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99	To the personnel of Dept. of Virology, add Postgraduate Student: Hisashi Ogura (小 倉 寿)

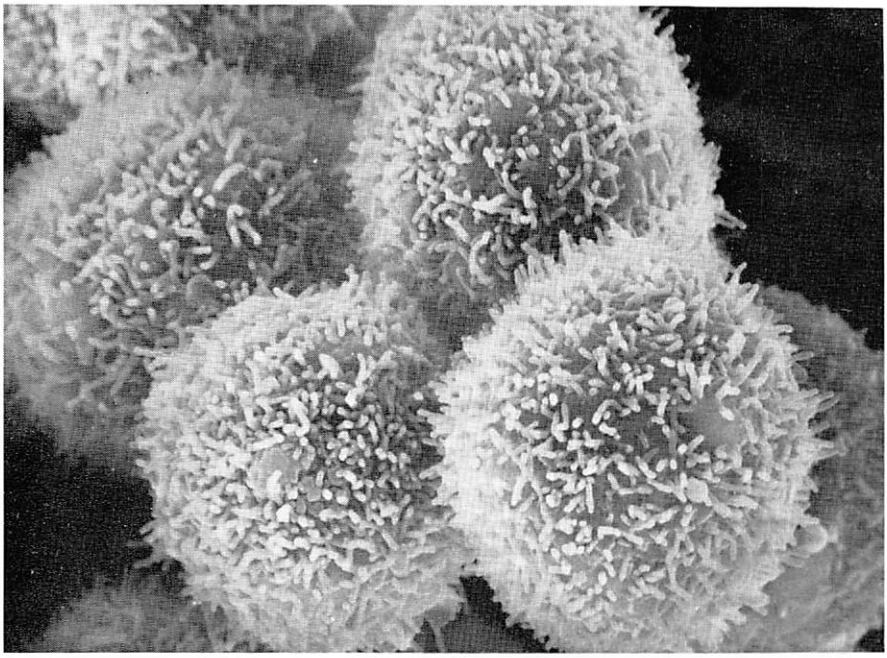

Photo by Dr. T. Goshima

DEPARTMENT OF MOLECULAR BIOLOGY

GENERAL SUMMARY

1. History

Since 1962, when our laboratory started, our efforts had been concentrated on the mechanism of transcription and its regulation using bacterial cells and bacteripphages. Thereafter our research work had been concerned with DNA-dependent RNA polymerase, *in vitro* transcription and repression of the lac-operon, expression of bacteriophage, initiation signals at the promoter and so on. After a while in 1966 a new research project on Rous sarcoma virus was started, since a unique process whereby viral RNA should be reversely transcribed into DNA, predicted by Temin, was very atractive with respect to the molecular entity acting in such an unique elementary process. Consequently these two problems became our main thema. Furtheremore, young biologists, afer training in these main thema, have recently been ambitiously setting about their own problems. Since these problems will be described in their own abstracts, only the studies on transcription done in our laboratory during the period from 1970 - 1975 are summarized.

2. Function of the sigma factor

When a unique subunit structure of E. coli RNA polymerase was found by Burgess et al (1969), function of the σ-factor was thought to be essential in recognition of the initiation signal at the promoter region. On this point our investigations made it clear that the σ-free core enzyme was almost completely inactive on native DNA without any denatured region, and that once added with o it induced a high activity on native DNA concerting with core enzyme. Therefore it is established that the binding region preferable for the core enzyme is single stranded DNA and σ functions in separating hydrogen bonds of duble stranded DNA (Ishihama et al, 1971; Kameyama et al, 1971). These attempts also brought us some technical progress. A new and simple method for isolating holoenzyme from core enzyme was developed. According to this device holoenzyme was eluted at 0.15 M KCl through DNA-cellulose column in the presence of 10 mM MgCl2, while core enzyme was eluted at 0.25 M KCl (Mukai and Iida, 1973). Using the holo enzyme thus obtained, kinetic studies to determine the step when the o-facter is released from the initiation complex, were carried out. The results clearly indicated that the o release from the initiation complex took place at least at or before the step of the dinucleotide complex (Mukai, 1974).

3. Effect of KCl on T7 transcription

On the other hand precise conditions for the *in vitro* transcription on T7 DNA, giving the same mRNA as *in vitro*, were explored. The concentration of KCl showed different effects on RNA synthesis depending on the DNA species used as template. The specific effect of the KCl concentration on the initiation rate

depending on the DNA species was observed only when the initiation complex was formed in the presence of a unique combination of three nucleotide triphosphates : (ATP, GTP, CPT) for T7 DNA. Further studies revealed that at higher KCl than 0.2M the RNA complementary to the r-strand of early regions of T7 DNA was exclusively synthesized, the specificity of which is the same as the early *in vivo* transcription in *E. coli* infected with T7 phage. The starting nucleotide sequences of RNA synthesized using T7 DNA in the presence of 0.2 M KCl were also investigated. The results indicated that the major nucleotide sequence of ATP-starting RNA was pppApUpCpGp----- and that of GTP-starting RNA was pppGpRyp-----. On the other hand, at low KCl (0.02 M), heterogeneous sequences were observed (Matsukage, 1972 ; Iida and Matsukage, 1974). These major starting sequences at 0.2 M KCl were proved to be identical to those of in vivo T7 early mRNA by other investigators.

