Construction and Characterization of a Chimeric Plasmid Composed of DNA from *Escherichia coli* and *Drosophila melanogaster*†

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ABSTRACT: A chimeric plasmid has been constructed in vitro from colicin E1 factor (Col E1), nontransmissible R-factor RSF-1010, and *Drosophila melanogaster* DNAs by the sequential action of *Escherichia coli* endonuclease RI(Eco RI) and T4 phage DNA ligase. The chimeric plasmid was assembled in two stages—first, a composite plasmid consisting of Col E1 and RSF 1010 was constructed, followed by partial digestion of the composite with Eco RI (in order to open one of the susceptible cleavage sites) and ligation with an Eco RI-digested *D. melanogaster* DNA preparation. The chimeric plasmid was selected and amplified in vivo by sequential transformation of *E. coli* C with the ligated mixture, selection of transformants in medium containing streptomycin plus colicin E1, followed by amplification in the presence of chloramphenicol and purification of the extracted plasmid by dye-buoyant density gradient centrifugation in ethidium bromide–CsCl solution. Treatment of the chimeric plasmid with Eco RI yields three fragments with mobilities corresponding to the linear forms of the constituents—Col E1, mol wt 4.2×10^6, RSF 1010, mol wt 5.5×10^6 and *D. melanogaster* DNA, mol wt 4.0×10^6. The buoyant densities of the three constituents are respectively 1.706, 1.719, and 1.697 g/cm^3, while the buoyant density of the composite factor is 1.712 and that of the chimeric plasmid is 1.705. *Serratia marscesens* endonuclease R (Sma) which introduces a single cut in Col E1, but not in RSF 1010, converts the chimeric plasmid to a single linear molecule (mol wt 13.7×10^6) and sequential digestion with both Sma and Hin III yields two distinct fragments, mol wt 3.7 and 10.0×10^6, respectively; this implies that the two sites are unique and occur at distinctly different positions. Sequential digestion with both Hin III and Eco RI reveals that the Hin III cut is in the *D. melanogaster* segment; neither Col E1 nor RSF 1010 contain sites susceptible to digestion with Hin III. In the presence of chloramphenicol, the chimeric plasmid continues to replicate for 9 hr while bacterial chromosomal DNA replicates at a much slower rate. As in the case of the composite plasmid, continued synthesis in the presence of chloramphenicol suggests that the replicator of Col E1 is functional in the chimeric plasmid as well. Examination of the chimeric plasmid by partial denaturation mapping permits identification of its constituents, each of which presents a characteristic profile. The *D. melanogaster* segment reveals a wealth of detail at the molecular level pertaining to the distribution of AT-rich regions.

The possibility of cultivating DNA from *Xenopus laevis* in cells of *Escherichia coli* by attachment to a tetracycline resistance factor used as vehicle has recently been demonstrated by Morrow et al. (1974). In order to clone eucaryotic genome fragments and to amplify them more efficiently the approach taken in our studies has been to utilize the chloramphenicol-resistant DNA synthesis which characterizes the mode of replication of Col E1 (Clewell, 1972; Hershfield et al., 1974; Tanaka and Weisblum, 1975). In this approach a composite factor has been prepared in which genes conferring resistance to streptomycin and colicin E1 were covalently linked; this plasmid was found to exhibit the same chloramphenicol-resistant DNA synthesis as the constituent Col E1. In the present report, we describe the enlargement of this composite plasmid by stepwise partial digestion with Eco RI and attachment of unselected DNA from an Eco RI digest of a *Drosophila melanogaster* DNA preparation.[1] The chimeric plasmid prepared in this manner exhibits chloramphenicol-resistant DNA synthesis as expected and its structure is characterized by cleavage with specific bacterial restriction endonucleases followed by electrophoretic analysis of the fragments produced, as well as by banding in CsCl gradients and by partial denaturation mapping.

Materials and Methods

Detailed instructions for the preparation of the composite plasmid and its linear form suitable for use as replicator have been described, together with a detailed description of endonuclease purification, digestion conditions, and electrophoretic fractionation of resultant fragments by the agarose ethidium bromide method (Tanaka and Weisblum, 1975).

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[1] Abbreviations used are: Eco RI, restriction endonuclease RI from *E. coli;* Sma, restriction endonuclease R from *S. marscesens;* Hin III, restriction endonuclease III from *H. influenzae;* composite (Eco RI) refers to all products obtained by treatment of the composite factor with Eco RI, or other enzyme indicated within the parentheses; composite (Sma, Eco RI) means all products obtained by sequential treatment of the composite factor with Sma and Eco RI; chimera (Hin III, Sma) Eco RI refers to the products obtained by sequential digestion with Hin III and Sma, purification of the stated product followed by digestion with Eco RI. Dro, *Drosophila melanogaster.*

Hin III was prepared by the method of Smith and Wilcox (1970) except that the bulk of the enzyme was found to be eluted stepwise from phosphocellulose with 0.3 *M* rather than 0.2 *M* KCl. The early 0.3 *M* KCl fractions were found to contain a mixture of Hin II plus Hin III while the later fractions were found to contain Hin III alone. Sma was prepared as described previously except that in subsequent preparations of the enzyme the activity emerged from the DEAE-cellulose column between 0.2 and 0.4 *M* NaCl; its activity was obscured by the trailing edge of another nuclease which extensively degraded the substrate. Presumptive active fractions therefore had to be pooled by these criteria.

The chimera was prepared in two stages.

Stage 1: Digestion of D. melanogaster DNA. The reaction mixture contained Tris-HCl (pH 7.4), 90 m*M*; $MgCl_2$, 10 m*M*; *D. melanogaster* (embryo) DNA preparation, 2.2 μg; Eco RI, 5 μl, in a final volume of 50 μl. The mixture was incubated at 37° for 1 hr and the reaction terminated by heating at 65° for 5 min; 10 μl containing 0.2 μg of linear composite factor was added to the Eco RI digest of *D. melanogaster* DNA and the mixture placed on ice.

Stage 2: Ligation. The ligase reaction was performed in a final volume of 100 μl. The sample described in step 1 was placed on ice and the following were added: $MgCl_2$, 50 m*M*, 10 μl; dithiothreitol, 0.1 *M*, 10 μl; ATP, 0.5 m*M*, 10 μl; water, 10 μl; T4 ligase, 100 units/ml, 1 μl. The reaction mixture was incubated on ice for an additional 18 hr. $CaCl_2$, 1 *M*, 3 μl was added immediately prior to addition of the ligated mixture to competent cells for transformation, final $CaCl_2$ concentration, 30 m*M*.

