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DNA DIRECTED PEPTIDE SYNTHESIS

I. A Cell-free System for Regulation of the Synthesis of β-Galactosidase.

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Abstract——A cell-free system for sustaining an efficient enhancement of amino acid incorporation primed by *E. coli* DNA and φ80 phage DNA has been devised.

The essential procedures are the preparation of S30 extract by sonic disintegration of *E. coli* cells and its preincubation with supplements of energy-generating system and amino acids.

The amino acid incorporation is efficiently stimulated with addition of calcium ion in the reaction mixture. The system thus established is able to synthesize 2 to 3μg of protein by either 80μg of *E. coli* DNA or 38μg of φ80p*lac* DNA. The prominent effect of calcium addition may be explained as follows; calcium ion stimulates a certain step of translation, resulting in the stimulation of transcription by coupling with translation, thus enhances the overall process of protein synthesis.

In addition, an enzyme protein, α-peptide of β-galactosidase is also synthesized by φ80p*lac* DNA in this reaction system and the activity of β-galactosidase is detected by α and ω complementation of β-galactosidase protein.

Finally, the specific repression of β-galactosidase synthesis by a partially purified repressor and partial derepression by 10^{-3}M IPTG* have also been accomplished.

INTRODUCTION

Molecular mechanism of gene expression has been extensively studied according to the central working hypothesis that DNA directs the synthesis of RNA, which in turn directs the synthesis of protein. The validity of results in these studies may be accepted more precisely when these processes are fully realized in an *in vitro* system, in which biologically active protein is synthesized by use of well characterized components under controlled conditions.

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*Abbreviations.
IPTG : Isopropyl-β-D-thiogalactoside. ONPG : Ortho-nitrophenyl-β-D-galactoside. PEP : Phosphoenolpyruvate. PEPkinase : Phosphoenolpyruvate kinase. PCA : Perchloric acid. PolyU : Polyuridilic acid. aa : amino acid.

The molecular mechanism of RNA polymerase reaction has been eagerly studied in many places including our laboratory and now the correspondence between the ***in vitro*** and ***in vivo*** reactions has been established in several points. It has been recognized that the following three points about transcription process have to be elucidated in connection with protein synthesis in a cell-free system.

(1) How can we evaluate the biological activity of RNA which is synthesized in the well characterized ***in vitro*** system by purified RNA polymerase and DNA?

(2) What kind of effects may be brought on transcription, on its operon-reading and regulation, when it is coupled with translation?

(3) How can RNA synthesized ***in vitro*** be translated more and more efficiently? What factors will govern the coupling between the two processes?

With these aims, we have attempted to find suitable conditions for a DNA directed synthesis of an enzyme protein.

MATERIALS AND METHODS

(1) *E. coli* strains

XA35 (i^-z^{del}) ; This strain was derived from strain M15 described by Ullmann, Jacob and Monod,[1] and obtained from Dr. Beckwith. It has a deletion of about one third of the α-portion of the *z*-gene, and was used for preparation of S30 extract.

21i^+ (i^+z^{del})[2]; This strain has normal i-gene and almost the same deletion as XA35, and was kindly supplied by Dr. Zubay. It was used for preparation of lactose repressor.

1402-1 (ϕ80p***lac***) ; This strain is ϕ80p***lac*** lysogen and was kindly supplied by Dr. Ohshima[3].

3102 (ϕ80) ; This strain is ϕ80 lysogen.

B. ; This strain was used for *E. coli* DNA preparation.

(2) Reagents

Phosphoenolpyruvate monopotassium salt (C. F. Boehringer & Soehne GmbH Co.), phosphoenolpyruvate kinase (C. F. Boehringer & Soehne GmbH), reconstituted ^{14}C-protein hydrolysate (^{14}C-RPH) (The Radiochemical Center), cytidine 5'-triphosphate-2-^{14}C 20.6mc/mmol (Schwarz BioResearch Inc.), IPTG (Mann Research Laboratories Inc.), ONPG (Carbiochem. Co.), Chloramphenicol (Sankyo Co.), RNase (Worthington Co.) DNase (Worthington Co.), CM-Sephadex C50 (Pharmacia Co.), polyuridilic acid (Carbiochem. Co.), and ^{14}C-phenylalanine (The Radiochemical Center).

(3) Preparation of S30 extract

The medium used for bacterial growth was 10g of polypeptone, 1.5g of yeast extract, 2g of meat extract and 3g of NaCl per liter, or minimal glycerol supplemented with 0.1% casamino acids (Difco) and 10μg per ml of vitamin B_1. There was no difference between these two media. 100ml of preculture medium was inoculated with *E. coli* XA35 grown on the surface of an agar slant, and aerated by shaking over night at 37C. This was added to 10 l of a fresh medium and it was vigorously

aerated at 37C. In the case of rich medium several drops of silicon oil (Shinetsu Chemical Ind. KM68F1) was added for diminishing foams. At intervals the cell turbidity was determined spectrophotometrically to follow cell growth. Aeration was stopped at 5 x 10^8 cells per ml and cells were chilled by adding 0.5 volume of crushed ice. Cells were harvested and washed twice in 3 volume of 10 mM Tris acetate (pH 8.0), 14 mM Mg acetate, 60 mM potassium acetate and 6 mM β-mercaptoethanol (buffer F). Ordinarily the yield was 7 to 8 g of packed cells, wet weight per 10 l of the medium. The preparation of cell extract was started immediately after cells were obtained. All procedures, unless specified, were carried out at 2 to 3C. Fifteen g of washed packed cells were suspended in 1.3 vol of buffer F (w/v) and sonicated (10^3V. 60 mA) for 3 min. With this condition the turbidity of cell suspension was decreased by 80%. The resulting lysate was centrifuged at 10,000 rpm for 20 min and the supernatant solution was further centrifuged at 30,000×g for 20 min. Then the supernatant solution was decanted and again centrifuged at 30,000×g for 30 min. The upper two-thirds of the supernatant solution was taken by pipetting and stored. To 10 ml of the extract thus obtained were added 0.5 ml of 2M Tris acetate (pH 8.0 at 37C), 0.2 ml of 0.14 M Mg acetate, 0.08 ml of 0.1 M ATP, 0.225 ml of 0.4 M PEP, 100μg of PEP kinase, 0.004ml of β-mercaptoethanol and 0.04 ml of 20 amino acids mixture (2.5 μmol each amino acid/ml) as described by Nirenberg.[4] The preincubation was carried out at 37C for 60 min and dialysed against two 1 liter portions of buffer F at 0C for 5 hr. The cell extract thus prepared is referred to as the S30 extract. After dialysis the S30 extract was divided into 1 ml aliquots, frozen quickly in dry ice acetone mixture and stored at minus 70C until needed. Little loss in activity could be detected after storage at this temperature for one month. Thawing was done only once.