4. *In vivo* analysis of *B. subtilis* germination

As a model of positive control of transcription, studies on the germination process of bacterial spores were carried out. As the first attempt the rate of *in vivo* RNA synthesis during germination of *Bacillus subtilis* was analyzed. Using a pulse labeling technique with ^{3}H-uridine, the rate of RNA synthesis increased gradually for 20 min (G_1), then reached a definite level which was maintained up to 45 min(G_2) after onset of germination, and finally increased thereafter explosively(G_3). Since these three distinct phases (G_1, G_2, G_3), based on the rate of RNA syntheses, were interpreted as a reflection of uniqueness in regulatory mechanism during germination, further investigations were carried out to specify them by addition of antibiotics. Chloromycetin (100 μg/ml) had no inhibitory effect on G_1 and G_2 if added at time 0 and 10 min, but it inhibited G_3 if added at 20 min. In accordance with this result G_3 was more sensitive to refamycin (11 μg/ml) than G_2. These observations seem to imply that the germination step is divided into three phases ; in the G_1-phase a molecular entity responsible for RNA synthesis is activated to a definite level, then in the G_2-phase the activated entity operates without de novo synthesis of any RNA synthesizing molecule, and finally in the G_3-phase a different entity for RNA synthesis is abruptly synthesized. On this aspect further investigations are now in progress (Kameyama and Matsuda). Such perspectives led us easily to studies on purification and characterization of DNA-dependent RNA polymerase of B. subtilis spores (Nomura, 1974).

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

ABSTRACT

(1) **Two active forms of DNA-dependent RNA polymerase purified from *Bacillus subtilis*.**
T. Kameyama, T. Nomura and M. Ohya

In the germination step of the *Bacillus subtilis* spore, all of the capacities for macromolecular synthesis must be activated. Therefore, the activation at the transcriptional level may play an important role as a trigger. On this aspect it has been thought that DNA-dependent RNA polymerase(RPase), as a molecular entity of transcription, pre-exists in dormant spores. However, there has been little information about RPase purified from *B. subtilis* spores.

In this paper our attempts to purify RPase from *B. subtilis* spores as well as vegetative cells are briefly reported. Then purified enzyme preparations as vegetative cells are purified enzyme preparations are analyzed for comparison of their enzymatic properties. As a conclusion from our results, it can be shown that two different molecular forms of RPase exist in vegetative cells whereas only one is present in dormat spores.

B. subtilis spores(100-120g) were pretreated with 8 Murea and washed. The washed spores and vegetative cells were further treated with egg white lysozyme. After incubation, they were mechanically disrupted by an RIBI Cell Fractionator(Sorvall) under high pressure. The crude extracts were subjected to a series of chromatographs through DEAE-Sephadex A50, DNA-cellulose and phosphocellulose columns. Active preparations thus obtained were further purified by glycerol gradient centrifugations, repeated several times under various conditions. The final preparations exhibited high specific activities; about 300 and 600 fold for vegetative cells and spores respectively.

On account of a great difference between their activities to native and denatured DNA used as template, complete holoenzymes seemed to be contained in these preparations. Sedimentation coefficients were determined as 16S and 15S for spores and cells respectively. The optimal activities were represented at 2.5 mM Mn^{2+} and below 4mM Mg^{2+} by spores, and at 1.8 mM Mn^{2+} and 7mM Mg^{2+} by cells. As shown in the Figure, the reaction by the enzyme from spores using the bacteriophage SPO1 DNA was activated dramatically just at about 0.28 M KCl, while that by the T4 DNA showed a very small activity at the same concentration of KCl. All the results obtained suggested that these enzymatic differences might be more or less due to different enzyme molecules contained in the purified preparations. In order to examine this posibility a small volume of the preparation from cells was carefully analyzed by sedimentation through a longer distance in a glycerol gradient. The centrifugal pattern revealed two active peaks separable from each other. In addition to this one of the peaks behaved just as the enzyme obtained from spores(S), judging by optimal divalent cations, preference for DNA template, activation on SPO1 DNA just at 0.28 M KCl and so on. This spore-type RPase isolated from vegetative cells was named Sv to distinguish it from V as a main component of cellular RPase.

Further analysis by polyacrylamide gel-electrophoresis in the presence of sodium dodecyl sulfate revealed that these three forms consisted of common subunits(β, β' and α), and also that

a specific subunit, σs for S- and Sv-type and σv for V respectively, was contained in each enzyme form. The fact that the subunits of Sv completely conincide with those of S and are distinguished from V by the specific subunit, well supports the results already described with respect to their enzymatic properties.

