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EDITED BY

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ASSISTANT EDITOR
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Circulating Nucleic Acids in Higher Organisms[1]

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I. Introduction

Since the early transformation experiments of Griffith (1928) intensive studies have been made of the molecular aspects of bacterial genetics, prompted by the finding of Avery *et al.* (1944) that the im-

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portant transforming factor was DNA. It is now clear that DNA is able to leave bacteria and enter other bacteria, and the mechanisms and situations involved are readily understood.

Bacterial DNA can move from members of one strain to those of another by means of conjugation or transduction, which does not strictly involve extracellular release of DNA. In conjugation a newly replicated DNA molecule passes from one bacterium to another via a conjugation tube or F pilus (see, e.g., Davis *et al.*, 1973). Similarly, in the case of transduction, bacterial genes are transferred from one bacterial strain to another via a bacteriophage. In the case of generalized transduction bacterial genes, or extrachromosomal elements such as the F factor, may be transferred by lysis of the host cell and release of a fragment of host chromosome enveloped in a phage protein coat. On contact with the host bacterium, the DNA is injected into the cell, and a process similar to transformation follows.

Bacteria possess several extrachromosomal factors including the F factor (the first fertility factor), R factors (antibiotic resistance transfer factors), and bacteriocinogenic factors, which are distinguishable by their molecular weight. F and R factors and some bacteriocinogens have a molecular weight of $6–140 \times 10^6$, corresponding to 100 to 200 genes, while many bacteriocinogens have a molecular weight of $4–5 \times 10^6$—about 15 genes. These factors are termed plasmids and can be transferred by conjugation from a bacterium of one strain to another of the same strain, or from bacteria of one strain to those of another, hence the ability of one strain of bacteria to confer drug resistance to previously sensitive strains (see Novick, 1969; Wolstenholme and O'Connor, 1969; Willets, 1972).

Normally, transformation is demonstrated by providing one strain of bacteria with purified DNA from another strain. Pieces of DNA having a molecular weight of 3×10^5 to 1×10^7 or more have been shown to induce transformation. The double-stranded DNA enters the recipient bacterium, one strand is rapidly hydrolyzed, and the other participates in recombination.

Transformation also has been achieved by providing one strain of bacteria with nonpurified DNA found in the culture medium of another strain. In the first studies reporting the presence of extracellular DNA in the culture medium of bacteria it was not clear whether the DNA was released by living cells or was due to cell lysis (Catlin, 1960; Smithies and Gibbons, 1955; Ottolenghi and Hotchkiss, 1960; Campbell *et al.*, 1961; Takahashi, 1962; Demain *et al.*, 1965). However, in two series of elegant experiments, Ottolenghi and Hotchkiss

(1962) demonstrated that living cells were responsible for the release of highly active transforming DNA. Similarly, Ephrati-Elizur (1968) found a spontaneous release of transforming DNA by early exponential- and stationary-phase cells of *Bacillus subtilis*. This extracellular DNA was also shown not to be due to cell lysis. The extracellular DNA was localized on the outer walls of the bacteria by fluorescent vital staining, an observation confirmed by electron microscopy. Further studies (Borenstein and Ephrati-Elizur, 1969) showed that the release of DNA from synchronously replicating germinating spores, based on the relative frequencies of various gametic markers, had a polarity of release that resembled the order of replication of the chromosome. Moreover, the results indicated a marked correlation between DNA synthesis and DNA release, suggesting that the released DNA may have been newly synthesized.

More recent observations on bacteria in stationary phase (Stroun and Anker, 1972b) showed that the process of DNA release by living bacteria was governed by a precise homeostatic mechanism.

In addition to bacteria, blue-green algae also were seen to release a transforming principle into the growth medium (Herdman and Carr, 1971), which later proved to be DNA (Herdman, 1973).

It is clear from the foregoing remarks that spontaneous release of nucleic acid from prokaryote cells occurs, and that the DNA so released is capable of actively transforming a proportion of the recipient cells. It is pertinent therefore to ask the question, Is there a spontaneous release of nucleic acid material from eukaryote cells? If the answer to this question is in the affirmative, supplementary questions may be posed: (1) Can other eukaryote cells take up this nucleic acid? (2) Can released nucleic acids circulate in eukaryote systems? (3) To what purposes can this released nucleic acid be ascribed?

