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A REQUIREMENT FOR GENE-SPECIFIC DEOXYRIBONUCLEIC ACID FOR THE CELL-FREE SYNTHESIS OF β-GALACTOSIDASE

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The following article (pp. 659–666) describes the preparation and some properties of a cell-free system from *E. coli* that catalyzes the *de novo* synthesis of induced β-galactosidase.[1] The system requires the presence of a particle and supernatant fraction obtained by centrifugation at 105,000 × *g*, inducer, an energy source, amino acids, and the nucleoside di- and triphosphates. The cell-free system also catalyzes the incorporation of C^{14} amino acids into protein. The rate of increase in enzyme activity is proportional to the rate of C^{14} labeling of protein that is precipitable by anti-sera to β-galactosidase. The incorporation of amino acids as well as enzyme synthesis is inhibited by treatment with RNase and by the presence of chloramphenicol. One of the surprising observations made was that the synthesis of β-galactosidase as well as the incorporation of C^{14}-leucine into protein was sensitive to DNase. The present communication represents an extension of these observations and presents evidence for the requirement for gene-specific DNA in the cell-free system. A preliminary account of this work has appeared.[2]

Materials and Methods.—Cell-free extracts from pre-induced cells of *E. coli* were prepared as described[1] except that the buffer used was 0.01 *M* Tris-HCl pH 7.5, 0.01 *M* Mg acetate, and 0.001 *M* β-mercaptoethanol. The reaction mixture for synthesis of β-galactosidase was as described except that glutathione (5 μmoles/ml) was included. Other methods were as described in ref. 1. The reaction mixture for the synthesis of β -galactosidase was composed of the following in μmoles/ml: Tris buffer, pH 7.5, 100; Mg acetate, 4; $MnCl_2$, 2; ATP, 10; methyl-β-D-thiogalactopyranoside (TMG), 5; PEP, 10; PEP kinase, 100 μg; UTP, GTP, CTP, UDP, GDP, CDP, 0.03 each; GSH, 5; L-amino acids, 50 μg, representing a mixture which reflects the amino acid composition of β-galactosidase described by Wallenfels and Arens.[3]

Conditions for in vivo inhibition experiments: *E. coli* was grown overnight in the synthetic medium with 0.5% glycerol. The cells were diluted tenfold in fresh medium and distributed in culture tubes equipped with aeration tubes and incubated at 37°C. Early log phase cells were X irradiated, with aeration at room temperature, using a G.E. Maxitron X-ray machine operated at 250 kvp and 30 ma with 3 mm of added aluminium filtration (hvl, 0.34 mm of Cu). The samples were mounted in a Lucite frame 15 cm from the target and received an X-ray dose of 2,100 r/min. Each experimental dose point consisted of two such tubes, one which was X irradiated and a control that was shielded from the X-rays. Immediately after irradiation TMG was added to a concentration of 5×10^{-4} *M* and incubation continued. One ml samples were withdrawn to prechilled test tubes and kept on ice for later assay. To these tubes 0.1 ml of toluene and 10 μg of sodium desoxycholate were added. The tubes were incubated at 37°C for 10 min with frequent shaking and assayed for enzyme formation and total protein. Cell-free preparations were also

made from pre-induced X-irradiated cells after washing and resuspension in distilled water to a concentration of 5×10^9 cells/ml, using the same procedure described for normal cells.

Preparation of crude nucleic acid fraction (N.A.): A crude nucleic acid fraction was prepared from the 100,000 $\times$ *g* supernatant by the addition of 5% streptomycin sulfate in 0.05 *M* Tris buffer, pH 8.4, at 0° with stirring.[4] The stirring was continued for 60 min and the precipitate was collected by centrifugation, then dissolved and dialyzed for 18 hr against the same buffer. After dialysis a small amount of insoluble material was removed by centrifugation.

