Cultured Mammalian Cell Deoxyribonucleic Acid as a Template for *in Vitro* Protein Synthesis*

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ABSTRACT: DNA from established or primary cells cultured *in vitro* stimulates incorporation of amino acids in a cell-free system. Such deoxyribonucleic acids (DNA's) are active only after denaturation. Ribonucleic acid (RNA) polymerase is not required and the effect is relatively resistant to actinomycin.

These facts indicate that the single-stranded DNA acts directly as a template rather than through intermediate RNA synthesis. This messenger activity of DNA can be attributed to streptomycin bound to the deoxyribonucleic acid during growth in an antibiotic-containing medium.

It has been clear for some time that there are restrictions on the type of polynucleotide chain which can serve as a template for *in vitro* protein synthesis. Thus, naturally occurring messenger RNA,[1] virus RNA, or synthetic polynucleotides will stimulate amino acid incorporation in an *Escherichia coli* cell-free system while ribosomal RNA and soluble RNA are very poor templates. Likewise, single-stranded DNA is inactive even though one of the two strands has a base sequence identical with that of the messenger RNA which is an active template both *in vivo* and *in vitro*. Although the basis of these restrictions remains obscure, some exceptions have been reported. In particular, denatured DNA isolated from HeLa cells grown in tissue culture shows substantial activity in stimulating amino acid incorporation (Holland and McCarthy, 1964). It is the purpose of this communication to demonstrate that this and other DNA, isolated from similar sources, acts directly as a template without intervening RNA synthesis. In addition, it will be shown that acquisition of this activity by the DNA results from the exposure of the cells to streptomycinoid antibiotics apparently resulting in combination of the antibiotic with the DNA molecules.

Materials and Methods

HeLa cells and mouse L cells were grown as monolayers on glass in a medium containing 7% calf serum, 0.1% yeast extract (Difco), and 0.1% Proteose peptone no. 3 (Difco) in Hanks' balanced salt solution buffered by 0.06% sodium bicarbonate or in Eagle's medium. In both cases the medium contained also 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 25 units/ml of mycostatin. Similar methods were used for the cultivation of embryonic chick cells isolated from 10-day fertilized eggs.

DNA was prepared from the nuclei of washed cells by the pronase method of Berns and Thomas (1965) or the method of Marmur (1961). DNA was extracted from coliphage T2 and RNA from coliphage f2 by phenol extraction. Salmon sperm DNA was a commercial product of Calbiochem. DNA was denatured by boiling for 3 min in distilled water at a concentration of 100–200 μg/ml followed by chilling in ice.

RNA polymerase, having a specific activity of 20 units/μg of protein, was prepared from *E. coli* as described by Chamberlain and Berg (1962).

Deoxyribonuclease was a crystalline preparation from Worthington Biochemical Corp. electrophoretically purified to remove traces of ribonuclease. Actinomycin D was kindly provided by Merck Sharp and Dohme Co. Poly U, triphosphates, phosphoenolpyruvate (Na salt), PEP kinase, and pronase were obtained from Calbiochem. Streptomycin sulfate (Squibb) and neomycin sulfate (Upjohn) were commercial products. [^{14}C]-Phenylalanine (333 μc/μmole), [^{14}C]valine (185 μc/μmole), and [^{14}C]isoleucine (222 μc/μmole) were all obtained from New England Nuclear Corp.

The cell-free system was prepared by the method previously described (Holland and McCarthy, 1964) which is modified from the procedure of Matthaei and Nirenberg (1961). After preparation of the extract in the French pressure cell by disruption at 10,000–13,000 psi and removal of the debris by centrifugation at 30,000*g* for 15 min, the resulting S30 supernatant was dialyzed at 4° for 24 hr *vs.* a large volume of buffer containing 0.01 M Tris–HCl, pH 7.8, 0.06 M KCl, 0.006 M mercaptoethanol, and 10^{-4} M magnesium acetate. Thus, the procedure differs mainly by the use of very low magnesium concentrations. This serves to dissociate ribosomes to 30S and 50S subunits and liberate pre-

* *Received February 4, 1966.* This investigation was supported in part by Public Health Service Research Grant, GM 12449, from the National Institute of General Medical Sciences and by the National Science Foundation Grant, GB 2301.

