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*CHROMOSOMALLY DIRECTED PROTEIN SYNTHESIS**

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The different kinds of specialized cells of a higher organism differ among themselves in the kinds of enzymes and other proteins which they contain. Nonetheless, each possesses in the chromosomes of its nucleus the complete genomal DNA containing information concerning the manufacture of all of the kinds of enzymes contained in all of the kinds of cells of that creature. It is clear, therefore, that there is in the nucleus some mechanism for the control of genetic activity, a mechanism responsible for that orderly repression and derepression of gene activity which makes possible development. The present work concerns the control of genetic activity.

Genetic activity consists in the direction of DNA-dependent RNA synthesis, the RNA thus formed containing in coded form the message appropriate for the ribosomal synthesis of a particular protein. For the study of the control of genetic activity, we first establish an *in vitro* system for the conduct of chromosome-dependent RNA synthesis. We then couple our chromosomal RNA-generating system to a messenger RNA-dependent ribosomal protein synthesis system.

With our coupled system established we next focus attention upon a particular gene and its product-protein, in our case the gene of the pea plant responsible for the production of the reserve globulin of pea cotyledons. This protein is synthesized in developing pea cotyledons, and is not synthesized by such other pea plant tissues as buds or roots. It will be shown that a specific protein, pea seed globulin, is synthesized by our coupled system in response to the presence of cotyledon chromatin, and is not synthesized in response to the presence of pea bud chromatin. Thus, the control of genetic activity characteristic of the living cell is preserved in the isolated chromatin. It will be further shown that such control is exerted by the histone component of the chromosome.

Materials and Methods.—Preparation of chromatin: Chromatin was prepared by the methods of Huang and Bonner.[1] The organ or tissue (in this work, of pea plants, var. Alsaka) is first

ground in a blendor, in a grinding medium composed of sucrose, 0.25 *M;* tris, pH 8, 0.05 *M;* $MgCl_2$, 0.001 *M*. The homogenate after filtration through cheesecloth and miracloth is centrifuged at 4,000 × *g* for 30 min and the supernatant discarded. The crude chromatin, which contains 90–95% of the tissue DNA, is scraped from the underlying starch pellet, resuspended in 0.05 *M* tris, pH 8, and repelleted for 15 min at 10,000 × *g*. This step is repeated twice. The crude chromatin is then layered on 1.9 *M* sucrose and centrifuged at 22,000 rpm for 120 min in the SW-25 Spinco head. The pellet, resuspended in dilute saline citrate (0.016 *M*), constitutes the purified chromatin which has been freed of contaminating protein by this procedure. In the case of chromatin of pea buds, we have found it advantageous to use the crude chromatin dialyzed against dilute saline citrate without further purification.

RNA polymerase: RNA polymerase was prepared from log phase cells of *E. coli* strain B by the method of Chamberlin and Berg[2] and purification of the enzyme carried through ammonium sulfate fractionation to their fraction 3. The freshly made enzyme at this stage possessed a specific activity of approximately 160 mμm AMP incorporated into RNA/10 min/mg enzyme protein, a dependency ratio (activity in presence of added DNA/activity in absence of added DNA) of 40- to 100-fold and a 280/260 ratio of 1.2 to 1.5.

Preparation of ribosomes: The *E. coli* ribosomal system was prepared from cells ground with alumina according to the procedure of Nirenberg and Matthaei.[3] The resulting homogenate was centrifuged at 30,000 × *g* for 60 min and the supernatant recentrifuged for a further 20 min. This supernatant was then incubated for 45 min at 37°C in the presence of a complete protein synthesis reaction mixture, including 19 amino acids, ATP, and phosphoenolpyruvate and its kinase. Ribosomes were then pelleted from the preincubation mixture, resuspended, repelleted, and finally resuspended and exhaustively dialyzed. The supernatant from the initial ribosomal pellet, containing activating and other soluble enzymes, as well as *E. coli* transfer RNA, was exhaustively dialyzed and then freed of *E. coli* DNA and its RNA polymerase by 18 hr of centrifugation at 105,000 × *g*. We have found the optimum composition of the ribosomal system to consist of, per 50 μg ribosomal protein, 70 μg protein of supernatant fraction supported by 20 mμm of RNA generated by the *E. coli* polymerase system.

