Binding of Complementary Oligonucleotides to Free and Aminoacyl Transfer Ribonucleic Acid Synthetase Bound Transfer Ribonucleic Acid*

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ABSTRACT: Complementary oligonucleotides have been tested for their ability to hybridize to various sequences on $tRNA^{Ile}$ from *Escherichia coli* B. Strong binding is observed of complementary oligomers to the anticodon section and to the ACC sequence at the 3′ terminus. Strong binding of complementary oligomers to the dihydrouridine loop does not occur, possibly because of limitations placed by the three dihydrouridine residues in this loop. When $tRNA^{Ile}$ is complexed with the isoleucyl-tRNA synthetase, oligomers cannot be hybridized to either the anticodon section or to the ACC sequence at the 3′ terminus. This suggests that both the anticodon region and the 3′ terminus are covered or shielded in some way by the enzyme and, therefore, that the synthetase–tRNA interaction may encompass regions which are quite far apart in the tRNA.

The tight and specific binding of each aminoacyl-tRNA synthetase with its cognate tRNA presents the obvious question of the nature of the physical basis underlying the marked specificity and the large free energy of association. The challenge of this question has provoked a variety of studies on the interaction of modified tRNA molecules with their synthetases. Although considerable progress has been made (see reviews of Yarus, 1969, Zachau, 1969a,b, Mehler, 1970, and Chambers, 1971), many of these experiments suffer from the difficulty that the modification may alter portions of the tRNA conformation other than just the local region modified. Differences in the synthetase–tRNA interaction may therefore result from the change in overall RNA structure.

Not long ago, Uhlenbeck *et al.* (1970) and Lewis and Doty (1970) introduced a new method for investigating the conformation of nucleic acids. In particular, it was shown that the unpaired and unshielded regions of tRNA and of 5S RNA can hybridize complementary tri- and tetranucleotides (Uhlenbeck *et al.*, 1970; Lewis and Doty, 1970). Thus, with these probes, the exposed and unpaired segments of a molecule can be deduced and conclusions concerning the secondary and tertiary structure follow naturally. Specifically, this approach has provided evidence for the existence of the cloverleaf secondary structure of tRNA as well as for additional tertiary structure (Uhlenbeck *et al.*, 1970; O. C. Uhlenbeck, 1972).[1]

We report here the application of the oligonucleotide hybridization technique to a natural synthetase–tRNA complex. By comparing the ability of different regions on free and synthetase-bound tRNA to hybridize their complementary oligomers, one ideally can learn which sections of the tRNA are, and which sections are not, covered or blocked by the enzyme. Thus, the general geometric relationships between the tRNA and its synthetase can be studied. A variety of oligonucleotides which complement different segments of $tRNA^{Ile}$[2] (*E. coli* B) are first tested for their ability to hybridize to the uncomplexed nucleic acid. The cloverleaf arrangement of the base sequence of this tRNA, as determined by Yarus and Barrel (1971), is shown in Figure 1. Having identified oligomers which strongly bind to their complementary sites on $tRNA^{Ile}$, the nucleic acid is then complexed with IRS and presented with the same oligomers. The results obtained suggest that both the anticodon section and the 3′-terminal ACC sequence are blocked in the complex by IRS. The implications of these findings are discussed.

Materials and Methods

IRS was purified from *Escherichia coli* B according to previously published schemes (Baldwin and Berg, 1966; Eldred and Schimmel, 1972). Active enzyme concentration was determined by the standard ATP–PP_i isotope-exchange assay whereby one unit of enzyme activity corresponds to the amount which catalyzes the incorporation of 1 μmole of PP_i into ATP in 15 min under standard reaction conditions (Calendar and Berg, 1966). The molar concentration of enzyme was calculated from the fact that completely active enzyme has 72.8 units/nmole (Baldwin and Berg, 1966). Enzyme activity was used to determine concentration because some loss of activity of initially purified enzyme occurred during storage. Although loss of exchange activity may not necessarily reflect a corresponding loss in ability to bind tRNA (Iaccarino and Berg, 1969), it is shown below that complete elimination of oligonucleotide binding to $tRNA^{Ile}$ does not

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[2] Abbreviations used are: $tRNA^{Ile}$, isoleucine tRNA; $tRNA^{Tyr}$, tyrosine tRNA; $tRNA_f^{Met}$, formylmethionine tRNA; $tRNA^{Val}$, valine tRNA; IRS, isoleucyl-tRNA synthetase.

TABLE I: Tests and Controls for Binding to Anticodon Sequence UGAU.

Triplets	Approx Assocn Constant (M^{-1})
AUC	7,100
AUA	–
AUU	–
AUG	–
Tetramers	
AUCA	120,000
AUAC	–
AUAA	–
AUCC	20,000
AUCG	130,000
AUGC	–
AUCU	50,000

occur until somewhat greater than stoichiometric amounts (as determined by ATP–PP_i activity) of enzyme are added.