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[1] Abbreviations used: DNA and RNA, deoxyribo- and ribonucleic acids; PEP, phosphoenolpyruvate; UTP, CTP, and GTP, uridine, cytidine, and guanosine triphosphates.

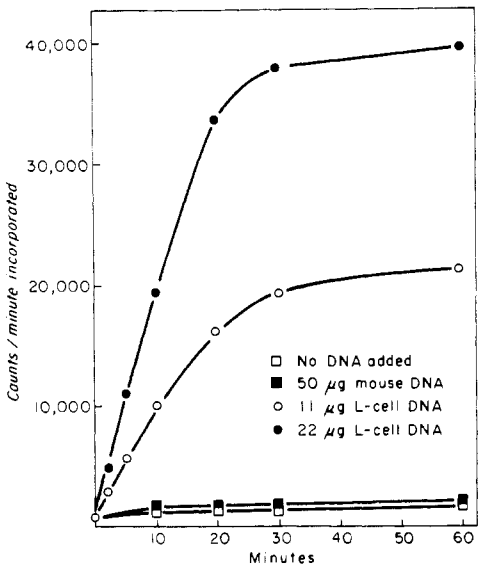

FIGURE 1: Stimulation of [^{14}C]isoleucine incorporation by mouse L cell DNA. Reaction mixtures (0.1 ml) contained 50 μmoles of NH_4Cl, 12 μmole of $Mg(CH_3COO)_2$, 6 μmoles of β-mercaptoethanol, 20 μmoles of Tris, pH 7.8, 0.1 μmole of GTP, 1 μmole of ATP, 5 μmoles of phosphoenolpyruvate (Na salt), 20 μg of PEP kinase, 0.02 μmole each of all [^{12}C]-amino acids, 0.2 μc of [^{14}C]isoleucine, and 2 mg of S30 protein. Denatured mouse DNA or mouse L cell DNA were added as indicated. Samples (0.1 ml) were removed at intervals and assayed for hot trichloroacetic acid insoluble radioactivity as described.

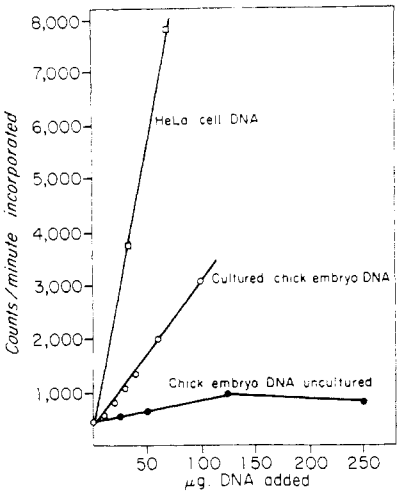

FIGURE 2: Effect of *in vitro* cultivation on the activity of chick cell DNA in stimulating amino acid incorporation. Reaction mixtures (0.5 ml) as in Table I containing 0.01 μmole of each [^{12}C]amino acids, 0.1 μc of [^{14}C]isoleucine, 1 mg of S30 protein, and various amounts of denatured HeLa cell DNA or chick cell DNA isolated either directly from 10-day embryos or from the same cells after cultivation *in vitro* for 10 days.

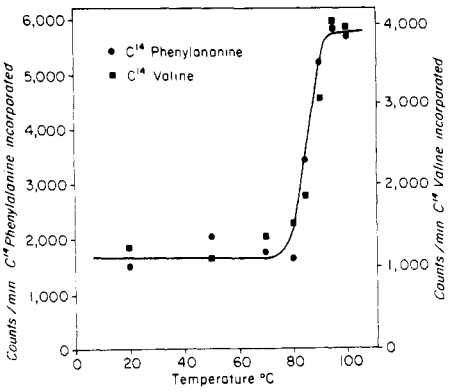

FIGURE 3: Effect of heating HeLa cell DNA on its ability to stimulate [^{14}C]phenylalanine or [^{14}C]valine incorporation. Reaction mixtures (0.5 ml) containing 0.01 μmole each of all [^{12}C]amino acids and 0.1 μc of [^{14}C]phenylalanine or [^{14}C]valine, 1 mg of S30 protein, and 30 μg of HeLa cell DNA. The DNA was previously heated for 5 min at the indicated temperatures in 0.1 M KCl at a concentration of 200 μg/ml, fast cooled, alcohol precipitated, and redissolved in 0.01 M KCl at 500 μg/ml.