Transformation and selection were as described for the composite factor except the *E. coli* C-1a (provided by W. F. Dove) was used as host because of possible K-restriction of the *D. melanogaster* DNA. Following growth in enriched broth medium and selection with streptomycin, 10 μg/ml, and colicin E1, 10 units/cell, a 10-ml sample of the transformed culture was diluted 10-fold into 100 ml of M-9 medium supplemented with 2% Casamino acids and 0.5% glucose, and following growth for 2 hr in this medium further incubated in the presence of chloramphenicol, 170 μg/ml, for 12 hr in order to amplify selectively plasmids utilizing the Col E1 replicator. A lysate prepared from this culture revealed the presence of a prominent slowly migrating band tentatively identified as supercoils with molecular weight greater than 9.7×10^6. Cells from the selection medium were cloned. Of 16 clones picked, 12 yielded a slower moving fraction while four yielded band patterns indistinguishable from those of cells carrying the composite factor. Of the former, a single clone was chosen for further study. In these studies, all laboratory ware which came in contact with living organisms was heat sterilized.

Partial denaturation mapping of the chimeric plasmid was performed by the method of Inman and Schnös (1970). Molecular weights of fragments were calculated following coelectrophoresis and comparison of mobilities with those of fragments of λcb2 DNA obtained with Eco RI used as internal standards as before (Tanaka and Weisblum, 1975). In these measurements, the molecular weights of the λcb2 (Eco RI) fragments were taken as 13.7, 4.5, 3.7, 2.9, and 2.3×10^6 starting from the origin, and they are based on the data of Allet et al. (1973). Additional points on the calibration curves were provided by pSC101, 5.8×10^6, and by the composite factor, 9.7×10^6. Unless stated to the contrary, molecular weights given in the text are the electrophoretic molecular weights; these figures agree within 10% with molecular weights deduced from contour length measurements made with the electron microscope.

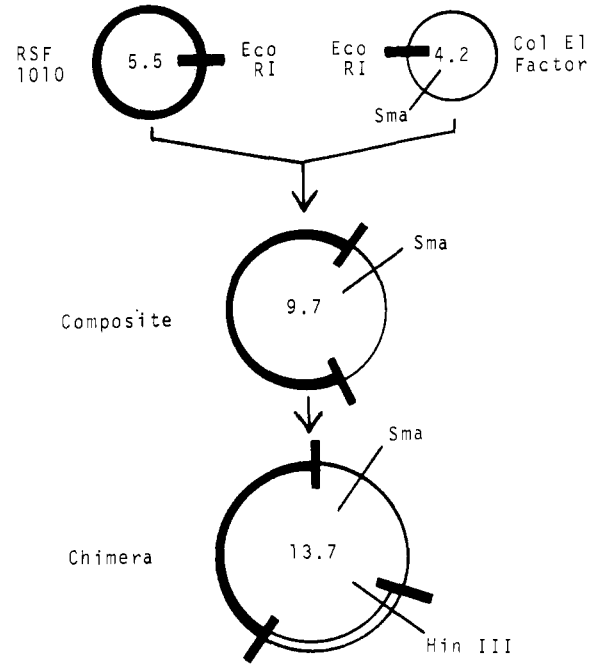

FIGURE 1: Construction of a chimeric plasmid, schematic outline. Plasmids RSF 1010 and Col E1 factor were digested with Eco RI and joined with ligase. Transformants obtained using the ligase mixture were selected with streptomycin plus colicin E1, cloned, and one clone, found to contain a composite plasmid, was used. The composite plasmid was partially digested with Eco RI and linear forms of the composite factor, purified by electrophoresis, were ligated with an unfractionated Eco RI digest of *D. melanogaster* DNA. The ligase mixture was used for transformation as above and transformants, selected for streptomycin plus colicin E1 resistance, were screened for augmentation of molecular weight. One such plasmid, mol wt 13.7×10^6, was obtained from one transformant clone. The approximate sites of cleavage of Eco RI (dark bands) as well as Sma and Hin III are as indicated. The segment containing the Hin III site represents DNA from *D. melanogaster*.

Results

1. General Plan. The general plan for construction of the chimera is described below and summarized schematically in Figure 1. The plasmid was assembled in two stages; the first stage, involving ligation of Col E1 and R-plasmid RSF 1010 along their respective unique Eco RI sites, has been described (Tanaka and Weisblum, 1975). This preliminary construction, which yielded a composite factor, was performed in order to strengthen the subsequent selection step, since the resistance conferred by Col E1 alone was not sufficiently strong; selection of transformants with colicin E1, in our hands, yielded too many resistant (chromosomal) mutants. Use of the composite plasmid allowed us to select for both streptomycin and colicin E1 resistance. The composite factor, converted to the linear form by partial digestion with Eco RI, was fractionated by agarose gel electrophoresis and the largest linear species recovered in purified form following excision and elution from the agarose gel. The linear forms were next ligated with a tenfold excess of Eco RI-digested *D. melanogaster* DNA. *E. coli* cells, made competent for transformation by $CaCl_2$ treatment, were incubated with the ligase reaction mixture and transformants were selected with streptomycin and colicin E1. Following selection and amplification, a lysate prepared from the culture was

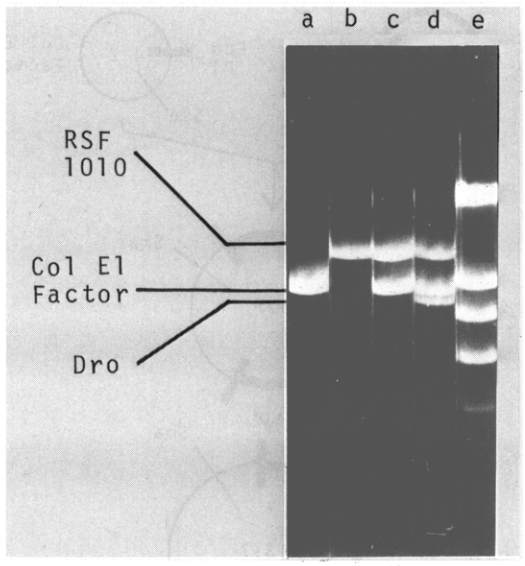

FIGURE 2: Eco RI digestion of the chimera and its constituents. The samples shown are: (a) Col E1, (b) RSF 1010, (c) composite factor, (d) chimera, and (e) phage λcb2 (Eco RI) molecular weight standards, 13.7, 4.5, 8.7, 2.9 and 2.3 × 10^6 starting at the top of the gel.

