ACCUMULATION OF REPLICATIVE INTERMEDIATES AND CATENATED FORMS OF THE COLICINOGENIC FACTOR E_1 IN E.COLI DURING THE REPLICATION AT ELEVATED TEMPERATURES.

Werner Goebel and Jürgen Kreft+

Institut für Mikrobiologie und Molekularbiologie,
Universität Hohenheim, D 7ooo Stuttgart 7o, Germany

Received October 30,1972

SUMMARY: When cultures of an E.coli wild strain are incubated at temperatures of 47° C to 49°C the replication of the chromosomal DNA is almost completely blocked. However, different complex DNA forms of the plasmid Col E_1 can be isolated from a colicinogenic E.coli wild strain after incubation at these temperatures. After incubation at 47° C a large amount of catenated Col E_1 DNA forms is generated. Incubation at 49° C leads mainly to the accumulation of replicative intermediates of Col E_1 DNA as shown by pulse-chase experiments. In addition the nature of these complex Col E_1 DNA molecules has been revealed by electron microscopy.

INTRODUCTON: The DNA replication of the bacterial plasmid colicinogenic factor E_1 (Col E_1) exhibits some remarkable features,which distinguish it from the replication of the chromosome of E.coli and of several other plasmids, known as sex factors. Col E_1 DNA replication continues in the presence of chloramphenicol (1), an antibiotic which inhibits the initiation of chromosomal DNA replication (2). Polymerase III, but not polymerase I seems to be required for the duplication of the bacterial chromosome and several sex factors (3,4,5). In contrast the Col E_1 plasmid requires DNA polymerase I but not polymerase III for its replication and/or maintenance (4,5). Moreover, Col E_1 DNA continues to replicate at the restrictive temperature in a temperature-sensitive dna B^- mutant, where the synthesis of chromosomal DNA and of various sex factors stops immediately (6,7). Under the latter conditions complex DNA molecules can be detected by electron microscopy and by various physicochemical techniques (6,8). Recently we observed that Col E_1 DNA replicates in E.coli at elevated temperatures (47 - 49° C) where no incorporation of radioactive DNA precursors into high molecular weight chromosomal DNA is observed. Under these conditions the formation of

+ Present address: Biologisches Institut der Universität D 7ooo Stuttgart 6o, Ulmer Straße 227, Germany

catenated DNA molecules and the accumulation of replicative intermediates of Col E_1 DNA can be demonstrated.

MATERIALS AND METHODS: The isolation of Col E_1 DNA from E.coli JC 411 (Col E_1) was performed by the lysozyme-brij technique which has been described extensively in various instances (6,9). The grids for the electron microscopy were prepared by the technique of KLEINSCHMIDT and ZAHN (1o). Modifications and further experimental details are given in the legends to the figures.

RESULTS AND DISCUSSION: Supercoiled Col E_1 DNA in a cell lysate obtained by the lysozyme-brij 58 technique can be enriched by pelleting the chromosomal DNA, still bound to cell wall and membrane fragments, by relatively low speed centrifugation (8ooo x g, 1o min, 4° C). The resulting "cleared lysate" is subsequently centrifuged in a cesium chloride-ethidium bromide (CsCl-EtBr) density gradient to equilibrium (11). Fractionation of the gradient reveals a denser band containing the supercoiled DNA (I) and a less dense band (II) containing the residiual chromosomal DNA fragments still present in the "cleared lysate" (Fig. 1 A).

The amount of radioactivity present in the first band related to the total amount of radioactivity incorporated into acid insoluble material, can be taken as a measure for the content of supercoiled plasmid DNA of the cell. E.coli JC 411 (Col E_1) (6) was incubated at various increasing temperatures and the DNA was labelled for 1 h with ^{3}H-thymidine. Table 1 indicates that the incorporation into chromosomal DNA drops sharply above 45° C, whereas the incorporation into supercoiled plasmid DNA decreases only slightly with increasing temperatures. This results in an apparently increased ratio of plasmid DNA to chromosomal DNA. CsCl-EtBr gradient centrifugation of lysates from E.coli JC 411 (Col E_1) labelled at 47°C shows in addition to the supercoiled plasmid DNA (I) and the chromosomal DNA (II) the appearance of labelled DNA at an intermediate density region (Fig. 1 B).