TABLE I: Effect of Mg^{2+} Concentration in Dialysis on the Ability of the S30 Fraction to Function in Polypeptide Synthesis.[a]

Nucleic Acid Addn	cpm Incorpd	
	10^{-2} M Mg^{2+} S30	10^{-4} M Mg^{2+} S30
None	80	100
8 μg of poly U	7,770	19,800
50 μg of f2 phage RNA	504	810
30 μg of HeLa cell DNA	1,800	2,790

[a] The reaction mixture contained the following, each in 0.5-ml amounts: 25 μmoles of NH_4Cl, 6 μmoles of $Mg(CH_3COO)_2$, 3 μmoles of β-mercaptoethanol, 10 μmoles of Tris, pH 7.8, 0.05 μmole of GTP, 0.5 μmoles of phosphoenolpyruvate (Na salt), 10 μg of pyruvate kinase, 0.01 μmole each of all [^{12}C]amino acids, 0.1 μc of [^{14}C]phenylalanine, and 0.05 ml of S30 containing 0.5 mg of S30 protein dialyzed either in 10^{-2} M Mg^{2+} or 10^{-4} M Mg^{2+}. The S30 protein was added last to the reaction mixture which was then incubated at 37° for 30 min.

existing messenger RNA, which is probably destroyed during dialysis. As shown by the data in Table I, the resulting cell-free system is considerably more active for stimulation by poly U, f2 RNA, or HeLa DNA compared with one prepared in parallel using 10^{-2} M magnesium acetate during dialysis. The dialyzed S30 fraction was stored frozen in aliquots in sealed tubes at

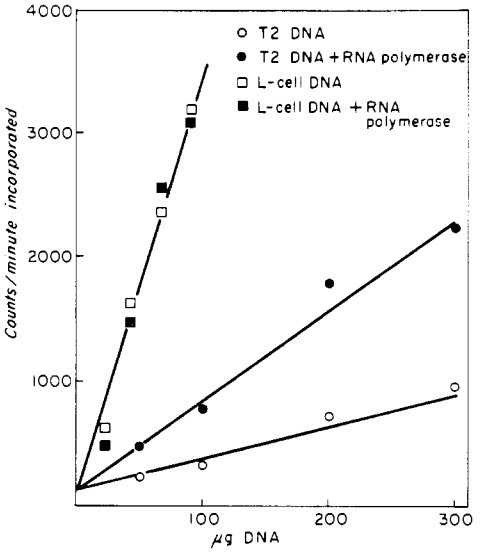

FIGURE 4: The influence of RNA polymerase on amino acid incorporation directed by T2 DNA and L cell DNA. Reaction mixtures (0.5 ml) contained 25 μmoles of NH_4Cl, 6 μmoles of $Mg(CH_3COO)_2$, 3 μmoles of β-mercaptoethanol, 10 μmoles of Tris, pH 7.8, 0.5 μmole each of ATP, CTP, GTP, and UTP, 2.5 μmoles of phosphoenolpyruvate (Na salt), 10 μg of PEP kinase, 1 mg of S30 protein, 0.01 μmole each of all [^{12}C]amino acids, 0.1 μc of [^{14}C]phenylalanine, and various amounts of either denatured L cell DNA or native T2 DNA. Where indicated, 50 units of purified RNA polymerase was also present.

