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Transcription and Translation of Cloned *Drosophila* DNA Fragments in *Escherichia coli*†

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ABSTRACT: The expression of three unique DNA fragments from *Drosophila melanogaster* which have been inserted into *Escherichia coli* (*E. coli*) via the plasmid, pSC 101, was studied. The hybrid plasmid DNA molecules containing *Drosophila* DNA were transformed into the minicell producing strain of *E. coli*, X1411. *Drosophila* DNA-directed RNA synthesis was studied by hybridizing newly synthesized RNA isolated from the minicells with various DNA fragments which were immobilized on nitrocellulose filters. RNA was synthesized as readily from the inserted *Drosophila* DNA as from the original bacterial plasmid, pSC 101. In one case, transcription appeared to be initiated preferentially on one of the two strands of a *Drosophila* DNA fragment regardless of the orientation of that *Drosophila* DNA fragment with respect to the pSC 101 sequences. Two of the three *Drosophila* DNA fragments did not induce the synthesis of new polypeptides in minicells as detected by autoradiography of [^{35}S]methionine-labeled polypeptides on polyacrylamide gels. The third *Drosophila* DNA fragment caused the synthesis of one additional polypeptide of 29 000 daltons. When an 8200 base pair portion of the third inserted *Drosophila* DNA (63% of the total *Drosophila* insertion) was removed by digestion with the restriction enzyme, Eco R_I, this new polypeptide was no longer synthesized by minicells containing the remaining *Drosophila* DNA. When the 8200 base pair fragment was placed back into its parent plasmid as an inversion, the new polypeptide did not reappear. In addition, the presence of some, but not all, of the *Drosophila* DNA insertions affected the relative synthesis of the six polypeptides coded for by the parent plasmid, pSC 101.

Recently, techniques have become available for introducing eukaryotic DNA fragments into *Escherichia coli* via covalent linkage to circular DNA plasmids (Morrow et al., 1974; Wensink et al., 1974; Glover et al., 1975; Tanaka et al., 1975; Kedes et al., 1975; Chang et al., 1975). In this paper we have investigated the extent to which *E. coli* can synthesize RNA and protein from three random DNA fragments of *Drosophila melanogaster* which have been inserted into *E. coli* via a plasmid which codes for tetracycline resistance (Wensink et al., 1974).

One inserted *Drosophila* fragment of 2.9 kb[1] is a moderately repetitive sequence which is repeated 90 times per cell and hybridizes in situ to the chromocenter and to at least 15 different chromomeric regions of the four *Drosophila* polytene chromosomes. The other two fragments (8.8 and 12.65 kb) contain only single copy sequences and hybridize to only one chromomeric region each. Plasmids containing each of these three fragments have been transformed into a strain of *E. coli* which produces minicells, i.e., small particles which bud off the end of the bacterial cell as a result of aberrant cell division (Frazer and Curtiss, 1975). They are too small to contain the bacterial chromosome but do contain plasmid DNA molecules as well as RNA polymerase, ribosomes, and the substrates and other enzymes necessary for transcription and translation (Frazer and Curtiss, 1975). Thus expression of plasmid DNA molecules in vivo can be studied without interference from the bacterial chromosome by incubating isolated minicells containing the appropriate plasmid with radioactive precursors such as [^{3}H]uridine or [^{35}S]methionine.

In this paper we present evidence that RNA is synthesized from cloned *Drosophila* DNA fragments. Also, additional polypeptides are occasionally observed whose synthesis may be directed by the *Drosophila* DNA sequences.

Experimental Procedure

Bacterial Strains and Plasmids. The minicell producing strain of *E. coli* K-12, X1411, was obtained from R. Curtiss III and has the following genetic markers: F^-, prototroph, T_6^s, $minA^-$, λ^-, $minB^-$, str^s. Plasmid DNA molecules used were those described by Wensink et al. (1974).

Media. Bacteria were grown in L broth (Lennox, 1965). Minicells were incubated in the minimal salts medium described by Freifelder and Freifelder (1968) supplemented with

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[1] Abbreviation used: kb, kilobases or kilobase pairs; BSG, buffer salt gelatin; Tris, tris(hydroxymethyl)aminomethane; SSC, standard saline citrate; EDTA, ethylenediaminetetraacetic acid; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

0.5% glucose and 0.87 mM of each of the amino acids except methionine. Isolation and centrifugation were done in buffer salt gelatin (BSG) medium (Curtiss, 1965).

Transformation to Tetracycline Resistance and Isolation of Plasmids. Transformation was carried out using a slight modification of the procedure of Wensink et al. (1974). After the 42 °C heat pulse step, the cells were incubated for 10 min at 25 °C followed by the addition of 2 mL of L broth and further incubation for 1–2 h at 37 °C. The cells were then placed in a Petri dish and mixed with nutrient agar (Miller, 1974) supplemented with tetracycline (20 μg/mL). Tetracycline resistant colonies appeared overnight at 37 °C. Experiments using bacterial cells isolated from such colonies were carried out in accordance with the P2 physical containment conditions specified by the Federal Register National Institutes of Health Guidelines for Recombinant DNA Research (1976).

Plasmids were isolated by CsCl-ethidium bromide density gradients as described previously (Wensink et al., 1974).

Isolation of Minicells. The minicell purification procedure of Kool et al. (1974) was slightly modified. Cultures of X1411 were incubated overnight at 37 °C in 80 mL of L broth containing 16 μg/mL of tetracycline (Pfizer). Cultures were twice centrifuged at 1000*g* for 5 min, and the supernatant was then centrifuged at 10 000*g* for 15 min. The pellets were resuspended in 2 mL of BSG. Minicells were isolated from the suspended pellets by three successive sedimentations through 40-mL linear gradients of 5–20% (w/v) sucrose in BSG. The gradients were centrifuged at 5000 rpm in an SW 25.2 rotor for 15 min. After the third sucrose gradient, the minicells were suspended in 2 mL of minimal salts medium with 0.5% glucose, 16 μg/mL tetracycline, and 0.87 mM amino acids except methionine. This purified minicell fraction (OD = 1.0) contained about 10^{10} minicells/mL and less than one bacterium per 10^7 minicells.