II. Released DNA from Cells of Eukaryotes

Various indirect pieces of evidence indicating the possible movement of informative molecules out of eukaryotic cells have appeared in the literature. They range from studies with graft hybrids on intact organisms (e.g., Stroun *et al.*, 1963) to experiments with eukaryote cells in culture (Bendich *et al.*, 1965, 1971; Roosa and Bailey, 1970; Roosa, 1971; Reid and Blackwell, 1970, 1971). However, while such movement may have been implied, it was not shown to have occurred. More recently, evidence has accrued indicating conclusively that DNA can leave living, intact, eukaryote cells.

A. Release of DNA from Nonstimulated Cells

1. *Release of DNA from Frog Auricles*

Studies on the auricles of frog hearts offer the first concrete example of DNA release from eukaryote organ (Anker and Stroun, 1972a; Stroun and Anker, 1972a,b; Stroun *et al.*, 1977). This material was chosen because of its ease of handling and ability to survive for several days in Ringer's medium as evidenced by continued pulsation of the auricles. Blood-free, sterile auricles were incubated for various periods of time in sterile Ringer's solution. The supernatant was removed and centrifuged at 165,000 *g* for 12 hours to eliminate all cellular contaminants. DNA was isolated (Marmur, 1961) from both the supernatant and the auricles. Supplementary passage of the supernatant DNA through a hydroxyapatite column greatly improved its purity, allowed it to be colored by diphenylamine, and increased its sensitivity to DNase (Anker *et al.*, 1976b; Stroun *et al.*, 1977). It was identified as DNA because (1) it had a typical ultraviolet light absorption maximum and minimum, (2) similar amounts were identified by both ultraviolet light absorption and by deoxyribose assays with indole (Ceriotti, 1952) and diphenylamine (Giles and Myers, 1965), and (3) more than 95% of the material was digested by DNase but remained insensitive to RNase and pronase. The released DNA was shown to be double-stranded by the hyperchromic effect after heat denaturation and elution on hydroxyapatite columns.

As discussed by Stroun and Anker (1972b), Anker *et al.* (1976b), and Stroun *et al.* (1977), the DNA present in the incubation media was not due to the presence of dying or dead cells for the following reasons:

1. The amounts of DNA (representing from 1 to 2% of the cellular DNA) present in the medium after varying periods of incubation (2, 4, 12, or 24 hours) were similar, but if the DNA were from dead or dying cells, the amount would be expected to increase with time.
2. When the incubation medium was changed every 4 hours, similar amounts of DNA were detected in each of the successive solutions. This constant renewal of released DNA is suggestive of an active mechanism which slows down, and even stops, when there is a high frequency of short incubation times, for example, after 10 periods, each of 1 hour.
3. It is unlikely that the release was due to experimental stress, since it took more than 1 hour to obtain a maximum concentration of

extracellular DNA. Moreover, auricles taken from their medium six times in a row and resuspended each time in the same medium released much less DNA than auricles submitted to the same treatment but resuspended each time in fresh medium.

4. Labeling studies indicated a delayed release of newly synthesized DNA which had a higher specific activity than cellular DNA after a long period of labeling.

5. Few repeated genes copies were present in the released DNA, in contrast to the high frequency expected if the DNA were lost from dead cells.

6. After 24 hours of incubation the auricles beat with nearly the same rhythm as at the start of the experiment.

7. All cells seemed to be viable, since they readily incorporated uridine-^{3}H into RNA during the last hour of a 24-hour incubation period, as shown autoradiographically.

8. Auricles cut in three, thereby increasing the wounded surface 4-fold, continued to beat regularly, yet did not release more DNA than intact auricles, indicating that tissue wounded during ablation of the ventricle was not the source of the extracellular DNA.

9. Regular changing of the incubation medium lengthened the survival time rate of the auricles.

10. Virtually no DNA was measured from medium in which 5% of the auricles incubated had been previously killed by heating to 50°C in the medium.

11. Microscopic examination of the pellet after centrifugation of the supernatant revealed no intact cells and very few cell fragments, which represented not more than 1×10^{-6} of the total cell preparation assuming each fragment represented a whole cell. This was expected in view of the syncitial organization of the frog heart.

Separation on a cesium chloride gradient indicated that the general AT/GC ratio was the same for the cellular and the extracellular DNA; both DNAs banded at a density of 1.700 gm/cm^3. This eliminates the possibility that the released DNA was of mitochondrial origin. The molecular weight of the released DNA was not as homogeneous as that of the cellular DNA and banded on a sucrose gradient with a peak at 13S and a shoulder at 8S.