Analytical procedures: Analysis of RNA, DNA, and protein were done by the methods standard to our laboratory.[1, 4] Determination of radioactivity incorporated into RNA or protein was accomplished by 5× repeated washing of the sample with cold 10% TCA, after which it was dissolved in 0.2 *N* NH_4OH, and pipetted on to and dried on a glass planchet for counting.

Immunochemical detection of pea seed globulin: Authentic pea seed globulin was prepared from mature pea seeds, var. Alaska, by the method of Danielsson.[5] Potent antisera were developed in chickens by repeated immunization with the globulin over a period of six months. A single lot of pooled antiserum was used for all of the work here reported. The amount of antiserum required to maximally precipitate a given amount of globulin (slight antibody excess) was determined by standard quantitative precipitin methods and used thereafter. Synthesis of globulin both *in vivo* and by the *in vitro* ribosomal system was detected as follows: to the reaction mixture containing soluble C^{14}-labeled proteins, 0.5 mg of authentic carrier globulin (except in the case of pea cotyledons *in vivo*, which contain endogenous globulin) was added the appropriate amount of antiserum protein. Precipitate formation was then allowed to develop maximally (1 hr at 37°C). The precipitated complex, containing approximately 2.5 mg of protein (ratio of pea globulin to antibody, 1:3), was centrifuged off and washed by five successive complete resuspensions and recentrifugations in cold 0.15 *M* NaCl. The complex was then dissolved in 0.2 *N* NH_4OH and counted.

Experimental Results.—Isolated chromatin possesses the ability to conduct DNA-dependent RNA synthesis, using as substrate the four riboside triphosphates,[6] and contains bound chromosomal RNA polymerase.[1] However, chromatin, pretreated at 60°C for 5 min to inactivate its endogenous polymerase and other enzymes so that it can serve only as template, can support RNA synthesis by the purified polymerase of *E. coli*, as is shown in Table 1. In addition, the RNA synthesis supported by chromatin in response to added *E. coli* RNA polymerase consists of molecules which are largely free, that is, do not remain bound to and sedi-

TABLE 1

ACTIVITY OF CHROMATIN OF DEVELOPING PEA COTYLEDONS AND OF PEA DNA IN SUPPORT OF RNA SYNTHESIS BY *E. coli* RNA POLYMERASE

System*	RNA synthesis ($\mu\mu$m nucleotide/10 min)
125 μg deproteinized DNA: *coli* polymerase present	2,220†
37.5 μg deproteinized DNA: *coli* polymerase present	2,030
12.5 μg deproteinized DNA: *coli* polymerase present	740
125 μg DNA in form of native chromosomal nucleohistone: *coli* polymerase present	25
125 μg DNA as whole chromatin: *coli* polymerase present	760
125 μg DNA as whole chromatin heated at 60°C for 5 min: *coli* polymerase present	940
125 μg DNA as whole chromatin: no added *coli* polymerase	17

* Reaction mixture includes tris, pH 8, 20 μm; $MnCl_2$, 0.5 μm; $MgCl_2$, 2 μm; 8-C^{14}-ATP, 0.13 μm, 1.5 μc/μm; GTP, CTP, and UTP each 0.2 μm; β-mercaptoethanol, 6 μm; and chromatin or DNA and *E. coli* polymerase (approx. 10 μg) as indicated, all in 0.5 ml final volume. Incubation at 37°C.
† Incorporation by polymerase alone subtracted.

ment with the chromatin, as does RNA synthesized by chromosomal RNA polymerase.[7]

The data of Table 1 show not only that whole chromatin, in this case of developing pea cotyledons, supports RNA synthesis by *E. coli* RNA polymerase, but that, in addition, the nucleohistone component of such chromatin prepared by the methods of Bonner and Huang[4] and in which DNA and histone are complexed in very nearly equivalent amounts is almost totally inactive in the support of RNA synthesis. Since this nucleohistone component (in which the histone greatly stabilizes the DNA against melting[4]) includes the bulk of the DNA of pea cotyledon chromatin, only a small fraction of the DNA of such chromatin would appear to be present in an active state. The data of Table 1 show in fact that under conditions in which DNA is limiting, the DNA of pea cotyledon chromatin is as active in the support of RNA synthesis as about one-tenth as much deproteinized whole genomal pea DNA.