Preparation and Isolation of Labeled RNA from Minicells. Purified minicells were diluted 2.5-fold with minimal salts medium (Freifelder and Freifelder, 1968) plus 0.5% glucose, 0.5% Difco Casamino acids, 1 μg/mL thiamine, and 12 μg/mL tetracycline and incubated 20 min at 37 °C with gentle shaking. [^{3}H]Uridine (specific activity, 40 Ci/mmol from New England Nuclear) was added to a final concentration of 50 μCi/mL and the incubation continued for 7 min. Labeled RNA was isolated by the method of Cohen and Hurwitz (1968), except that the cells were brought up in 3 mL of buffer (rather than 6 mL) after washing, and the final dialysis of RNA was against 2 × SSC (0.3 M NaCl–0.03 M trisodium citrate).

Preparation and Isolation of Eco R_I-DNA Fragments. Plasmid pDm 4 DNA, 200 μg/mL, was cleaved by the restriction enzyme, Eco R_I, by incubating at 37 °C for 30 min with gentle shaking in 100 mM Tris-HCl (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$ and 1 unit Eco R_I (Miles Laboratory) per 20 μg of DNA.

The Eco R_I-cleaved DNA was electrophoretically resolved into two bands on preparative 0.5% agarose gels, 5-cm diameter, using the conditions of Sharp et al. (1973). The bands were cut from the gel and the DNA was purified from the agarose by KI gradient centrifugation (Blin et al., 1975) and dialyzed against 2 × SSC.

DNA–RNA Filter Hybridization. Denatured DNA was immobilized on 2.3-cm diameter nitrocellulose filters with a pore size of 0.45 μm (Type B-6, Schleicher and Schuell) and labeled RNA was hybridized to the immobilized DNA as described by Jakovcic et al. (1975).

Preparation and Isolation of Labeled Protein. Purified minicells suspended in the supplemental minimal salts medium were incubated 30 min at 37 °C with aeration. The L-[^{35}S]methionine (New England Nuclear, Boston, Mass.) with a specific activity of 350–400 Ci/mmol was then added to the minicells in a final concentration of 60 μCi/mL and incubated 90 min at 37 °C with aeration. Protein synthesis was monitored by the incorporation of L-[^{35}S]methionine into acid-insoluble material. The minicells were centrifuged at 12 000*g* for 10 min and washed with minimal salts medium. The pellet was suspended in 100 μL of 0.05 M Tris-HCl (pH 8.0)–1% 2-mercaptoethanol–25% sucrose–3.7% EDTA. Then 50 μL of 5 μg/mL lysozyme in 0.25 M Tris-HCl (pH 8.0) was added, the mixture was incubated for 15 min at 7 °C, and the cells were lysed by treatment with 0.5% sodium dodecyl sulfate for 30 min at 37 °C. The lysate was centrifuged at 10 000*g* for 15 min, and the supernatant used for further experiments.

Polyacrylamide Gel Electrophoresis of Protein. The proteins of the minicell lysates were separated by electrophoresis on 4–10% discontinuous polyacrylamide gels with sodium dodecyl sulfate using a modification of the method of Laemmli (1970). A volume of 250 μL of the lysate was mixed with 100 μL of a 0.2 M phosphate buffer (pH 7.4) containing 0.1% sodium dodecyl sulfate, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.002% bromophenol blue. A 20–50-μL aliquot of this mixture was applied to slab gels containing sodium dodecyl sulfate which were prepared as follows. A 10% separating gel (0.15 M phosphate buffer, pH 7.2, 0.15% sodium dodecyl sulfate, 10% acrylamide, 0.27% bisacrylamide, 0.075% (v/v) *N,N,N′,N′*-tetramethylethylenediamine (Temed), and 0.75 mg/mL ammonium persulfate) was poured and allowed to set overnight before electrophoresis. A 4% stacking gel (0.16 M phosphate buffer, pH 5.8, 4% acrylamide, 0.11% bisacrylamide, 0.2% Temed, 1.5 mg/mL ammonium persulfate) was poured the following morning. The reservoir buffer was 0.1 M phosphate buffer (pH 7.2)–0.1% sodium dodecyl sulfate. Gels were run at 90 mA until the dye front had gone 10 cm. After electrophoresis, the proteins were fixed in the gel with 50% trichloroacetic acid for 30 min and then stained with 0.1% Coomassie brilliant blue in 50% trichloroacetic acid for a minimum of 2 h. The gels were destained by diffusion overnight at 60 °C, dried by the method of Fairbanks et al. (1965), and autoradiographed in contact with Du Pont Cronex safety x-ray film.

Electron Microscopy and Measurement of DNA Molecules. DNA molecules were prepared for microscopy using the Kleinschmidt procedure described by Davis et al. (1971) and observed in a Hitachi HU-125E electron microscope. Molecular lengths were measured directly on 8 × 11 prints using φX174 single-stranded DNA or φX174 RF duplex DNA as internal length standards. Measurements were carried out using a Hewlet-Packard 9107A digitizer and 9100B calculator generously provided by Dr. Charles Wunder.

Results

Transformation of Plasmids into Minicell-Producing Strains of E. coli. The plasmids used in this study are those described by Wensink et al. (1974). The parent bacterial plasmid is pSC 101, a 9.2 kb plasmid which codes for tetracycline resistance (Cohen et al., 1973). *Drosophila* DNA fragments were inserted at the single Eco R_I-cleavage site of pSC 101 by the poly(dA):poly(dT) joining technique, and the relative locations of the pSC 101 *Drosophila* DNA boundaries and Eco R_I-cleavage sites of one of the hybrid plasmids, pDm 4, are shown in Figure 1. Table I summarizes the relevant properties of these plasmids.

TABLE I: Summary of the Relevant Properties of the Plasmid DNA Molecules Used.[a]

	pSC 101	pDm 1	pDm 2	pDm 4
Total size	9.2 kb	12.1 kb	18.0 kb	21.85 kb
Size of *Drosophila* DNA insert		2.9 kb	8.8 kb	12.65 kb
No. of Eco R_I sites	1	0	4	2
Repetitiveness[b]		90.0	3.5	2.0
No. of *Drosophila* polytene bands[c]		16	1	1

[a] Wensink et al., 1974. [b] Determined by renaturation kinetics. [c] To which the fragment hybridizes.