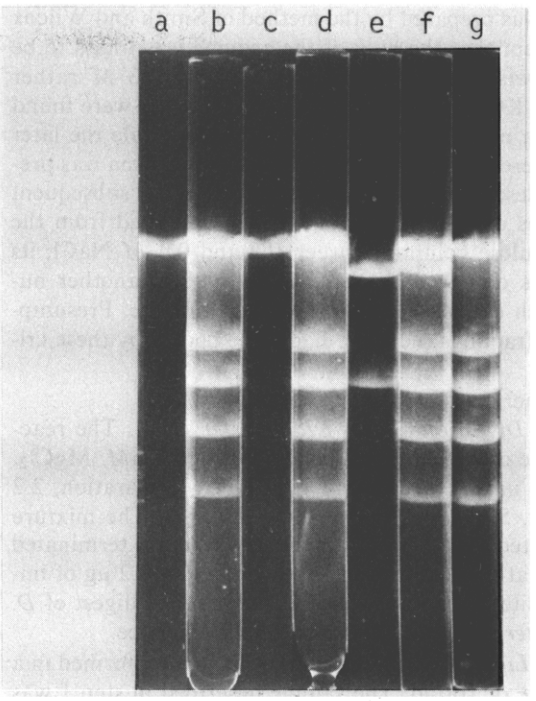

FIGURE 3: Hin III and Sma digestion of the chimeric plasmid. The chimera plasmid was digested with Hin III and Sma, separately and together. The digests were fractionated either alone or in the same gel together with an Eco RI digest of phage λcb2 DNA used as source of internal molecular weight standards. The samples shown are: (a) chimera (Sma), (b) chimera (Sma) plus λcb2 (Eco RI), (c) chimera (Hin III), (d) chimera (Hin III) plus λcb2 (Eco RI), (e) chimera (Hin III, Sma), (f) chimera (Hin III, Sma) plus λcb2 (Eco RI), and (g) phage λcb2 (Eco RI) molecular weight standards as in Figure 2e.

found to contain a preponderance of a slower moving plasmid band, presumably of higher molecular weight than the parental composite plasmid. The culture was streaked out and 16 clones were isolated. Twelve of these were found to contain a band which migrated more slowly than the composite factor suggesting that the plasmid(s) present in these clones contained additional DNA. One of these clones was selected for further study.

2. Digestion of the Chimera with Eco RI. The putative chimera, the composite factor, and their constituents (RSF 1010 and Col E1) were treated with Eco RI. The results are shown in Figure 2. Col E1 and RSF 1010 were converted to their respective linear forms (Figure 2a and b). The composite factor was converted to two linear forms with mobilities corresponding to the 4.2 and 5.5 × 10^6 linear forms of Col E1 and RSF 1010 respectively (Figure 2c), while the chimera yielded, in addition to these two expected fragments, a third fragment running slightly ahead of Col E1 and corresponding to a molecular weight of 4.0 × 10^6 (Figure 2d). The simplest composition to infer is one in which the chimera consists of a single copy, each, of Col E1, RSF 1010, and, as a result of the cloning step, a *discrete D. melanogaster* DNA fragment. According to this interpretation, the chimera would be expected to have a molecular weight of 13.7 × 10^6, the sum of the molecular weights of each of its putative constituents. Finally, for molecular weight calibration, the λcb2 (Eco RI) fragments are also included (Figure 2e).

3. Conversion of the Chimera to the Linear Form with Sma and Hin III. Conversion of the supercoiled to the linear form would be useful in establishing the molecular weight of the chimera. From our previous studies (Tanaka and Weisblum, 1975), it was known that Col E1 contained a single Sma-susceptible site, cleavage at which permitted conversion of Col E1 to a 4.2 × 10^6 linear, and of the composite factor to a 9.7 × 10^6 linear molecule. The chimera was therefore digested with Sma yielding a single band with mobility expected of a 13.7 × 10^6 linear (Figure 3a and b). From earlier studies (unpublished), as well as from data described in detail below, we also knew that the composite factor was insusceptible to Hin III; however, treatment of the chimera with Hin III converted it to a linear form with mobility indistinguishable from that generated by Sma (Figure 3c and d). In order to establish whether the Sma and Hin III cleavage sites were indeed distinct, the chimera preparation was treated with Hin III and Sma sequentially. This digestion produced two fragments with mol wt 3.7 and 10.0 × 10^6, respectively (Figure 3e and f). These results further implied that the Sma and Hin III cuts, each of which alone converted the chimera to a 13.7 × 10^6 linear, occur at distinctly different sites.

4. Localization of the Sma and Hin III Cuts within Segments of the Chimera. Digestion of the chimera with Eco RI yielded three fragments with mol wt 5.5, 4.2, and 4.0 × 10^6, respectively corresponding to RSF 1010, Col E1, and, tentatively, a discrete *D. melanogaster* DNA fragment (Figure 4a). Digestion of the chimera with Hin III and Eco RI sequentially produced a band pattern, shown in Figure 4b. The mobilities of the RSF 1010 and Col E1 bands remained identical in the two samples, while the fastest band (Figure 4a), tentatively identified as *D. melanogaster* DNA, was not present in the (Hin III, Eco RI) digest (Figure 4b) and a slightly faster band with mol wt 3.8 × 10^6 was seen instead; other cleavage product(s), molecular weight less than 0.2 × 10^6, could not be visualized in this gel owing to their small size with consequent higher mobility and decreased fluorescence yield. By this analysis a Hin III cut is localized within the *D. melanogaster* segment.

Digestion of the chimera with Eco RI and Sma sequentially produced a band pattern shown in Figure 4c. Fluorescent bands corresponding to RSF 1010 and *D. melanogaster* DNA were present in both samples. The intermediate band (Figure 4a) corresponding to Col E1 was absent

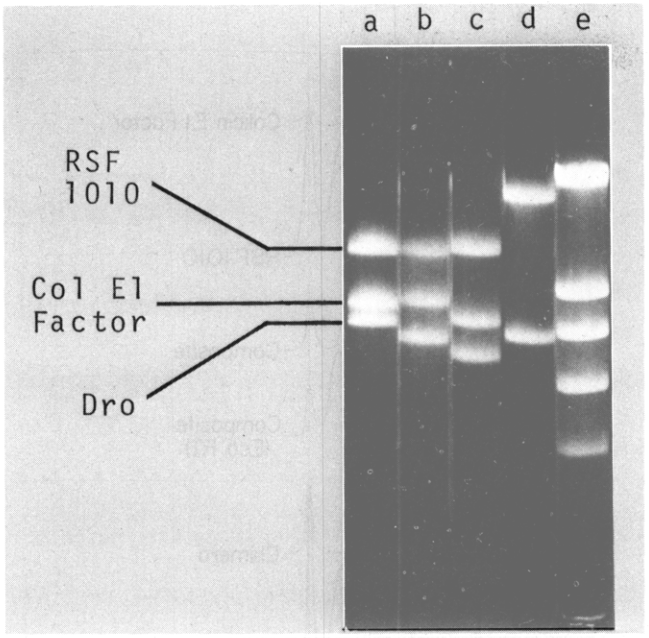

FIGURE 4: Localization of the Sma and Hin III sites in specific segments of the chimera. The samples shown are: (a) chimera (Eco RI), (b) chimera (Hin III, Eco RI), (c) chimera (Sma, Eco RI), (d) chimera (Hin III, Sma), and (e) phage λcb2 (Eco RI) molecular weight standards, as in Figure 2e.