Preparation of DNA from whole cells: The method used is essentially a combination of the methods of Zamenhof *et al.*[5] and Kirby.[6]

Approximately 10 gm (wet weight) of cells were washed 3 times with 0.15 *M* NaCl and suspended in 10 ml of distilled water. The cell suspension was added slowly to 100 ml of 15% Dupanol at pH 6.4 and the mixture was stirred for 3 hr at room temperature. To the viscous material was added 2 volumes of 95% ethyl alcohol, and the stringy precipitate was lifted out and washed once with 75% ethyl alcohol and extracted twice with 50 ml of 1.5 *M* NaCl. The extract was then precipitated with $1^1/_2$ volumes of 95% ethyl alcohol and the gelatinous material wound on a glass rod and lifted out. This material was dissolved in 50 ml of 0.15 *M* NaCl. The precipitation with alcohol was repeated twice. Thirty μg/ml of pancreatic RNase was added to the mixture and incubated at 37°C for 30 min. At the end of the incubation period an equal volume of 90% phenol was added and the mixture was emulsified on a wrist-action shaker for 60 min. The resulting emulsion was centrifuged at 2500 $\times$ *g* for 30 min and the upper viscous layer removed by suction. The phenol extraction was repeated. The DNA solution was precipitated with $1^1/_2$ volumes of 95% ethyl alcohol and dissolved in 0.15 *M* NaCl. This alcohol precipitation was repeated four times. The resulting DNA contained essentially no RNA and no detectable protein. The 280 to 260 mμ ratio was 0.517 and exhibited 38% hyperchromicity upon heating to 95°C. The T_M[7] value was 90.5°C. Ultraviolet (UV) irradiation of supernatant and DNA was carried out as described by Kameyama and Novelli.[8] Other materials and methods were as described in the preceding paper except that measurements of radioactivity were made by the method of Mans and Novelli.[9]

Results and Discussion.—The observation that DNase inhibited both the synthesis of β-galactosidase as well as the incorporation of C^{14}-leucine into protein suggested that DNA may play a direct role in protein synthesis. Allfrey and Mirsky[10] had previously observed a DNase inhibition of nuclear phosphorylation in a preparation from lymphocyte nuclei. However, this inhibition could be overcome by DNA, RNA, or any one of a number of polyanions, indicating that in their system DNA did not play a specific role. Thus it was of interest to establish whether the observed effect of DNase in our system reflected a specific requirement for DNA in the synthesis of protein.

Since we previously reported that the induced synthesis of β-galactosidase by whole cells was sensitive to exposure to UV irradiation[8] and it is well known[11] that DNA replication is similarly sensitive, attempts were made to inactivate the DNA in our preparations. The bulk of the DNA in the cell-free system is present in the 100,000 $\times$ *g* supernatants. Therefore, these were exposed to UV irradiation and then tested for ability to catalyze the synthesis of β-galactosidase. The results of such an experiment are presented in Table 1.

Using a combination of induced or constitutive particles with constitutive or induced supernatant, it is evident that UV irradiation of the supernatant either destroyed or greatly reduced the ability of the system to synthesize β-galactosidase. If DNA prepared from the constitutive strain is added to a reaction containing UV-irradiated supernatant, enzyme synthesis is either partially or totally restored. When the native DNA is irradiated the ability to restore enzyme synthesis to the inactivated system is similarly destroyed. These data, consistent with the observa-

TABLE 1

EFFECT OF UV IRRADIATION OF SUPERNATANT AND DNA ON ENZYME SYNTHESIS

Additions	Δ Enzyme units/60 min/ml
$P_c + S_i$	115
$P_c +$ UV S_i	−17
$P_c +$ UV $S_i + DNA_c$	25
$P_c +$ UV $S_i +$ UV DNA_c	0
$P_c +$ none $+ DNA_c$	0
$P_i + S_c$	226
$P_i +$ UV S_c	127
$P_i +$ UV $S_c + DNA_c$	366
$P_i +$ UV $S_c +$ UV DNA_c	0
$P_i +$ none $+ DNA_c$	0

Reaction mixture as given in *Methods*. Abbreviations: P_i, S_i, P_c, S_c—induced particles and supernatant, and constitutive particles and supernatant, respectively. Amounts in mg of protein: P_i, 4.2; S_i, 1.4; P_c, 5.2; S_c, 0.7. DNA_c indicates DNA extracted from the constitutive mutant of *E. coli*.

tion of the DNase inhibition of enzyme synthesis, go further in indicating that loss of enzyme synthesis as a consequence of UV irradiation can be restored with DNA and suggest that DNA may play a rather direct role in the synthesis of the induced enzyme. This finding is in accord with the observations of Nisman and Fukuhara,[12] who have shown a similar participation of DNA in the synthesis of β-galactosidase in a cell-free preparation from a constitutive mutant.