If these results could be accepted as true, an outline of the transcriptional regulation during germination of *B. subtilis* might be sketched as follows; i) spore-type RPase, pre-existing in dormant spore, is activated at first, ii) then it transcribes genes in a sequence to mRNA, iii) a specific mRNA is translated giving a new subunit such as σv for V-type RPase, iv) when σv is accumulated, σs in S-type RPase can be replaced by σv resulting in V-type RPase, and v) finally this V-type RPase operates in a fashion unique to the vegetative step. These are perspectives of our current investigations now in progress.

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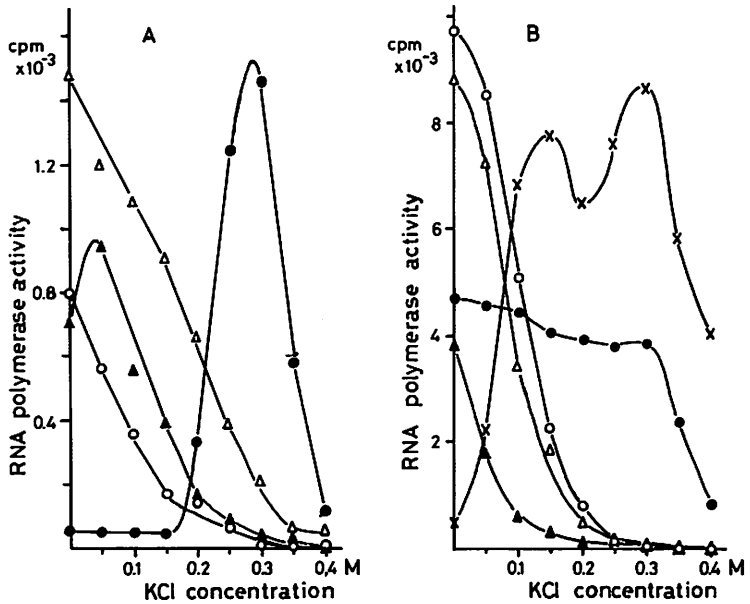

Figure. Effects of KCl concentration on the RNA polymerase reaction directed by various DNA.

(A): Using the spore RNA polymerase (5.5 μg), enzyme activities were measured by the standard assay system containing different DNA in the presence of varied concentrations of KCl. ^{3}H-UTP (sp. act.: 33,000cpm/mμ mole) was used.

(B): The vegetative cellular RNA polymerase (11.9μg) was used. Others were the same as (A), except ^{3}H-UTP (sp. act.: 17,600). The reaction was carried out for 20 min at 37C. The results obtained by T4 DNA (—○—○—), SPO1 DNA (—●—●—), *B. subtilis* DNA (—△—△—), and T7 DNA (—▲—▲—) are shown. The result using *E. coli* RNA polymerase with SPO1 DNA (—×—×—) is also shown in Figure B.

(2) On the DNA polymerase alpha from mouse myeloma MOPC 104E.

A. Matsukage

The process of DNA replication appears to involve synthesis of DNA in the form of relatively short pieces, later to be joined, and these contain RNA tract on the 5'-end. Since all mammalian DNA polymerases require a poly- or oligonucleotide primer molecule for activity, it is reasonable to predict that the RNA tract serves as a primer during the synthesis of the DNA pieces. The biochemical mechanism of such events is unknown and we may ask the question, what are the signals that terminate synthesis of the RNA primer and initiate action of the DNA polymerase?

The aim in the present study was to determine whether DNA polymerase (DPase) alpha, thought to have a main function in DNA replication, contains enough information so that it can recognize specific polynucleotide tracts as initiation signals for DNA synthesis.

DPase alpha was isolated and purified with a sequence of column chromatography of ion-exchangers, DEAE cellulose, phosphocellulose and hydroxyapatite. Second DEAE-cellulose column chromatography made it possible to separate DPase alpha into three active forms, A, B and C[1]. Each form was further purified by zone sedimentation in glycerol gradient, affinity chromatography on DNA cellulose and polyacrylamide gel-electrophoresis under non-denaturing conditions. The DNA polymerase, thus obtained, sediments at about 7S and the molecular weight was estimated as 100,000 to 150,000 daltons. The A form enzyme contained 135,000, 120,000 and 106,000 dalton polypeptides, whereas, the β form contained 58,000 and 49,000 polypeptides in addition to 135,000 and 120,000 dalton polypeptides. The relationship among these polypeptides is under investigation.