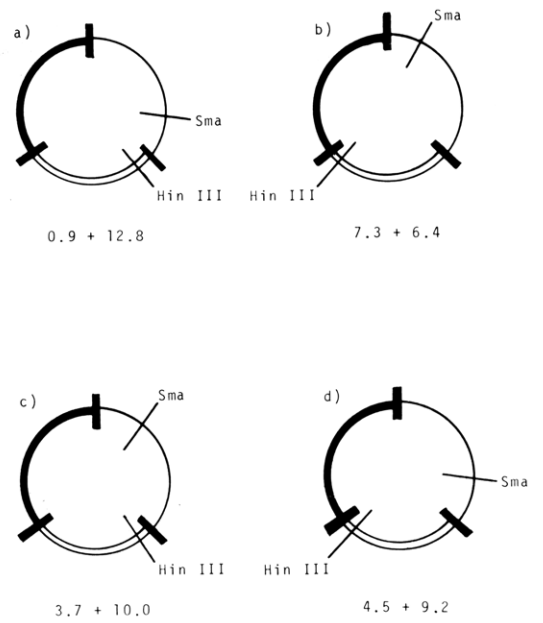

FIGURE 5: Four possible relative positions of the Hin III and Sma sites in the chimera—schematic outline. The Hin III and Sma sites can assume one of four possible relative orientations. The four orientations and the molecular weights expected by combined Hin III plus Sma digestion of each are indicated below the respective model in the figure.

from the (Sma, Eco RI) digest, and instead a faster band with mol wt 3.5×10^6, corresponding to the band described previously in the (Sma, Eco RI) digest of the composite factor, was found. On the basis solely of the data presented above, however, one cannot rule out an interpretation according to which the Col E1 and *D. melanogaster* bands (Figure 4a) were further digested by Sma to produce the two faster migrating bands shown in Figure 4c plus at least two faster bands which have migrated off the gel.

Finally, for comparison, the two (Hin III, Sma) fragments, mol wt 3.7 and 10.0×10^6, as well as the λcb2 (Eco RI) fragments (Figure 4d and e) are shown.

5. Mapping the Relative Positions of the Sma and Hin III Sites within the Chimera. From the foregoing we conclude that there are present a single Sma site in the Col E1 segment of the chimera, and a single Hin III site in the *D. melanogaster* segment. The relative positions of these two sites were next mapped. From the data presented above (Figure 4a and b) as well as from results previously reported (Tanaka and Weisblum, 1975), we infer that the Sma site in Col E1 occurs at a distance of 0.7×10^6 (17% of the distance) from the Eco RI site while the Hin III site in the *D. melanogaster* DNA occurs at a distance 0.2×10^6 (5% of the segment length) from the Eco RI site. Since the Col E1 and *D. melanogaster* segments are continuous in the chimera, there are a priori four possible ways in which these segments can be joined to RSF 1010, neglecting the relative orientation of RSF 1010. These are depicted in Figure 5 together with the corresponding molecular weights of the fragments that would be expected by (Hin III, Sma) digestion of the chimera. From the data presented in Figures 3 and 4, we concluded that (Hin III, Sma) digestion bisected the chimera into two fragments with molecular weights in the ratio of 2:1, thus we can immediately rule out models a and b in Figure 5. Because the molecular weights of the fragments expected from models c and d in Figure 5 fall within 10% of each other, an unambiguous distinction between these two models was not immediately possible. Although accurate determination of the molecular weights of the two fragments produced by (Hin III, Sma) digestion of the chimera is possible, such measurements require coelectrophoresis of the unknown with known internal standards in the *same* gel. By making a comparison of the electrophoretic mobilities of suitable digestion fragments in the same gel, the same end could be achieved, as follows: In order to discriminate between the models c and d, the smaller (Hin III, Sma) fragment was first purified. According to model c (as outlined in Figure 6), this fragment would be expected to have a mol wt of 3.7×10^6 which upon treatment with Eco RI would yield a fragment mol wt of 3.5×10^6 as the major product; according to model d (as outlined in Figure 7), the (Hin III, Sma) fragment would be expected to have

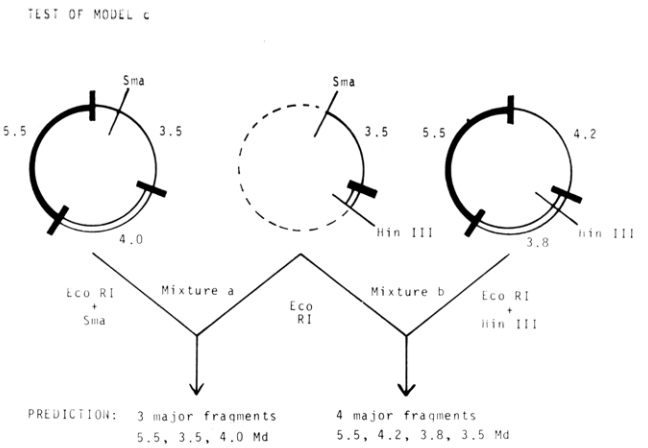

FIGURE 6: A test of model c—schematic outline. Two fragment mixtures were prepared: Mixture a, chimera (Sma, Eco RI) plus smaller (Hin III, Sma) linear fragment (Eco RI), and mixture b, chimera (Hin III, Eco RI) plus smaller (Hin III, Sma) linear fragment (Eco RI). If model c is correct, mixture a would be expected to yield three major fragments and mixture b, four major fragments, on electrophoresis.

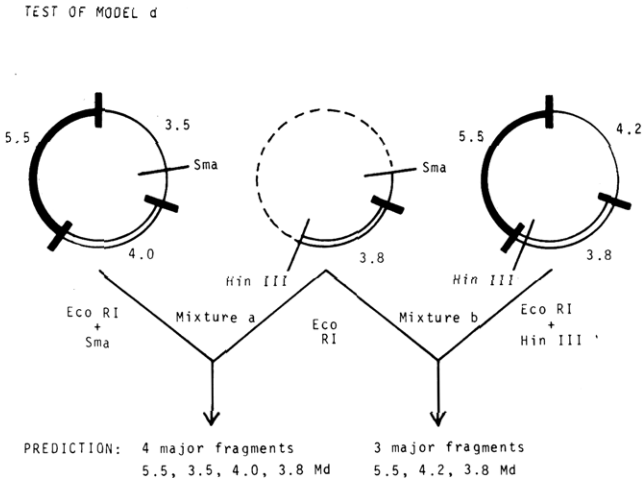

FIGURE 7: A test of model d—schematic outline. According to model d, the two fragment mixture prepared and fractionated by electrophoresis as in Figure 6 would be expected to yield four major fragments from mixture a and three major fragments from mixture b.