X-ray studies: Another method of damaging DNA is by the use of X-rays. However, it had been previously reported by Barron *et al.*[13] that the induced synthesis of galactozymase and maltazymase in yeast was resistant to 300,000 r and by Chantrenne[14] that the induced synthesis of catalase was similarly resistant to X irradiation. More recently, however, Clayton and Adler[15] reported that the synthesis of induced catalase in *Rhodopseudomonas spheroides* was as sensitive to X irradiation as viability. Pauly[16] reported that the synthesis of induced lysine decarboxylase in *Bacillus cadaveris* is similarly sensitive to X irradiation. These investigations prompted us to try X irradiation as a means to inhibit the induced synthesis of β-galactosidase. The results of such an experiment are shown in Figure

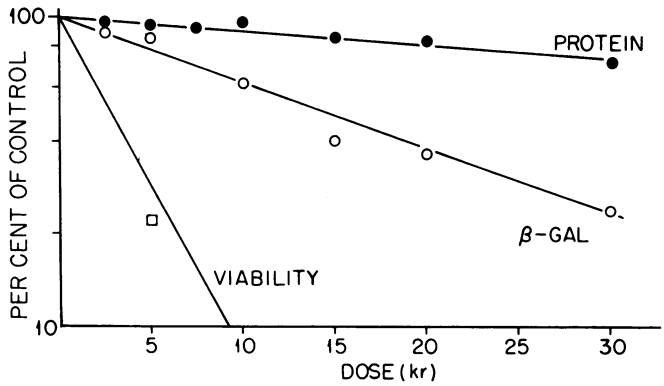

FIG. 1.—Effect of X or irradiation on the induced synthesis of β-galactosidase. Conditions as described in *Methods*. The values shown here were obtained 60 min after X-ray treatment and induction. Cells were grown at 37°C with aeration by bubbler tube, using 0.5% final concentration of glycerol as the carbon source. At time of X irradiation and induction, cell concentration was approximately 1×10^8 cells/ml. Aeration continued during the time of X irradiation.

1. These data show that the synthesis of β-galactosidase is sensitive to X irradiation over the dose range of 10–30 kr. It is evident that enzyme synthesis is more sensitive than general protein synthesis, although less sensitive than viability.

Cell-free preparations made from X-irradiated cells were found to have lost the capacity for enzyme synthesis (Table 2). If particles and supernatants from X-irradiated cells were mixed with their normal counterparts it became evident that the X-ray treatment had caused damage to some component in the supernatant.

TABLE 2

COMPARISON OF DAMAGE IN PARTICLES AND SUPERNATANT DERIVED FROM X-IRRADIATED CELLS

Additions	Δ Enzyme units/60 min/ml
$P_n + S_n$	156
$P_x + S_x$	5
$P_n + S_x$	26
$P_x + S_n$	142

The reaction mixture was as described in the text. Incubation was at 37°C with agitation in the presence of a 95% N_2 − 5% CO_2 atmosphere. 0.1 ml samples were withdrawn and diluted with 0.9 ml of cold extracting medium and then assayed for β-galactosidase activity. The reaction mixture contained (as mg/ml of protein): Particles from untreated cells (P_n), 1.93; supernatant from untreated cells (S_n), 0.83; particles from X-irradiated cells (P_x), 1.82; and supernatant from X-irradiated cells (S_x), 0.62.