(4) Preparation of DNA's

E. coli DNA was prepared from *E. coli* B by a modification of the procedure of Marmur[5] as described elsewhere[6]. Its molecular weight was estimated as 5×10^6 daltons.

The source of *E. coli* lactose gene was from phage φ80p*lac* DNA. This phage was isolated by Ohshima *et al*[3] and has *E. coli* lactose gene ($i^+o^+z^+y^+a^-$) incorporated near the immunity region of φ80 phage genome. Nevertheless the phage is not defective and can grow normally in a sensitive host without helper phages. So it is easy to obtain the phages in large quantities. To obtain the phage lysate *E. coli* 1402-1, a lysogen of φ80p*lac* was induced by ultraviolet irradiation. The resulting lysate had 2 to 3×10^{10} plaque forming titer per ml and was infected to *E. coli* W1485 in large scale.

The lysate thus obtained was containing 1×10^{11} plaque forming titer per ml with a little contamination of *lac*$^-$ plaque, less than 2~3% (see Y. Iida *et al.*[7]). The phage lysate was concentrated by liquid-polymer-phase technique (Watanabe and August[8]). Further purification of the phage and DNA extraction from it was described elsewhere[7].

ϕ80 DNA was prepared by the same procedure. Before use all DNA's were dialysed against 20 mM tris acetate (pH 8.0), 1mM EDTA and then against 20 mM tris acetate (pH 8.0) only.

(5) Incubation conditions of amino acid incorporation

The incubation mixture contains: 50 mM Tris acetate (pH 8.0), 5 mM β-mercaptoethanol, 80 mM potassium acetate, 12.5 mM Mg acetate, 2 mM ATP, 0.5 mM each of GTP, CTP, and UTP, 20 mM PEP (potassium salt), 50μg/ml PEP kinase, 0.05 to 0.2 mM each of 20 amino acids mixture containing ^{14}C-amino acids (about 10^4 cpm/one mμmol of amino acid), 5 to 8 mM $CaCl_2$, 300 to 400μg/ml *E. coli* DNA, or 120 to 160μg/ml ϕ80 or ϕ80plac DNA, and 5 to 8 mg/ml protein as S30 extract.

Final volume was 0.25ml. All components except S30 were mixed together and preincubated for 3 min at 37C. The reaction was started by addition of the S30 extract. The incubation was ordinarily for 30 min at 37C. At the end of the incubation the reaction tubes were chilled in an ice bath, then added 250μg of bovine serum albumin as carrier, and PCA to final concentration of 3.5%. The tubes were placed in a boiling water bath for 10 min to hydrolyse aminoacyl-tRNA, then were chilled in ice for 30 min. After centrifugation the supernatant solution was discarded and the precipitates were washed three times in 3.5% PCA. The precipitates were dissolved in 80% formic acid and put into a planchet, dried and counted in a thin window gas flow B-scaler.

(6) Assay for β-galactosidase activity

Since the source of S30 extract was XA35 (i^-z^{del}) which was an α-acceptor, complementation[9] would take place between this α-accepting peptide contained in the extract and those operator proximal peptide synthesized in the cell-free system.

After the reaction mixture for DNA directed amino acid incorporation was incubated at 37C as described above, the reaction tubes were further incubated for 1.5 hours or more at 28C for complementation reaction of β-galactosidase. Then 1.25ml of the solution for β-galactosidase assay was added to the tubes, which finally contained 0.1M sodium phosphate buffer (pH 7.2), 0.14M β-mercaptoethanol, 0.52mg/ml of ONPG and 0.01% marzonin as an antiseptic (final vol. was 1.5ml).

The tubes were incubated at 28C until enough yellow color was developed. At the end of the incubation, the tubes were added one drop of glacial acetic acid to precipitate the protein, thus decreasing the back ground absorption due to turbidity, chilled in ice, and centrifuged at 3C. One ml of the supernatant solution was pipetted and added to 0.5ml of 2M Na_2CO_3. The optical density was determined at 420mμ.

(7) Preparation of partially purified IPTG binding substance.

E. coli 21i^+(i^+z^{del}) was cultured in minimal glycerol supplemented with 0.1% casamino acids (Difco) and 20μg/ml vitamin B_1 with violent aeration. Cells were harvested at late log phase (1.5×10^9 cells/ml) and washed twice in 10 mM Tris HCl (pH 8.0), 10 mM Mg acetate, 60 mM KCl, 6 mM β-mercaptoethanol and 0.2 mM sodium EDTA (B_2d') and stored at minus 20C. All steps were carried out at 5C. One hundred g of frozen cells were thawed and suspended in 150ml of B_2d' and disrupted through

RIBI cell fractionator at 20,000 psi. Then 100ml of B_2d' was added to the lysate and pH was adjusted to 7.8, and the resulting solution was centrifuged at 12,000rpm for 40 min. The supernatant was further centrifuged at 75,000×g for 2.5 hours to remove ribosomes.

To the high centrifuged supernatant was added solid ammonium sulfate to 30% saturation adjusting pH to 7.5 with 1M Tris solution. After 40 min of stirring, the precipitate was collected by centrifugation at 12,000 rpm for 60 min, suspended in 20 ml of 20 mM Tris-HCl (pH 7.0), 40 mM KCl, 10 mM Mg acetate, 0.2 mM sodium EDTA, 6 mM β-mercaptoethanol (BII+0.04 M KCl) and dialysed overnight against two 1 liter portions of BII containing 0.04 M KCl. The resulting precipitates were removed by centrifugation, and the solution was mixed with 100 ml of CM-sephadex C50 which had been bufferized in BII containing 0.04M KCl for 2 days.