Chromatin-dependent RNA synthesis will next be coupled to a messenger RNA-dependent ribosomal system. For this purpose ribosomes are borrowed from *E. coli*.[8] The mode of their preparation, described under *Materials and Methods*, makes the system highly dependent upon exogenous messenger RNA. Toward the achievement of this end, preincubation of the crude system in a complete protein synthesis reaction mixture contributes importantly, as is shown in Table 2. Protein synthesis by the ribosomal system is highly dependent, as shown in Table 3, upon both added DNA and upon added RNA polymerase. Pea plant DNA is as effective in this function as is that of bacteriophage T2 or T4. The protein synthesized by the ribosomal system in response to RNA formed by the DNA-depend-

TABLE 2

DEPENDENCY OF RIBOSOMAL PROTEIN SYNTHESIS SYSTEM ON EXOGENOUS MESSENGER RNA AS AFFECTED BY PREINCUBATION OF RIBOSOMAL SYSTEM IN COMPLETE PROTEIN SYNTHESIS REACTION MIXTURE

System*	C^{14}-Leucine Incorporation into Protein ($\mu\mu$m/60 min/mg ribosomal protein) Preincubated	Not preincubated
Ribosomal system alone	88	251
Ribosomal system + pea DNA + RNA polymerase	844	754

* Reaction mixture contains tris, pH 8, 50 μm; $MgAc_2$, 2 μm; $MnCl_2$, 0.5 μm; KCl, 20 μm; β-mercaptoethanol, 3 μm; 18 amino acids, each 0.02 μm; C^{14}-leucine, 6.8 μc/μm, 0.075 μm; ATP(K), 1 μm; GTP, CTP, UTP each 0.1 μm; 50 μg ribosomal protein; 69 μg 105,000 × *g* supernatant protein; pea DNA, 100 μg; and approx. 20 μg *E. coli* RNA polymerase, all in total volume of 0.3 ml. Incubation at 37°C.

TABLE 3

DEPENDENCE OF PROTEIN SYNTHESIS BY *E. coli* RIBOSOMAL SYSTEM ON PEA AND *E. coli* RN POLYMERASE

Additions to ribosomal system*	C^{14}-Leucine Incorporation into Protein ($\mu\mu$m/mg ribosomal protein/30 min)
Pea DNA, 125 μg; RNA polymerase	1,158
T2 DNA, 125 μg; polymerase	828
T4 DNA, 125 μg; polymerase	756
Polymerase; no DNA	100
Pea DNA, 125 μg; no polymerase	102
No DNA; no polymerase	104

* Reaction mixture includes components specified in Table 2.

TABLE 4

SUPPORT OF RIBOSOMAL PROTEIN SYNTHESIS BY CHROMATIN-DEPENDENT RNA SYNTHESIS*

System†	C^{14}-Leucine Incorporation into Protein (cpm/30 min) Two-step expt.	One-step expt.
Cotyledon chromatin; RNA polymerase: ribosomal system	505	721
Cotyledon chromatin; ribosomal system: no polymerase	87	133
Bud chromatin + RNA polymerase: ribosomal system	182	924
Bud chromatin; ribosomal system: no polymerase	122	110

* In the two-step experiment, RNA is generated by chromatin for 30 min, the chromatin removed by centrifugation, and the ribosomal system added to supernatant. In the one-step experiment, all ingredients are present simultaneously.

† Reaction mixture includes components specified in Table 2 except for C^{14}-leucine. In this case, uniformly labeled leucine, 0.02 μm, 131 μc/μm, was used. Chromatin containing 250 μg DNA used in each case.

ent RNA generating system is largely soluble, i.e., is released from the ribosomes. Of the protein synthesized, 65–90 per cent (av. 75 per cent) remains in solution after removal of the ribosomes by centrifugation.