Since the supernatant contains both DNA and RNA, a crude nucleic acid preparation was prepared from normal supernatant by streptomycin precipitation and tested for its ability to restore activity to the inactive supernatant. These results are shown in Table 3. It is evident that the crude nucleic acid fraction is able

TABLE 3

EFFECT OF ADDED NUCLEIC ACID ON ENZYME SYNTHESIS IN X-RAY INACTIVATED PREPARATION

Additions	Δ Enzyme units/60 min/ml
$P_x + S_x$	0
$P_x + S_x$ + nucleic acid (N.A.)	168
$P_x + S_x$ + RNase-treated N.A.	177.5
$P_x + S_x$ + DNase-treated N.A.	−3.0
$P_x + S_x$ + *P. saccharophila*, N.A.	−4.5

Reaction mixture as described in *Methods*. 1.82 mg of particle protein and 0.62 mg of supernatant protein were used. The amount of nucleic acid used was equivalent to 30 μg of DNA on the basis of absorbance at 2600 A.

to restore the capacity for enzyme synthesis to the inactive preparations. Samples of the crude nucleic acid were treated with RNase or DNase. The nucleases were inactivated by repeated phenol treatment, and the nucleic acid was recovered by alcohol precipitation. It is clear that RNase had no effect on the activity of the nucleic acid, whereas DNase destroyed all activity. This experiment shows that X irradiation of the cells causes damage to cellular DNA leading to a loss of ability to synthesize enzyme. This loss can be restored by addition of a nucleic acid fraction that is sensitive to DNase. That the effect is not simply that of a polyanion is evident from the fact that a similar nucleic acid fraction from *Pseudomonas saccharophila*, an organism that cannot make β-galactosidase, is completely inactive.

Further evidence for the species-specificity of the DNA reversal of X-ray inhibition is shown in Table 4. In this and in all subsequent experiments the DNA's have been prepared by the detergent-phenol method and treatment with RNase, as described in *Methods*. The data of Table 4 emphasize that a variety of mam-

TABLE 4

COMPARISON OF THE EFFECT OF VARIOUS NATIVE DNA'S ON THE X-RAY DAMAGED SYSTEM

Additions	Δ Enzyme units/90 min/ml
$P_x + S_x$	0
$P_x + S_x$ + DNA_c	159
$P_x + S_x$ + DNA (calf thymus)	−12
$P_x + S_x$ + DNA (rat liver)	3
$P_x + S_x$ + DNA (rat spleen)	−4
$P_x + S_x$ + DNA (mouse liver)	0

Reaction mixture as described in the text. 2.13 mg of particle protein (P_x) and 0.78 of supernatant protein (S_x) were added. DNA was added to a concentration of 50 μg/ml.

malian DNA's are incapable of restoring activity to the X-ray damaged system, but DNA prepared from the constitutive mutant of *E. coli* is fully active.

It therefore became important to determine whether the effect observed, besides being species-specific, was gene-specific. For this purpose DNA's were prepared from a variety of *E. coli* mutants: (1) inducible, but noninduced cells (DNA_{ni}), (2) pre-induced cells (DNA_i), (3) constitutive cells (DNA_c), and (4) from two species of lactose-negative cells ($i^+ z^- y^+$) ($i^- z^- y^-$) (DNA_{lac}^-). These DNA's were then tested for the ability to restore enzyme synthesis to the X-ray damaged system. The results of a typical experiment are shown in Table 5. Only DNA from the con-

TABLE 5

EFFECT OF VARIOUS DNA'S ON ENZYME SYNTHESIS IN THE X-RAY INACTIVATED PREPARATION

Additions	Δ Enzyme units/60 min/ml
$P_x + S_x$	5
$P_x + S_x$ + DNA_{ni}	15
$P_x + S_x$ + DNA_i	119
$P_x + S_x$ + DNA_{lac}^-	−2.5
$P_x + S_x$ + DNA_c	170
$P_x + S_x$ + DNA_c − TMG	64
$P_x + S_x$ + DNA_i, H + C	−12.5

Conditions as described in Table 4. Abbreviations for solutions: DNA_{ni}, extracted from non-induced cells; DNA_i, extracted from fully induced cells; DNA_{lac}^-, extracted from the lactose-negative mutant which cannot synthesize β-galactosidase; DNA_c, extracted from the constitutive mutant; and DNA_i H + C extracted from fully induced cells, heated to 100°C for 10 min and cooled by placing in an ice bath.

stitutive mutant or from pre-induced cells was capable of restoration of enzyme synthesis. DNA from the lactose-negative strains were completely ineffective. A small though definite effect is seen when DNA from noninduced cells is used. The experiment clearly demonstrates that the reversal of X-ray inhibition is gene-specific in a cell-free system presumably without DNA replication.