After 40 min with occasional stirring, the resin was poured onto a Büchner funnel fitted with glass filter to remove unabsorbed materials. Then the resin was suspended in 100 ml of BII containing 0.09M KCl and poured onto the Büchner funnel after 40 min of gentle stirring. This washing was repeated until brown color of the resin was washed off.

Then the resin was suspended in 20ml of BII containing 0.3M KCl and the filtrated solution was stored. This elution was repeated once more, and the two portions of resulting filtrate were collected, and concentrated to 2 ml in a collodion bag under negative pressure.

The concentrated fraction was dialysed against B_2d' supplemented with 10% glycerol and rapidly frozen in 0.5ml aliquots and stored at minus 70C. This fraction was assayed for its ability to bind IPTG by the equilibrium dialysis method as described by Gilbert and Müller-Hill[11].

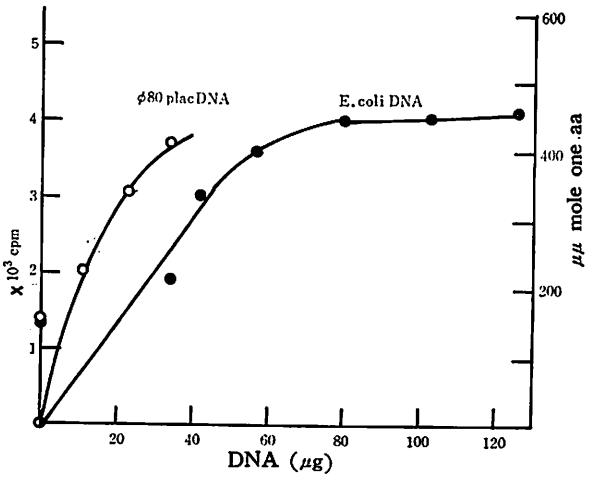
RESULTS

(A) Cell-free amino acid incorporation programmed by exogenous *E. coli* and temperate phage DNA's.

The template of DNA dependent cell-free systems[12][13][14] for amino acid incorporation reported so far was mainly DNA derived from T-even coliphages. It seems curious that DNA from *E. coli* from which the extract for the reaction system was prepared had very poor template activity, whereas DNA derived from T-even coliphages stimulated a high degree of amino acid incorporation in the same system. DNA derived from temperate phages, such as λ and φ80 also had very poor template activity in these systems. It is therefore necessary to develop a suitable incorporation system which is highly stimulated by *E. coli* DNA, because this study intends to have a DNA dependent cell-free system which can synthesize the enzymes of *E. coli* lactose operon.

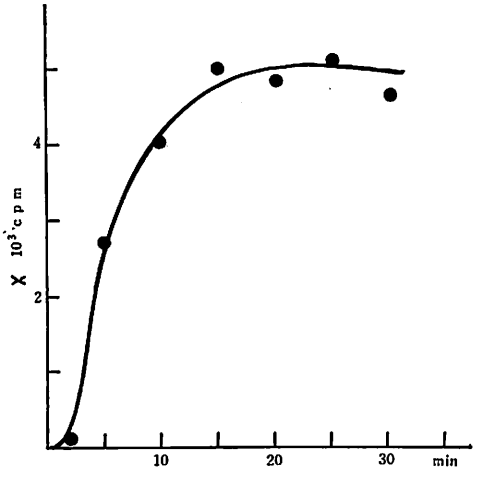
As shown in Table 1 (a) (b), we developed the system which responds favorably to DNA derived from *E. coli* and temperate phages, such as φ80 and φ80p*lac* by

Fig. 1. (a) The dependence of amino acid incorporation upon DNA concentration



Each reaction mixture contains 12.5 mM Mg acetate, 5 mM $CaCl_2$ 1.25 mg of S30 protein and indicated amount of DNA. Other components are as described in Materials and Methods (5). Incubation was at 37C for 30 min. Each point represents net cpm from which 1,360 cpm incorporated in the absence of DNA was subtracted.

(b) The kinetics of amino acid incorporation directed by *E. coli* DNA



Each reaction mixture contains 80 μg of *E. coli* DNA, 1.25 mg of S30 protein, 12.5 mM Mg acetate and 5 mM $CaCl_2$. Other components are as described in Materials and Methods (5). Incubation time is as indicated. Cpm in the absence of DNA at each reaction time is subtracted from each point.

Table 1. Summary of amino acid incorporation programmed by *E. coli* and φ80*plac* DNA's

(a) *E. coli* DNA 115 μg

System	+DNA	−DNA	mμmole one aa stimulated
1) complete(aa 0.3 mM each)	18,478 cpm	4,066 cpm	0.96(100)
2) complete + 300 μg chloramphenicol	1,190	1,208	<0 (<0)
3) complete + 10 μg DNase	1,684	1,650	<0.04(<4)

0.96 mμmole one aa =19mμmole total aa = 33% Expression of *E. coli* DNA

(b) φ80plac DNA 38μg

System	+DNA	−DNA	mμmole one aa stimulated
1) complete (aa 0.4 mM each)	27,981cpm	5,213cpm	1.52
2) complete (aa 0.2 mM each)	10,243	1,877	0.93 (100)
3) complete−GTP, CTP, UTP	2,350	1,723	0.07 (8)
4) complete−PEP, PEPkinase (ATP, 0.5 mM)	1,370	1,258	0.01 (1)
5) complete+ 300 μg of chloramphenicol	922	544	0.04 (4)
6) complete+10 μg DNase	861	751	0.01 (1)

1.52mμmole one aa = 30.4mμmole total aa = 160% Expression of φ80plac DNA

Each reaction mixture contains 12.5mM Mg acetate, 6mM $CaCl_2$, 0.8mg of S30 protein and DNA's as indicated. Other components in the complete system are as described in Materials and Methods (5). Specific activity of ^{14}C-amino acid mixture was 15,014 cpm per mμmol of one amino acid. Incubation was at 37C for 30 min.

preparing the S30 extract as described in Materials and Methods (3) and improving the reaction system especially by adding calcium ion to the system. The degree of amino acid incorporation stimulated by these DNA's is almost equal to that of T-even coliphage DNA' s.

