*STIMULATION OF PROTEIN SYNTHESIS IN VITRO BY DENATURED DNA**

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Following the recognition of a role for ribosomes in protein synthesis,[1] and the evolution of a messenger RNA theory,[2] it has been well established[3] that certain types of RNA and synthetic polyribonucleotides can serve a coding function in protein synthesis. Current evidence suggests that only one of the two strands of DNA serves as a template for synthesis of messenger RNA.[4] It is obvious, therefore, that the other strand of DNA should exhibit a nucleotide sequence resembling that of messenger RNA. The present study was initiated to determine whether single-stranded DNA might be capable of directly programing protein synthesis on *E. coli* ribosomes in a manner analogous to messenger RNA action, or whether the 5-methyl group of thymine and the lack of a 2′ hydroxyl group in deoxyribose imposes steric or configurational blocks to translation of single-stranded DNA. In the assay of Nirenberg and Leder,[5] the 2′ hydroxyl group is apparently indispensable for codeword recognition by sRNA's since oligodeoxynucleotides may not be substituted for the corresponding oligoribonucleotides. On the other hand, Takanami and Okamoto demonstrated the formation of polyribosome complexes by denatured DNA and *E. coli* ribosomes *in vitro*, and Szer and and Ochoa showed that polyribothymidylate promotes phenylalanine incorporation by *E. coli* ribosomes.[6]

Materials and Methods.—*E. coli* strain K12 was grown at 37° in Difco brain-heart infusion broth and harvested by centrifugation while still in the log phase of growth. Cells were suspended in 2 or 3 vol of the standard buffer of Matthaei and Nirenberg;[7] 0.01 *M* tris·HCl, pH 7.8; 0.01 *M* magnesium acetate; 0.06 *M* KCl and 0.006 *M* mercaptoethanol. The frozen cells were disrupted in a French pressure cell at 10,000–13,000 psi. The supernatant solution, after removal of cell wall debris by centrifugation at 30,000 *g* for 15 min (S30), was dialyzed against the above standard buffer, modified by reduction of the magnesium acetate concentration to 10^{-4} *M*, for 24 hr at 4°. The dialyzed S30 fraction was placed into sealed tubes in small aliquots and stored frozen at −85°C or below until used.

Deoxyribonuclease I was a crystalline preparation obtained from Worthington Biochemical Co., electrophoretically purified to remove traces of ribonuclease. Pancreatic ribonuclease was a crystalline preparation from Boehringer & Soehne, boiled before use to inactivate traces of deoxyribonuclease. Unlabeled amino acids, ATP, CTP, UTP, CTP, phosphoenolpyruvate Na salt (PEP), PEP kinase, and poly U, were obtained from Calbiochem, as were salmon sperm DNA and calf thymus DNA and the enzyme pronase. Puromycin was a generous gift from Lederle Laboratories, and chloramphenicol was obtained from Parke-Davis and Co. Actinomycin D was kindly provided by Merck, Sharp and Dohme Co. C^{14} yeast protein hydrolysate (850 μc/mg) and C^{14} L-phenylalanine (141 mc/mM) were obtained from Schwarz, and C^{14} L-valine (200 mc/mM) was obtained from New England Nuclear Corporation.

Two methods were used for the extraction of DNA. Nuclei prepared from animal tissues or intact cells from tissue cultures were lysed in a solution containing 0.2% sodium dodecyl sulfate, 50 μg/ml pronase, 0.001 *M* EDTA, and 27% w/v sucrose[8] by incubation at 37° for several hours. Following 2 extractions with equal volumes of phenol at 60°, DNA was precipitated several times from ethanol. The final precipitate was dried in a stream of nitrogen to remove ethanol, dissolved in a small volume of distilled water, and frozen until used. Alternatively, DNA was recovered from the phenol water interphase after applying the Scherrer and Darnell procedure for quantitative extraction of RNA[9] and purified by an additional phenol extraction and multiple

ethanol precipitations. Apurinic acid was prepared by incubating DNA at pH 1.5 for 15 hr at 37°.[10] T2 phage DNA was a generous gift from Mr. R. Crouch.