The hybrid plasmids were originally isolated by transformation into *E. coli* strain HB101 ($r_K^-m_K^-$ rec A^-) and, therefore, are not modified when isolated from this strain. Initially, we attempted to transform plasmids isolated from HB101 into the *E. coli* minicell-producing strain P678-54 ($r_K^+m_K^+$). Transformation to tetracycline resistance was 10^3 times less efficient than with HB101, but we anticipated that those few transformants which did occur would contain the original plasmids that had escaped restriction either because they had been modified or because they had entered a restriction$^-$ mutant cell. However, when plasmids were isolated from these transformants, they were found to be of variable length and in all cases were smaller than the original plasmid used in the transformation. A second *E. coli* minicell-producing strain, X1411, which is also $r_K^+m_K^+$, was provided by Roy Curtiss III and was transformed to tetracycline resistance with the same low efficiency as P678-54. However, plasmids isolated from this strain were identical with the original transforming plasmid as determined by restriction enzyme mapping, length measurement, and heteroduplex analysis in the electron microscope. In addition an overnight culture of strain X1411 produced two to three times more minicells than did strain P678-54. Therefore all subsequent studies were done with minicells isolated from strain X1411.

Construction of pDm 4-1 and pDm 4-i8. Two new plasmids were constructed in vitro from pDm 4. As shown in Figure 1 and Table I, the restriction enzyme, Eco R_I, cleaves pDm 4 into two fragments of 13.65 and 8.2 kb, respectively. Both of these Eco R_I sites are within the *Drosophila* DNA insertion since the original Eco R_I site in pSC 101 is destroyed by the formation of the poly(dA):poly(dT) boundary during the construction of the hybrid plasmid in vitro.

When duplex DNA is digested with Eco R_I, termini with cohesive ends of four nucleotides are generated (Boyer et al., 1973) which can be sealed by DNA ligase (Mertz and Davis, 1972). Thus when pDm 4 is incubated with Eco R_I followed by DNA ligase, a variety of linear and circular DNA molecules are obtained. Since linear molecules of pSC 101 are ten times less efficient in transforming *E. coli* to tetracycline resistance than are circular molecules (Cohen et al., 1973), the linear molecules in the ligated mixture were not removed from the mixture prior to transformation. Length measurement of 250 circular DNA molecules which resulted from Eco R_I-DNA ligase incubation gave 42% 8.2 kb monomer circles, 40% 13.65 kb monomer circles, 6% 16.4 kb dimer circles (8.2 + 8.2 kb), 7% 21.85 kb dimer circles (8.2 + 13.65 kb), and 5% 27.3 kb dimer circles (13.65 + 13.65 kb). Without further purification after the DNA ligase reaction, the mixture of ligated DNA molecules was used to transform *E. coli* strain HB101 to tetracycline resistance. Only those circular molecules carrying the pSC 101 sequence will transform *E. coli* to tetracycline resistance. Thus the 8.2 kb monomer and dimer circles contain only *Drosophila* DNA and can be ignored. Twenty-eight transformants were randomly selected and plasmid DNA was isolated from each. Four possessed circular plasmids of 21.85 kb and the remainder had circular plasmids of 13.65 kb. The first 13.65 kb plasmid to be characterized, pDm 4-1, gave the expected structures in the electron microscope when heteroduplexed with pSC 101 and pDm 4 DNA and was selected for further study.

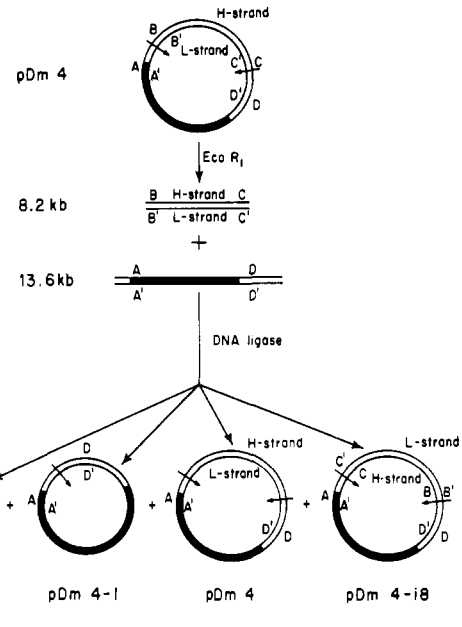

FIGURE 1: Diagram illustrating the construction of plasmids pDm 4-1 and pDm 4-i8. Plasmid pDm 4 was cleaved with Eco R_I as described under Experimental Procedure and the enzyme inactivated after the incubation by heating to 65 °C for 10 min. The resulting DNA fragments were ligated at a concentration of 5 μg of DNA/mL in 30 mM Tris-HCl, pH 8.1, 4 mM $MgCl_2$, 0.2 mM dithiothreitol, and 1 unit/mL of T4 DNA ligase (Miles Laboratory) at 16 °C for 10 h.

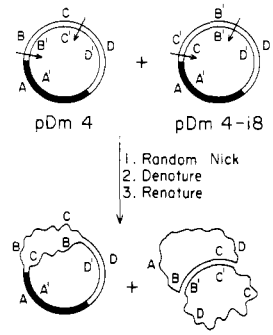

FIGURE 2: Schematic diagram of heteroduplex DNA molecules formed between pDm 4 and pDm 4-i8.

Plasmids from the four transformants containing a 21.85 kb plasmid were then screened by heteroduplex analysis with pDm 4 for one that possessed the 8.2 kb fragment as an inversion of the original pDm 4. Figure 2 shows the expected result of a heteroduplex between pDm 4 and a plasmid that contains an inverted 8.2 kb fragment. Two of the four 21.85 kb plasmids gave the heteroduplex structures shown in Figure 2 proving that they contained the 8.2 kb inversion. Heteroduplex formation of the other two 21.85 kb plasmids with pDm 4 gave no new structures indicating that the 13.65 and 8.2 kb