It has been shown that catenated DNA molecules consisting of supercoiled and open circular parts are found in such intermediate positions in CsCl-EtBr gradients (12). Sedimentation

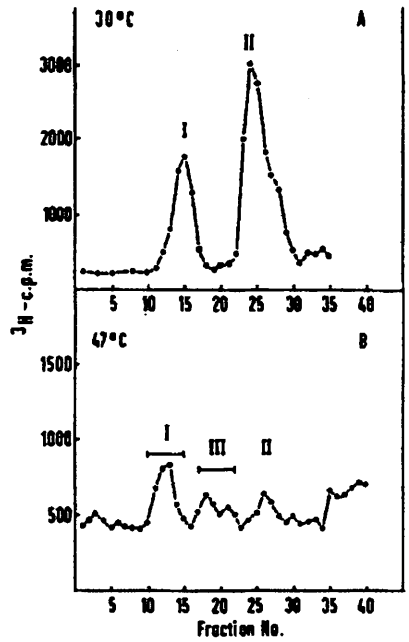


Fig. 1 CsCl-ethidium bromide densitiy centrifugation of cleared lysates of E.coli JC 411 (Col E_1) grown at 30°C and at 47°C.
Cultures of E.coli JC 411 (Col E_1) were labelled in the presence of 250 ug/ml deoxyadenosine for 1 h with 5 uCi/ml ^{3}H-thymidine (spec.act. 20.4 Ci/mmol) at 30°C and with 10 uCi/ml at 47°C. Phosphate-buffered minimal medium (6) was used for culturing the strains. Cleared lysates were prepared by the lysozyme/Brij 58 procedure (9) and directly centrifuged in CsCl-EtBr gradients (11). Fractions of the gradients were collected in small tubes, from which aliquots were taken for the determination of the radioactivity (6). ^{32}P-labelled supercoiled Col E_1 DNA was added to each CsCl-EtBr gradient as a density marker to indicate the position of supercoiled DNA (I) and relaxed circular DNA (II).
A. E.coli JC 411 labelled at 30° C
B. E.coli JC 411 labelled at 47° C.

analysis of the DNA from the intermediate band in neutral sucrose gradients demonstrated the presence of various DNA species (Fig. 2 A). Their sedimentation values are in agreement with catenated forms consisting of supercoiled and relaxed Col E_1 DNA (8,13). In the dense supercoiled band (I) monomeric supercoiled Col E_1 DNA and DNA species with sedimentation values (31 S, 37 S, 43 S) previously found for oligomeric forms of Col E_1 DNA can be detected (Fig. 2 B) (14,15). However, sedimentation analysis under these conditions does not allow to distinguish between supercoiled

TABLE 1

Incorporation of ^{3}H-thymidine into the chromosomal and Col E_1 DNA of JC 411 (Col E_1) at various growth temperatures.

Temperature during the labelling period	Incorporation of ^{3}H-radioactivity into the total DNA in cpm +	% of incorporation of ^{3}H-radioactivity into total DNA as compared to 100 % incorporated at 30°C ++	% of ^{3}H-incorporation into plasmid DNA related to the total incorporation
30°C	$5,3 \times 10^{5}$	100 %	1.1 - 1.3 %
40°C	$4,8 \times 10^{5}$	90 %	1.4 - 1.6 %
43°C	$4,3 \times 10^{5}$	75 - 80 %	1.5 - 1.7 %
45°C	$2,4 \times 10^{5}$	40 - 50 %	2.5 - 3.5 %
47°C	$3,7 \times 10^{4}$	6 - 8 %	10 - 13 %
49°C	$1,0 \times 10^{4}$	1 - 3 %	40 %

+ The values represent acid-insoluble counts/min of 0.1 ml of the total lysates (2.0 ml) of one experiment; 5 uCi/ml ^{3}H-thymidine was added for 1 h at the indicated temperature.