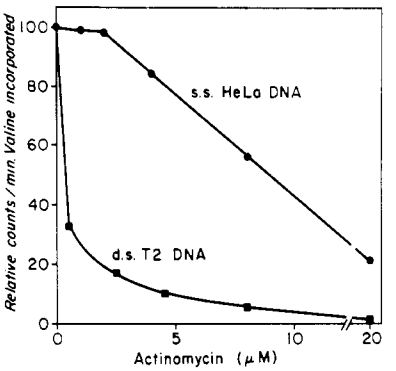

FIGURE 5: The effect of actinomycin on the stimulation of [^{14}C]phenylalanine incorporation by HeLa DNA or T2 DNA. Reaction mixtures (0.5 ml) were used as in Figure 4, including 1 mg of S30 protein and 50 μg of native T2 DNA or 50 μg of denatured HeLa cell DNA. RNA polymerase (80 units) was present in mixtures containing T2 DNA.

−85° or below until used. It was not preincubated or treated with deoxyribonuclease.

The reaction mixture of Table I was modified from that of Matthaei and Nirenberg (1961) by the omission of UTP and CTP and by the substitution of NH_4Cl for KCl. All components were added prior to the addition of the S30 fraction. After incubation at 37° for 30 min the reaction mixture was diluted, precipitated with trichloroacetic acid, heated to 90° for 15 min, and centrifuged. The protein precipitate was redissolved in 0.05 N NaOH, reprecipitated, and redissolved. The final trichloroacetic precipitate was collected on a membrane filter, dried, and counted in a Packard scintillation counter. One microcurie was equivalent to *ca.* 10^6 cpm.

Results

Source of Active DNA. DNA with the ability to stimulate amino acid incorporation may be isolated from a number of cell types grown in tissue culture. Most of the previously reported observations (Holland and McCarthy, 1964) were made with DNA from human HeLa cells, but other cell culture sources provide DNA of similar activity. Figure 1 illustrates an experiment in which denatured DNA from mouse L fibroblasts was added to an amino acid incorporating system at various levels. The stimulation is proportional to the amount of added DNA and the incorporation of labeled amino acid continues for *ca.* 30 min. Similar results were obtained with DNA isolated from other continuous line cell cultures.

In addition, cells put into primary culture rapidly undergo a change in which their DNA becomes active; *e.g.*, if DNA is prepared directly from embryonic chick cells and also from a parallel group of cells cultivated for 10 days in Eagle's medium, only the latter preparation has activity. This is demonstrated in Figure 2 where it is also apparent that DNA from the primary culture does not have the same activity as that prepared from HeLa cells grown in this medium for many generations. Primary cultures derived from other sources, such as human amniotic membrane and mouse embryos, provided similar results although in each case the specific activity acquired by the DNA did not approach that of HeLa or L cell DNA.

Necessity for Denaturation. If DNA participates as a template rather than a primer for RNA synthesis, it is to be expected that it will be active only when single stranded (Holland and McCarthy, 1964). This may be demonstrated by following the activity of samples of native HeLa cell DNA after they have been heated to different temperatures (Figure 3). The activity increases at a temperature corresponding to that of denaturation of the DNA and is complete at 90–95°. Separate experiments showed the T_m of the DNA to be 85° in this solvent. The stimulatory activity for both phenylalanine and valine incorporation increased in parallel. It will be noted that unheated DNA has some activity perhaps implying that native DNA is partially effective or that it is already partially denatured. The basis for this effect will be discussed in a later paragraph.

Necessity for RNA Synthesis. Since the cell-free system used is a crude one, it is clearly possible that the activity of these DNA's in stimulating protein synthesis

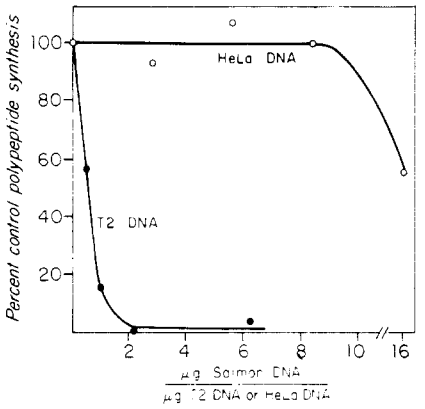

FIGURE 6: Inhibition of [^{14}C]phenylalanine incorporation by the addition of denatured salmon DNA. Reaction mixtures (0.5 ml) as in Figure 4 including 50 units of RNA polymerase, 35 μg of either native T2 DNA or denatured HeLa DNA, and various amounts of denatured salmon DNA.

results from an effect on intermediate RNA synthesis rather than template activity. Such a coupled system in which DNA stimulates amino acid incorporation has been studied in detail particularly by Wood and Berg (1962, 1964). The requirements for this effect, over and above those for protein synthesis itself, are double-stranded or native DNA, RNA polymerase, and all four ribonucleotide triphosphates. It has already been shown that for stimulation by HeLa cell DNA, denatured DNA is more active (Figure 3) and that only ATP and GTP are required (Holland and McCarthy, 1964). Other contrasts between the two types of DNA stimulation will be documented below.