Template-primer specificity was studied using all kinds of oligo- and poly-nucleotides with homogeneous deoxy- or ribonucleotides. Polynucleotide was used as a template and poly- or oligo-nucleotide as a primer[2]. Among these combinations, with the DNA template, relatively high activity was observed with poly(dT), poly(dA) and poly(dC) in combination with appropriate primers, but not with poly(dI). The highest activity observed was with poly(dT) as template and oligo(rA) as primer and the priming effect of oligo(rA) resulted in more than 10 fold higher activity than with any of the other combinations involving an RNA primer. All polyribonucleotides have almost no activity as template.

In the reaction where a very small amount of oligo(rA) (0.063 μg or 10 pmoles) was added in combination with poly(dT), 95 pmoles of dAMP were incorporated after 60 min incubation at 37C. This means that an average of 9.5 nucleotides were polymerized with each primer molecule. In contrast with this, oligo(dA) with poly(dT) represented only a very small priming activity for the incorporation of dAMP. These observations indicated that oligo(rA) with poly(dT) at the 5' end promotes the incorporation of additional dAMP on dAMP residue at the 3'-end

of the nascent chain.

The binding affinity between the enzyme and a template-primer complex was examined in the experiment where a comparison was made of the ability of various template-primer combinations to compete for available enzyme molecules when template-primer systems were present in the same reaction mixture. (^{3}H)poly(dG) synthesis directed by poly(dC): poly(dI) was reduced 92% by the poly(dT): oligo(rA) system. Poly(dT): oligo(rA) was more inhibitory to (^{3}H)poly(dG) synthesis than the other template-primer combinations tested, and (^{3}H)poly(dA) synthesis directed by poly(dT): oligo(rA) was not inhibited more than 30% by any of the combinations. This strong binding ability of the enzyme to poly(dT): oligo(rA) is thought to be an important factor for the high template-primer activity of this system.

Single stranded calf thymus DNA alone was a poor template-primer for DPase alpha. However, after addition of poly(dT) tract to the 3' end with the enzyme, terminal deoxynucleotidyl transferase, and oligo(rA), the denatured DNA could be replicated. The other combination of the polydeoxynucleotide tract and the complementary oligoribonucleotide were ineffective in promoting replication of this denatured DNA. The DNA formed in this system was of relatively short chain and contained all four deoxynucleotides in about the same relative amounts.

The results described above indicate that DPase alpha has the ability to specifically recognize the poly(dT): oligo(rA) complex and to initiate DNA synthesis from this RNA-primer. This complex suggests the initiation signal of DNA synthesis *in vivo*.

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(3) On the DNA polymerase gamma from mouse myeloma MOPC 104E.

A. Matsukage

It is now well established that mammalian cells contain three classes of DNA polymerases (alpha, beta and gamma) in addition to mitochondrial polymerase and terminal deoxynucleotidyl-transferase. Each class could be isolated from the other by using a sequence of chromatographys of cell extracts on DEAE-cellulose and phosphocellulose columns[1].

DNA polymerase alpha, which is composed of at least three types of activities as described in an accompanying report, has a molecular weight of about 150,000, and is active with pancreatic DNase treated (activated) calf thymus DNA but not with poly(rA):oligo(dT) as a template primer. DNA polymerase beta has a molecular weight of about 30,000 and localizes mainly in

the nuclear fraction. This enzyme is active with both activated DNA and poly(rA):oligo(dT), and with the latter template-primer dTMP was incorporated into the polymer.

The third one (gamma), which was originally detected by using poly(rA):oligo(dT), but is not active with activated DNA, was extensively purified[2]. After phosphocellulose column chromatography, the enzyme was purified through hydroxyapatite column chromatography, affinity chromatography on a DNA-cellulose column and by zone sedimentation in glycerol gradient. The final preparation was purified about 20,000 folds from the crude extract. Electrophoresis in polyacrylamide gel under non-dissociating condition revealed that 80% of the protein in the preparation migrated as one protein band which coincided with the DNA polymerase activity. The molecular weight of this enzyme determined by three methods ranges between 200,000 - 300,000 (average 250,000). Polyacrylamide gel electrophoresis of the denatured protein in the presence of sodium dodecyl sulfate and urea revealed that the enzyme protein was composed of three polypeptides whose molecular weights are 72,000, 68,000 and 50,000 daltons, respectively, as seen in the figure. The molar ratio of these polypeptides is about 1:1:2, and therefore the molecular weight is estimated to be 240,000.