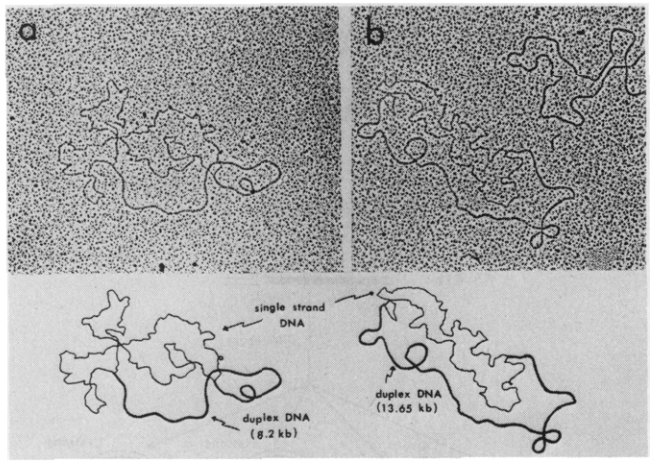

FIGURE 3: Electron micrographs of heteroduplex DNA molecules formed between pDm 4 and pDm 4-i8. (a) Heteroduplex molecule in which the duplex portion is 8.2 kb in length (type II). (b) Heteroduplex molecule in which the duplex portion is 13.65 kb in length (type I). See text for interpretation.

TABLE II: Length Measurements of Heteroduplex DNA Molecules Formed between pDm 4 and pDm 4-i8.[a]

Hetero-duplex	No. of Molecules Measured	Duplex Region (kb)	Single-Strand Regions (kb)
Type I	26	13.45 ± 0.32	8.38 ± 0.35
Type II	6	8.48 ● 0.30	13.69 ± 0.37

[a] Circular duplex DNA molecules of pDm 4 and pDm 4-i8 (10 μg/mL each) in 0.1 N NaOH were heated at 65 °C for 10 min which introduced nicks in approximately 20% of the supercoiled molecules. The solution of denatured molecules was then made 150 mM Tris-HCl, pH 7.2, 0.1 N HCl, and 50% formamide with a final DNA concentration of 5 μg/mL. After 2 h at 45 °C, the DNA was mixed with φX 174 RF I DNA (duplex) and φX 174 plus strand DNA (single stranded) as internal reference molecules and spread in 50% formamide as described by Davis et al. (1971).

fragments had been ligated in the same orientation as the original pDm 4. Figure 3 shows electron micrographs of the two classes of heteroduplex structures expected between pDm 4 and pDm 4-i8, one of the plasmids containing the 8.2 kb inversion, and Table II gives length measurements of the duplex and single stranded regions of the heteroduplex molecules. Plasmids pDm 4-1 and pDm 4-i8 which had been isolated from HB101 were then transformed into the minicell-producing strain X1411. Plasmid DNA was isolated from individual transformants of strain X1411 and shown to be the same as the original plasmids, pDm 4-1 and pDm 4-i8, by Eco R_I cleavage and heteroduplex analysis.

Transcription of Drosophila DNA Sequences in E. coli. Our first attempts to isolate *Drosophila* DNA-specific RNA involved fractionating by polyacrylamide gel electrophoresis RNA which had been labeled with [^{3}H]uridine and purified from minicells containing various plasmids. In all cases tried, the ^{3}H label was distributed throughout the gel and showed no distinct banding pattern, which suggests that stable RNA species were not being synthesized. No distinction could be made between [^{3}H]RNA from minicells containing pSC 101 and [^{3}H]RNA from minicells containing the pDm plasmids by this technique.

Therefore RNA products synthesized by minicells in the presence of [^{3}H]uridine were studied by filter hybridization. [^{3}H]RNA isolated from minicells containing pSC 101, pDm 4, pDm 4-1, and pDm 4-i8 was hybridized with various Eco R_I generated DNA fragments immobilized on nitrocellulose filters. Under conditions of DNA excess, 35% of the RNA labeled in a 7-min pulse of minicells from X1411 (pDm 4) hybridized to immobilized pDm 4 DNA. Attempts to vary the hybridization conditions so that a larger percentage of RNA hybridized were unsuccessful.

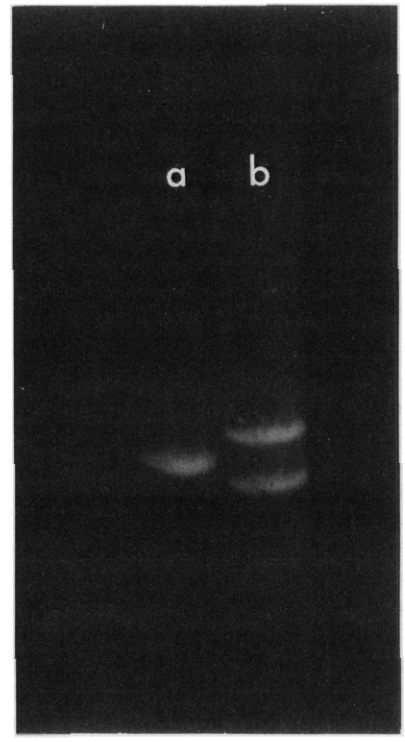

FIGURE 4: An 0.85% agarose gel after electrophoresis of (a) Eco R_I cleaved pSC 101 and (b) Eco R_I cleaved pDm 4 showing the separation of the 8.2 and 13.65 kb DNA fragments. The gels were run in the presence of 2 μg/mL ethidium bromide under the conditions of Sharp et al. (1973) and photographed through an orange filter using an ultraviolet lamp.

The two fragments generated by Eco R_I digestion of pDm 4 were separated on agarose gels as shown in Figure 4 and removed from the agarose by KI centrifugation as described under Experimental Procedure. The faster moving band in gel b of Figure 4 is the 8.2 kb fragment containing only *Drosophila* DNA and the slower band is the 13.65 kb fragment which contains the entire pSC 101 sequence and the remaining *Drosophila* sequence of pDm 4. When the purified 8.2 kb fragment is subjected to alkaline CsCl buoyant density centrifugation similar to that used to separate the two strands of mitochondrial DNA (Clayton and Teplitz, 1972), it likewise separates into two well-resolved species as shown in Figure 5. Since there was no radioactive label in the 8.2 kb fragment, the DNA in each fraction was quantitated spectrophotometrically after neutralization and dialysis to remove the CsCl. The small shoulder adjacent to the light strand was not characterized but probably is a degradation product of the heavy strand. Fractions 2–4 and 9–11 were pooled and one-half of each peak was centrifuged again in a second alkaline CsCl gradient. Each pooled peak sedimented individually to the same fractions under the same centrifugation conditions as in the first gradient with little or no detectable contamination of the other peak. Thus fractions 2–4 and 9–11 were called respectively the H strand and the L strand and used for hybridization experi-