The specificity of the DNA found in the supernatant has been demonstrated by hybridization studies (Stroun and Anker, 1972b), and the possibility of selfing has been excluded by hybridizing on filters, according to Gillespie and Spiegelman (1965).

Additional information (Anker *et al.*, 1976b; Stroun *et al.*, 1977) was obtained by the C_0t curve hybridization method (Britten and Kohne, 1968).

The differences in the renaturation curves of the cellular DNA and of the released DNA showed that the reiterated sequences of newly synthesized cellular DNA was absent from the medium, at least in the form of multiple copies. A preferential release of unique DNA was also indicated by the hybridization of unlabeled cellular DNA with DNA-^{3}H released from labeled cells. Indeed, the hybridization curve appeared to follow second-order kinetics with a high half-C_0t value indicating that the released DNA-^{3}H was highly complex and not composed of numerous copies of simple sequences. The fact that the hybridization curve of the released DNA-^{3}H reached a plateau before that of the cellular DNA-^{3}H whether the mass ratio of labeled DNA to unlabeled DNA was 1 : 100 or 1 : 1000 can be explained if it is assumed that 50% of the released DNA consisted of unique sequences present in the cell genome. The complexity of the released DNA shown by the C_0t curves ruled out direct microbial contamination.

2. *Release of DNA from Human Lymphocytes*

Since the release of DNA by organisms as diverse as nondividing bacteria and frog auricles *in vitro* seemed to be regulated by the same homeostatic mechanism (Stroun and Anker, 1972b), human cells were also tested for a similar regulatory mechanism (Anker *et al.*, 1975a,b, 1976b). Nonstimulated human lymphocytes obtained by Ficoll Isopaque gradient separation were cultured in TC 199 medium in the presence or absence of 20% homologous serum of the same blood group as the blood cell donor. After incubating the lymphocytes for varying periods of time, the cells were removed and the medium centrifuged at 165,000 *g* to remove all possible contaminating cell debris. Lymphocytes were counted at zero time and after each incubation, and their viability was tested by the ability to exclude trypan blue. DNA was isolated both from the lymphocytes and the supernatant by phenol extraction followed by passage through hydroxyapatite columns in the presence of 3 *M* potassium chloride (Anker *et al.*, 1975b, 1976b). Once purified from the associated protein, the DNA from the supernatant exhibited the typical characteristics described for frog auricles in Section II,A,1.

The DNA released into the supernatant did not emanate from dying or dead cells as shown in several ways: (1) Reasons 1 through 5 as discussed for frog auricles; (2) although in some cases as many as 25% of the incubating lymphocytes die in the absence of serum, the amount

of DNA released in these cultures was similar to that found in cultures with serum, where cell death was negligible; (3) the same amount of extracellular DNA was found (about 2% of the cellular DNA) whether cell recovery was almost complete (99%) or whether 25% of the cells were lost, showing that dead cells and cell fragments were not responsible for the DNA in the supernatant but were pelleted out by centrifugation; and (4) conservation of the functional cellular integrity of the lymphocytes during similar time periods to those used in the above experiments was confirmed by the capacity of previously incubated lymphocytes to increase DNA synthesis on phytohemaglutinin (PHA) or concanavalin-A stimulation in the same way as nonincubated lymphocytes.

Separation on a cesium chloride gradient, sedimentation on a sucrose gradient, C_0t curves of the renaturation of released DNA, and hybridization of this DNA with an excess of cellular DNA showed characteristics similar to those of the DNA released from frog auricles.

Thus it appears that a specific DNA is released from healthy, nonstimulated human lymphocytes.

3. *Released DNA Complexes*

The foregoing data indicate strong similarities between the DNAs released from frog auricles and from nonstimulated lymphocytes. The DNA was released as a complex and not as naked DNA since: (1) The released DNA was resistant to DNase prior to purification, suggesting a protective coat which can be removed only by extensive deproteinization procedures; (2) the DNA was not only complexed to proteins, but also to additional components, which resulted in changed physical characteristics of the complex such that it could not be pelleted even after 12 hours of centrifugation at 165,000 *g*.