Enzymological studies of this enzyme represents the following results. The Km value is relatively low (1.9×10^{-7}M of dTTP in the presence of Mn^{2+} and poly(rA):oligo(dT) as a template-

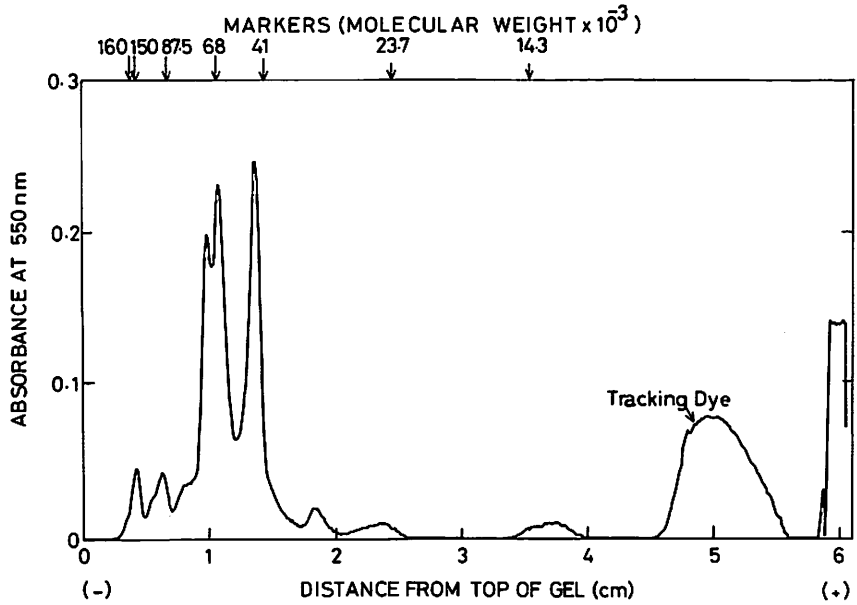

Figure 1. Sodium dodecyl sulfate-Urea polyacrylamide gel electrophoresis of purified DNA polymerase gamma. The experiment is carried out using the preparation after the zone sedimentation step. The figure is made by tracing the gel stained with coomassie brilliant blue. The molecular weights of polypeptides were determined by using several standard proteins electrophoresed in accompanying gels.

primer). The optimal pH is 7.0 - 7.5 in the presence of Mn^{2+} and poly(rA):oligo(dT) and 7.8 - 8.3 in the presence of Mg^{2+} and poly(dA):oligo(dT). The optimal concentration of the divalent cation is 0.5 mM for Mn^{2+} or 2 mM for Mg^{2+}. In the presence of Mn^{2+}, DNA polymerase gamma has relatively high activity with poly(rA):poly(dT) in addition to poly(rA):oligo(dT) and also in the presence of Mg^{2+} with poly(dA):oligo(dT), poly(dA):poly(dT) or poly(dC)·oligo(dG). But, the enzyme has little activity with activated DNA.

DNA polymerase gamma is also present in the normal tissues such as mouse or calf liver, and is not inactivated with antiserum to murine leukemia viral reverse transcriptase. These observations suggest that the enzyme is not of viral origin but rather of cellular origin. In mouse liver, 84% of DNA polymerase gamma activity is localized in the postmitochondrial membrane fraction while the rest is in the nuclear fraction. The biological significance of this enzyme is now under investigation.

References

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2) Matsukage, A., Bohn, E. W. and Wilson, S. H. : On the DNA polymerase III of mouse myeloma : Partial purification and characterization. Biochemistry, *14*, 1006-1020, 1975.

(4) Studies on type-C RNA tumor virus.

K. Iida

1. The effect of toyocamycin on the synthesis of avian myeloblastosis viral RNA

Toyocamycin, a nucleoside antibiotic, is an adenosine analogue and inhibits the processing of ribosomal precursor RNA to ribosomal RNA[1] as well as the propagation of virulent viruses. To clarify the effect of toyocamycin on RNA synthesis of avian myeloblastosis virus (AMV) and its growth, the following experiments were carried out.

After incubation of myeloblast cells in a suspension culture with ^{32}P-phosphate or ^{3}H-uridine, the culture fluid was fractionated by sucrose gradient centrifugation. As shown in the Figure, toyocamycin inhibited the incorporation of ^{3}H-uridine into viral fractions and a small amount of radioactivity was found in lighter fractions than in normal viral particles.

The analysis of extracted RNA by sucrose gradient centrifugation indicated that any fraction obtained from a toyocamycin treated culture contained only a RNA of small size while viral particles from the control culture contained both small and large RNA's. Toyocamycin was less inhibitory to the synthesis of the cellular transfer RNAs and the small RNA in AMV particles than to the processing of ribosomal precursor RNA and the synthesis of large RNA of AMV. The figure also shows that the synthsis of the abnormal particles defective in large RNA continued for several hours after adding toyocamycin. Similar results

were obtained by treatment with 5-azacytidine, which is incorporated into RNA instead of cytidine and inhibits growth of RNA phage. 5-Azacytidine inhibited ^{3}H-uridine incorporation into AMV particles and resulted in the production of light abnormal particles similarly containing only small RNA as toyocamycin.

2. Homology between RNA of avian myeloblastosis virus and cellular DNA, and polyadenylate stretch in the viral RNA.

The existence of homology (or partial homology) between RNA of type-C tumor virus and cellular DNA was reported in various systems. The homology region of large AMV RNA was shown to be rich in adenine by the hibridization method combined with pancreatec ribonuclease digestion.