In the complete reaction mixture *E. coli* DNA (Table 1 (a)-1) or φ80p*lac* DNA (Table 1 (b)-1) stimulates the incorporation more than four to five times of amino acids over that of background which was incubated without addition of DNA and these values can be calculated as two to three μg of protein synthesized *de novo*.

If it is assumed that each amino acid was incorporated equally, it can be estimated that 60% and 160% of information carried on *E. coli* DNA and φ80p*lac* DNA respectively was translated into protein in the complete system in 30 min.

It is clear that conditions which allow RNA synthesis is prerequisite since the incorporation was reduced more than 90% when UTP, GTP and CTP were omitted (Table 1 (b)-3), and DNase completely inhibited the incorporation (Table 1 (a)-3 and (b)-6). The reaction was also completely inhibited by addition of 300μg of chloramphenicol (Table 1 (a)-2 and (b)-5) and 10μg of RNase in the reaction mixture. The reaction system completely depends on an energy generating source (Table 1 (b)-4).

Thus it can be concluded that the protein synthesis occured by bringing to completion of the whole process of gene expression; DNA→RNA→Protein.

Fig. 1(a) shows the effect of DNA concentration on amino acid incorporation. The amount of incorporation reached rapidly to a plateau with about 30μg of φ80p*lac* DNA, whereas it increased gradually in proportion to the amount of added *E. coli* DNA, until saturation was attained with 60μg of *E. coli* DNA.

All experiments below were performed with this amount of DNA which gave the saturated level of incorporation, and varied somewhat depending on S30-extract used. Fig. 1(b) shows the kinetics of amino acid incorporation directed by *E. coli* DNA. The amount of amino acids incorporated increased only for 15 to 20 min.

(B) Examination of the incorporation system.

(a) The effect of monovalent cation

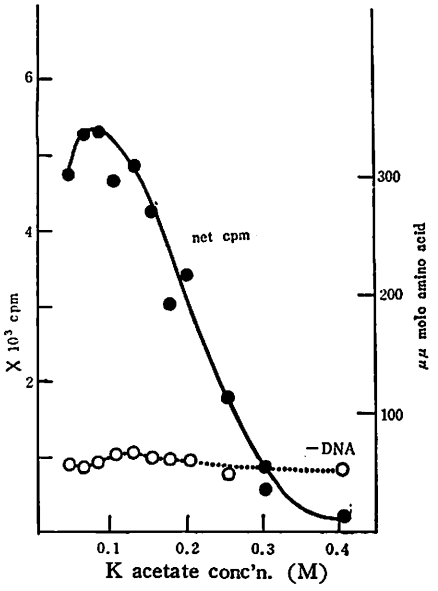
As monovalent cation, potassium ion was used. There was no recognizable difference between ammonium ion and potassium ion. The optimal concentration of potassium ion was 80 mM, and at higher concentration amino acid incorporation greatly decreased (Fig. 2(a)).

(b) Divalent cation

For amino acid incorporation reaction, Mg^{2+} ion is indispensable. The optimal concentration in this reaction system was at near 13 mM and at higher concentrations a slight decrease in amino acid incorporation was observed (Fig. 2(b)).

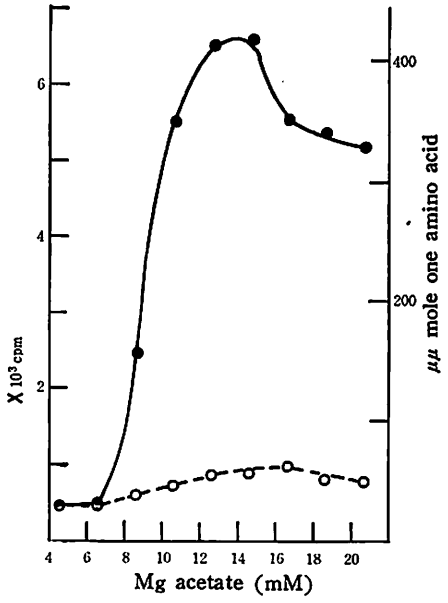
It has been reported that high Mg^{2+} concentration results in misreading in translation of messenger RNA[15,16,17], and the presence of formylmethionyl-$tRNA_F$ lowers the optimal concentration of Mg^{2+} ion[18,19], so we tried tetrahydrofolic acid, a formyl donor to the initiator methionyl-$tRNA_F$. But its addition to the reaction system had

Fig. 2. (a) Dependence on concentration of potassium



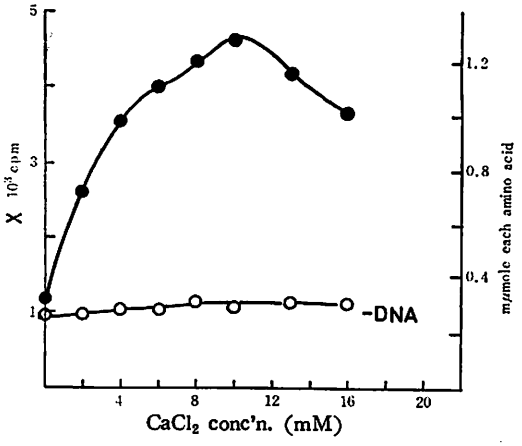
Each incubation mixture contains 69 μg of *E. coli* DNA, 1.25mg of S30 protein, 12.8 mM Mg acetate, 8 mM $CaCl_2$ and the indicated concentration of potassium acetate. Other components are as described in Materials and Methods (5). Incubation was at 37C for 30 min. Solid circles indicate the amount of amino acids incorporated programmed by DNA, from which those incorporated in the absence of DNA (open circles and dotted line) was subtracted (net cpm).

(b) Dependence on concentration of magnesium



Each incubation mixture contains 96μg of *E. coli* DNA, 2mg of S30 protein, 7.3 mM $CaCl_2$ and the indicated concentration of magnesium acetate. Other components are as described in Materials and Methods (5). Incubation was at 37C for 30 min. Solid circles indicate the net cpm and open circles indicate the amount of amino acids incorporated in the absence of DNA.