If active DNA is heated to 100°C and rapidly cooled, a method which produces single-stranded DNA, it loses the capability to reverse X-ray damage. It is also of interest to note that when the constitutive DNA (DNA_c) is used, there is no requirement for inducer. The observation that more β-galactosidase is synthesized with DNA_c in the presence of inducer may indicate that the i^+ gene of the supernatant (S_x) is still partially active. An examination of the kinetics of enzyme appearance (Fig. 2) illustrate this point. Enzyme synthesis with or without inducer is identical for the first 30 min. If inducer is present, synthesis continues at the same rate, but without inducer it ceases. At other times, if inducer is absent, after 30 min synthesis does not stop, but continues at a reduced rate.

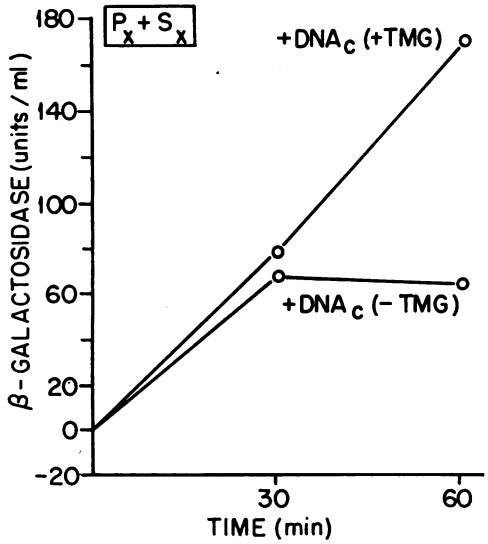

FIG. 2.—Effect of TMG on enzyme synthesis with constitutive DNA in the X-ray inactivated system. Conditions identical to those described for Table 5.

An insight to the role of DNA in these inhibited cell-free systems can be gained by the data of Table 6. In this case, using X-ray inhibited particles and supernatant (P_x, S_x) supplemented with constitutive DNA, 159 units of β-galactosidase are synthesized in 90 min. By contrast, an identical incubation mixture with the exception of the nucleoside di- and triphosphates is essentially inactive. The requirement for nucleoside triphosphates in this system may be a reflection of the observations of Volkin and Astrachan[17] and Volkin[18] who discovered an RNA species in phage-infected cells of *E. coli* which has the base composition of the phage DNA. They have suggested that this RNA species may be directly involved in the synthesis of phage protein. Hurwitz,[19] Weiss and Nakamoto,[20] Stevens,[21] and Ochoa *et al.*[22] have described in a variety of species an enzyme that carries out a DNA-dependent synthesis of RNA. This enzyme, called RNA polymerase, together with a DNA primer and nucleoside triphosphates, synthesizes an RNA species which has a base composition reflecting the base composition of the priming DNA species.

These findings in conjunction with the requirement for the nucleoside triphosphates in the cell-free synthesis of both ornithine transcarbamylase[23] and β-galactosidase are consistent with the hypothesis that the code in DNA for the amino acid sequence in a protein is transmitted through the activity of RNA polymerase to a specific RNA, called messenger RNA,[24] which then participates directly in protein synthesis. If this RNA is considered to be unstable and short lived, then

TABLE 6

REQUIREMENT FOR NUCLEOSIDE DI- AND TRIPHOSPHATES FOR THE DNA-DEPENDENT SYNTHESIS OF β-GALACTOSIDASE

Additions	Δ Enzyme units/90 min/ml
$P_x + S_x$	0
$P_x + S_x$ + DNA$_c$	159
$P_x + S_x$ + DNA$_c$ − XTP, XDP	8

Conditions as described in Table 4.

these observations are consistent with the observations of Riley *et al.*[25] that in whole cell studies a stable, cytoplasmic gene product does not accumulate and that, therefore, functional DNA must be present at all times to maintain synthesis of β-galactosidase.