The cell-free system used in these experiments does contain appreciable amounts of RNA polymerase activity (Holland and McCarthy, 1964). However, the addition of denatured HeLa cell DNA does not stimulate RNA synthesis to a greater degree than other denatured DNA samples, themselves inactive, in stimulating amino acid incorporation (Holland and McCarthy, 1964). Thus, the effect on amino acid incorporation cannot be attributed to a large increase in RNA synthesis. On the other hand, in spite of the intrinsic polymerase activity of the system, it is possible to demonstrate a requirement for RNA polymerase in the stimulation of [^{14}C]phenylalanine incorporation by native T2 DNA (Figure 4) in agreement with Wood and Berg (1962). In parallel experiments in which denatured L-cell DNA was added, the incorporation of amino acids was independent of the addition of RNA polymerase, again suggesting that the denatured DNA was a direct template.

A similar conclusion may be reached from the results of an experiment comparing the effect of actinomycin on protein synthesis directed either by native T2 DNA or denatured HeLa DNA. The former effect mediated by RNA synthesis is highly sensitive to this agent (Hurwitz *et al.*, 1962) while HeLa DNA remains active even in the presence of 2 μM actinomycin (Figure 5). Actinomycin has more affinity for native DNA than denatured DNA (Kahan *et al.*, 1963) and would, therefore, be expected to inhibit the activity of DNA in priming RNA synthesis more than its activity as a single-stranded template.

In their studies of DNA-dependent polypeptide synthesis, Wood and Berg (1964) demonstrated that denatured DNA from many sources was inactive. This cannot be attributed to a lack of RNA synthesis for, in fact, denatured DNA has a higher affinity for RNA polymerase. This affinity is so high that single-stranded DNA strongly inhibits the stimulation of protein synthesis by double-stranded DNA. The role of RNA synthesis and RNA polymerase in amino acid stimulation may, therefore, be studied by adding denatured DNA as an inhibitor. It is shown in Figure 6 that the addition of low levels of denatured salmon DNA to a T2 DNA-stimulated system is very inhibitory as described by Wood and Berg (1964). On the other hand, a parallel experiment with HeLa cell DNA shows that an eightfold excess of denatured salmon DNA may be added without any effect on amino acid incorporation. Since RNA polymerase is limiting and salmon DNA is itself inactive, a competitive effect is to be expected if RNA synthesis plays any role in polypeptide formation.

Role of Streptomycinoid Antibiotics. It can be shown by experiments to be described that the peculiar activity of HeLa cell and other DNA's is not an intrinsic property of the DNA itself but rather a result of a factor

TABLE II: Effect of Deoxyribonuclease Treatment on the Ability of Deoxyribonucleic Acid to Stimulate Amino Acid Incorporation.[a]

Addn	cpm Incorpd
(1) None	840
(2) 30 μg of denatured HeLa cell DNA	16,900
(3) 30 μg of denatured HeLa cell DNA, deoxyribonuclease treated and boiled	2,800
(4) Same as 3 but with 50 μg of denatured salmon DNA added after boiling and cooling	61,400
(5) 30 μg of native HeLa cell DNA	3,200
(6) 30 μg of native HeLa cell DNA, deoxyribonuclease treated and boiled	2,820
(7) Same as 6, but with 50 μg of denatured salmon DNA added after boiling and cooling	28,900