(c) Dependence on the concentration of calcium



Each incubation mixture contains 81μg of *E. coli* DNA, 1.2 mg of S30 protein, 12.5 mM Mg acetate and the indicated concentration of $CaCl_2$. Other components are as described in Materials and Methods (5). Incubation was at 37C for 30 min. Solid circles indicate the net cpm. Open circles represent the amount of amino acids incorporated in the absence of DNA.

neither effect on Mg^{2+} dependency of the system nor on activity of β-galactosidase detected in the system as described later.

It seems curious that Ca^{2+} ion stimulates amino acid incorporation directed by *E. coli* DNA. This phenomenon was first reported by Lederman and Zubay.[20] Our system does not completely depend on Ca^{2+} ion since it exhibits a considerable amount of amino acid incorporation in the absence of this ion. The degree of stimulation exhibited by the ion fluctuated from a preparation of S30 extract to another, but ordinarily it stimulated 3 to 10 times of amino acid incorporation primed by *E. coli* DNA at the optimal concentration compared to that in the absence of this ion. On the contrary the optimal concentration of the ion was always constantly at near 10 mM with a shoulder at near 6mM (Fig. 2(c)).

We are interested in which step of protein synthesizing process was effected by the ion.

Fig. 3(a) shows the effect of Ca^{2+} ion on the RNA synthesis in this reaction system. In the complete reaction system the stimulation effect of the ion on RNA synthesis was exhibited with two peaks of incorporation of ^{14}C-CMP, one at 6 mM of Ca^{2+} ion and the other at 10 mM of the ion. These optimal concentrations of Ca^{2+}ion coincide well with those in amino acid incorporation reaction.

But as shown in Fig. 3(a) the stimulation effect of Ca^{2+} ion was not detected when energy generating source and amino acids were omitted from the complete reaction mixture.

Furthermore the calcium effect was also eliminated when ribosome fraction was removed from S30 extract by a high speed centrifugation. The stimulation effect reappeared when the ribosome fraction was again mixed with the supernatant (Fig. 3 (b)). These results suggest that the stimulation effect of Ca^{2+} ion on transcription was performed by coupling the transcription with translation.

Fig. 3(c) shows the effect of calcium ion on polyU directed polyphenylalanine synthesis to test the effect of Ca^{2+} ion on the system confined to translation. This experiment was carried out in the presence of 15 mM of Mg acetate. The incorporation showed a maximum at 10 mM of $CaCl_2$. Similar effect of calcium ion was first reported by Gordon *et al*[21]. They studied the effect of calcium ion on polyU directed polyphenylalanine synthesis by purified ribosomes and transfer enzymes in the absense of Mg^{2+} ion, giving 9mM of Ca^{2+} ion as the optimal concentration.

These results suggest the mechanism of calcium effect on DNA directed amino acid incorporation as follows ; first Ca^{2+} ion stimulates some steps of translation process, at least at 10mM. Then synthesis of messenger RNA may be accelerated by coupling with the stimulated translation. Thus the overall process of amino acid incorporation is stimulated.

(C) Cell-free synthesis of specific protein—DNA directed synthesis of α-portion of β-galactosidase.

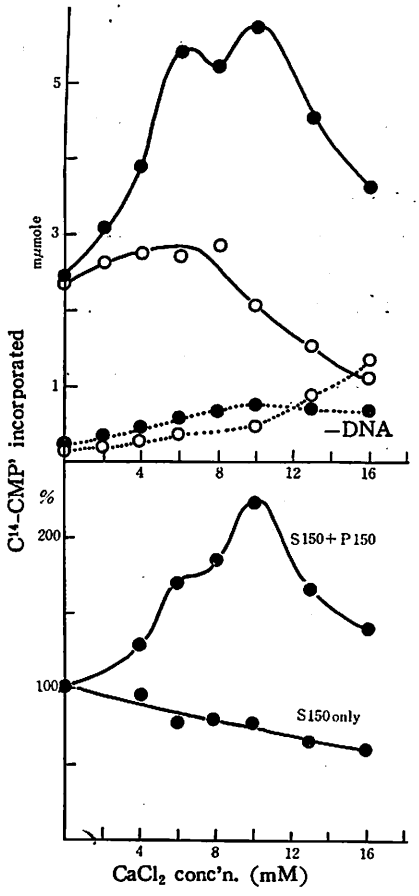


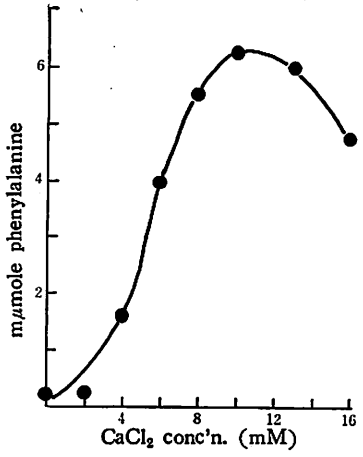
Fig. 3. (a) Effect of calcium on transcription

In each reaction mixture cold amino acid mixture is substituted for ^{14}C-amino acid mixture and ^{14}C-CTP for ^{12}C-CTP. 81μg of *E. coli* DNA was added. Other components are as described in Materials and Methods (5). Incubation is at 37 C for 5 min. Solid circles and line represent the net amount of CMP incorporated in the complete reaction system. Open circles and solid line represent the net amount of CMP incorporated in the system in which PEP, PEPkinase and amino acid mixture are deleted from the complete reaction mixture. Dotted lines represent CMP incorporated in the absence of DNA.

(b) Effect of calcium on transcription

In this experiment, S30 extract was centrifuged at 150,000g for 2 hr and two thirds of the supernatant solution was taken and used as ribosome-free extract (S150). The precipitate was suspended in buffer F and used as ribosome fraction (p 150). Other reaction components are as described in Fig. 3(a). The amount of incorporation in the absence of calcium was represented as 100%. All points indicate the net amount of CMP incorporated.