The reaction mixture described in Table 1 was modified from that of Matthaei and Nirenberg[7] by substituting NH_4Cl for KCl as suggested by the data of Conway,[11] by omitting UTP and CTP and increasing the level of GTP. All ingredients of the reaction mixture were added prior to the addition of dialyzed S30 fraction. After incubation the reaction was stopped by the addition of 1 drop of 1 *N* NaOH. The mixture was diluted to 3 ml, and 3 ml of 10% TCA were added, followed by heating to 90° for 15–20 min. The protein precipitate was collected twice by centrifugation with intervening dispersal in NaOH and finally filtered onto a membrane filter. Samples were washed with 5% TCA, dried, and counted in a Nuclear-Chicago scintillation counter where 1 μc of C^{14} was equivalent to approximately 10^6 cpm.

HeLa cells and L cells were grown as monolayers on glass in a medium consisting of 7% calf serum, 0.1% yeast extract (Difco), and 0.1% Proteose peptone #3 (Difco) in Hanks' balanced salt solution buffered by the addition of 0.06% sodium bicarbonate and containing 100 units/ml penicillin, 100 μg/ml steptomycin, and 25 units/ml mycostatin.

Results.—Various modifications of the *in vitro* system of Nirenberg and Matthaei[3] were used in attempts to stimulate protein synthesis with denatured DNA from a wide variety of organisms. Regardless of whether washed *E. coli* ribosomes or crude supernatants were employed, all the DNA preparations tested initially failed to stimulate amino acid incorporation significantly. Finally, an *E. coli* S30 fraction was employed which had been dialyzed against a salt solution with a magnesium concentration of 10^{-4} *M* in order to dissociate endogenous messenger RNA from ribosomes. Under these conditions, it was found that denatured HeLa cell DNA produced marked stimulation of amino acid incorporation.

Table 1 shows that 100 μg of heat-denatured HeLa cell DNA stimulated phenyl-

TABLE 1

EFFECT OF VARIOUS NUCLEIC ACIDS ON C^{14} PHENYLALANINE INCORPORATION

Additions	Cpm incorporated
None	1,110
100 μg Poly U	135,000
100 μg Chick embryo DNA	1,300
100 μg Bacterial DNA, Providence strain	1,240
100 μg Mouse embryo DNA	2,250
100 μg Rabbit liver DNA	2,290
100 μg HeLa cell DNA, batch #4	71,200
100 μg HeLa cell DNA, batch #4 + 2 μg DNAase	5,830
100 μg Mouse sarcoma I DNA	2,570
100 μg HeLa cell RNA	982

The reaction mixture contained in 0.5 ml 25 μmoles NH_4Cl, 6 μmoles $Mg(CH_3COO)_2$, 3 μmoles β-mercaptoethanol, 10 μmoles tris pH 7.8, 0.05 μmoles GTP, 0.5 μmoles ATP, 2.5 μ-moles phosphoenolpyruvate Na salt, 10 μg PEP kinase, 1 mg S30 protein, 0.01 μmoles each of all amino acids except phenylalanine, 0.5 μc of C^{14} phenylalanine. All DNA's added were denatured by boiling in H_2O for 5 min and rapidly cooling. The S30 protein was added to the mixtures last, and incubation was at 37° for 60 min.

alanine incorporation more than 60-fold, whereas DNA extracted from bacteria, mice, chicks, and rabbits stimulated very slightly or not at all. It is also shown that 100 μg of total HeLa cell RNA failed to stimulate incorporation in this system although tobacco mosaic virus RNA and f2 phage RNA do stimulate phenylalanine incorporation severalfold. The stimulation by HeLa cell DNA is sensitive to 2 μg of highly purified deoxyribonuclease.