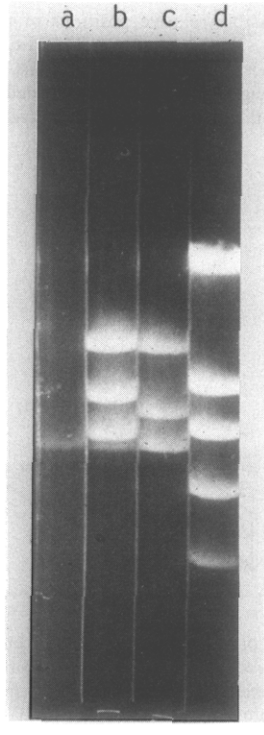

FIGURE 8: Discrimination between model c and model d. Fragment mixtures prepared and fractionated by electrophoresis were: (a) chimera (Hin III, Sma) smaller fragment (Eco RI), (b) chimera (Hin III, Sma) smaller fragment (Eco RI) plus chimera (Hin III, Eco RI), (c) chimera (Hin III, Sma) smaller fragment (Eco RI) plus chimera (Sma, Eco RI), and (d) phage λcb2 (Eco RI) molecular weight standards as in Figure 2e.

a mol wt of 4.5×10^6 which upon treatment with Eco RI would yield a fragment of mol wt 3.8×10^6 as the major product. At this point, model c appears to provide a better fit to the data.

The (Hin III, Sma) Eco RI fragment was prepared, mixed with a (Sma, Eco RI) digest and with a (Hin III, Eco RI) digest of the chimera, respectively, as outlined in Figures 6 and 7, and each of the mixtures was fractionated electrophoretically. If the (Hin III, Sma) Eco RI fragment is derived mainly from the Col El segment (model c), we would expect to find three major bands in the (Sma, Eco RI)-digest (mol wt 5.5, 4.0, and 3.5×10^6) and four major bands in the (Hin III, Eco RI) digest (mol wt 5.5, 4.2, 3.8, and 3.5×10^6), while if the (Hin III, Sma) Eco RI fragment is derived mainly from the *D. melanogaster* DNA segment (model d), we would expect to find four major bands in the (Sma, Eco RI) digest (mol wt 5.5, 4.0, 3.5, and 3.8×10^6) and three major bands in the (Hin III, Eco RI) digest (mol wt 5.5, 4.2, and 3.8×10^6). The results, Figure 8, show clearly the patterns of three and four bands consistent only with model c, and allow us to deduce that this model is the correct one. The mobility of the (Hin III, Sma) Eco RI fragment (Figure 8a) clearly differs from that of any of the fragments in the (Hin III, Eco RI) digest (Figure 8b) while it is indistinguishable from that of the fastest fragment in the (Sma, Eco RI) digest (Figure 8c). This conclusion is corroborated below by electron microscopy of partially denaturated Hin III and Sma chimera linears.

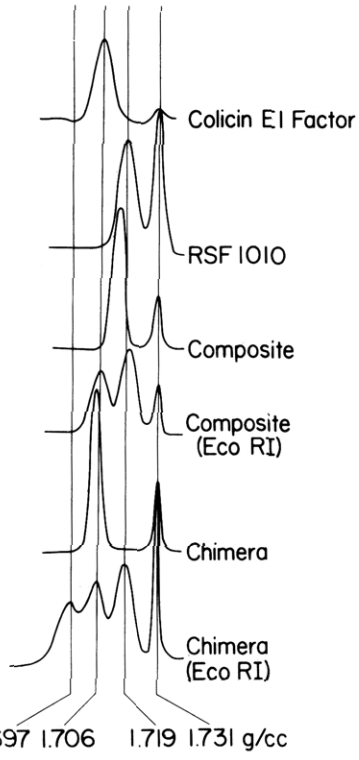

FIGURE 9: Analysis of chimera and its constituents by CsCl density gradient centrifugation. The chimera and its constituents, with and without Eco RI treatment, were analyzed by centrifugation in CsCl density gradients. *M. luteus* DNA (1.731 g/cm^3) was included as internal standard in all the samples.

6. Buoyant Density Analysis of the Chimera. In order to characterize further the *D. melanogaster* DNA segment, the chimera and its constituents were analyzed by density gradient centrifugation (Figure 9). Col E1 and RSF 1010 were found to have buoyant densities 1.706 and 1.719, respectively, while the composite plasmid (density 1.712) yielded two peaks upon Eco RI digestion, with densities which correspond to those of the Col E1 and RSF 1010 constituents, respectively. The chimera was found to have buoyant density 1.705 and upon Eco RI digestion yielded three peaks, two of which corresponded to Col E1 and RSF 1010, and a third peak had a density of 1.697. This peak presumed to represent DNA from the *D. melanogaster* preparation had a density which agrees well with the value 1.699 ± 0.006 reported for the main band fraction of DNA from this organism by Travaglini et al. (1972).

Table I: Summary of DNA Contour Lengths and Molecular Weights Determined by Electron Microscopy.[a]

DNA Sample	Length (μm)	Molecular Weight
Col E1 (Sma)	2.40 ± 0.05	4.30×10^6
RSF 1010 (Eco RI)	3.15 ± 0.06	5.65×10^6
Composite (Sma)	5.57 ± 0.07	9.99×10^6
Chimera (Sma)	7.90 ± 0.06	14.16×10^6
D. melanogaster Eco RI segment	2.35	4.21×10^6

[a] Molecules were linearized by the restriction enzymes shown in parentheses. Native lengths were measured against phage P4 DNA used as standard (length, 4.10 μm, mol wt 7.35×10^6; unpublished). The length of the *D. melanogaster* segment was determined from the difference between the chimera and the sum of the Col E1 and RSF 1010 segment lengths. The molecular weights calculated from the measured contour lengths agree within less than 10% with the electrophoretic molecular weights summarized in Figure 13.

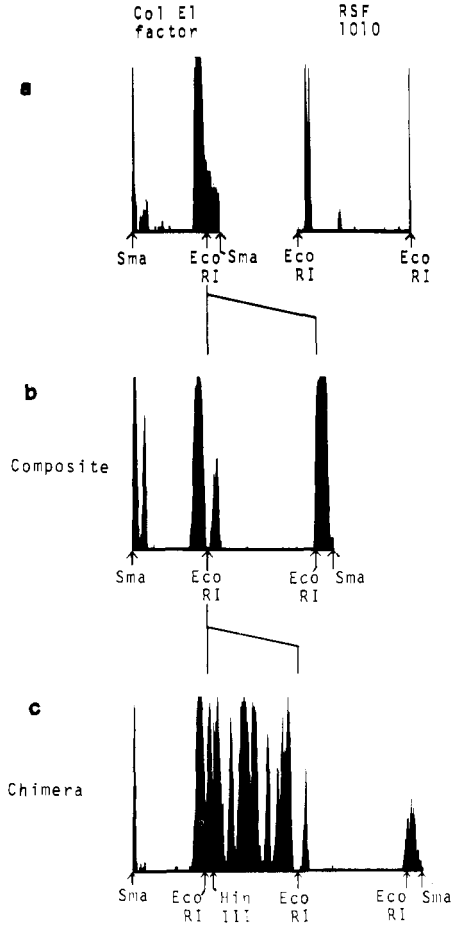

FIGURE 10: Construction of the composite factor and chimera: analysis by partial denaturation mapping. The fraction of DNA molecules denatured is plotted as a function of distance along the plasmid starting at a specific site determined by Sma or Eco RI, as indicated. The molecules were not denatured to the same degree so that certain peaks are not equally developed in all samples. Contour lengths of the plasmids are presented in Table I as well as in Figures 11 and 13. (a) Col E1 (Sma) and RSF 1010 (Eco RI), (b) composite (Sma), and (c) chimera (Sma). The bracketed regions above b and c indicate, respectively, the mode of insertion of RSF 1010 into Col E1, and of Dro into the composite.