++ The range of percent given represents values from three independent experiments.

oligomeric and supercoiled catenated molecules. At 49° C, the incorporation of ^{3}H-thymidine is considerably reduced, which indicates that the rate of replication of Col E_1 DNA is slowed down at this temperature. We have therefore considered this temperature as convenient for obtaining replication intermediates. When ^{3}H-thymidine was added to the culture for 1 h essentially the same Col E_1 DNA forms were obtained as at 47°C with a reduced yield. However, when the culture was labelled for only 1 min, most of the labelled material banded at an intermediate density in a CsCl-EtBr gradient. When this DNA from the intermediate band was sedimented through neutral sucrose gradients, DNA was detected which sedimented slightly faster than supercoiled monomeric Col E_1 DNA in a rather heterogenenous distribution. To reveal whether this DNA represents replicative intermediates

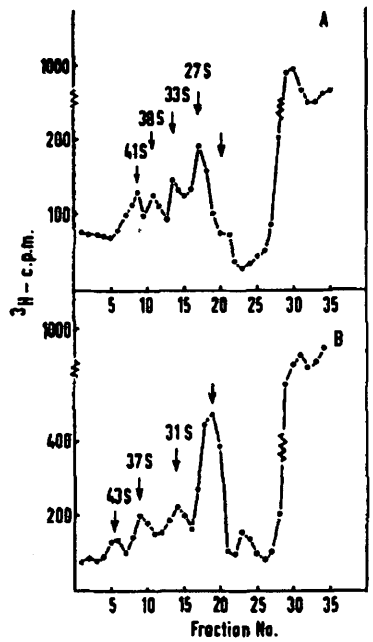


Fig. 2 Neutral sucrose density gradient centrifugation of the DNA isolated from the intermediate density region (III) and from the heavy density region (I) of the CsCl-EtBr gradient. The fractions of the two regions marked III and I in Fig. 1 B were isolated, dialysed and sedimented through a 5 % to 2o % linear sucrose gradient (SW 65, 45 ooo r.p.m., 2o°C, L 2-5o centrifuge). Centrifugation was carried out for 12o min. S-values are related to the internal 23 S marker (supercoiled ^{32}P-Col E_1 DNA). The arrows indicate the position in the gradients of this marker DNA.

---

pulse-chase experiments were carried out. Cultures of E.coli JC 411 (Col E_1) at a cell density of 5 x $1o^8$ cells/ml were pulse labelled for 1 min at 49° with ^{3}H-thymidine. The cells were poured on ice to stop further incorporation and washed twice with ice-cold medium. One half of the cells was processed immediately, the other half was subsequently incubated for 1 h at 3o° C in a medium containing no ^{3}H-thymidine but an excess of non-radioactive thymidine. Plasmid DNA was extracted and analysed as described. Fig. 3 A indicates that after the 1 min pulse a high amount of radioactivity is found in the intermediate density region of the CsCl-EtBr gradient and disappears after 1 h chase at 3o°C (Fig. 3 D). The sedimentation analysis demonstrated that the