(c) Effect of calcium on polyU directed phenylalanine incorporation



All reaction mixtures contain 49g of polyuridilic acid, 15 mM Mg acetate and indicated concentration of calcium. ^{14}C-phenylalanine and 19 cold amino acids except phenylalanine are substituted for ^{14}C-amino acid mixture. Other components are as described in Materials and Methods (5). Incubation was at 37C for 30 min. Net amount of ^{14}C-phenylalanine incorporated is represented.

It is important to examine the biological activity of peptides which are synthesized in this cell-free system. Thus it was attempted to synthesize β-galactosidase, the structural gene of which is situated most proximal to the operator of lactose operon. Now the author overcomes two difficulties to carry out such experiment. One is the concentration of lactose operon DNA to be used as template, and the other is that the molecular weight of β-galactosidase is so large (β-galactosidase has a molecular weight of 540,000[22] and is a tetramer containing identical subunits, the molecular weight of which is 125,000) that it may be difficult to synthesize any detectable amounts of complete molecules *in vitro* (about one thousand amino acids must be polymerized in correct sequence *in vitro*.).

The former difficulty may be overcome by DNA derived from φ80p*lac* phage which was mentioned in some detail in Materials and Methods (4). Lactose operon occupies little less than 10 per cent of this phage DNA, because the molecular weight of DNA from φ80 phage is 3×10^7 daltons and that of *E. coli* lactose region is 4×10^6 daltons. On the other hand DNA which belongs to lactose region occupies about 0.1 per cent of whole bacterial DNA. Thus φ80p*lac* DNA was accounted giving us DNA which contains the lactose genes in concentration of about a hundred times greater than *E. coli* DNA. Genetic analysis of the phage tells us the presence of the following genotype; i^+, p^+, o^+, z^+, y^+ and a^-[7].

Furthermore, as reported by Y. Iida et al,[7] the lactose genes of φ80p*lac* DNA was transcribed by purified *E. coli* RNA polymerase, and the lactose specific RNA occupied about 20 to 30% of the total RNA synthesized in this *in vitro* reaction.

The polypeptide chain of a monomer of β-galactosidase has been divided into three parts designated α, β, and ω by intracistronic complementation (Ullmann *et al*[1]). Alpha part is adjacent to operator and synthesis of β-galactosidase begins from this part and proceeds in order of β and ω. When the extract from XA35 which has a deletion in α-part but has intact β and ω parts is mixed with that from 2A238 which is a deletion mutant in ω part but has intact α part, and the mixture is incubated at 28C for several hours, a high level of β-galactosidase activity is detected. Of course, each extract by itself has no activity of the enzyme at all.

By the same way it may be possible to detect the β-galactosidase activity by the process of intracistronic complementation between the α-deleted peptides contained in S30 extract and α-part of peptides which is synthesized *in vitro* programmed by φ80-p*lac* DNA. Rough estimate indicates that the α-part contains about 250 to 300 amino acid residues. So it is only necessary to have a correct sequence of about three hundreds amino acid residues to obtain a successful complementation. DeVries and Zubay[10] already reported cell-free synthesis of active α-peptide by the same way.

α-Peptide synthesis was carried out and the enzyme activity was measured as described in Materials and Methods (6). At the same time a rough estimate of total protein synthesis was done by ^{14}C-amino acid incorporation. In Table 2 the results of these experiments are summarized. Each reaction system was repeated three times in one experiment, but fluctuation among the experimental values was rather small, and

Table 2. β-Galactosidase activity of *in vitro* synthesized α-peptide by intracistronic complementation

System	$A_{42.}(=E)$	$\triangle E(E-E_{noDNA})$	%Expression	%^{14}C-aa incorporated
1. no DNA	0.051	—	—	—
2. ϕ80 DNA 36μg	0.051	0	—	—
3. ϕ80*plac* DNA 38μg	0.243	0.192	100	100 (0.93mμmole one aa)
4. ϕ80*plac* DNA 38μg + 4 $\times 10^{-4}$M IPTG	0.210	0.160	84	—
5. 〃 〃 +10μg DNase	0.063	0.012	6	<0
6. 〃 〃 +10μg RNase	0.060	0.010	5	—
7. 〃 〃 +300μg C. P.	0.048	0	0	<0
8. 〃 〃 −GTP, CTP, UTP	0.081	0.032	16	4
9. 〃 〃 −amino acids	0.084	0.034	18	—
10. 〃 〃 −PEP, PEPkinase	0.051	0	0	6

−ONPG(0.003−0.01) was substracted
Assay time of β-galactosidase activity for 40 hrs at 28C

Each reaction mixture contains 1.0 mg of S30 protein from XA 35, 12.5 mM Mg acetate, 6 mM $CaCl_2$, 50 mM each amino acid and DNA as indicated in the table. Other components are as described in Materials and Methods (5). After incubation at 37C for 30 min the incubation mixtures are allowed to stand for 2.5 hours at 28C to promote complementation. Then β-galactosidase activity is measured as described in Materials and Methods (6) and colorimetry is performed after 40 hr of incubation at 28C.

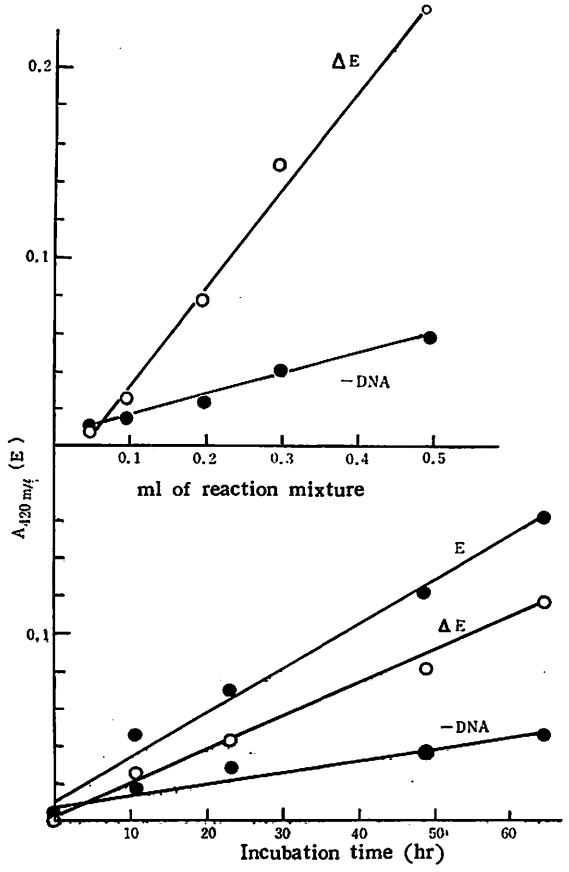


Fig. 4. (a) The same α-peptide synthesis as Table 2 was carried out in the complete system scaled up six times. ϕ80*plac* DNA added was 152μg per ml of reaction mixture. After the complementation reaction at 28C indicated volume of reaction mixture was added to the reaction mixture for β-galactosidase assay. Colorimetry was performed after 42 hours. The definition of $\triangle E$ is described in the text.

