gozes *et al.* reported reduction in the synthesis of tubulin during postnatal development of rat cerebrum nuclei and proposed that it was controlled at the level of transcription[4]. Studies are in progress on tubulin synthesis in rat cerebrum compared with cerebellum during postnatal development.

Brain tissues contain the membrane-bound colchicine-binding activity[5]. In the rat brain, 45% of the total colchicine-binding activity was found in the particulate fraction. By sonication of the brain homogenate for 10 seconds, approximately one-half of the particle-bound tubulin was solubilized in the 3–10 day-old rat brain. The percentage of solubilized tubulin decreased with age until its content was only 10% of that in the adult brain. By further sonication for 60 seconds, almost all tubulin was solubilized in the young rat brain, while in the adult 30% of the particle-bound tubulin was still not solubilized. The increase of the tightly membrane -bound tubulin would result from the development of synapses in which tubulin is supposed to play an important role.

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(8) Studies on the circular dichroism of polypeptides and proteins.

T. Kontani

The circular dichroism (CD) of α-helix in the far ultraviolet region was calculated according to the method of Bayley *et al.*[1),2)]. Since detailed conformation of α-helix is dependent on the structural parameters such as the number of residue, n, the pitch per residue, p, and the rotational angle of a residue around the helical axis, θ, CD must be calculated on conformations generated by varying these parameters. The calculated CD shown in Fig. 1 is dependent on n in the $\pi\pi^*$ region (around 195 nm), and the dependence of CD on p and θ is also significant in the $\pi\pi^*$ (around 208 nm) and the $n\pi^*$ region (around 222 nm).

The calculation was then extended to the CD spectra of a coiled-coil of two α-helices (αHCC), which may be classified into two types, i. e., one with two parallel polypeptide chains and the other with two anti-parallel polypeptide chains. The calculated CD patterns of these two αHCC were almost the same and showed a slightly smaller peak in the $\pi\pi^*$ region than that of α-helix (Fig. 2). The calculated CD spectra of αHCC and α-helix were compared with the experimental spectra of tropomyosin (a typical coiled-coil molecule) and maleylated tropomyosin (an α-helical molecule), and agreement was good in the wavelength above 200 nm, but not below 200 nm. The cause of the disagreement is not clear at present. Since the CD pattern of α HCC is not different from that of α-helix, an experimental CD spectrum may not be utilized to distinguish αHCC structure from α-helix nor to find the directions of two chains. According to the analysis of the calculation, the difference in the CD pattern of αHCC and α-helix is not attributed to the conformational difference, but to the interaction of two chains of αHCC. When the distance of the two chains of αHCC was varied, little difference was detected presumably because the change in the interaction was too small to affect the CD spectrum.

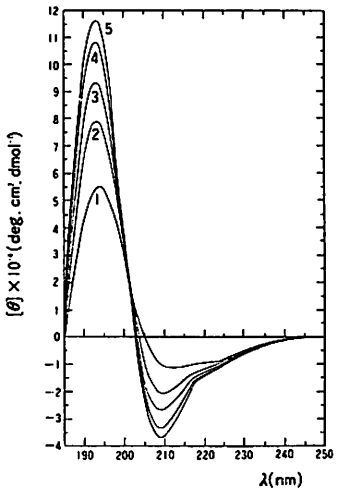

Fig. 1. CD spectra as a fuction of n. p=1.5A. θ=100°. 1 : n=10, 2 : n=15, 3 : n=20, 4 : n=30, 5 : n=40.

Finally, the CD spectra of globular proteins were analyzed by the matrix rank method[3),4)] to see whether or not these spectra could be expressed by superposition of the reference spectra of three secondary structures in protein, i. e., α-helix, β-structure, and irregular conformation. Eleven globular proteins were available for the analysis because their CD spectra and the three dimensional structures from X-ray analysis had already been published. The result showed that the experimental spectra were success-

fully reproduced by the superposition of three reference spectra using the corresponding content of the secondary structures in the wave length region of 210–240 nm. Therefore the reference spectra in this region were determined by the least squares method. The reference spectra thus obtained, however, were not good, since the agreement of the calculated spectra weighing the given extent of the three secondary structures of each protein to the experimental one was poor. The reason for this discrepancy is due to the uncertainty of the experimental contents of the secondary structures. Thus the least squares method was carried out again taking both of the reference spectra and the contents of the secondary structures of each protein as unknown parameters (in computation, either of them were fixed alternatively and the caluculation was iterated until both values converged). By using three reference spectra obtained (Fig. 3), the agreement of the experimental CD spectra to the computed one was satisfactory. The secondary structure of *E. coli* RNA polymerase was estimated to be composed of 34% α-helix, 34% β-structure and 32% irregular conformation based on the reference spectra obtained by the latter method.

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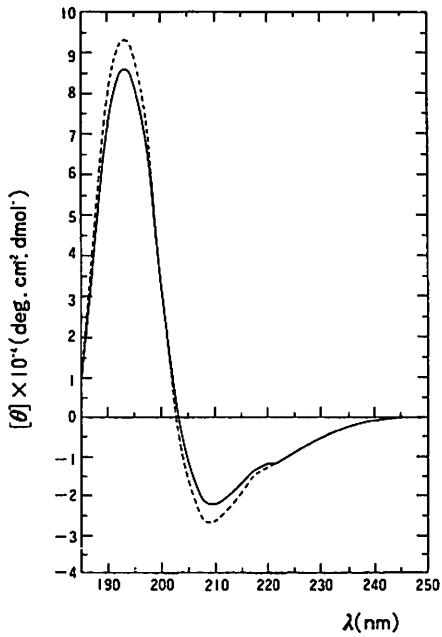

Fig. 2. CD spectra of αHCC (which has a parallel arrangement of two chains). Broken line: α-helix, n=20. Solid line: αHCC, n=20 x 2.

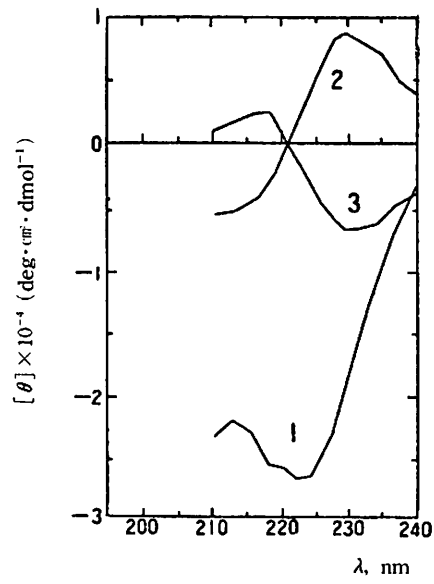

Fig. 3. Reference spectra obtained by the least squares method. 1: α-helix, 2: β-structure, 3: irregular conformation.