7. Analysis of the Chimera by Partial Denaturation Mapping. The technique of partial denaturation mapping (Inman, 1966; Inman and Schnös, 1970) permits direct visualization in the electron microscope of AT-rich regions in single DNA molecules, because of the relative ease with which such regions are denatured. The patterns obtained by this method are highly characteristic of the DNA sample. The chimera was, therefore, characterized by this technique in order to establish the topography of the denaturable sites.

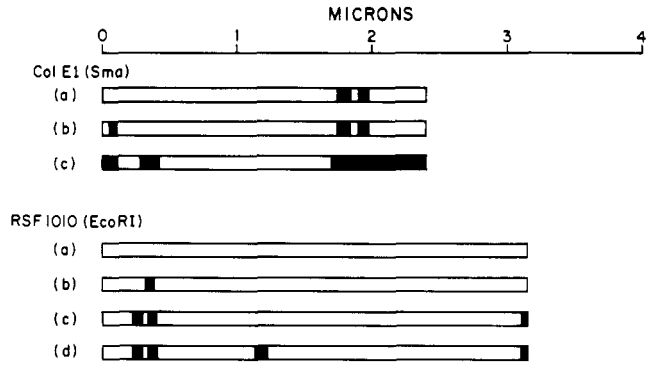

FIGURE 11: Grand average denaturation maps of Col E1 and RSF 1010. The black regions indicate characteristic denatured segments observed at increasing parallel degrees of denaturation labeled a-d. Col E1 and RSF 1010 circular forms were converted to linear molecules with Sma and Eco RI, respectively. Note that at the degree of denaturation labeled "(a)", RSF 1010 is still native whereas Col E1 contains denatured regions.

Native lengths of linear Col E1, RSF 1010, composite, and chimera were first measured and all samples exhibited sharp length distributions (Table I). As expected the length of the composite (5.57 μm) was not significantly different from the sum of the Col E1 and RSF 1010 length (5.55 μm) and we will use the latter value for computational purposes. The difference in length between chimera and composite (2.35 μm) should correspond to the inserted *D. melanogaster* DNA segment. According to length measurements, the ratio of Col E1/RSF 1010/*D. melanogaster* DNA is 1:1.31:0.98 which is in close agreement with the electrophoretic size estimates of 1:1.31:0.95 given in Figure 13.

In order to discriminate between the *E. coli and D. melanogaster* components, the identity of the composite was first established. By examination of the denaturation maps of Col E1 and RSF 1010 individually (Figure 10), the contributions of these two components to the composite factor can readily be identified.

Both Col E1 and RSF 1010 have characteristic denaturation profiles (Figure 11), which can be used to deduce the position and relative polarity of these two segments within the composite. The histogram average of denatured sites in Col E1 (Figure 10a) corresponds to a degree of denaturation between (b) and (c) in Figure 11 while the pattern for RSF 1010 (Figure 10a) corresponds to a degree of denaturation between (c) and (d) in Figure 11. The denaturation histogram for the composite (Figure 10b) must begin and end with a Col E1-like pattern because both the composite and Col E1 circles were cut with Sma (RSF 1010 is resistant to Sma); this fact together with alignment of denatured sites shows that the composite contains RSF 1010 DNA between 1.99 and 5.14 μm in a total length of 5.55 μm. Thus the Sma and Eco RI sites in Col E1 must be separated by 17% of Col E1 length and the relative position of the Eco RI sites are indicated in Figure 10b. The small discrepancies between the maps of the Col E1 and RSF 1010 constituents and the composite are due to slight differences in degree of denaturation. For instance, the denatured site at 0.3 μ observed in the composite (peak 2, Figure 10b) is not well developed in the Col E1 pattern (Figure 10a) but is observed

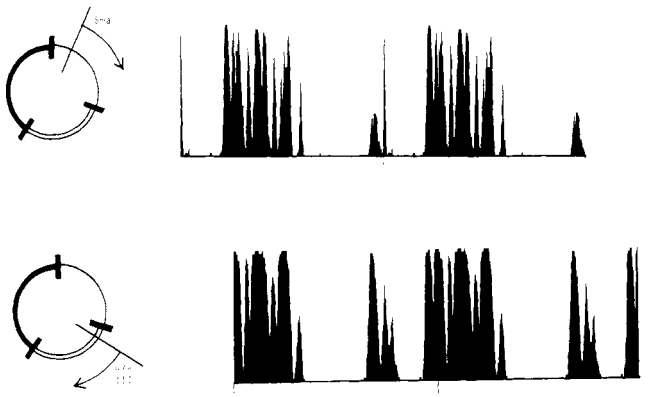

FIGURE 12: Localization of the Sma and Hin III sites in the chimera by comparison of their respective partial denaturation maps. Partial denaturation maps of the Sma and Hin III linears of the chimera were prepared. Each map was mounted twice in tandem in order to facilitate comparison along the entire length. The Sma origin is 2.16 μm distant from the Hin III origin, and the data shown are in clear agreement with model c (Figures 5 and 6).

at a somewhat higher degree of denaturation (Figure 11). Similarly the double site at 0.3 μm in the RSF 1010 pattern (Figure 10a) is observed as a single denatured site at a slightly lower degree of denaturation (Figure 11) and corresponds to the peak at 2.3 μm (peak 4, Figure 10b) in the composite.

When the chimera and composite patterns are compared (Figure 10c and b) one can deduce the position of the *D. melangaster* segment because, again, both circles have been cut with Sma and the characteristic patterns can be aligned. Figure 10c shows the position of the *D. melanogaster* (bounded by Eco RI sites). The absence of the 0.3 μm peak (in the Col E1 factor segment) and the decreased size of the extreme right peak in the chimera pattern are again due to a slightly lower degree of denaturation in this sample.