Table 2 shows that 2 other batches of denatured DNA from HeLa cells caused comparable stimulation of incorporation. DNA from mouse L cells in culture is also active in contrast to DNA of crab testes, salmon sperm, and calf thymus. Native HeLa cell DNA stimulated phenylalanine incorporation, but not as effi-

TABLE 2

EFFECT OF VARIOUS DNA'S ON C^{14} AMINO ACID INCORPORATION

Additions	Cpm incorporated
1. None	1,470
2. 200 μg HeLa cell DNA batch #3, native	15,400
3. 200 μg HeLa cell DNA batch #3, denatured	56,200
4. 200 μg HeLa cell DNA batch #4, denatured	43,300
5. 200 μg Salmon sperm DNA, denatured	1,680
6. 200 μg Calf thymus DNA, denatured	2,010
7. 100 μg Crab testis DNA, *Cancer sp.*, denatured	2,460
8. 150 μg Mouse L-cell DNA, denatured	56,900
9. None	782
10. 200 μg HeLa cell DNA batch #4, denatured	41,100

Reaction mixture as described in Table 1. Expts. 1-8 contained 0.01 μmoles each C^{12} amino acid except phenylalanine, plus 0.5 μc C^{14} phenylalanine. Expts. 9 and 10 contained 0.01 μmoles each C^{12} amino acid and 0.5 μc C^{14} protein hydrolysate. Incubation 60 min at 37°C.

ciently as did the same batch of DNA after heat denaturation. This native DNA probably contained melted regions since it was dissolved in distilled water prior to addition to the reaction mixture. Table 2 also demonstrates (expts. 9 and 10) that denatured HeLa cell DNA stimulates incorporation of a mixture of C^{14} amino acids (protein hydrolysate) into acid-insoluble polypeptide.

Figure 1 illustrates the kinetics of C^{14} protein hydrolysate incorporation by *E. coli* ribosomes with and without addition of DNA. It can be seen that DNA-mediated incorporation is linear for at least 30 min. Figure 2 compares stimulation of C^{14} phenylalanine and C^{14} valine incorporation by various amounts of poly U and HeLa cell DNA. It is apparent that incorporation is a linear function of added DNA in the range of 10–250 μg.

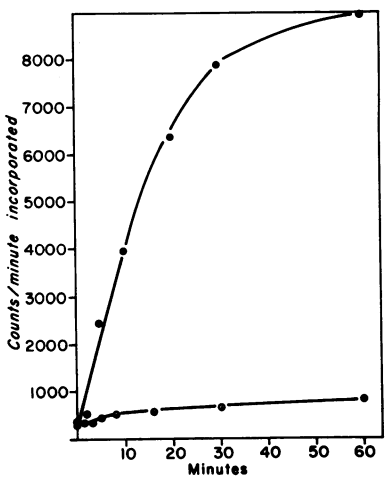

FIG. 1.—Rate of incorporation of amino acids. Reaction mixture as in legend to Table 1. Total volume 1.0 ml containing 0.04 μmoles of each C^{12} amino acid and 0.5 μc C^{14} protein hydrolysate. 0.1-ml samples were removed at intervals and assayed for TCA-precipitable radioactivity as described in *Materials and Methods*. ○ = No added DNA. ● = 200 μg denatured HeLa DNA, batch #6, added at zero time.

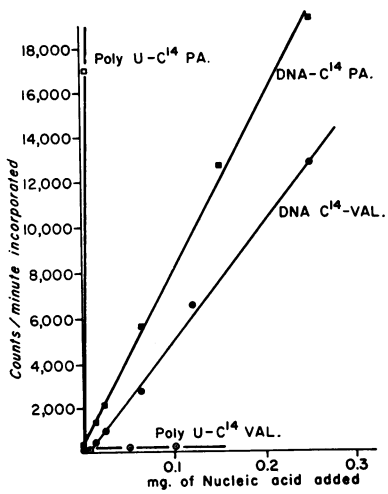

FIG. 2.—Effect of various amounts of poly U or HeLa cell DNA on C^{14} valine and C^{14} phenylalanine incorporation. Conditions as in legend to Table 1. Each incubation contained 0.01 μmoles of each amino acid and 0.2 μc of either C^{14} phenylalanine or C^{14} valine. Incubation 20 min at 37°C.