Examination of the released material by electron microscopy (Gahan *et al.*, 1977), using both negative staining and platinum-shadowing methods, revealed material similar in form to chromatin (Fig. 1A) having strand diameters of 16–99 nm, compared to 20–23 nm for spreads of interphase chromatin (Dupraw, 1966; Dupraw and Bahr, 1969). The large fibers were comprised of bundles of fibrils, each fibril being about 16–20 nm in diameter and the overall fiber size depending on the number of fibrils present. There were also many fiber fragments varying from 17 nm to 15 μm in length, which may have been derived from the network of material since they were of similar diameter (Fig. 1B). Digestion with protease or trypsin resulted in the long, large strands being rendered to fragments, and the final strand width was about 6 nm. Similar strand widths were obtained after puri-

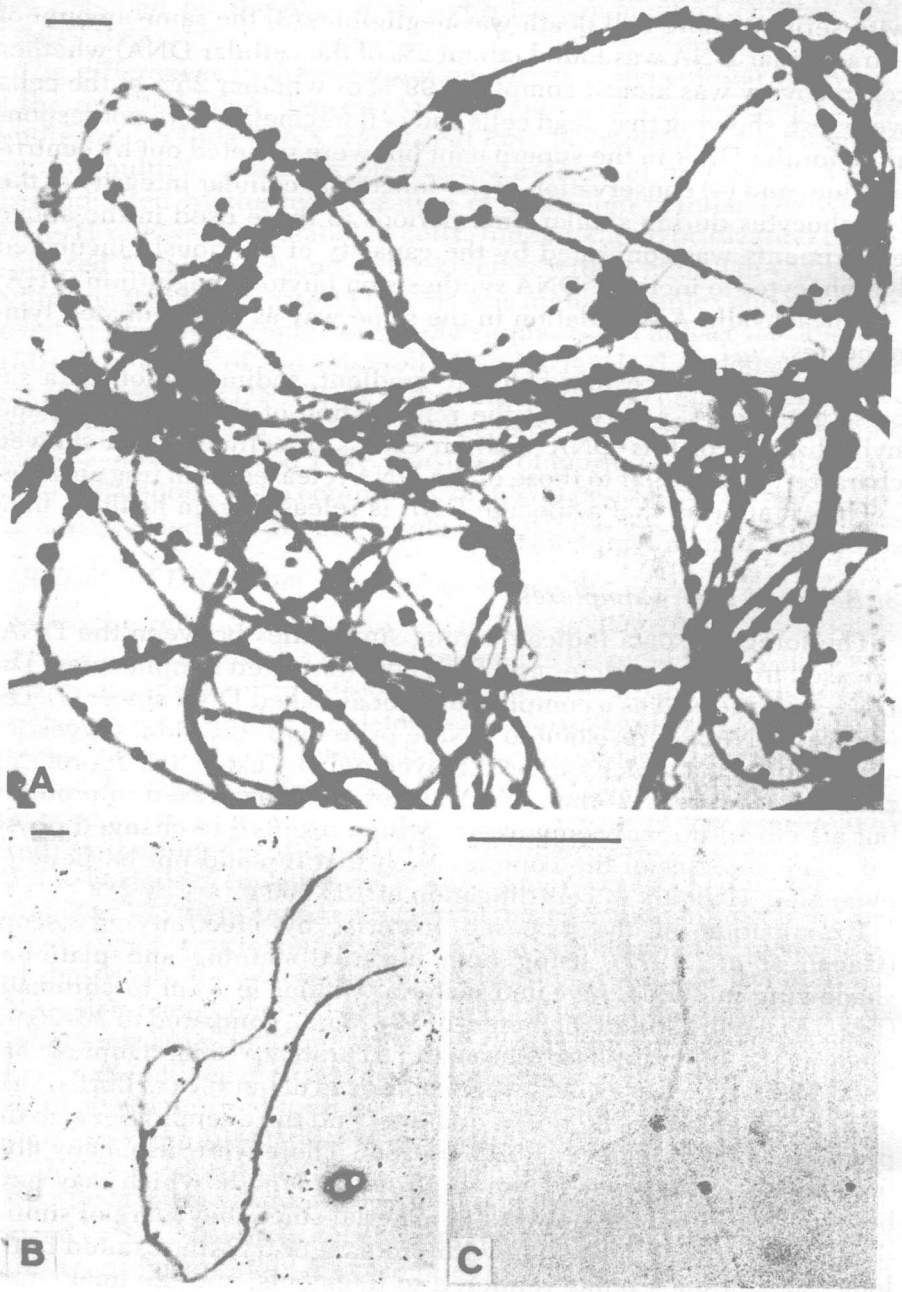

FIG. 1. Material present in the supernatant of the incubation medium from a frog auricle experiment (see Section II,A,1) after centrifugation for 12 hours at 165,000