[a] As described in Table I, 0.5-ml reaction mixtures contained 0.01 μmole each of [^{12}C]amino acid, 0.15 μc of [^{14}C]phenylalanine and 1 mg of S30 protein. Where noted, DNA's were treated at 37° for 30 min with 10 μg/ml of deoxyribonuclease in Tris, 10^{-2} M, and $MgCl_2$, 10^{-2} M, and subsequently boiled for 2 min to destroy the enzyme.

associated with those DNA's. As shown in Table II, pretreatment of denatured HeLa cell DNA with deoxyribonuclease abolishes activity. On the other hand, this treatment releases a factor which can activate a normally inactive DNA. This may be demonstrated by boiling the deoxyribonuclease-treated preparation and adding salmon DNA. By the same means, it may be shown that native HeLa DNA does not lose activity on deoxyribonuclease treatment. Thus, the double-stranded molecule has no template activity itself but does stimulate amino acid incorporation through an associated factor. This explains the initial activity of unheated DNA in Figure 2.

The factor associated with cultured cell DNA can be shown to be streptomycin derived from the growth medium. Thus, cells grown in the absence of streptomycin contain inactive DNA. The effect may be duplicated by exposing the purified DNA to streptomycin *in vitro* or by adding streptomycin or other related antibiotics to the cell-free protein-synthesizing system (McCarthy and Holland, 1965).

Discussion

The formation of complexes between ribosomes and DNA has often been reported. Thus, native or denatured DNA can associate with calf thymus ribosomes (Naora, 1962). This resulted in a slight stimulation of amino acid incorporation (Frenster *et al.*, 1961), although it was not clear whether RNA synthesis was involved. Later Takanami and Okamoto (1963) demonstrated the association of single-stranded DNA, particularly that of ϕX 174, with ribosomes. Other DNA's were relatively inactive unless treated with formaldehyde to alter their secondary structure. A more well-defined complex is that studied by Byrne *et al.* (1964) in which ribosomes are bound to DNA by means of a nascent strand of messenger RNA. This is presumably the normal situation and reflects the activity of native DNA in stimulating protein synthesis by means of transcription. The requirements for this DNA-dependent polypeptide synthesis have been extensively studied (Wood and Berg, 1962, 1964) and it has been the purpose of this report to compare and contrast them with the effect of HeLa cell and other DNA's.

There are two main differences which serve to distinguish the two roles of DNA. For stimulation through intermediate RNA synthesis the DNA must be in a double-stranded or native form (Wood and Berg, 1964). On the other hand, in the present experiments only denatured DNA is active. The requirement for RNA synthesis implies the necessity for RNA polymerase and all four ribonucleoside triphosphates. The participation of RNA synthesis, when denatured HeLa cell DNA is used, is excluded since neither UTP and CTP (Holland and McCarthy, 1964) or RNA polymerase are required. The insensitivities to actinomycin or competing denatured DNA are also strong arguments against intervening RNA synthesis. Finally, in experiments in which apurinic acid activated by neomycin was used, the amino acids incorporated proved to be those expected from the restricted code words in the apurinic acid rather than those in a putative complementary RNA molecule (McCarthy and Holland, 1965).

There remains little doubt, therefore, that denatured DNA can act directly as a template in a cell-free system and that this is effected by streptomycin or similar antibiotics. The special properties of DNA isolated from well-washed cells from tissue culture are due simply to the adsorption of these antibiotics to the DNA *in vivo*. This implies that these antibiotics penetrate the cells and probably also the nucleus where they associate with the DNA. This association is a very tenacious one and is resistant to boiling or alkali treatment (Holland and McCarthy, 1964). The presence of this foreign substance in such cells should certainly be borne in mind, both in *in vivo* experiments and in the analysis of cell components; *e.g.*, it is clear that many attempts to transform mammalian cells have been made with streptomycin-containing DNA.

The basis for the effectiveness of these antibiotics in promoting template activity by DNA has been discussed elsewhere (McCarthy and Holland, 1965). It is sufficient to conclude here that a generalized barrier to the translation of polynucleotide messages exists in the cell for ribosomal RNA, soluble RNA, and DNA. In each case this restriction may be circumvented by the addition of these antibiotic molecules, for soluble RNA and ribosomal RNA also stimulate protein synthesis *in vitro* in their presence (Holland *et al.*, 1966).

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