TABLE 3

EFFECT OF INHIBITORS AND NUCLEOSIDE TRIPHOSPHATES ON DNA-STIMULATED AMINO ACID INCORPORATION

Additions	Cpm incorporated
DNA omitted	1,190
None	12,200
GTP, UTP, CTP	13,200
Puromycin	1,520
Chloramphenicol	5,900

Reaction mixture as in Table 1. 100 μg denatured HeLa cell DNA batch #7 (pretreated with 0.3 *N* NaOH for 1 hr at 60°C to digest any accompanying RNA) was present in all but the first incubation. 0.01 μmoles all C^{12} amino acids and 0.2 μc C^{14} phenylalanine were present throughout. Where noted, 0.5 μmoles each of GTP, UTP, and CTP, 5 μg puromycin, or 50 μg chloramphenicol were also present. Incubation 30 min at 37°C.

DNA-mediated amino acid incorporation is sensitive to the usual inhibitors of protein synthesis. It can be seen in Table 3 that puromycin completely inhibited DNA stimulation, and chloramphenicol reduced incorporation to about 50 per cent of the normal level. The effect of chloramphenicol is more reminiscent of its partial inhibition of poly U-directed polyphenylalanine[3, 7] synthesis than of its complete inhibition of polypeptide synthesis mediated by RNA made on double-stranded DNA.[12] The addition of UTP and CTP does not increase the amino acid uptake as might be expected if the DNA effect were due to priming of RNA synthesis. Since the S30 fraction employed in all these studies was extensively dialyzed prior to use, the only major source of UTP and CTP would be degradation of nucleic acids to nucleoside mono- or diphosphates followed by kinase action.

RNA polymerase activity in the S30 fraction employed was measured in the presence of all 4 triphosphates (Table 4). Most of the ATP incorporated could be

TABLE 4

INCORPORATION OF C^{14} ATP INTO POLYNUCLEOTIDE BY *E. coli* S30 FRACTION

DNA added	Cpm ATP incorporated	mμMole C^{14} ATP incorporated/ hour/μg protein
None	795	9.4
50 μg T2 bacteriophage DNA, native	1,160	14.0
50 μg T2 DNA, denatured	1,050	12.6
100 μg HeLa cell DNA, native	1,050	12.6
100 μg HeLa cell DNA, denatured	870	10.5
75 μg Mouse L cell DNA, native	1,200	14.1
75 μg Mouse L cell DNA, denatured	980	11.9
5 μg DNAase added	120	1.4

The reaction mixture 0.25 ml contained 10 μmoles tris pH 7.9, 0.25 μmole $MnCl_2$, 1.0 μmole $MgCl_2$, 80 mμM each of ATP, CTP, GTP, and UTP, 0.05 μc C^{14} ATP, and 0.05 ml S30 containing 1 mg of protein.[13] Incubation 20 min at 37°C.

accounted for by *E. coli* DNA already present in the S30 fraction. Addition of T2, HeLa, or L cell DNA, either native or denatured, caused only slightly increased incorporation, while deoxyribonuclease greatly decreased ATP incorporation into acid-insoluble polynucleotide. It was not possible to remove *E. coli* DNA by adding deoxyribonuclease to the S30 fraction employed in these studies because of the effects the deoxyribonuclease would later have on added DNA. Table 4 also shows that denatured DNA was slightly less active as a template for RNA synthesis than was native DNA, in contrast to the greater ability of the denatured DNA to stimulate amino acid incorporation. The RNA polymerase activity revealed by the measurements and expressed in the units of Chamberlin and Berg[13] is comparable to that found by these authors in a crude supernatant fraction.