The $tRNA^{Ile}$ was purified to greater than 90% homogeneity from *E. coli* B unfractionated tRNA (Schwarz BioResearch), according to methods given elsewhere (Yarus and Berg, 1969; A. A. Schreier, 1972).[3] Purified $tRNA^{Ile}$ had an amino acid acceptor activity of about 1300 pmoles/ODU at 260 mμ, in 0.1 N NaOH. Concentrations of $tRNA^{Ile}$ were based on this relationship.

Tritium-labeled oligonucleotides were synthesized with polynucleotide phosphorylase according to Uhlenbeck *et al.* (1970). (These oligomers were synthesized in the laboratory of Dr. P. Doty (Harvard University) and Dr. I. Tinoco (University of California, Berkeley).) Dialysis experiments were conducted in a 100-μl dialysis cell (O. C. Uhlenbeck, 1972)[1] at 0°. Solutions investigated were made up in a solvent generally consisting of 0.01–0.02 M phosphate, 0.5–1.0 M NaCl, and 5–10 mM $MgCl_2$, at an apparent pH (glass electrode) of 6.5–6.9 at 0°. These variations in ionic concentrations are of no significant consequence, as far as the results reported here are concerned. The $tRNA^{Ile}$ concentrations employed generally fell in the range of 5–30 μM. The time of dialysis was usually greater than 40 hr, which is ample for the attainment of equilibrium with most oligonucleotides (see exception below). At the completion of dialysis, 2- to 5-μl samples were taken in duplicate or triplicate from each side of the membrane, mixed with 5 ml of Aquasol (New England Nuclear), and counted in a Packard scintillation counter.

Experimental Results

Hybridization of Free $tRNA^{Ile}$. Experimental results were analyzed in terms of the parameter R, where R = [free + bound (oligomer)]/[free (oligomer)]. Since total ($tRNA^{Ile}$) ≫ total (oligomer), R may be directly related to the apparent association constant of the oligomer–$tRNA^{Ile}$ interaction by the relationship (Uhlenbeck *et al.*, 1970): $K = (R - 1)/(tRNA^{Ile})_o$, where subscript o denotes total concentration. In Tables I and II K values are reported only for those cases where $R > 1.07$ ($K > 2500$). If $R \leq 1.07$, K is assigned the symbol –, although in so doing some weakly binding oligomers are not noted. The numerical values of K given represent the average of two or more determinations. Some of the weakly binding oligomers were subject to only one determination.

[3] Ph.D. thesis, M. I. T., in preparation.

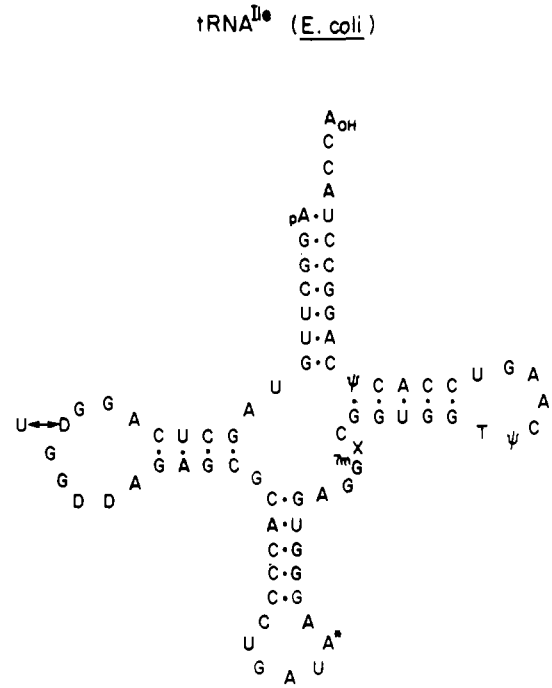

FIGURE 1: The sequence of the two major species of $tRNA^{Ile}$ (*E. coli* B) as determined by Yarus and Barrel (1971).

Table I gives the results of tests and controls for the binding of four trinucleotides to the anticodon sequence GAU, and of seven tetranucleotides to the sequence UGAU. It is clear that of the trimers tested, only the codon AUC associates strongly with $tRNA^{Ile}$. The wobble codon AUU, as well as the control trimers AUG and AUA, show much weaker associations with the tRNA. Similarly, of the tetramers AUCN, AUCA (the perfect matching complement), and AUCG bind the strongest. Sequence isomer controls AUAC and AUGC bind very poorly. In addition, the change of the C in AUCA to give AUAA results in no significant binding.

Table II gives K values for nine additional oligonucleotides which are complementary to other unpaired regions of $tRNA^{Ile}$. Only two of these show significant binding. UCAG

TABLE II: Hybridization Tests for Other Oligonucleotides.