TABLE III: Filter Hybridization of [^{3}H]RNA Extracted from Minicells.[a]

Type of DNA Immobilized (2 μg)	% of Input [^{3}H]RNA Counts			
	[^{3}H]RNA from Minicells of X1411 (pSC 101)	[^{3}H]RNA from Minicells of X1411 (pDm 4)[b]	[^{3}H]RNA from Minicells of X1411 (pDm 4-1)[b]	[^{3}H]RNA from Minicells of X1411 (pDm 4-i8)[b]
None	1.0	1.5	1.2	1.4
pSC101 DNA	36.0	15.7 (14.7)[c]		14.6 (13.6)
pDm 4 DNA		34.9 (34.9)		32.3 (32.3)
13.65 kb fragment of pDm 4		23.5 (21.8)	33.4	21.3 (20.2)
8.2 kb fragment of pDm 4	0.5	13.7 (13.1)	0.4	14.8 (12.1)
H strand of 8.2 kb fragment		9.6	0.2	10.2
L strand of 8.2 kb fragment		4.3	0.3	4.8

[a] The filter hybridization reactions were conducted as described by Jakovcic et al. (1975). [b] The amount of radioactivity applied to each filter was: X1411 (pSC 101), 15 400 cpm; X1411 (pDm 4), 23 300 cpm; X1411 (pDm 4-1), 47 300 cpm; X1411 (pDm 4-i8), 9800 cpm. [c] The numbers in parentheses are the percentages of [^{3}H]RNA that would hybridize if hybridization were proportional to only the size of the DNA fragment. They are calculated relative to the percentage of the RNA that hybridizes to pDm 4 DNA, 34.9% in the case of pDm 4 RNA, and 32.3% in the case of pDm 4-i8 RNA.

ments. The difference in the buoyant density of the two strands of the 8.2 kb fragment which enabled them to be separated by density centrifugation was fortuitous and initially unexpected.

Typical results of filter hybridization experiments are presented in Table III. Nitrocellulose filters containing 2 μg of the indicated DNA were soaked with a solution of 50% formamide, 5 × SSC, and labeled RNA and incubated overnight under paraffin oil in scintillation vials at 60 °C. When the RNA is incubated with the total plasmid DNA in the minicells during the labeling time, 32–36% of the RNA hybridizes to the DNA. As expected, almost none of the labeled RNA made in minicells containing either pSC 101 or pDm 4-1 DNA hybridizes to the 8.2 kb fragment (0.5 and 0.4%, respectively). Also when the two DNA fragments which result from Eco R_I cleavage of pDm 4 are individually immobilized on filters, the amount of RNA that hybridizes to the individual fragments (23.5% + 13.7% = 37.2%) is very close to the amount that hybridizes to the complete pDm 4 DNA molecule (34.9%). This result also occurs with RNA synthesized in minicells containing pDm 4-i8. The amount of RNA that hybridizes to the two fragments individually (21.3% + 14.8% = 36.1%) is similar to that which hybridizes to the complete pDm 4 DNA (32.3%). These data are consistent with the occurrence of random transcription and suggest that *Drosophila* DNA sequences are transcribed to the same extent as sequences of the parent plasmid, pSC 101.

This observation was further extended by hybridizing labeled RNA with the separated strands of the 8.2 kb fragment. As shown in the last two rows of Table III, approximately 70% of the RNA synthesized from the 8.2 kb fragment hybridizes with the H strand regardless of whether it is from minicells containing pDm 4 or minicells containing pDm-i8. These two plasmids contain identical sequences and differ only in the relative orientation of the 8.2 kb fragment with respect to the 13.65 kb fragment. As shown in Figures 1 and 2, when the 8.2 kb fragment is inverted in pDm 4-i8, one of the two strands (B′A′) must assume the position of its complementary strand (AB) in pDm 4 because of the antiparallel nature of duplex DNA. This inversion with respect to the two strands of the 13.65 kb fragment does not affect the relative amount of RNA that hybridizes to each of the two separated strands of the 8.2 kb fragment. Therefore more RNA is synthesized from the H strand than the L strand of the 8.2 kb fragment regardless of its orientation to the pSC 101 sequences. Explanations of this observation are considered in the Discussion section.

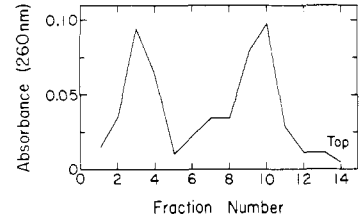

FIGURE 5: Separation of the two strands of the 8.2 kb DNA fragment of pDm 4 in an alkaline CsCl gradient. A total of 26 μg of the 8.2 kb DNA fragment of pDm 4 isolated from an agarose gel as described under Experimental Procedure was placed in 5 mL of 0.1 N KOH–0.1 M NaCl–0.01 M EDTA–and 0.01 M Tris base and brought to a final density of 1.75 g/mL by the addition of solid CsCl. After centrifugation at 38 000 rpm for 36 h in a 60 Ti rotor, fractions of 0.35 mL were collected from the bottom of the tube and dialyzed overnight against 4 L of 4 × SSC before determining the absorbance at 260 nm. Fractions 2–4 and 9–11 were pooled and rerun individually on a second identical alkaline gradient. Again the peaks in fractions 2–4 and 9–11 were pooled and called the H strand and L strand, respectively.

Translation of Drosophila DNA Sequences in E. coli. Purified minicells containing various plasmids were incubated with high specific activity [^{35}S]methionine for 90 min at 37 °C. The minicells were then lysed and treated with sodium dodecyl sulfate and the individual radioactive protein species separated on polyacrylamide gels containing sodium dodecyl sulfate and visualized by autoradiography.