The chimera was cleaved with Sma and Hin III separately to form the 13.7 $\times$ 10^6 linears described in Figure 3a–d, and partial denaturation maps prepared for each linear form. In order to facilitate comparison along the whole length of the chimera, two copies of each map were mounted in tandem; the results are presented in Figure 12. Although the Hin III linear was denatured to a greater degree than the Sma linear, the two maps fall into precise register; the Hin III site, as determined by electron microscopy, occurs at 8% of the *D. melanogaster* segment distance from the Eco RI site in agreement with the restriction endonuclease studies presented in Figures 5–8. Under the experimental conditions used, the partial denaturation pattern of the *D. melanogaster* segment is distinctly different from that of the composite factor and its constituents, and reveals a relatively large number of denaturable sites, consistent with the relative AT-richness of this segment inferred from the buoyant density determination shown in Figure 9. Moreover, the paucity of denaturable sites in the RSF 1010 segment is consistent with the relative GC-richness inferred from the buoyant density determinations. The foregoing data are summarized in Figure 13.

8. Replication of the Chimera. As in the case of the composite plasmid analyzed previously (Tanaka and Weisblum, 1975), replication of the chimera continues in the presence of chloramphenicol. Analysis of lysates prepared from chloramphenicol-treated cultures by the agarose–ethidium bromide electrophoresis method reveals the continued synthesis of plasmid DNA in the presence of chloramphenicol, while

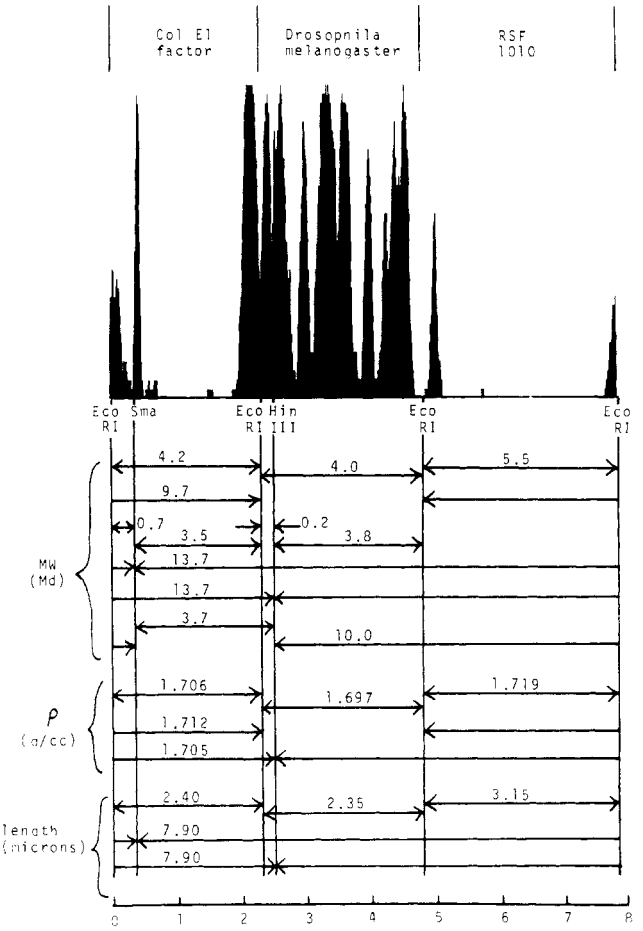

FIGURE 13: Summary of physical properties of the chimera. The physical properties of the chimera and its components are summarized in relation to the denaturation map. The molecular weights given are based on electrophoretic data. The map shown above is identical to that of the Sma linears shown in Figures 10c and 12 except that it starts at the Eco RI site indicated at the right end of Figure 10c.

analysis of the specific activity of the plasmid shows an increase during at least 9 hr growth in the presence of chloramphenicol (Figure 14). These results suggest that synthesis of the chimera is under control of the Col E1 replicator, and provide a demonstration of the useful application of the composite factor to the clonal selection and amplification of eucaryotic DNA fragments in cells of *E. coli*.

Discussion

Previous studies have demonstrated the possibility of constructing composite plasmids (Cohen et al., 1973; Herschfield et al., 1974; Tanaka and Weisblum, 1975). In the construction of the Col E1-RSF 1010 composite factor we have combined in a single molecule the desirable features of streptomycin-resistance which permits ease of selection with chloramphenicol-resistant DNA synthesis which permits the efficient preparation of plasmid DNA in relatively small volumes. In the present study, we have demonstrated the feasibility of enlarging the composite plasmid so as to make it possible to clone and amplify a fragment of eucaryotic DNA in a bacterial cell, and a detailed analysis of the fragment has been presented as part of this methodological study. One of the most useful features of this technique is the fact that yields between 0.1 and 1 μg/ml are obtained so that useful amounts of plasmid can be prepared efficiently in a 100-ml culture.

In the preparation of the linear composite by partial Eco RI digestion, less than 30% yield of the starting material is obtained, requiring also an additional purification step. Attempts are currently underway to alter one of the Eco RI sites in the composite factor so that it will be possible to prepare the linear replicator by limit, rather than partial, digestion. Moreover, additional manipulations would be minimized by preparing this component of the subsequent ligase reaction in the same reaction tube.

Reclosure of the composite linear to the relaxed circular or supercoiled form is a significant undesired side reaction of heterologous ligase reaction mixtures in which several-fold excess of eucaryotic DNA over composite linear DNA is used. Clearly, one is limited in the amount of eucaryotic DNA added, since too great an excess will inhibit transformation (Taketo, 1974). The molecular weight of the vehicle used for replication might be a more useful parameter to vary since, as the length of this component increases, the probability that the two ends find each other diminishes.

The partial denaturation map and buoyant density measurements reveal that the 4×10^6 DNA fragment inserted into the composite factor is clearly different from any molecular species likely to be present in the original composite factor preparation. On the basis of the evidence presented thus far we can state that we have cloned a DNA fragment presumed to have been present in the *D. melanogaster* DNA *preparation.* In principle, this DNA could have been derived from a microbial contaminant present in the embryos from which the DNA was prepared. We have performed in situ hybridizations with [^{3}H]cRNA prepared from the chimera as template, and find that the grains in the autoradiograms show highly localized distribution in both interphase nuclei and polytene chromosomes suggesting chromocentric origin of the cloned fragment. Results of the cytological analysis including renaturation profiles will be presented separately.

In principle, eight possible plasmids could have been constructed by using one copy each of the three elements which constitute the chimera. The two cloning steps, one in the preparation of the composite, and the second, in the preparation of the chimera, resulted in selection of only one of the eight possible isomers. It should be stressed that the selection of a single 4×10^6 *D. melanogaster* DNA sequence, also made possible by the cloning step, is tantamount to a purification which is impossible by currently available chemical or physical methods.