We now couple chromatin-dependent RNA synthesis to messenger RNA-dependent protein synthesis. This may be accomplished in two steps, as is shown by the data of Table 4. In the first step chromatin and RNA polymerase synthesize RNA. The chromatin is then centrifuged off at 16,000 $\times$ g and the ribosomal system added to the RNA-containing supernatant. As the data of Table 4 also show, however, it is more effective to simply combine chromatin, polymerase, and ribosomal system, and thus to directly couple RNA synthesis to protein synthesis. This is particularly true for the case of protein synthesis supported by bud chromatin.

We next focus upon the particular gene (in fact, genes[5]) and its product-protein, pea seed globulin, which we have selected for study. We consider first the extent to which globulin is synthesized by varied tissues of the plant. For the experiment of Table 5, 1- to 10-gm samples of each of a variety of pea plant tissues were ex-

TABLE 5

SYNTHESIS OF PEA SEED GLOBULIN *in vivo* BY VARIED ORGANS OF THE PEA PLANT

Organ	C^{14}-Leucine Incorporation into Protein: Total soluble protein (cpm)	Globulin-antibody precipitate (cpm)	Globulin/total protein (%)
Flower	17,500	510	2.9
Cotyledon	7,100	337	4.7
Older cotyledon	9,200	862	9.3
Roots	17,150	31	0.18
Apical bud	11,600	14	0.12
Apical bud	27,600	42	0.15

Tissues were separately incubated in L-leucine (1 μc/ml, 19 μc/μm) for 2 hr at 25°C, in the presence of penicillin, 6 μg/ml. Soluble protein obtained by dialysis of 105,000 $\times$ g supernatant of tissue homogenate.

cised, and each incubated aerobically with a small volume of buffer containing C^{14}-leucine and penicillin. After 2 hr of incubation, the tissue samples were ground (Virtis homogenizer), filtered through miracloth, and centrifuged for 120 min in the no. 40 Spinco head at 40,000 rpm. The clear supernatants were then freed of C^{14}-leucine by dialysis (four successive changes of 0.1*M* tris buffer, pH 8, 48 hr). To each supernatant representing the soluble proteins of that tissue, authentic carrier globulin and the appropriate amount of high-titer chicken antiserum was added. The specifically precipitated globulin was then centrifuged off, washed as described under *Materials and Methods*, and counted, as were aliquots of the whole supernatant. The data of Table 5 concern then the proportion which globulin-antiserum reacting material, known hereafter as globulin, constitutes of the total protein synthesized by the particular tissue during the experimental period. As is to be expected, globulin constitutes an important protein product of developing pea cotyledons, making up 4–10 per cent of total protein synthesized, the exact amount depending upon the exact stage at which the developing cotyledons are harvested.[5] The flower, too, produces a significant amount of globulin. For such organs as apical buds and root, however, the radioactivity of the globulin-antiserum precipitate constitutes only 0.1–0.2 per cent of total radioactive protein synthesized. A portion, or all, of even this amount may be due to nonspecific contamination of the carrier-precipitate by soluble, labeled but nonglobulin proteins. It may be concluded that pea apical buds synthesize little or no globulin.

For the study of globulin synthesis by the *E. coli* ribosomal system in response to chromosomally generated RNA, incubation mixtures were made on a sufficiently large scale to incorporate 5,000–50,000 cpm of C^{14}-leucine into protein. At the end of the incubation period, the cooled reaction mixtures were centrifuged 120 min in the no. 40 Spinco head at 40,000 rpm for removal of chromatin and ribosomes. The soluble proteins of the supernatant were dialyzed as outlined above. From each such reaction mixture of dialyzed soluble proteins added, authentic globulin was precipitated by the appropriate amount of antibody, just as with the *in vitro* reaction mixtures. The data of the example of Table 6 show that globulin is synthesized by the ribosomal system in response to the presence of chromatin of developing cotyledons. The chromatin of pea buds, although it supports protein

