# ISOLATION OF A REPLICATION-COMPLEX FROM EUKARYOTES

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SUMMARY

Treatment of the eukaryotic organism Tetrahymena pyriformis with low concentrations of Ethidium Bromide causes accumulation of a protein-nucleic acid complex consisting of a DNA polymerase, a RNA polymerase, a deoxyribonuclease and a RNA linked DNA fragment. The length of the RNA is about 30 nucleotides, while the DNA part is around 200 nucleotides long. Degradations with ribonucleases and deoxyribonucleases strongly indicate that the RNA exists in a non-hybrid structure with a homogenous base composition and that the DNA is single-stranded. The complex is purified 1100 fold from whole cells and sodium dodecyl sulphate acrylamide gel electrophoresis gives 9 defined bands. The polynucleotide in the isolated complex accounts for only $10^{-4}$ of the total cellular DNA.

As the complex contains some of the enzymes essential for discontinous DNA replication, in addition to a RNA linked Okazaki fragment, it is concluded that a highly purified replication complex has been isolated.

DNA in microbial systems is replicated by a discontinuous mechanism involving synthesis of RNA primed DNA fragments (1-6). Similar participation of RNA has been observed during replication of polyoma DNA in isolated nuclei (7). In both systems the RNA primer is found to be around 40 nucleotides long, while the length of the smallest DNA fragment is between 100-200 nucleotides. The mechanism involved in the DNA replication process still remains unclear, although genetic and enzymological studies have demonstrated that at least 8-10 proteins must be involved in the process (2, 3, 4, 8).

We have become interested in the initiation event of DNA synthesis in eukaryotic systems and have, from the organism Tetrahymena pyriformis, isolated a complex, which consists of a RNA linked DNA fragment in association with a DNA polymerase, a RNA polymerase, and a deoxyribonuclease. This complex is believed to represent a true replication complex.

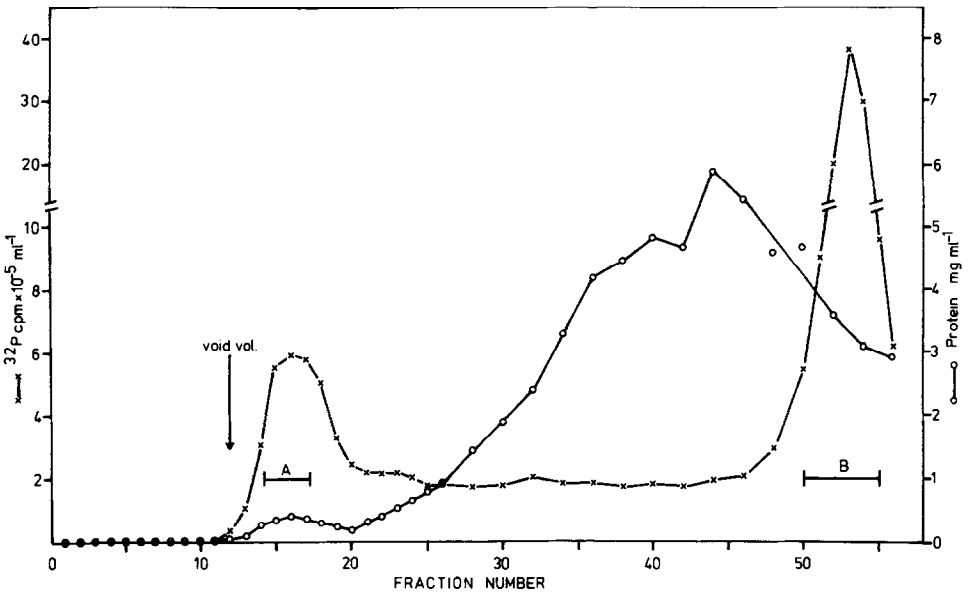


Fig. 1. Gel filtration on Sepharose 6B of fraction III from $|^{32}P|$ labelled Tetrahymena cells. A 100 ml cell culture was grown in defined medium (12, 13) in the presence of 2 mCi of $|^{32}P|$ phosphate. At a cell density of 40,000 cells/ml, Ethidium Bromide (8 µg/ml) plus $|^{32}P|$ phosphate (2 mCi) were added and the culture grown for 24 hrs at 27°C. The cells were harvested, mixed with 20g of unlabelled cells and fraction III prepared as described under Methods. 3 ml of fraction III were applied on a 2.0 x 18 cm column of Sepharose 6B and 1 ml fractions collected. The fraction at the void volume of the column is called fraction IV.