The biological functions specified by the *D. melanogaster* segment have not been determined. It would be of interest to establish whether complete gene sequences, including intact promotor function are present, and, if so, whether such sequences are capable of expression in a bacterial cell. It has so far been established by Morrow et al. (1974) that the ribosomal cistrons of *Xenopus leavis* are at least capable of transcription in minicells of *E. coli.*

In the present study, the methodology of chimera construction has been emphasized and the feasibility of the use of a composite replicating unit based on Col E1 demonstrated. This method should be applicable to the efficient preparation of other cloned fragments derived from eucaryotic cells. Moreover, the partial denaturation mapping technique (Inman, 1966; Inman and Schnös, 1970, 1971) permits direct visualization of AT-rich regions in single DNA molecules, while the use of fluorescent banding techniques has permitted identification of AT-rich regions in intact eucaryotic chromosomes (Weisblum and de Haseth, 1972; Weisblum, 1973; Weisblum and Haenssler, 1974). Application of both the cloning and partial denaturation mapping techniques reveals a wealth of detail in eucaryotic DNA in a form which makes it accessible to further study.

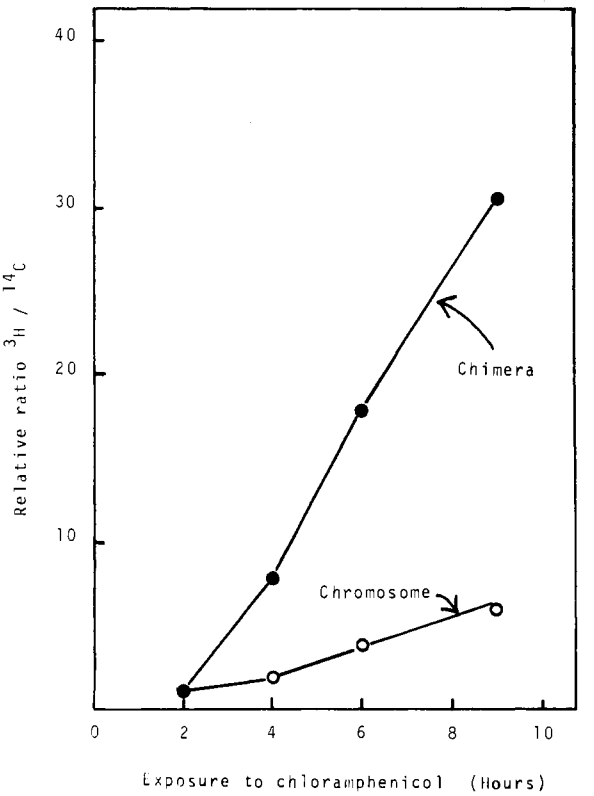

FIGURE 14: Replication of the chimera in the presence of chloramphenicol. Chloramphenicol (170 μg/ml) was added to a culture of *E. coli* C carrying the chimera growing in the presence of [^{3}H]thymidine. At times indicated, samples were withdrawn, mixed with [^{14}C]thymidine-labeled cells as internal standard, and the doubled-labeled lysate mixtures fractionated by the agarose–ethidium bromide electrophoresis method. The supercoil and chromosomal DNA bands were excised and the relative $^3H/^{14}C$ ratio was determined as a function of time.

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Use of a Specific Probe for Ovalbumin Messenger RNA to Quantitate Estrogen-Induced Gene Transcripts†

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ABSTRACT: DNA complementary to purified ovalbumin messenger RNA ($cDNA_{ov}$) was synthesized in vitro using RNA-directed DNA polymerase from avian myeloblastosis virus. This $cDNA_{ov}$ was then employed in hybridization assays to determine the effect of estrogen on the number of ovalbumin mRNA ($mRNA_{ov}$) molecules per tubular gland cell of the chick oviduct. The changes in $mRNA_{ov}$ were measured in immature chicks during primary stimulation, after hormone withdrawal and again following secondary stimulation of the chick oviduct with estrogen. The number of $mRNA_{ov}$ per tubular gland cell was also determined for egg-laying hen. Daily estrogen administration to the immature chick resulted in the growth of the oviduct, differentiation of epithelial cells to tubular glands, and a corresponding increase in the concentration of $mRNA_{ov}$ in the tubular gland cell from essentially zero before estrogen administration to 48,000 molecules per cell after 18 days of estrogen treatment. Upon withdrawal of estrogen from the chick, the $mRNA_{ov}$ concentration decreased to a level of 0–10 molecules/tubular gland cell after 12 days. Readministration of a single dose of estrogen to these chicks resulted in a dramatic and rapid increase in the concentration of $mRNA_{ov}$. Within 30 min, the $mRNA_{ov}$ concentration approximately doubled and by 29 hr the tubular gland cell concentration had reached 17,000 molecules. The initial transcription rate for the ovalbumin gene was 12 $mRNA_{ov}$ molecules/min. With these data, we have calculated that the half-life of the ovalbumin messenger RNA should be on the order of 40–60 hr and that the steady-state concentration of $mRNA_{ov}$ per tubular gland cell was 50,000 molecules. Similarly, each messenger RNA molecule was translated approximately 50,000 times during its lifetime in order to effect the necessary quantity of ovalbumin required for egg production. These data substantiate the hypothesis that estrogen exerts its primary action at the level of transcription to effect the synthesis of nascent mRNA molecules which in turn code for synthesis of hormone-induced proteins.

The cytodifferentiation of the chick oviduct under the influence of estrogen has proven to be an excellent system in which to study the induction of the specific messenger RNA molecule for the secretory egg-white protein ovalbumin (O'Malley and Means, 1974; Kohler et al., 1969; O'Malley et al., 1969; Oka and Schimke, 1969). When the immature chicks are given daily injections of either estradiol-17β or diethylstilbestrol, the oviducts begin to grow and differentiate into several new cell types. The predominant cell is the tubular gland cell which actively produces the major egg-white proteins.

No tubular gland cells exist in an immature chick prior to estrogen treatment. However, after approximately 20 days of daily estrogen injections this cell type comprises greater than 80% of the oviduct magnum (Kohler et al., 1969). Upon cessation of estrogen administration, the tubular gland cells become "inactive" and no longer secrete egg-white proteins. Readministration of estrogen results in the renewed synthesis of these proteins as well as their respective messenger RNAs (Rosenfeld et al., 1972; Means et al., 1972; Palmiter and Smith, 1973). Ovalbumin is the major egg-white protein and can reach concentrations as high as 60% of the total intracellular soluble protein of the oviduct in an active egg-laying hen (O'Malley et al., 1969; Harris et al., 1973).

We have previously employed viral RNA-directed DNA polymerase to transcribe a purified ovalbumin mRNA template and synthesize DNA complements of extremely high specific radioactivity (Harris et al., 1973). This radioactive complementary DNA ($[^3H]cDNA_{ov}$) can then be used as a sensitive hybridization probe to determine the gene dosage for ovalbumin in a chick oviduct cell. Our laboratory (Harris et al., 1973) as well as Sullivan et al. (1973) have recently utilized this technique to demonstrate that only one ovalbumin gene copy is present in the haploid chick genome. More importantly, this $[^3H]cDNA_{ov}$ can also be utilized as a sensitive probe in an RNA excess hybridization reaction to determine the concentration of ovalbumin messenger RNA in any given RNA population.

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