TABLE 5

EFFECT OF PRETREATMENT WITH RIBONUCLEASE, ALKALI, OR ACID ON ABILITY OF HeLa CELL TO STIMULATE POLYPEPTIDE SYNTHESIS

Template DNA employed	Cpm C^{14} amino acid incorporation	
1. None (control for expts. 2, 3)	1,520*	
2. HeLa cell DNA batch #2 (200 μg)	54,000*	
3. HeLa cell DNA batch #2 (200 μg) pretreated with 100 μg/ml RNAase	60,500*	
4. None (control for expts. 5, 6)	797†	
5. HeLa cell DNA batch #6 (250 μg)	59,982†	
6. HeLa cell DNA batch #6 (120 μg) pretreated with 0.3 *N* NaOH for 0 hr at 60°	54,100†	
7. None (control for expts. 8, 9)	702*	496‡
8. HeLa cell DNA batch #11 (110 μg)	27,600*	7,900‡
9. Apurinic acid yield from 220 μg HeLa cell DNA batch #11	2,476*	1,021‡

Reaction conditions as in Table 1 except that different C^{14} amino acids were employed as indicated below. Where employed, ribonuclease treatment was carried out for 1 hr at 37°, followed by phenol-SDS extraction at 60° and repeated reprecipitation of single-stranded DNA with ethanol.

* 1 μc C^{14} L-phenylalanine was used in each 0.5 ml reaction mixture.

† 1 μc C^{14} yeast protein hydrolysate was used in each 0.5 ml reaction mixture.

‡ 0.5 μc C^{14} L-lysine was used in each 0.5 ml reaction mixture.

To rule out the possibility that the stimulatory activity of HeLa cell DNA on protein synthesis was due to small amounts of contaminating RNA, some denatured DNA preparations were pretreated with ribonuclease and freed of the enzyme by phenol-SDS extraction at 60° before addition to the reaction mixture. Other preparations of RNA were incubated at 60° in 0.3 *N* NaOH for 1 hr, under which conditions DNA is completely denatured but not extensively degraded, and RNA is completely degraded to mononucleotides. It can be seen in Table 5 that such HeLa cell DNA preparations are still capable of stimulating amino acid incorporation. On the other hand, it is apparent from Figure 3 that the addition of small amounts of ribonuclease to the reaction mixture inhibited amino acid incorporation equally where the added polynucleotide was poly U or denatured DNA. This may reflect enzyme degradation of a polyribonucleotide product primed by added DNA or an effect on ribosomal RNA or transfer RNA. It appears from Table 5 that removal of purines from denatured HeLa DNA by acid treatment to yield apurinic acid destroyed nearly all ability to stimulate C^{14} phenylalanine or C^{14} lysine incorporation. Short tracts of thymidylate cannot, therefore, be responsible for the incorporation of phenylalanine. Denatured DNA primes homopolymer (poly A, poly C) formation more efficiently than does native DNA.[13, 14] On the other hand, incorporation of C^{14} lysine (codon AAA[3]) was not markedly higher than that of any other amino acid tested using either HeLa cell DNA or its apurinic acid. Poly A formation is not, therefore, a likely intermediary in DNA stimulation of amino acid incorporation.

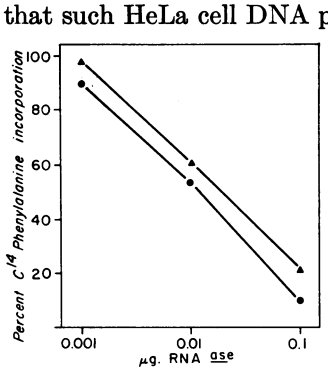

FIG. 3.—Effect of addition of ribonuclease on incorporation of C^{14} phenylalanine stimulated by poly U and HeLa DNA. Each incubation mixture contained 100 μg of poly U or 125 μg of HeLa cell DNA denatured and treated with NaOH, 0.01 μmoles of amino acid, and 0.5 μc C^{14} phenylalanine. Without ribonuclease added, 240,000 cpm were incorporated in the presence of poly U, and 35,000 cpm in the presence of HeLa cell DNA. Incubation 30 min at 37°C. ● = + Poly U. ▲ = + HeLa cell DNA.