## MATERIALS AND METHODS

Materials. Unlabelled nucleotides and Ethidium Bromide were purchased from Sigma Chemical Company. All $|^{3}H|$ labelled nucleosides and nucleotides were obtained from New England Nuclear Company. Aspergillus nuclease $S_1$ was a gift from Dr. Paul Berg and ribonuclease $T_1$ was obtained from Sankyo Company.

Culture of Cells and Enzyme Preparation. Tetrahymena pyriformis, strain GL, was grown in large batches as described earlier (9). For labelling experiments the cells were grown in a defined medium (10, 11). The cells were harvested by centrifugation, resuspended in four volumes of buffer T (10% sucrose, 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA and 2 mM mercaptoethanol) and the cell fragments collected by centrifugation (fraction I). After storage at -20°C, the material was thawed, four volumes of KPG buffer (10% glycerol, 20 mM K-$PO_4$, pH 7.2, 20 mM NaCl, 2 mM mercaptoethanol and

1 mM EDTA) were added and the homogenate centrifuged for 45 min at 150,000 x g (fraction II). After a 50-70% ammonium sulphate cut the precipitate was dissolved in 3 ml of KPG buffer, centrifuged for 45 min at 150,000 x g (fraction III) and applied on a Sepharose 6B column. The Sepharose fraction is called fraction IV.

Enzyme Assays. The DNA polymerase assays measure the incorporation of $|^3H|$ dTTP into an acid insoluble product (12). The standard incubation mixture contained in a volume of 100 µl the following: 2.0 µmoles Tris-HCl, pH 7.4, 0.6 µmole $MgCl_2$, 1.8 µmoles KCl, 0.2 µmole mercaptoethanol, 0.02 µmole EDTA, 10 nmoles each of dATP, dCTP, dGTP, 0.25 nmole of $|^3H|$-dTTP (spec. act. 3.1 mCi/µmole) and 15 µg heat denatured DNA. The RNA polymerase assays measure the incorporation of $|^3H|$ UTP into an acid insoluble product. The standard incubation mixture contained in a volume of 100 µl the following: 3.5 µmoles Tris-HCl, pH 7.5, 0.8 µmole $MgCl_2$, 3.5 µmoles KCl, 0.4 µmole mercaptoethanol, 0.01 µmole EDTA, 50 nmoles ATP, 25 nmoles each of CTP and GTP, 0.5 nmole of $|^3H|$ UTP (spec. act. 10.7 mCi/µmole) and 20 µg native DNA. The deoxyribonuclease assays measure the degradation of $|^3H|$ $d(T)_{100}$. The standard incubation mixture contained in a colume of 100 µl the following: 2.5 µmoles potassium phosphate buffer, pH 7.2, 0.5 µmole $MgCl_2$ and 0.8 nmole of oligo $d(T)_{100}$ (spec. act. 0.9 mCi/µmole). The degradation with the nuclease $S_1$ from Aspergillus were performed as already described (13).

Other manipulations. Acrylamide gel electrophoresis was performed according to the method of Weber and Osborn (14). Protein was determined by the method of Lowry (15).

## RESULTS AND DISCUSSION

The replication complex has been found to accumulate in cells exposed to Ethidium Bromide (EB) (5-10 µg/ml), and can easily be isolated by a two step lysis procedure followed by ammonium sulphate precipitation and gel