The hybrid between viral large RNA of AMV and DNA from avian cells infected with AMV was digested with ribonuclease T2 (Taka Diastase), since the ribonuclease T2 preferentially hydrolyzes the phosphodiester bond of adenosine nucleotide of RNA, rather than those from pancreatic rebonuclease A. After the first digestion with pancreatic ribonuclease A and ribonuclease T1, the hybride between ^{32}P-RNA of AMV and avian DNA was again treated with the ribonuclease T2. This final digestion resulted in decrease in the relative amount of adenine base from 65% to 39% in the undigested part of AMV RNA. The homologous part of large AMV RNA to cellular DNA was apparently richer in adenine and guanin (Adenine:39%, Guanine:45%, Cytidine:8%, Uracil:7%) than native viral large RNA (Adenine: 25%, Guanine:29%, Cytidine:23%, Uracil:23%). These results strongly suggest that the homologous part of AMV RNA compared to cellular DNA is rich in purine bases. However, further investigation is necessary, to obtain a conclusive result, since the purine-rich fraction of RNA might more effectively hybridize to DNA than the purine-poor fraction under the experimental conditions used (0.6 M NaCl - 0.06 M citrate, for 60 min. at 66C). These results also suggest the existence of polyadenylate stretch in the homologous part of the viral large RNA to cellular DNA, assuming the value of adenine base remaining in the resistant fraction decreased significantly while others either remained constant or increased.

It had been well known that the production of type-C viruses are induced, yet at a low frequency, spontaneously or by physical and chemical agents from a variety of non-virus producing cells. Recently, 5-bromodeoxyuridine and 5-iododeoxyuridine were found as more effective activators than those above[2)], and they not only increase the frequency of viral induction but also are effective on a variey of mammalian cells such as mouse, rat, cat, pig and others. Furthermore, cycloheximide and puromycin, known as inhibitors of protein synthesis, were also found to have potency for inducing type-C virus in murine cells.

The mechanism of type-C virus induction has not been well elucidated. In addition there is no evidence to show type-C viral induction in avian cells by either 5-bromodeoxyuridine or cycloheximide.

On these aspects, several conditions which can activate the type-C viral gene at a high frequency in avian cells are being explored.

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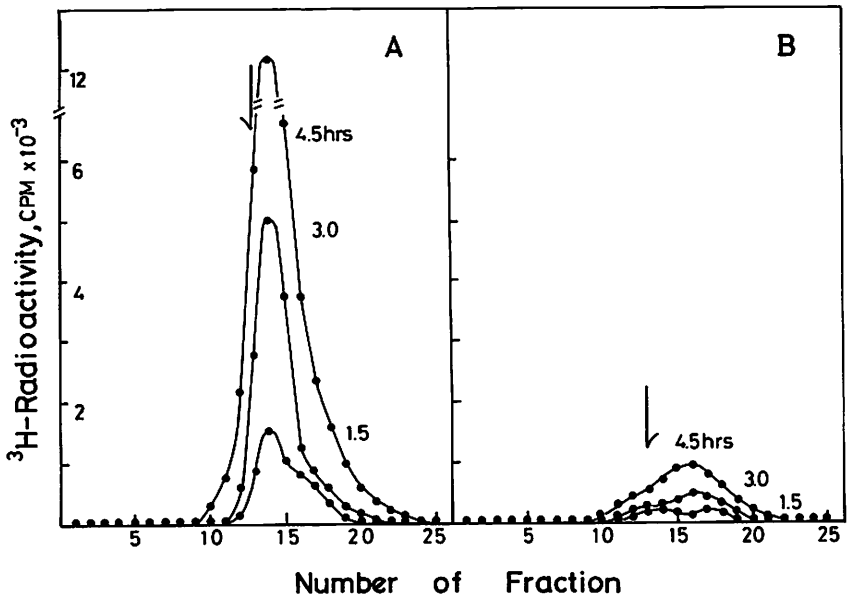


Figure. Sedimentation profiles of ^{3}H-uridine labeled particles obtained from chick myeloblast cells infected with AMV.
Particles, concentrated from culture medium, were layered onto sucrose gradient (15 - 60%) and centrifuged with SW 25.1 for 12 hrs. As an internal reference, AMV particles obtained from chick blood, were added to samples before running. The arrows indicate the position of the AMV.
(A) Myeloblast cells were incubated with ^{3}H-Uridine for 1.5, 3.0 and 4.5 hrs respectively in the absence of toyocamicin.
(B) They were incubated under the same conditions in the precense of toyocamycin (3.3 μg/ml).

(5) Culture of a single embryoid body from the testicular teratoma of a strain 129 mouse in a diffusion chamber.