(b) Time course of color development

The same α-peptide synthesis as Table 2 is carried out in the complete system scaled up five times. ϕ80*plac* DNA added was 152μg/ml of reaction mixture. After the complementation reaction at 28C for 1.5 hours 6.25 ml of reaction mixture for β-galactosidase assay were added. At the indicated time 1.4 ml of the mixture solution was pipetted and colorimetry was performed as described.

each reaction was repeated using at least two different S30 extract.

In the first column was indicated the increase of optical density at 420mμ (O.D. 420mμ) from which was subtracted that of O.D.420mμ in the same reaction mixture which was incubated without ONPG (E). E=0.051, the increase of O.D. 420mμ in the reaction with no addition of DNA was subtracted as a background from each values (E) cited in the first column and these values (*Δ*E) are given in the second column.

The complete system with 38μg of φ80p*lac* DNA gave a distinct yellow colour, showing the increase of *Δ*E=0.192. On the line 4, IPTG lowered the colour development, because it was confirmed that IPTG inhibited competitively the enzymatic hydrolysis of ONPG.

In order to ensure that colour development is due to ***de novo*** synthesis directed by added DNA carrying the *z*-gene, various control experiments were carried out. Almost no α activity was detected when DNase (line 5), RNase (line 6) or chloramphenicol (line 7) were added to the complete system, or UTP, GTP and CTP (line 8), amino acids (line 9) or energy generating system (line 10) were deleted from the complete system. Thus I can conclude that the α activity is due to the peptides synthesized ***in vitro via*** the whole process of gene expression, DNA→RNA→protein.

Fig. 4 (a) shows that the degree of colour development is proportional to the amount of α-peptides synthesized in this system within the range given in the figure.

Fig. 4 (b) shows that the rate of hydrolysis of ONPG is linear at least for 65 hrs reflecting the stability of α-peptide synthesized ***in vitro.***

(D) The effect of IPTG binding protein on synthesis of α-peptide in cell-free system.

Since Jacob and Monod proposed the operon theory,[23] regulation of lactose operon has been extensively studied, and it is of critical importance to purify the regulator substance, the repressor, and to clarify the mechanism of its action.

In 1966, Gilbert and Müller-Hill[11] devised a skillful technique to detect the repressor based on the hypothesis that repressor binds inducer, IPTG, and partially purified a IPTG binding protein. They proved that the protein was indeed a product of *i*-gene,[11] and was specifically bound to, or dissociated from the operator region of lactose operon in the absence or in the presence of IPTG respectively[24].

Zubay ***et al***[2] demonstrated that their IPTG binding protein could repress the synthesis of β-galactosidase in their cell-free system and the repression effect was lost by addition of 10^{-3}M IPTG.

Ohshima ***et al***[25] proved that messenger RNA synthesis of lactose operon by purified RNA polymerase was inhibited by 80% by the repressor fraction in the presence of ribosomes and some factors associated to ribosomes.

It was also tried to prove the effect of repressor in this cell-free system. IPTG binding protein was partially purified by the simplified method of Ohshima ***et al***[25] as described in Materials and Methods (7). The final fraction had 60 units/ml of IPTG

binding activity and 2.7 mg of protein per ml. Rough estimate indicates the purity of repressor in this fraction was 0.1 per cent, and the concentration of repressor was 20 $\mu\mu$mole per ml. The content of nucleic acid was less than 2%. The fraction was prepared from *z*-deletion mutant and had no β-galactosidase activity at all.

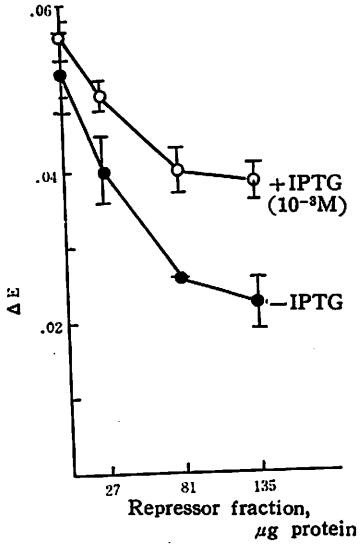
Varying amounts of this fraction with or without 10^{-3}M IPTG were mixed with the S30 extract prepared from XA 35 ($i^{-}z^{del}$) and these were added to preincubated incubation mixture containing 30μg of ϕ80p*lac* DNA and incubation and complementation were carried out as described in Materials and Methods (6). Then IPTG solution was added to those reaction tubes which had been incubated without IPTG, because of its inhibitory action on β-galactosidase activity, and ONPG solution was added to detect β-galactosidase activity. After 60 hrs of incubation colorimetry was carried out.

Fig. 5 shows the results of this experiment. The three volumes of IPTG binding fraction shown in the figure were estimated to be 0.2 $\mu\mu$mole, 0.6 $\mu\mu$mole and 1 $\mu\mu$ mole of repressor respectively. The amount of ϕ80*plac* DNA was 30μg and it can be calculated that the concentration of lactose operator was 1$\mu\mu$ mole. Thus the ratio of repressor to lactose operator in Fig. 5 was 0, 0.2, 0.6, and 1 respectively from left to right.