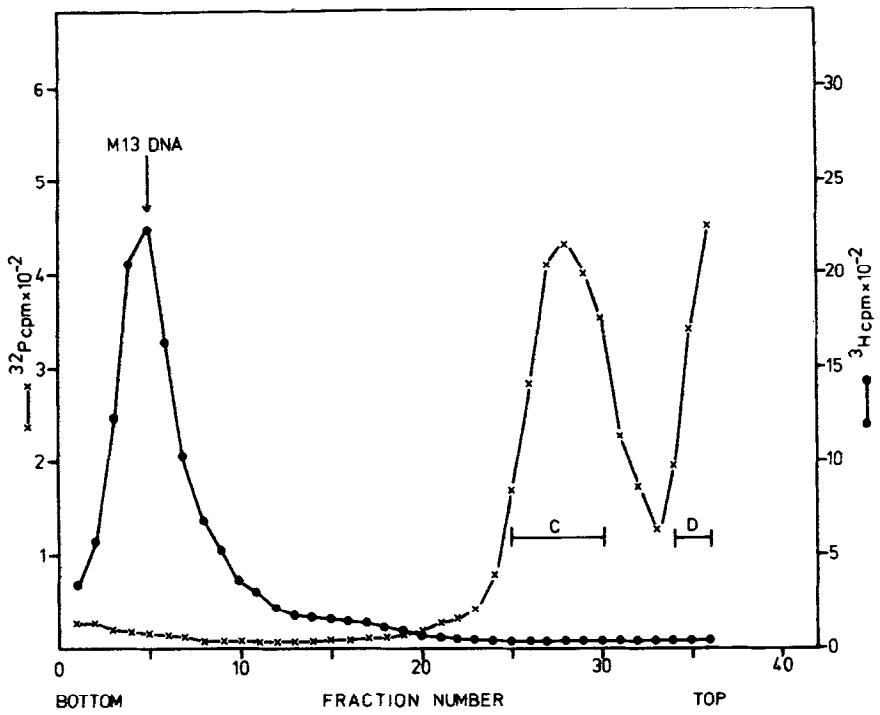


Fig. 2. Sucrose velocity sedimentation of the $|^{32}P|$ labelled complex in the presence of sarkosyl. To the $|^{32}P|$ labelled complex (fraction IV, 4,000 cpm) was added sarkosyl (1%) and $|^{3}H|$ M13 DNA marker (10,000 cpm). After sedimentation in a 5-20% sucrose gradient (1 mM NaCl, 10 mM Tris and 1 mM EDTA) for 4 hrs at 55 krpm in a Spinco SW56 rotor, fractions were collected and numbered from the bottom of the tube.

filtration on Sepharose 6B. Fig. 1 shows a typical pattern from gel filtration of a $|^{32}P|$ labelled preparation from EB treated cells. Two peaks of radioactivity are observed, one (peak A) with a molecular weight $\geq 2 \times 10^6$ at the void volume of the column, and one (peak B) with a molecular weight $\leq 10^5$. The material in peak A can be separated into a polynucleotide (C) and a protein part (D) by sucrose sedimentation in the presence of sarkosyl (Fig. 2). The polynucleotide fragment contains around 200 nucleotides, and consists, as seen from Table 1, of both RNA and DNA, while the radioactivity in fraction D is mainly due to phosphorylated histones (data not shown).

Digestion of the isolated polynucleotide by pancreatic RNase and DNase demonstrates that about 15% of the material is RNA, while the remainder is