Initially we were troubled by a large number of minor protein bands in the gels which appeared to be coded by the *E. coli* chromosome. We found that these bands could be reduced to a comparatively low level by purifying the minicells through three sucrose gradients instead of the usual two (Frazer and Curtiss, 1975) and by preincubating the purified minicells at 37 °C for 30 min to degrade the endogenous mRNA which originated from the *E. coli* chromosome. Even with these precautions, the occurrence of minor bands sometimes complicated the interpretation of the gel patterns. Preliminary experiments established that minicells incorporated radioactive methionine linearly into protein for about 90 min, after which incorporation ceased. Vigorous aeration during the incubation period stimulated the incorporation of [^{35}S]methionine two-to-threefold over that observed in nonaerated minicells.

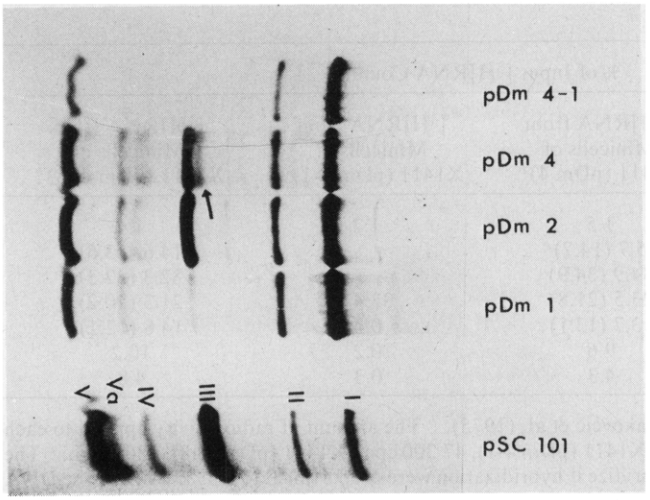

FIGURE 6: Autoradiogram of [^{35}S]methionine-labeled polypeptides separated on a sodium dodecyl sulfate–polyacrylamide slab gel as described under Experimental Procedure. The arrow points to an additional polypeptide detected in minicells isolated from X1411 (pDm 4). Molecular weights were determined by using cytochrome *c* (11 700), lysozyme (14 300), β-lactoglobulin (18 400), alcohol dehydrogenase (37 000), and serum albumin (68 000) as standards in parallel tracts of the slab gel (Weber and Osborn, 1969). Bands I, II, III, IV, Va, and V correspond to molecular weights of 44 000, 38 000, 28 500, 23 000, and 19 000, respectively.

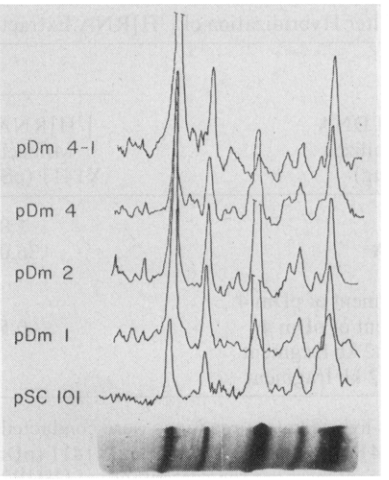

FIGURE 7: Densitometer tracings of the polypeptide bands of the autoradiogram shown in Figure 6. A photograph of the electrophoretic pattern of the pSC 101 coded polypeptides is shown at the bottom for reference.

Several experiments were conducted to compare the amount of total incorporation of [^{35}S]methionine into minicells containing various plasmids. Although there were variations from experiment to experiment, the presence in the plasmid of the three different *Drosophila* DNA fragments ranging in size from 2.9 to 12.65 kb did not consistently increase the amount of total incorporation over that observed with the parent plasmid, pSC 101. However, minicells containing any of the plasmids always incorporated at least ten times more radioactive label than minicells isolated from cells without a plasmid.

Figure 6 shows an autoradiogram of a polyacrylamide slab gel containing proteins extracted from minicells possessing the indicated plasmids. The right-hand column shows that the radioactive label is distributed among at least six different polypeptides in minicells containing the parent plasmid, pSC 101. These six bands vary in intensity, with the third band (III) from the top always containing the most radioactivity. The sum of the molecular masses of the pSC 101 coded polypeptides (173 500) accounts for about 50% of the pSC 101 genome using 110 as the average molecular weight of an amino acid. Figure 7 shows a densitometer tracing of autoradiograms of labeled polypeptides from minicells containing various plasmids.

Plasmid pDm 1 contains a 2.9 kb moderately repeated *Drosophila* DNA fragment (Table I) and does not induce the presence of additional polypeptide bands which are intense enough to be detected by autoradiography. The presence of the *Drosophila* DNA fragment does, however, greatly decrease the relative intensity of polypeptide III which is coded for by the pSC 101 sequences (see Figure 7). This decrease in intensity was seen in every preparation of pDm 1 containing minicells and its significance is not completely understood (see Discussion). Plasmid pDm 2 contains an 8.8 kb *Drosophila* DNA insertion, and it likewise does not appear to induce the presence of any new polypeptide bands as detected by autoradiography. In contrast to the pDm 1, gels of pDm 2 polypeptides are very similar to those of pSC 101. Some minor bands are faintly visible in the pDm 2 gel tract which are also visible after longer exposure times of the pSC 101 polypeptides and are thus not the result of the *Drosophila* DNA insertion.

Plasmid pDm 4 contains a 12.65 kb single copy sequence *Drosophila* DNA insertion and appears to induce the presence of an additional faint polypeptide band of 29 000 daltons (indicated by the arrow in Figure 6 and the shoulder of the third major peak in Figure 7). This band was consistently detected in more than 12 different minicell incubations over a 6-month period and with three independent transformant colonies of X1411 (pDm 4). It was not detected in very long exposure times of autoradiograms of labeled polypeptides from pSC 101, pDm 1, or pDm 2. As shown in the right-hand tract of Figure 6 and the top tracing of Figure 7, when the 8.2 kb fragment containing only *Drosophila* DNA is removed from pDm 4 to yield pDm 4-1, both the additional 29 000-dalton band and polypeptide III of pSC 101 are greatly reduced in intensity. When labeled proteins from pDm 4-i8 were separated on a gel, neither the additional 29 000-dalton band nor polypeptide III appeared. Rather the gel pattern of polypeptides from pDM 4-i8 is very similar to that observed with pDm 4-1 even though an additional 8.2 kb fragment is present in the plasmid. Formation of heteroduplex molecules between pDm 4 and pDm 4-i8 that was purified specifically from the minicell producing strain X1411 (pDm 4-i8) confirmed the expected structure of the pDm 4-i8 in strain X1411 and eliminated the possibility that pDm 4-i8 had undergone rearrangement when transformed into strain X1411.