There is pronounced difference in color development between the reactions with IPTG and those without IPTG. Both of these resulted in lowering of color development as the concentration of IPTG binding fraction increased, but without IPTG color development was more repressed. When 10^{-3}M IPTG was added, partial derepression occurred. The reasons of incomplete derepression are obscure but there may be two possibilities;

(1) IPTG binding fraction contained some inhibitor of protein synthesis such as

Fig. 5. The effect of IPTG binding fraction on synthsis of α- peptide in the cell-free system. See text.



nuclease.

(2) The repressor fraction was partially inactivated in the process of its preparation, or lost some essential factors, so IPTG bound to repressor could not deprive the repressor of its repression activity.

However the amount of repressor added in this experiment was not so enough that the repression was partial and the difference between repressed and derepressed reaction was a little.

But these results indicate that the IPTG binding fraction may have the three important characteristics as lactose repressor, IPTG binding, repression of β-galactosidase synthesis and derepression of it by the inducer.

DISCUSSION

The author has tried several ways to prepare S30 extract for cell-free protein synthesis. Each extract was examined into their ability both for RNA dependent and DNA dependent protein synthesis. Polyuridilic acid and MS-2 phage RNA as RNA template, and T_4 DNA, *E. coli* DNA and φ80 DNA as DNA template were tried. The S30 extract reported here gives the cell-free system which incorporate 20 to 40 mμmole of amino acids per 1 mg protein of the extract without distinction of the templates, RNA's or DNA's. This suggests that ability of the extract in translation sets limits to the amount of amino acids incorporated.

It seems most important how we disrupt cells for the preparation of the extract. Sonication, a French pressure cell, grinding with aluminum oxide or qualtz sand were tested to prepare the cell lysate, and it was concluded that mild sonication is the best way. The S30 extract prepared by this way are highly active with good reproducibility in amino acid incorporation programmed by any template cited above.

It is probable that activity of the extract is related to the degree of preservation of polysomes in the extract. We observed polysomes in the extract prepared by each way of disruption cited above in a model E Spinco ultracentrifuge equipped with Schlieren optics. Polysomes are well preserved in extract prepared by sonication, but little polysomes are observed in the extract prepared by disruption with alumina or qualtz sand. When the extract prepared by sonication is preincubated as described in Materials and Methods, most polysomes in it dissociate into monosomes and some into 30S and 50S ribosomal subunits[26,27,28,29,30], some of which are thought to be brought about by running-off of ribosomes from mRNA at termination sites.

It may be conceivable that these ribosomes dissociated from polysomes at termination sites are active in cell-free protein synthesis.

All cell-free systems reported up to this day except the one reported by Lederman *et al*[20], were poorly active in amino acid incorporation programmed by *E. coli* DNA or temperate phage DNA, but they are highly active by T-even DNA. We cannot find any reason for this difference. But in this system, there is no difference in the incorporation activity among these DNA's.

This system incorporated 20 to 30 mμmole of amino acids per 1 mg protein of S30 extract programmed by *E. coli* DNA or φ80 DNA. RNA polymerase activity was checked up in the system and estimated to be about 20 units per 1 mg protein of S30 extract. The amount of messenger RNA which was synthesized during 15 min, in which time amino acid incorporating reaction reached a plateau, was estimated to be 20 mμmole of nucleotides. This amount of messenger RNA is equivalent to the information for 7 mμmole of amino acids. So on the average the messenger RNA molecules were translated 3 to 4 times.

To see the effect of addition of purified RNA polymerase 20 to 200 units of highly purified 22S-RNA polymerase[31] were added to this system (1 to 10 times of endogenous enzyme). But amino acid incorporation was not stimulated at all, whereas RNA synthesis was stimulated comparably. Two possible interpretations of this fact may be considered;

(1) Purified RNA polymerase may have some defect, so that RNA synthesized in this system by it is inactive to participate in the translation process.

(2) The extract contained enough RNA polymerase that the translation machinery including initiation factors is fully operated. So it is the translation process that limits the ability of this system.

(3) For messenger RNA to be translated some unknown factors are indispensable, and these factors are insufficient. If (2) or (3) are possible we must try to remove the endogenous RNA polymerase from the extract to construct a system which is stimulated by purified RNA polymerase.

In agreement with the system reported by Lederman *et al*[20], my system is also stimulated by calcium ion. The transcription process is also stimulated by this ion. It is concluded that calcium ion may not directly stimulate transcription but it only stimulates some steps of translation process and as the result transcription is elevated by coupling with the stimulated translation process.

Shin and Moldave[33] first demonstrated the stimulation effect of ribosomes on transcription using a crude *E.coli* RNA polymerase-DNA complex. Revel *et al*[34] isolated a factor from a ribosome fraction, which also stimulated RNA polymerase reaction. Jones *et al*[39] also reached a conclusion that a significant portion of nascent RNA was released from RNA polymerase-template DNA complex by ribosomes. But these effects of ribosomes on transcription does not appear to be brought about by running of ribosomes on messenger RNA incorporating amino acids. On the contrary, the calcium effect can be explained as follows; As the ribosomes move along the nascent mRNA chain incorporating amino acids, the mRNA is released from the template DNA before its synthesis is completed, thus the transcription process is stimulated, as was suggested by Stent[35] as a hypothesis.

Subcellular system for enzyme synthesis were reported by Kameyama[36], Imai[37], DeVries[10] and Salser[38]. Also in this system, active α-peptide of β-galactosidase was synthesized. But it is possible that amino acid sequence of α-peptide of β-galactosidase

need not be so exact, as suggested by genetic data that point mutation at this portion of *z*-gene occurs less frequently. This may be one of the reasons that I succeeded.

Repressor function of IPTG binding protein was examined in this system. It was not proved so clearly, but it can be said "yes" with some confidence.

Further works about repressor function may be performed as follows.

(1) The same experiment as described above may be carried out using highly purified repressor.

(2) Whether or not *i*-gene produces only IPTG binding protein with molecular weight 150,000? This may be done by preparing S30 extract from a strain which deletes all parts of *i*-gene and using highly purified repressor.

(3) The degree of repression of peptides specific to β-galactosidase may be shown by detecting them by precipitation with β-galactosidase antibody.

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