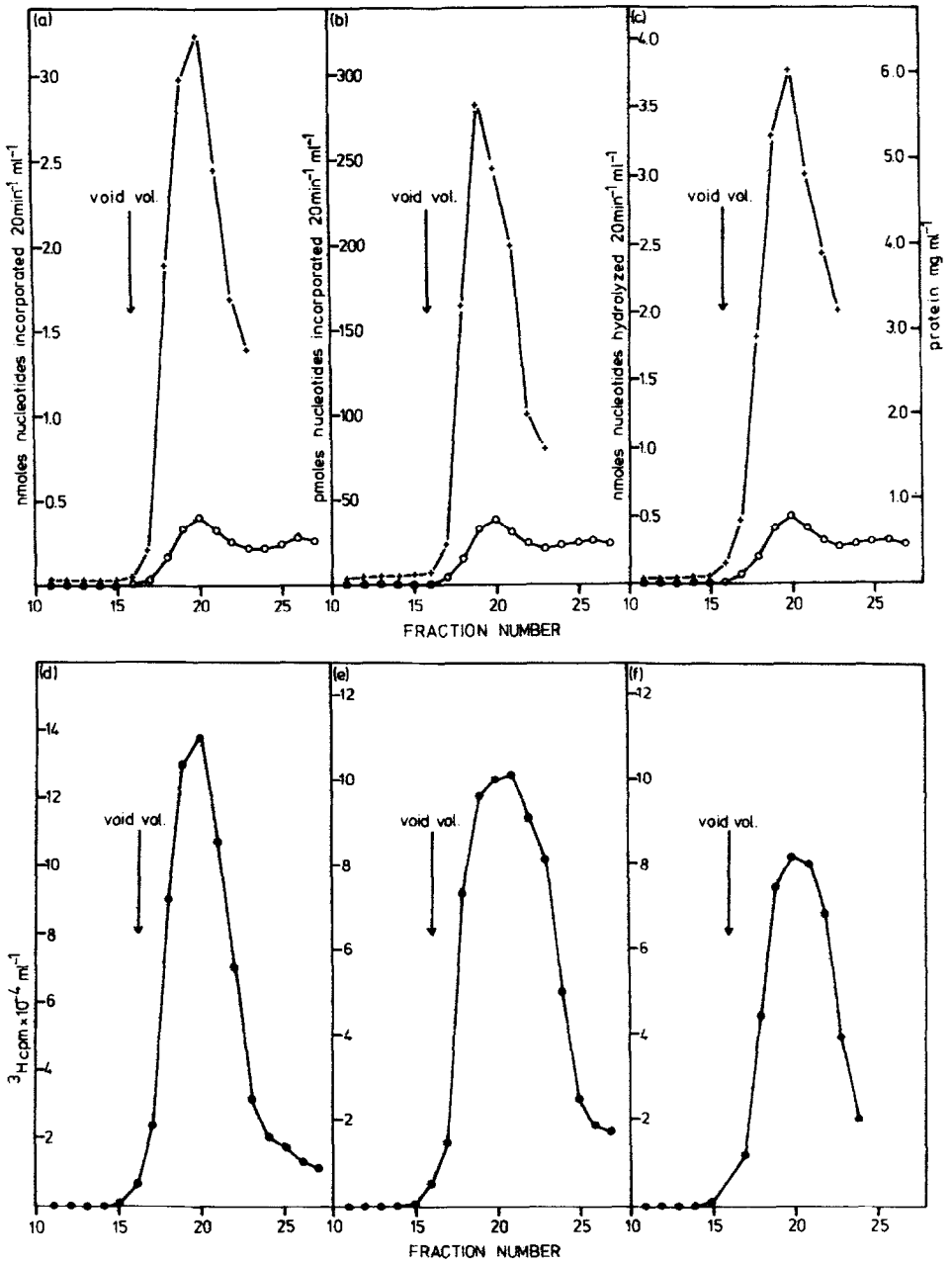


Fig. 3. Enzyme activities and polynucleotides present in the complex. Fraction IV was prepared from about 20 g of cells as described under Methods except that the 50-75% $(NH_4)_2SO_4$ cut was replaced by a 0-75% cut. In all cases (Fig.3a-f), 3 ml of fraction III were applied on a 2.0 x 18 cm Sepharose 6B column and 1 ml fractions were collected. Enzyme activities were measured around the void volume: (a) DNA polymerase, (b): RNA polymerase, (c): deoxyribonuclease activity, (d): acid insoluble $|^3H|$ thymidine counts around the void volume. Fraction III was prepared from cells grown and treated as mentioned under Fig. 1, except that 2 x 1 mCi of $|^3H|$ thymidine had replaced the $|^{32}P|$ phosphate, (e): acid insoluble $|^3H|$ uridine counts around the void volume. The cells were grown and treated as mentioned under Fig. 1, except 2 x 1 mCi of $|^3H|$ uridine had replaced the $|^{32}P|$ phosphate, (f): fractions from experiment (e) were hydrolyzed in 0.3 M KOH for 18 hours at 37°C before the samples were acid precipitated. All enzyme activities are measured as described under Methods.

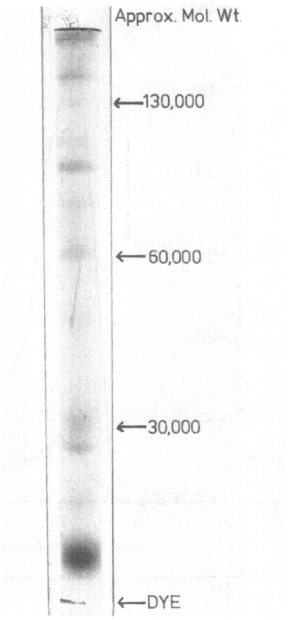


Fig. 4. Electrophoresis of fraction IV on sodium dodecyl sulphate acrylamide gels. Fraction IV was prepared as described under Methods and precipitated by 5% trichloroacetic acid. The precipitate was collected by centrifugation, dissolved in Carrier buffer (2% sodium dedocyl sulphate, 50 mM Tris, HCl pH 7.5, 1 mM EDTA, 40 mM Cleland's reagent and 8% sucrose) and applied on 7.5% acrylamide gels containing 1% sodium dodecyl sulphate (15).

found to be DNA (Table I). Electrophoresis on DEAE paper of the degradation product after incubation with ribonuclease $T_1$ gives about 8 defined bands. This observation strongly indicates that the RNA present in the fragment has a homogenous base sequence. The DNA part of the complex is fully degraded by deoxyribonuclease $S_1$ from Aspergillus, which shows that the DNA is single stranded (13). When this observation is considered together with the fact that the RNA is fully degraded by pancreatic RNase, it argues that the RNA is not present in a RNA-DNA hybrid but is covalently linked to DNA.