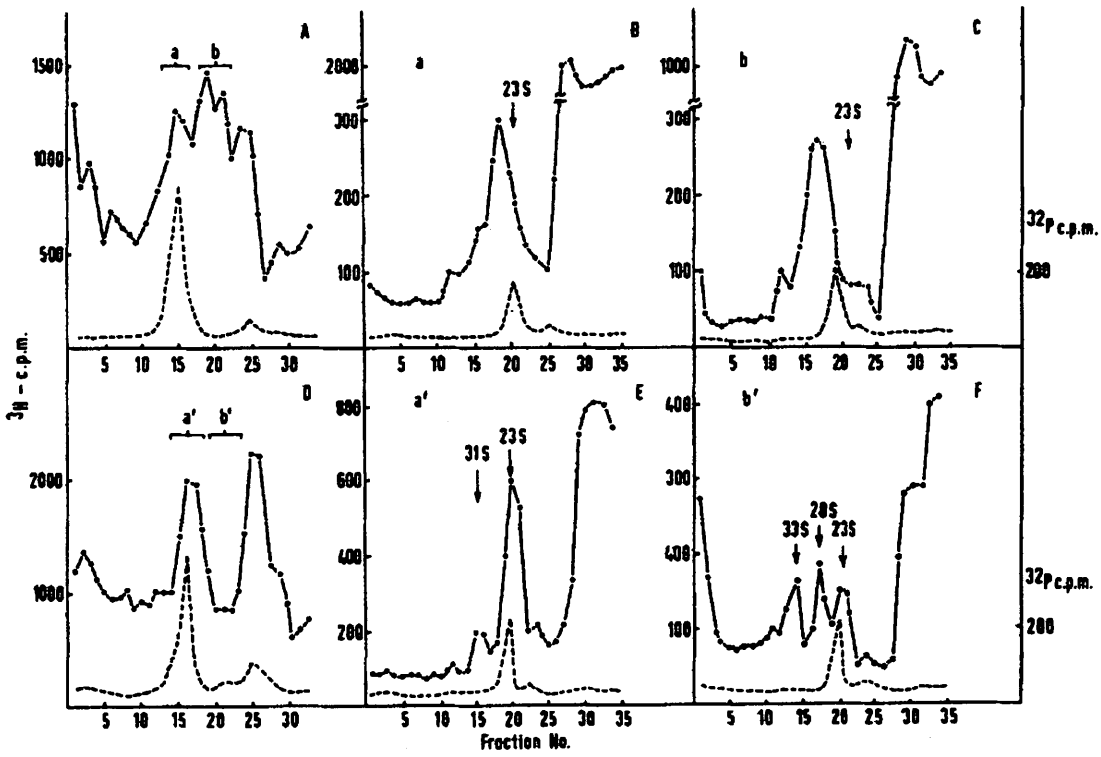
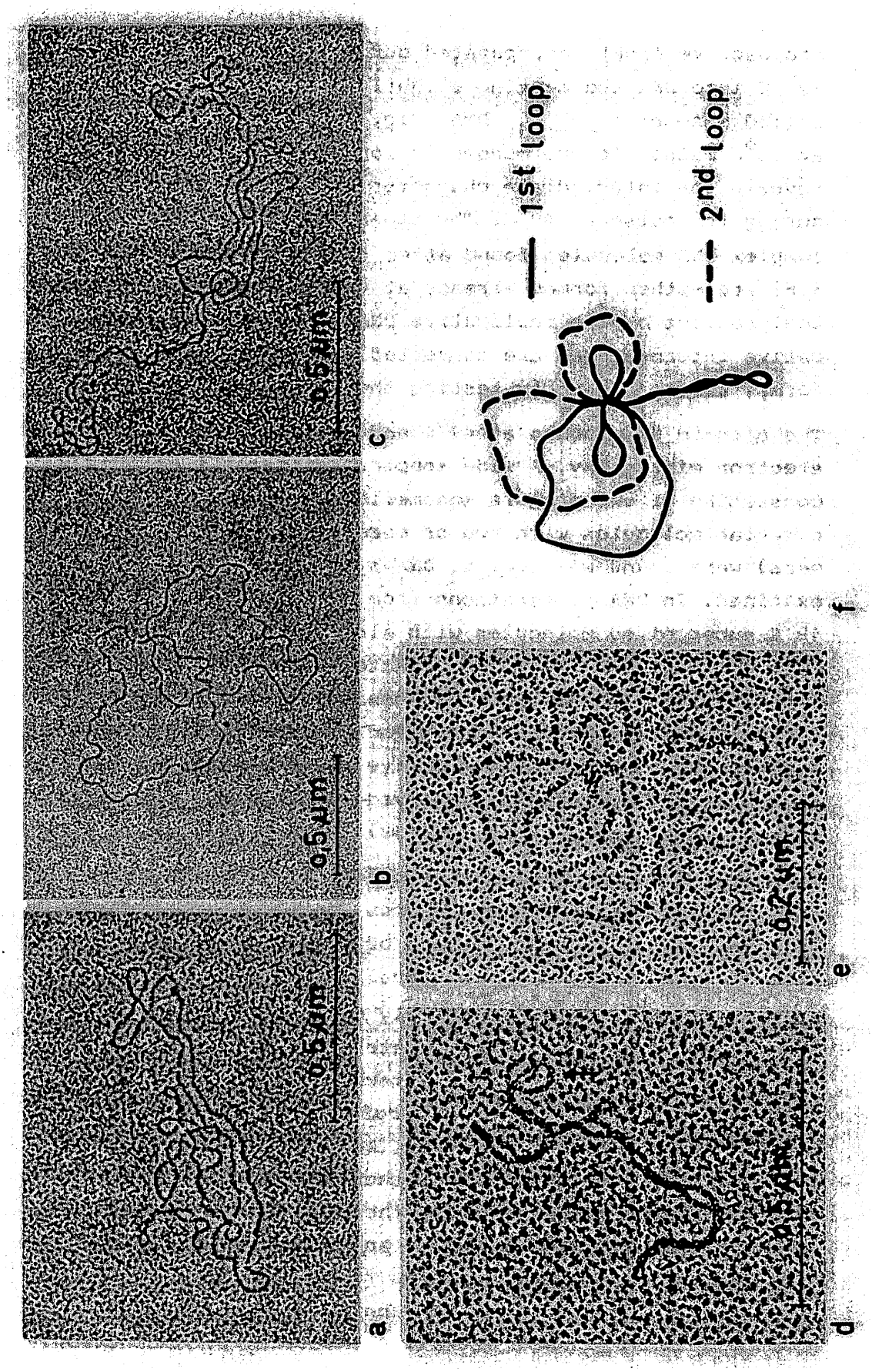