N. Satou and T. Nomura

Testicular teratoma occurs frequently in inbred strain 129 mice. The developmental studies on the primary testicular teratoma of the mouse showed that the processes of tissue formation beginning from undifferentiated embryonal cells and germ cells were observed. This observation was interpreted as

a demonstration of formation of three germ layers in testicular teratoma. When transplantable testicular teratoma was maintained as an ascites tumor, free-floating embryoid bodies similar to mouse embryo at 5 to 6 days of age were obtained in the peritoneal fluid. They were composed of morphologically undifferentiated embryonal cells enclosed by a single epithelium layer resembling embryonic endoderm. These embryoid bodies not only have tumorgenicity but also developmental capacity giving rise to a wide variety of tissues[1]).

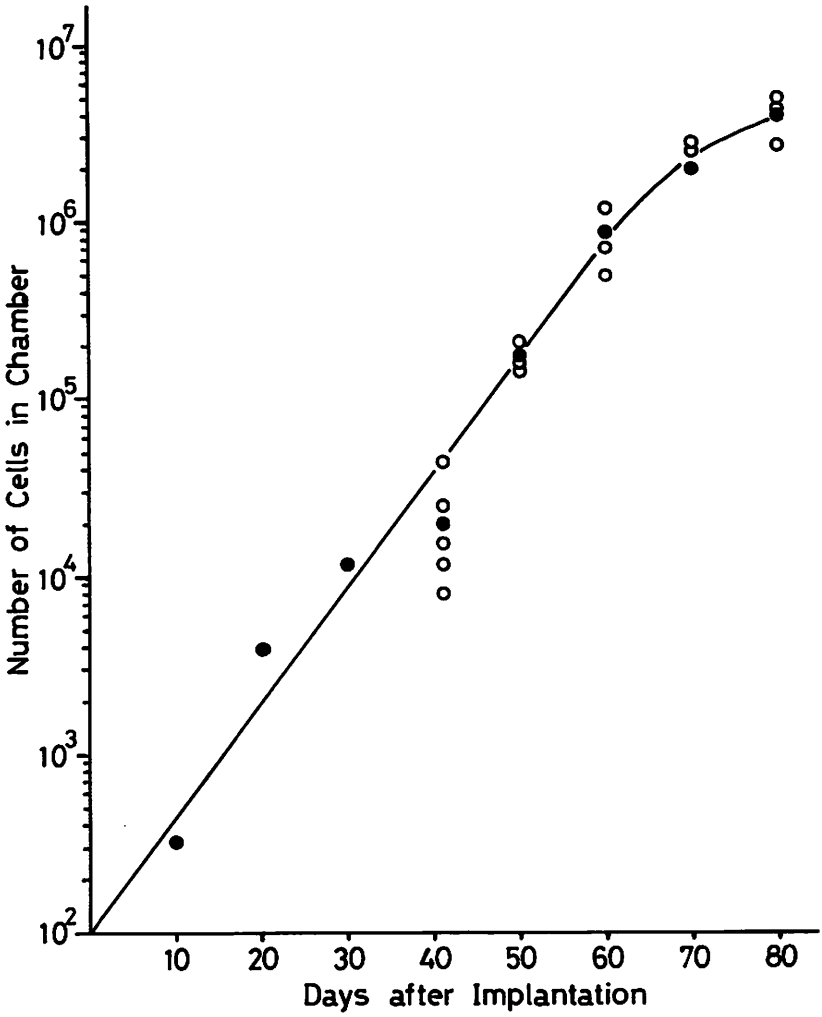
This report describes briefly proliferation of cells of an embryoid body in a diffusion chamber, and an instance that appears to imply a change in tumorgenicity of these cells cultured by chamber technique.

Embryoid bodies obtained from mouse peritoneal fluid were washed several times with Eagle's MEM medium and a single embryoid body was manipulated under a binocular microscope (×40) through a glass capillary connected to a syringe with a silicon microtube. With this device we can easily transfer the desired number of embryoid bodies into a diffusion chamber. In this experiment one embryoid body was transfered into a diffusion chamber (ϕ 16 mm × 5 mm) glued on both sides by two sheets of millipore filters (pore size : 0.45 μ) containing 0.5 ml of Eagle's MEM medium. The diffusion chamber was implanted into a mouse peritoneal cavity for *in vivo* culture. At every 10 days after implantation the chamber was then carefully taken out and the proliferated embryoid body in the chamber, which was filled with gelatinous matter, was trypsinized by one per cent trypsin to single cells. When peritoneal fluid was used instead of Eagle's MEM, the formation of gelatinous matter could be avoided, and it also resulted in lowering of the trypsin concentration to achieve complete dessociation of the proliferated embryoid body. As for the accesibility by trypsin, embryoid bodies maintained as a form of ascites tumor without the chamber technique were by nature resistant to dessociation even by one per cent trypsin. It was thus interesting that they now acquired a dramatic sensitivity to trypsinization once cultured in diffusion chamber.