In addition to the polynucleotide, the complex contains at least three enzyme activities (a DNA polymerase, a RNA polymerase and a deoxyribonuclease) which are coincident with the labelled material in peak A (compare Figs. 1 and 3). So far, it has not been possible to separate the three mentioned activities from one another by further chromatography on

TABLE I

Enzymatic Degradation of the Polynucleotides Isolated from the $|^{32}P|$ Phosphate Labelled Complex

| Incubation Mixture | Remaining Acid Insoluble Counts after Incubation for | |
|---|---|---|
| | 15 min | 30 min |
| $|^{32}P|$ Polynucleotide incubated at $^0$4 C in the presence of buffer | 2030 | 2020 |
| $|^{32}P|$ Polynucleotide incubated at 37$^0$C in the presence of buffer | 2090 | 2040 |
| $|^{32}P|$ Polynucleotide incubated at 37$^0$C with 0.2 µg DNase | 630 | 450 |
| $|^{32}P|$ Polynucleotide incubated at 37$^0$C with 0.2 µg RNase | 1830 | 1740 |
| $|^{32}P|$ Polynucleotide incubated at 37$^0$C with DNase and RNase | 80 | 75 |
| Background (Buffer at 37$^0$C) | 30 | 35 |

The $|^{32}P|$ labelled polynucleotide was separated from the protein by sedimentation in a 5-20% sucrose gradient (18 hrs at 40 krpm in a Spinco SW41 rotor) in the presence of 1% sarkosyl. The polynucleotide fragment was collected, precipitated by alcohol and redissolved in 10 mM Tris, pH 7.2, 1 mM EDTA and 6 mM $MgCl_2$ containing 100 µg/ml of heat denatured DNA plus 100 µg/ml of rat liver tRNA. For each digestion 100 µl of the mixture were used.

DEAE or Phosphocellulose. The overall purification of the complex is around 1100 fold from whole cells.

Electrophoresis on sodium dodecyl sulphate acrylamide gels demonstrates 9 defined polypeptide chains in the complex (Fig. 4). Of the total amount of protein, the histone band accounts for about half. The proteins with molecular weights of 150,000, 35,000 and 25,000 are possibly identical to the known subunits of mammalian RNA polymerase (16).

That the RNA linked DNA fragment accounts only for a very small fraction of total cellular DNA is seen from Table II, where the amount of $|^3H|$ labelled DNA is followed during the purification of the complex. The amount of radioactivity which is isolated represents less than $10^{-4}$ of the total labelling.

From the presented data it is logical to assume that the nucleic acid-

Table II
RESPIRATORY ACTIVITIES OF CONIDIA OF WILD-TYPE AND EXTRACHROMOSOMAL MUTANTS OF *N. CRASSA*

| Strain | Preculture Medium[a] | Specific Rate of Respiration[b] ($Q_{O_2}$, µl $O_2$ $hr^{-1}$ $mg^{-1}$ dry weight) | |
|---|---|---|---|
| | | Form of Respiration | |
| | | Terminal | Alternate |
| wild-type (74A8) | Y | 30±5 | 6±3 |
| | F | 4±7 | 30±5 |
| mutant: | | | |
| [mi-1]f$^-$ | Y | 0±3 | 25±3 |
| [mi-1]f$^+$ | Y | 0±3 | 40±7 |
| [mi-3] | Y | 1 | 22 |
| | F | 0.4 | 38 |
| [SG-1] | Y | 32±6 | 12±2 |

[a] Y, YEGCE; F, Fries'

[b] Conidia were obtained from 7- to 14-day-old cultures. Assays were with Fries' containing 2% glucose. Other procedures as in Table I.

factors. These results and additional (6) studies indicate that regulatory controls are altered in the extrachromosomal mitochondrial mutants of *N. crassa*. The genetics and regulation of respiration and fermentation will be discussed elsewhere (6) in terms of the dynamic repressor control model (7).

The mechanism(s) by which ammonia and nitrate serve in the regulation of the development of the mitochondrial respiratory systems remain to be determined. Hypothetically, these ions may act as co- or anti-repressors (6). Perhaps the regulation of the synthesis and catabolism of nitrate reductase by ammonia and

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