Requirement for RNA polymerase: The data of Table 7 illustrate the requirement for RNA polymerase for the synthesis of β-galactosidase. In this experiment, partially purified RNA polymerase was incubated with the ribonucleoside triphosphates, DNA_i and TMG. After incubation the samples were added to the β-galactosidase-synthesizing system and enzyme synthesis was measured. It is evident

TABLE 7

REQUIREMENT FOR RNA POLYMERASE IN THE SYNTHESIS OF β-GALACTOSIDASE

1st Incubation (RNA-synthesizing system)	2nd Incubation (DNase treatment)	3rd Incubation (Protein-synthesizing system) (Δ enzyme units/45 min/ml)
Complete + DNase	None	5
Complete	+ DNase	103
Complete	None	196
Complete − RNA polymerase	+ DNase	4

The reaction mixture (0.5 ml) for the first incubation contained: 5 μM of ATP and 80 mμM of the other 3 nucleotide triphosphates, 2 μM of $MnCl_2$, 4 μM of Mg acetate, 50 μM Tris buffer, pH 7.5, 2.5 μM of GSH, 30 μg of DNA_i, 5 μM of TMG and 50 units of RNA polymerase.[26] The reaction mixture was incubated at 37°C for 20 min under an atmosphere of 95% N_2 − 5% CO_2 with shaking.

For the second incubation 0.05 ml (100 μg) of DNase or distilled water was added and the incubation was continued for an additional 10 min.

The reaction mixtures were cooled to 0°C, and the materials for the third incubation were added. The reaction mixture added to the existing reaction for the third incubation was as follows: 50 μM of Tris buffer, pH 7.5, 4 μ M of Mg acetate, 5 μM of ATP, 10 μM of PEP, 100 μg of PEP-kinase, 50 μg of the L-amino acid mix.[3] 1.45 mg of particle protein and 0.58 mg of supernatant protein were added to initiate the final incubation, which was carried out under the same conditions as the first incubation. The final volume was 1 ml. 0.1 ml samples were withdrawn at various times for enzymatic assay. The initial β-galactosidase activity was 190 units/ml.

that if DNase is included during the RNA polymerase incubation, little or no enzyme synthesis is observed upon subsequent incubation with particles and supernatant. If, however, the RNA polymerase is allowed to act for 20 min and then the DNase is used to destroy the *E. coli* DNA, the product of the RNA polymerase is capable of bringing about the synthesis of β-galactosidase. This experiment proves that the RNA synthesized by RNA polymerase in the presence of pre-induced DNA does indeed contain information, and that this information can be transmitted to the protein-synthesizing system. If the DNase treatment is omitted after the RNA polymerase incubation (line 3, Table 7), enzyme synthesis is even better. Kinetic measurements indicate that in the presence of RNA polymerase enzyme synthesis proceeds linearly at least as long as 45 min. Details of the role of RNA polymerase and the locus of inducer action will be published in a subsequent communication.

Summary.—The experiments reported in this and the following article[1] demonstrate that a cell-free system can be prepared from pre-induced cells of *E. coli* that is capable of catalyzing the *de novo* synthesis of β-galactosidase. The system can be inactivated by either UV light or X irradiation and such inactivated systems can be restored to activity by the addition of DNA. In order for the DNA to be effective it must be prepared from cells that carry the structural gene that determines the synthesis of β-galactosidase. These observations suggested that a gene is capable of expressing itself in a cell-free system by giving its information to the protein-synthesizing system. Experiments with RNA polymerase have established that the structural information in the gene can be transmitted to a specific

RNA that is then capable of catalyzing enzyme synthesis in the absence of functional DNA. Two questions remain to be answered: (1) Why is it necessary to prepare the system, or even the DNA, from pre-induced cells and still have a requirement for inducer in the cell-free system? (2) What is the role of the inducer? Attempts to answer these questions will be made in subsequent communications.

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THE SYNTHESIS OF β-GALACTOSIDASE BY A CELL-FREE PREPARATION FROM ESCHERICHIA COLI

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During an investigation of the effect of ultraviolet irradiation on certain metabolic properties of *Escherichia coli*, a particle-bound fraction of β-galactosidase was observed.[1] The behavior of the particle-bound enzyme following various treatments suggested that the particle may be involved in enzyme synthesis. The existence of a ribosomal-bound fraction of β-galactosidase was also observed by Cowie *et al.*[2]