The Figure shows the number of cells proliferated of a single embryoid body in a diffusion chamber. Cells implanted as a single embryoid body proliferated logarithmically for 60 days. The rate of cell division up to this time was determined to be 2.3 times/10 days. After 60 days, cells grew at a lower rate (one time/10 days). In this Figure 100 cells per one embryoid body were plotted at time 0. Through the whole period of the experiment, all the cells were unstainable to Nigrosine, and thus the number of cells that died seemed to be small under the conditions used.

At the 70th day, when number of cells in a diffusion chamber reached the 10^6 level, their tumorgenicity was tested. Five male mice of strain 129 each received intraperitoneally 10^6 cells as cultured above. In a control experiment 4 groups of the same number of mice each received 10^4, 10^3, 10^2 and 10^1 embryoid bodies respectively. This experiment showed that all the

mice, implanted by cultured cells in chambers, survived at least up to the 65th day and also did not show any pathological evidence of ascites tumor. On the contrary in any control groups all the mice, receiving 10^4, 10^3, 10^2, and 10^1 embryoid



Figure

PROLIFERATION OF ONE EMBRYOID BODY IN DIFFUSION CHAMBER

The diffusion chamber was made of a plastic cylindrical ring (ϕ16 mm × 5 mm) and two millipore filters (pore size 0.45 μ), and contained 0.5ml of Eagle's MEM. One embryoid body (about 10^2 cells) was taken up and transfered into the germ free diffusion chamber under a binocular microscope (×40) in aseptic conditions. The chamber was then surgically implanted into the peritoneal cavity of strain 129 mice. After *in vivo* culture, 3 - 4 mice were sacrificed at every 10 days to estimate the cell number in the chamber up to the 80th day. Inorder to estimate viable cells all the contents of the chamber were treated with 1% trypsin and stained with Nigrosine. The number of cells at the 10th, 20th and 30th day were estimated by mixing up the contents of 3 or 4 chambers. For the estimation of the number of cells at the 10th day, 10 embryoid bodies were used in the chamber.
(o) : number of cells counted in each chamber, (●) : average value of 3 or 4 chambers.

bodies, died without exception on the average at the 31st, 37.5th, 41st, and 43rd day respectively. Since 10^6 cells corresponds to 10^4 embryoid bodies, if tumorgenicity was retained in these cells cultured for 70 days by the chamber technique, the subjected mice could develop tumors and die before 65 days. This result strongly suggests that tumorgenic potency is lost regardless of enough proliferated cells when a single embryoid body is implanted as a form sealed by millipore filters in the peritoneal cavity of a mouse.

These results lead us easily into another problem of determining when tumorgenicity of embryoid bodies is lost during *in vivo* culture in chambers. Further investigations should be designed from the viewpoint that an *in situ* interaction of a single embryoid body with native tissues might be required for the maintenance of its morphological and/or biological integrities.

Reference

1) Embryonic potency of embryoid bodies derived from a transplantable testicular teratoma of the mouse. Leroy C. Stevens. Developmental Biology, *2*, 285-297, 1960

(6) **Regulation of microtubule protien synthesis during postnatal development of rat cerebellum.**

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Tubulin, the colchicine-binding protein of microtubules, has been found in many eukaryotic tissues and appears to play an important role in maintaining cell structure and mediating a variety of cellular events including mitosis, motility, and the secretion of granular products. For example, neurotubules, microtubules occuring in central nervous tissues, are components of the axon and are believed to play an essential role in axon function and in brain differentiation.

In chick embryo brain, the amount of tublin increases from 20% of total proteins at 5-7 days of development to 42% at 13 days, and is followed by gradual decrease to 25% in the adult. Similar changes of tubulin concentration during the development of rat cerebellum is now being analysed, because this development occurs postnatally and this is easily examined. During this process it has been shown by histological studies that the external granule layer, where the primitive matrix cells proliferate, differentiates into granule, stellate and basked cells, and granule cells extend T-shaped axons giving parallel fibers in the molecular layer.

Based on these results, studies on the regulation of tubulin synthesis at the levels of transcription and translation are in progress. In *in vitro* study using DNA-dependent RNA polymerase obtained from rat brain, the synthesis of tubulin mRNA on chromatin isolated from the cerebellum cells at various developmental stages is analysed by hybridization with the DNA probe having a complemental polynucleotide segment to tubulin m-RNA. Among three molecular species of DNA-dependent RNA

polymerase in mammals, RNA polymerase II is presumed to transcribe DNA into mRNA, but has not been shown to transcribe DNA or chromatin template asymmetrically and selectively. Therefore, the following questions arise : (1) Is the enzyme not complete? (2) If the enzyme is complete, then are the other chromatin factors or non-chromatin factors necessary? (3) Is the chromatin structure essential for the specific transcription? Since these problems should be solved, the molecular apparatus of transcription should be carefully analyzed not only as a whole but also with a specific aim toward the synthesis of tubulin.