TABLE 6

SYNTHESIS OF PEA SEED GLOBULIN BY RIBOSOMAL SYSTEM OF *E. coli* IN RESPONSE TO VARIED TEMPLATES FOR RNA SYNTHESIS

Template for RNA synthesis	C^{14}-Leucine Incorporation into Protein[a]		
	Total soluble protein (cpm)	Globulin/antibody precipitate (cpm)	Globulin/total protein (%)
Apical bud chromatin[b]	15,650	16	0.10
Apical bud chromatin[c]	41,200	54	0.13
Cotyledon chromatin[c]	23,600	341	1.45
Cotyledon chromatin[c]	14,000	226	1.61
DNA of bud chromatin[d]	15,200	60	0.40
DNA of bud chromatin[d]	14,200	72	0.51
DNA of cotyledon chromatin[d]	5,600	22	0.39
DNA of cotyledon chromatin[d]	50,100	157	0.31

[a] Reaction mixtures included components of Table 2 but on scale 15–40× greater.
[b] Chromatin not subjected to 60°C pretreatment.
[c] Chromatin pretreated at 60°C, 5 min, in dilute saline citrate, 0.015 *M*.
[d] DNA prepared by deproteinization of purified chromatin.

synthesis equally as well as does that of pea cotyledons, does not cause the formation of any globulin detectable above the level of the background.

Although the chromatin of pea buds does not support the synthesis of globulin, the gene or genes responsible for such synthesis is of course present in it. Table 6 includes data from experiments in which protein synthesis by the ribosomal system was supported by the presence of DNA prepared by deproteinization of pea bud or of pea cotyledon chromatin. The amount of radioactivity in the globulin complex prepared from such reaction mixtures, approximately 0.4 per cent of total protein formed, is appreciably greater than that formed either by the pea-bud-chromatin-primed ribosomal system or by pea buds *in vivo*.

Discussion.—We acknowledge that the immunochemical methods here used for the separation and determination of pea seed globulin are not rigorous in the sense that we have not chemically established that the product protein is, in fact, globulin. This is a task for the future. In the present work we are, however, concerned with comparison between the amounts of anti-globulin-reacting material formed in the living cell and in the chromosomally supported ribosomal system, and in the similar comparison between the products of ribosomal systems supported by different chromatins. Used in this manner the immunochemical assay of globulin would be a rigorous one were it not for the background: the small amount of activity associated with the complex when carrier globulin is precipitated with its antibody from protein mixtures containing little or no globulin, as in the case of the proteins of pea buds, pea roots, etc. Whether this background represents nonspecific absorption or a small amount of synthesis of globulin or other cross-reacting material, we do not know. The differences in proportion of globulin formed as between systems supported by cotyledon chromatin, pea bud chromatin, and deproteinized DNA from pea bud or cotyledon chromatin are in any case well beyond the range of this background.

The protein produced by the ribosomal system in response to pea whole-genomal DNA includes a substantial proportion of globulin, in the examples of Table 6, 4 or 5 times above the background. It may be that the genes for synthesis of globulin constitute a larger-than-average proportion of the pea genome. Our immunochemical methods of globulin detection are, however, inadequate to establish this point rigorously.

The results presented above show not only that messenger RNA-dependent protein synthesis can be supported by RNA formed by chromatin-dependent RNA synthesis, but also that during the isolation of chromatin there is preserved to a considerable degree the control of genetic activity characteristic of the particular tissue in life. Cells in which globulin synthesis is repressed yield chromatin which does not support globulin synthesis in our coupled system, and vice versa. It is true, however, that protein synthesis as supported by chromatin of developing pea cotyledons *in vitro* does not result in a proportion of globulin as high as is characteristic of pea cotyledons *in vivo*. This may be due to some derepression of normally repressed genes during the preparation of pea cotyledon chromatin. It may result also from some fractionation of the genome during chromatin preparation, although as is shown in Table 6, the DNA of cotyledon chromatin is approximately as effective as that of pea buds in support of globulin synthesis. It is clear, however, that the repressed state of the genes responsible for globulin synthesis is preserved

during the preparation of pea bud chromatin. What agent is responsible for such repression? It has been shown that removal of protein from pea bud chromatin derepresses the genes for globulin synthesis. The principal protein of chromatin is histone, although other proteins are also present. That it is removal of histone which is responsible for derepression is indicated by the facts (Table 1; see also refs. 1, 4, and 9) that the nucleohistone component of chromatin, in which DNA is fully complexed with histone, is inert in support of RNA synthesis and that ability of chromatin to support RNA synthesis is greatly increased by the specific removal of histone.[1] The histone component of chromatin is, then, a repressor of genetic activity.