Fig. 3 Analysis of Col E_1 DNA of E.coli JC 411 (Col E_1)pulse labelled at 49°C and chased at 30°C.
A culture of E.coli JC 411 (Col E_1) was labelled at 49°C for 1 min with 10 uCi/ml ^{3}H-thymidine in the presence of 250 ug/ml deoxyadenosine. Incorporation of radioactivity was stopped by pouring the culture in ice-cold minimal-medium. Cells were collected by centrifugation and washed twice with ice cold minimal medium. One half of these cells were lysed immediately by the procedure described in Fig.1. The other half was resuspended into fresh medium supplemented with 500 ug/ml thymidine and incubated at 30°C for 1 h. The cleared lysates of both cultures were centrifuged to equilibrium in CsCl-EtBr gradients. Supercoiled ^{32}P-labelled Col E_1 DNA was added as an internal marker. Fractions of the heavy density regions (a and a') and the intermediate density regions(b and b') were pooled, dialysed and sedimented through a neutral 5 % to 20 % linear sucrose gradient as described in Fig. 2.
A. CsCl-EtBr gradient of the cleared lysate of cells pulse labelled for 1 min at 49° C. B. and C. sucrose gradients of fractions a and b of the CsCl-EtBr gradient of A.
D. CsCl-EtBr gradient of the cleared lysate of cells pulse labelled for 1 min at 49°C and chased for 1 h at 30°C.
E. and F. sucrose gradients of fractions a' and b' of the CsCl-EtBr gradient of D. ---^{32}P-Col E_1 DNA.

radioactive label incorporated during the pulse period at 49° C into DNA sedimenting slightly faster than supercoiled monomeric Col E_1 DNA (Fig. 3 B,C) can be chased at 30°C mainly to the monomeric form (Fig. 3 E). This reveals the intermediate character of the DNA labelled during the pulse at 49°C. The other faster sedimenting complex DNA molecules found after the chase period (Fig. 3 F) are either formed already at 49°C but are masked in the gradient by the replicative DNA, or some of the replicative intermediates are converted into these complex forms. We are presently testing these two possibilities.

The plasmid DNA formed at 47°C and 49°C was examined by electron microscopy. A wide spectrum of catenated forms consisting of two or more monomeric molecules but no circular molecules with two or more unit lengths (oligomers) were found when Col E_1 DNA synthesized at 47°C was examined. In DNA preparations from cultures incubated at 49°C supercoiled molecules with already replicated double loops of various sizes were detected (Fig. 4). Such molecules were first found by Jaenisch et al. in SV 40 infected animal cells and regarded as replicative intermediates (16). Similar replicative intermediates of Col E_1 DNA have been recently observed in minicells of E.coli carrying the Col E_1 factor (17,18).

The results described indicate that Col E_1 replication continues at elevated temperatures where the replication of the bacterial chromosome and of several sex factors stops completely. At these unusual temperatures complex Col E_1 DNA molecules, especially catenated forms, are synthesized. The formation of catenated structures by recombination, one possible mechanism, would presume that this process takes place preferentially above 47° C in E.coli. This seems rather unlikely. Besides, recent results in our laboratory have shown that such catenated molecules are also obtained at the elevated temperature in rec$^-$ mutants (rec A$^-$, rec B$^-$ and rec C$^-$) carrying the Col E_1 factor. These results favor again a replication mechanism for the formation of these complex DNA molecules rather than a mechanism involving recombination.