Discussion

This study investigates to what extent *E. coli* can synthesize RNA and protein from random fragments of *Drosophila* DNA which have been inserted into the plasmid pSC 101 via the poly(dA):poly(dT) joining method (Wensink et al., 1974). The results indicate that RNA is as readily synthesized from the *Drosophila* DNA portion of the recombinant plasmid as from the original parent plasmid portion, a result observed by others as well (Morrow et al., 1974; Kedes et al., 1975; Chang et al., 1975). Furthermore in the case of the 8.2 kb fragment of pDm 4, seventy percent of the hybridizable RNA hybridizes to the H strand regardless of the orientation of the 8.2 kb fragment

within the plasmid. This suggests either (i) that H-strand RNA is more stable than L-strand RNA or (ii) that RNA is synthesized preferentially from the H strand. However, it seems unlikely that RNA synthesized from one strand of a *Drosophila* DNA fragment would be more susceptible to bacterial ribonucleases than RNA synthesized from the other strand. Rather it seems more likely that transcription occurs preferentially on the H strand regardless of the relative orientation of the 8.2 kb fragment. This suggests that transcription of the H strand is not the result of "read through" across the pSC 101 *Drosophila* DNA boundary but rather involves the recognition of specific initiation signals within the 8.2 kb fragment by the *E. coli* transcription machinery. If transcription of this fragment were the result of "read through" and controlled by a promotor in the pSC 101 sequence, then one would expect the opposite strand to be preferentially transcribed when the fragment orientation is reversed. As Table III shows, this does not happen. Another possibility that cannot be completely eliminated by these data is that L strand of the 8.2 kb *Drosophila* fragment contains a terminator signal which stops the *E. coli* RNA polymerase shortly after "reading through" across the boundary. This seems unlikely because it requires that both strands of the original pSC 101 be transcribed in opposite directions in the region of the single Eco R_1 site. This "read-through" transcription of the L strand would have to be stopped in one direction with one orientation of the 8.2 kb fragment and stopped in the other direction by the other orientation. If RNA synthesis of the 8.2 kb fragment were the result of completely random copying of both DNA strands, then equal amounts of RNA would be expected to hybridize to the two separated strands.

The data do not indicate whether the RNA synthesized by the bacteria is bona fide RNA also synthesized by *Drosophila* or whether it is merely nonsense RNA synthesized because of the accidental presence of sequences in the H strand that the *E. coli* transcriptional machinery recognizes. Differing results have been obtained when eukaryotic DNA is copied by *E. coli* RNA polymerase in vitro. When *Xenopus laevis* DNA which codes for 18S and 28S ribosomal RNA is used as a template for transcription in vitro, *E. coli* RNA polymerase primarily copies the wrong strand (Reeder and Brown, 1970). Likewise, the L strand of rat liver mitochondrial DNA (Tabak and Borst, 1970) and *Xenopus laevis* mitochondrial DNA (Dawid, 1972) is preferentially copied by *E. coli* RNA polymerase in vitro, although the moderately stable RNA species extracted from the mitochondria hybridize predominately (but not exclusively) to the H strand. On the other hand, it has been reported that *E. coli* RNA polymerase can synthesize hemoglobin mRNA in vitro from chromatin isolated from Friend's cells (Gilmour et al., 1975). So perhaps, promotor regions of prokaryotes and eukaryotes are occasionally similar enough that a prokaryote RNA polymerase can recognize a eukaryotic start signal. Certainly the simplest explanation of the above data is to postulate the presence of a sequence in the 8.2 kb *Drosophila* fragment that is recognized by the bacterial RNA polymerase as an initiation signal for transcription of the H strand.

The parent plasmid pSC 101 codes for at least six different polypeptides whose genes comprise about 50% of the pSC 101 genome. The insertion of foreign DNA sequences at the Eco R_1 site of pSC 101 does not abolish the synthesis of any of the six polypeptides but sometimes affects the relative amounts of each that are synthesized. For example, insertion of the 2.9 kb *Drosophila* DNA fragment in pDm 1 decreases the relative amount of polypeptide III coded for in the pSC 101 sequence while having little or no effect on the other five. On the other hand, the insertion of the 8.8 kb fragment in pDm 2 does not significantly affect the relative amount of the polypeptide III. Both pDm 1 and pDm 2 were constructed by the poly(dA):poly(dT) joining method (Wensink et al., 1974), but the poly(dA):poly(dT) boundary probably is not responsible for the decreased synthesis of polypeptide III in pDm 1 since it is not seen with pDm 2. Rather it seems more likely that the primary sequence of the 2.9 kb fragment itself is responsible.

The *Drosophila* DNA present in pDms 1 and 2 does not induce the synthesis of additional polypeptides as detected by autoradiography while one extra polypeptide is observed with pDm 4. Three possible explanations for the occurrence of this extra polypeptide band are that the 12.65 kb *Drosophila* insert of pDm 4 (i) alters posttranslational processing of pSC 101 coded polypeptides, (ii) induces increased synthesis of pSC 101 coded polypeptides normally not present or present in only a few copies per cell and therefore not detected by autoradiography, or (iii) serves as a template for the *E. coli* transcription-translation machinery. The data do not distinguish between these possibilities. The least likely seems to be that the *Drosophila* insert is affecting posttranslational events of the pSC 101 coded polypeptides. Possibility ii is supported by the observation that pDm 1 decreases the relative synthesis of polypeptide III. Therefore a foreign DNA insertion might also increase the relative synthesis of a polypeptide. Possibility iii is supported by the recent report that a specific yeast DNA fragment cloned in a nonrevertable *E. coli* histidine auxotraph allows the bacterium to grow in the absence of histidine (Struhl et al., 1976) suggesting that the yeast fragment is being expressed. And since *Drosophila* appears to have as much as 20–30 times more DNA than is necessary to code just for structural genes (Davidson and Britten, 1973), it might be expected that only a small fraction of the cloned fragments would be expressed.