Even though we now see that the histone of the chromosome is one agent responsible for genetic repression, the principal questions concerning control of genetic activity remain unanswered. How does nature turn genes off and on—put on or take off histones? How is programming of genetic activity exerted? We cannot yet answer these questions. We do, however, have a system with which such matters can be studied. It appears probable, too, that the present type of system in which the well-standardized, readily preparable components of *E. coli*, such as ribosomes and polymerase, are used as tools for the investigation of chromosomal activity may be of general utility in the study of development in higher organisms.

Summary.—The ability of chromatin, isolated from varied tissues of the pea plant, to support DNA-dependent RNA synthesis is enhanced in the presence of the RNA polymerase of *E. coli*. We have coupled such chromatin-dependent RNA synthesis to a messenger RNA-dependent ribosomal protein synthesis system, the latter also derived from *E. coli*. The material synthesized by the ribosomal system under the direction of chromatin isolated from developing pea cotyledons includes a protein characteristic of such cotyledons, the pea seed reserve globulin. Chromatin of pea buds, which do not synthesize pea seed globulin *in vivo*, does not support the synthesis of such globulin by the isolated ribosomal system. Hence the control of genetic activity characteristic of the living cell is, to an appreciable extent, preserved in the isolated chromatin. This control is exerted by the histone of the chromosome. Thus, the removal of histone from pea bud chromatin, in which the genes for globulin synthesis are repressed, yields DNA which supports globulin synthesis.

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*SOME OBSERVATIONS ON THE ENZYME HYDROGENASE OF DESULFOVIBRIO DESULFURICANS**

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The purification and the properties of hydrogenase of *Desulfovibrio desulfuricans* have been described in earlier papers.[1-4] It was previously observed that both the crude extract and the purified enzyme were completely inactive in the absence of cysteine[1, 2] or dithionite[3] even when these preparations were incubated with hydrogen for several hours. The present communication describes the preparation of hydrogenase which is active without any special activating procedure or additions as assayed by the dye (methylene blue or benzyl viologen) reduction[1, 2] or the deuterium exchange method.[5] This finding is different from the results reported earlier.[1-4] With this preparation it is possible to study the reversibility of the CO inhibition of hydrogenase of *D. desulfuricans* by light and the sulfhydryl nature of the enzyme. Such studies have so far been complicated because of the requirement of dithionite[3] or cysteine[1, 2] for activation. Dithionite prevents reversal of CO inhibition by light.[6]

Experimental.—A phosphate extract of the acetone-treated *D. desulfuricans* (strain Hildenborough, NCIB No. 8303, which is being maintained in lyophilized state in this laboratory) cells was prepared according to the procedure of Sadana and Morey.[2] The crude extract was dialyzed against 0.05 *M* phosphate, pH 7.2, for 48 hr and used without any further purification unless otherwise indicated. Oxygen-free hydrogen was prepared by passage through a Deoxo purifier (Baker and Co., Inc., Newark, New Jersey) and then over hot copper. Carbon monoxide-hydrogen mixtures (v/v) were prepared by mixing the appropriate quantities of H_2 and CO.

The hydrogenase was assayed by the deuterium exchange method[5] or by the methylene blue reduction method[2] at 35°C. The reaction flasks were of either 30-ml or 90-ml volume, depending on the number of gas samples to be removed from the flask. Quartz flask was used for study of the reversibility of CO inhibition by light. The D_2O concentration of the liquid phase was 20%. The pH of the system was 8.0 unless otherwise indicated. The flask was evacuated for 10 min while the solution was frozen in dry ice and then filled with oxygen-free hydrogen or with the required CO-H_2 mixture.

For the experiments on light reversibility, a 2,000-watt bulb (Westinghouse pro-