0.5 µm
0.5 µm
0.5 µm
1st loop
2nd loop
0.2 µm
0.5 µm
a
b
c
d
e
f

This is in agreement with the results of previous investigations on the fomation of complex Col E_1 DNA in temperature sensitive DNA replication mutants of E.coli and in P.mirabilis (8, 14). At 49° C Col E_1 DNA replication in E.coli apparently is slowed down so far that replicative intermediates can be isolated. They resemble the replicative forms found for SV 4o DNA and for Col E_1 DNA in minicells of E.coli. Rolling-circle Col E_1 structures like the ones isolated from minicells (18) could not be detected by us.

ACKNOWLEDGEMENTS: The authors wish to thank Prof. Dr. Lingens for his interest and support of this work. Dr. H. Frank (Tübingen) is thanked for his valuable assistance with the electron microscope.
This investigation was supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES:

(1) Clewell, D.B., J. Bacteriol. 110, 667 (1972)
(2) Lark, K., Ann.Rev.Biochem. 38, 569 (1969)
(3) Gefter, M.L., Hirota, Y., Kornberg, T. Wechsler, J.A., and Barnoux, C., Proc.Natl.Acad.Sci.,U.S., 68, 3150 (1971)
(4) Kingsbury, D.T., and Helinski, D.R., Biochem.Biophys.Res. Comm. 41, 1538 (1971)

Fig. 4 Complex molecules of Col E_1 DNA from JC 411 (Col E_1) purified by CsCl-EtBr density gradient centrifugation. The intermediate density fractions were collected and diluted 1 : 5 with o,o2 % cytochrome c in 1 M ammonium acetate. The grids were prepared by the procedure of Kleinschmidt and Zahn (1o). The hypophase was bidistilled water. The grids were positively stained with uranylacetate, rotary shadowed with platinium-palladium (Pt-Pd) and examined in a Siemens Elmiskop 1 (magnification 2o.ooo x). Contour lengths were measured on large scale prints.
a. Catenated dimer (open circular/open circular)
b. Catenated dimer (open circular/supercoiled)
c. Catenad trimer (supercoiled/open circular/supercoiled)
d. Replicative form of Col E_1 DNA from JC 411 (Col E_1) grown at 49°C. The small replicated loops are indicated by an arrow.
e. Further replicated molecule. (The replicated loops are probably folded back during the preparation of the grids).
f. Schematic representation of the molecule from e. The parts belonging to one replication loop were determined by measuring the contour lengths.

(5) Goebel, W., Nature 237, 67 (1972)
(6) Goebel, W., Europ. J. Biochem. 15, 311 (197o)
(7) Goebel, W., and Schrempf, H., Biochim.Biophys.Acta 262, 32 (1972)
(8) Goebel, W., Biochim. Biophys.Acta 232, 32 (1971)
(9) Clewell, D.B. and Helinski, D.R., Proc. Natl.Acad.Sci., U.S., 62, 1159 (1969)
(1o) Kleinschmidt, A.K., and Zahn, R.K., Z.Naturforsch. 14b, 77o (1959)
(11) Radloff, R., Bauer, W. and Vinograd, J., Proc.Natl.Acad.Sci., U.S., 57, 1514 (1967)
(12) Hudson, B., and Vinograd, J., Nature 216, 647 (1967)
(13) Wang, J.C., Biopolymers 9, 489 (197o)
(14) Bazaral, M. and Helinski, D.R., Biochem. 7, 3513 (1968)
(15) Goebel, W., and Helinski, D.R., Proc. Natl.Acad.Sci., U.S., 61, 14o6 (1968)
(16) Jaenisch, R., Mayer, A., and Levine, A.J., Nature 233, 72 (1971)
(17) Inselburg, J., and Fuke, M., Proc.Natl.Acad.Sci., U.S., 68, 2839 (1971)
(18) Inselburg, J., and Fuke, M. Proc.Natl.Acad.Sci., U.S., 69, 89 (1972)