When the 8.2 kb fragment is removed from the 12.65 kb *Drosophila* fragment, the additional polypeptide disappears and pSC 101 coded polypeptide III greatly decreases in intensity. Again the presence of specific foreign DNA sequences, as well as their relative orientation to the pSC 101 sequences, appears to affect the expression of structural genes within the pSC 101 DNA, apparently by influencing the transcription-translation of these genes or the posttranslational processing of their products. It should be emphasized that, although these results are difficult to interpret, they have occurred reproducibly over many experiments and we are confident that the purified minicells contain a homogeneous population of plasmids whose sequence organization has been correctly identified.

Recently Chang et al. (1975) have reported on the transcription and translation of hybrid plasmids constructed between pSC 101 and mouse mitochondrial DNA. They also find that one strand of the eukaryotic (mitochondrial) DNA is preferentially transcribed by the *E. coli*.

We and Chang et al. (1975) have observed a similar gel pattern of polypeptides synthesized from the parent plasmid, pSC 101, although our assignment of the molecular weights of these polypeptides based on the migration of five standard proteins in parallel slots of the slab gel differs somewhat from theirs. Although we cannot explain this discrepancy, it should be noted that we used a different *E. coli* minicell-producing strain (X1411) than they did (X1274) and that our strain of pSC 101 was obtained from their laboratory over 2 years ago and may have undergone alterations since then.

They reported a large decrease in the amounts of several of the pSC 101 coded polypeptides synthesized when the moto-

chondrial DNA was inserted at the R_1 site. We, however, observed a significant decrease in only one polypeptide (III) with only one of the three *Drosophila* DNA insertions (pDm 1). We also did not observe the large increase in low-molecular-weight peptides that they did with the mitochondrial DNA insertions.

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References

Bishop, J. O. (1974), *Cell 2,* 81-86.

Blin, N., Gabain, A. V., and Bujard, H. (1975), *FEBS Lett. 53,* 84-86.

Boyer, H. W., Chow, L., Dugaiczyk, A., Hedgpeth, J., Goodman, H. M. (1973), *Nature (London), New Biol. 244,* 40-44.

Britten, R. J., and Davidson, E. H. (1969), *Science 165,* 349-357.

Brown, D. D., and Sugimoto, K. (1973), *Cold Spring Harbor Sym. Quant. Biol. 38,* 501-506.

Chang, A. C. Y., Lansman, R. A., Clayton, D. A., and Cohen, S. N. (1975), *Cell 6,* 231-244.

Clarkson, J. G., and Birnstiel, M. L. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 451-460.

Clayton, D. A., and Teplitz, R. L. (1972), *J. Cell. Sci. 10,* 487-493.

Cohen, S. N., Chang, A. C. Y., Boyer, H. W., and Helling, R. B. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3240-3244.

Cohen, S. N., and Hurwitz, J. (1968), *J. Mol. Biol. 32,* 15-23.

Curtiss, R., III (1965), *J. Bacteriol. 89,* 28-40.

Davidson, E. H., and Britten, R. J. (1973), *Q. Rev. Biol. 48,* 565-613.

Davidson, E. H., Graham, D. E., Neufield, B. R., Chamberlin, M. E., Amenson, C. S., Hough, B. R., and Britten, R. J. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 295-302.

Davis, R. W., Simon, M., and Davidson, N. (1971), *Methods Enzymol. 21D,* 413-428.

Dawid, I. B. (1972), *Dev. Biol. 29,* 139-151.

Fairbanks, G., Jr., Levinthal, C., and Reeder, R. H. (1965), *Biochem. Biophys. Res. Commun. 20,* 393-399.

Federal Register (1976), National Institutes of Health Recombinant DNA Research Guidelines, Part III, Vol. 41, pp 38126-38483.

Frazer, A. C., and Curtiss, R., III (1975), *Curr. Top. Microbiol. Immunol. 69,* 1-85.

Friefelder, R. D., and Freifelder, D. (1968), *J. Mol. Biol. 32,* 15-23.

Gall, J. G., Cohen, E. H., and Atherton, D. D. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 417-421.

Gilmour, R. S., Windass, J. D., Affara, N., and Paul, J. (1975), *J. Cell. Physiol. 85,* 449-458.

Glover, D. M., White, R. L., Finnegan, D. J., and Hogness, D. S. (1975), *Cell 5,* 149-157.

Grunstein, M., Schedl, P., and Kedes, L. H. (1973), in *Mol. Cytogenet., Proc. Annu. Biol. Div. Res. Conf., 26th, 1973,* 115-122.

Hough, B. R., and Davidson, E. H. (1972), *J. Mol. Biol. 70,* 491-509.

Jakovcic, S., Casey, J., and Rabinowitz, M. (1975), *Biochemistry 14,* 2037-2042.

Kedes, L. H., Cohn, R. H., Lowry, J. C., Chang, A. C. Y., and Cohen, S. N. (1975), *Cell 6,* 359-369.

Kool, A. J., van Zeben, M. S., and Nijkamp, H. J. J. (1974), *J. Bacteriol. 118,* 213-224.

Laemmli, U. K. (1970), *Nature (London) 227,* 680-685.

Lennox, E. S. (1965), *Virology 1,* 190-206.

Mertz, J. E., and Davis, R. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3370-3374.

Miller, J. H. (1974), in Experiments in Molecular Genetics, Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 443.

Morrow, J. F., Cohen, S. N., Chang, A. C. Y., Boyer, H. W., Goodman, H. M., and Helling, R. B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1743-1747.

Peacock, W. J., Brutlag, D., Goldring, E., Appels, R., Hinton, C. W., and Lindsley, D. L. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 405-416.

Rae, P. M. M. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1018-1022.

Reeder, R. H., and Brown, D. D. (1970), *J. Mol. Biol. 51,* 361-377.

Sharp, P. A., Sugden, B., and Sambrook, J. (1973), *Biochemistry 12,* 3055-3063.

Struhl, K., Cameron, J. R., and Davis, R. W. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1471-1435.

Tabak, H. F., and Borst, P. (1970), *Biochim. Biophys. Acta 217,* 356-364.

Tanaka, T., Weisblum, B., Schnös, M., and Inman, R. B. (1975), *Biochemistry 14,* 2064-2071.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406-4412.

Wensink, P. C., Finnegan, D. J., Donelson, J. E., and Hogness, D. S. (1974), *Cell 3,* 315-325.