If DNA were acting indirectly as a template for RNA polymerization, this system should show some of the characteristics of that described by Wood and Berg[12] in which native T2 phage DNA primes RNA synthesis, which then stimulates protein synthesis by ribosomes added either simultaneously or subsequently. However, it can be seen in Table 6 that although native T2 phage DNA does stimulate the S30

TABLE 6

EFFECT OF ADDITION OF NUCLEOSIDE TRIPHOSPHATES ON DNA STIMULATION OF C^{14} VALINE INCORPORATION

Additions	Cpm incorporated
None	106
None + triphosphates	94
100 μg T2 DNA, native	735
100 μg T2 DNA, native + triphosphates	2,806
100 μg T2 DNA, denatured	110
100 μg T2 DNA, denatured + triphosphates	119
100 μg HeLa cell DNA, native	1,930
100 μg HeLa cell DNA, native + triphosphates	1,390
100 μg HeLa cell DNA, denatured	7,260
100 μg HeLa cell DNA, denatured + triphosphates	6,490

Conditions as in Table 1. Incubation mixtures contained 0.01 μmoles all amino acids and 0.1 μc C^{14} valine. T2 DNA was denatured by boiling for 5 min in H_2O. HeLa DNA (batch #4) was denatured in the same way and treated with NaOH for 1 hr at 60°C to digest any accompanying RNA. Where indicated, 0.5 μmoles each of ATP, GTP, UTP, and CTP were added in addition to the normal amounts of ATP and GTP. Incubation 60 min at 37°C.

protein-synthesizing system employed in our studies, it requires added nucleoside triphosphates for maximal activity. Furthermore, T2 phage DNA had no activity after heat denaturation as reported by Wood and Berg.[12] In contrast, nucleoside triphosphates do not augment the stimulation by HeLa cell DNA, and denatured HeLa cell DNA is actually more active than native DNA (Table 6). While actinomycin D abolished stimulation by native T2 DNA, presumably by preventing RNA synthesis, its effect on the denatured HeLa cell DNA and poly U was marginal (Table 7). All of these observations argue against the possibility of RNA synthesis as an intermediate step in the stimulation caused by denatured DNA prepared from mammalian cells in culture.

TABLE 7

EFFECT OF ACTINOMYCIN D ON DNA-STIMULATED AMINO ACID INCORPORATION

Nucleic acid addition	Cpm incorporated
1. None	326
2. None + actinomycin	357
3. 50 μg Poly U	59,800
4. 50 μg Poly U + actinomycin	50,600
5. 100 μg HeLa cell DNA, denatured	14,400
6. 100 μg HeLa cell DNA, denatured + actinomycin	10,800
7. None	20
8. None + actinomycin	31
9. None + triphosphates	45
10. None + triphosphates + actinomycin	29
11. 100 μg HeLa cell DNA, denatured	4,340
12. 100 μg HeLa cell DNA, denatured + actinomycin	3,700
13. 100 μg T2 DNA, native	217
14. 100 μg T2 DNA, native + actinomycin	107
15. 100 μg T2 DNA, native + triphosphates	1,380
16. 100 μg T2 DNA, native + triphosphates + actinomycin	109

Reaction mixture as described in Table 1. All incubations contained 0.01 μmoles all amino acids, and in preparations 1–6, 0.3 μc C^{14} phenylalanine, and in preparations 7–16, 0.1 μc C^{14} valine. Where noted, 2.5 μg of actinomycin D and 0.5 μmoles each of ATP, GTP, UTP, and CTP were also present. HeLa cell DNA batch 7 was denatured by boiling for 5 min and treated with NaOH. Incubation 30 min at 37°C.

Discussion.—The results presented above clearly indicate that denatured DNA from certain cells grown in cell culture causes stimulation of *in vitro* polypeptide synthesis which is quite unlike the RNA-mediated stimulation reported by Wood and Berg[12] and by Byrne *et al.*[15] for native T2 phage DNA. It must be concluded that the denatured DNA from these cultured cells either associates with ribosomes directly and acts as a template for protein synthesis, or it is causing some indirect stimulation by serving as a single-stranded primer for an active polyribonucleotide. The latter mechanism cannot be excluded, but major differences exist between the present system and that documented by Wood and Berg.[12] In the first place, these authors found denatured DNA to be completely inactive in stimulation of amino acid incorporation, although active for RNA synthesis. Indeed, they showed that single-stranded DNA is an inhibitor of protein synthesis mediated by double-stranded DNA by virtue of its very high affinity for RNA polymerase.[12] HeLa cell DNA becomes more active after denaturation. The effect of HeLa cell DNA is not dependent on the addition of UTP and CTP, whereas the presence of these precursors greatly amplifies stimulation by native T2 DNA. While the system of Wood and Berg is sensitive to actinomycin, that using HeLa cell DNA is not. The presence of active template RNA as a contaminant of some DNA preparations is excluded by the retention of activity after alkaline digestion and by sensitivity to deoxyribonuclease. No accumulation of deoxyribonuclease-resistant activity was detected after preliminary incubation to allow RNA synthesis[12] and subsequent addition of amino acids and protein-synthesizing system (unpublished results).

Even if the effects reported here are mediated indirectly by RNA synthesis, it would still be of considerable interest to determine why only certain cells yield active DNA. In any case, it is difficult to explain why DNA from mouse L cells in cell culture was highly stimulatory, whereas DNA from mouse embryos or mouse peritoneal tumor cells was nearly inactive. Studies in progress suggest that other animal cell and tissue cultures yield DNA with stimulatory activity. The biochemical basis for the differences between DNA's which possess or do not possess such activity is being explored by studies of the degree of methylation and the sites of methylation[16] of the various DNA's. An understanding of the chemical differences between the two classes of DNA might allow extension of these studies to viral or bacterial DNA. Whether direct participation of DNA in protein synthesis has any role *in vivo* remains obscure, although such a role would prove attractive for models concerned with evolution of the earliest forms of life.

Finally, these findings question the validity of assays of messenger RNA in mixed nucleic acid preparations based upon stimulation of the uptake of amino acids into polypeptide by a cell-free system.

Summary.—Denatured DNA from cultured HeLa cells and mouse L cells greatly stimulated amino acid incorporation into polypeptides by an *E. coli* cell-free extract *in vitro*. Denatured DNA from other sources failed to exhibit this effect. This stimulation differs in several respects from previously reported systems in which native DNA stimulated polypeptide synthesis indirectly by acting as a template for RNA synthesis. These HeLa cell and L cell DNA's have the apparent characteristics of template material, although an indirect effect involving RNA synthesis cannot be rigorously excluded by the evidence thus far obtained.

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*PROPAGATION OF AN INTRANUCLEAR INCLUSION-FORMING AGENT FROM HUMAN CONDYLOMA ACUMINATUM**

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In an attempt to propagate an agent from tissues of *condyloma acuminatum*, a series of biopsies were taken from a young Negro woman who had typical lesions on the mucous membrane surface of the vagina. The tissue was minced with sharp scissors and treated with 0.5 per cent trypsin for 40 min. The fragments were then sedimented by centrifugation, the trypsin was removed, and the pieces were resuspended in double-strength Eagle's medium[1] containing 15 per cent fetal bovine serum.