Oligomer	Approx Assocn Constant (M^{-1})
UCG	–
GGU	200,000
CCU	–
UUA	–
CUU	–
UCA	–
ACCU	–
UCAG	6,400
CCAC	

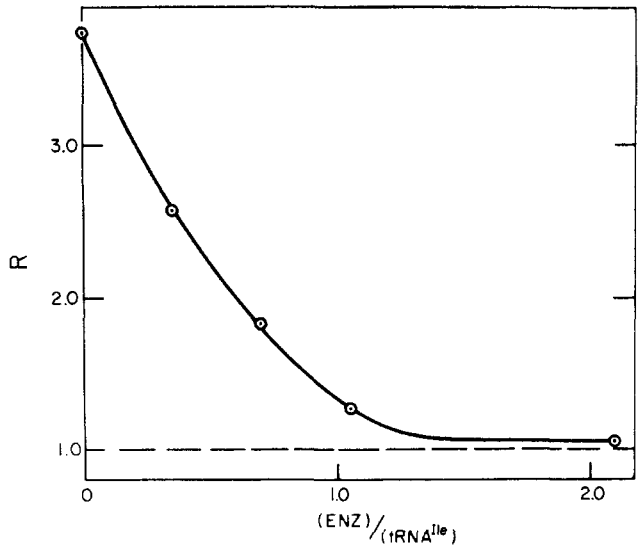

FIGURE 2: Effect of IRS on the hybridization of GGU to $tRNA^{Ile}$. The value of R is plotted *vs.* the approximate ratio $(IRS)_o:(tRNA^{Ile})_o$. The concentration of $tRNA^{Ile} \approx 12\ \mu M$, except for the last point at $(IRS)_o:(tRNA^{Ile})_o \approx 2.1$; in this case $(tRNA^{Ile})_o \approx 5.6\ \mu M$ and the value of $R - 1$ obtained was multiplied by 2 and added to 1.0 to give the value plotted in the figure. Control experiments indicated that the value of R for the binding of GGU to 5.6 μM $tRNA^{Ile}$, in the absence of IRS, is approximately what is expected based on the value for the binding to 12 μM $tRNA^{Ile}$.

($K = 6400$) is the antisequence for the CUGA on the 5′ side of the anticodon loop. GGU ($K = 200{,}000$) doubtless binds to the ACC of the 3′-terminal ACCA sequence (O. C. Uhlenbeck, 1972).[1] However, in this case the tabulated K value may be not as accurate because of the known self-aggregation of G-rich oligomers (Lewis, 1971) which in turn prevents complete equilibration with respect to GGU monomers. The oligomers ACCU and CCAC complement sections of the dihydrouridine loop if the base at position 17 is U and not D (see Figure 1). However, according to Yarus and Barrel (1971) the more abundant species of $tRNA^{Ile}$ contains a D.

Association to IRS-Bound $tRNA^{Ile}$. The effect of IRS on the association of oligomers to $tRNA^{Ile}$ was investigated with the three oligomers which bind tightly: AUCA, AUCG, and GGU. Figure 2 gives a plot of R for GGU binding *vs.* $(IRS)_o/(tRNA^{Ile})_o$. It is seen that the value of R in the absence of IRS is 3.7 ($(tRNA^{Ile})_o \approx 11\ \mu M$) and the addition of IRS causes a decrease in R which is proportional to the amount of IRS added until $R \approx 1.0$.

The effect of IRS on the hybridization of AUCA and of AUCG to $tRNA^{Ile}$ is given in Table III. Values of R are tabulated for various ratios of $(IRS)_o:(tRNA^{Ile})_o$. The first row of each column gives the value of R obtained in the absence of IRS, while the value obtained in the presence of IRS is given by the entry in one of the subsequent rows of the column. It is seen that IRS strongly inhibits the binding of oligomers to the anticodon loop. Control experiments employing bovine serum albumin instead of IRS show that at comparable protein concentrations this protein has no significant effect on hybridization to the anticodon. In addition, in order to verify that the diminished binding of AUCA and of AUCG in the presence of IRS is not due to degradation of the tRNA or the oligomer by an impurity in the enzyme preparation, the value of R was measured after dialysis times of about 48 and 72 hr, for a case (row 2 of Table III) in which the $tRNA^{Ile}$ is only partially saturated with enzyme. In the

TABLE III: Effect of IRS on the Hybridization of Oligonucleotides to the Anticodon of $tRNA^{Ile}$ Values of R.

$(IRS)_o:(tRNA^{Ile})_o$	AUCA		AUCG			
0	2.6[a]	1.7[b]	2.5[a]	2.7[c]	1.8[b]	2.8[a]
0.6	1.5[a]		1.7[a]			
1.0				1.2[c]		
1.3		1.1[b]			1.2[b]	
2.1						1.0[a]

[a] $(tRNA)^{Ile} \approx 11\ \mu M$. [b] $(tRNA^{Ile}) \approx 5.6\ \mu M$. [c] $(tRNA^{Ile}) \approx 12\ \mu M$.

presence of a nuclease activity the value of R would be expected to decrease with time. Instead, the value for both AUCA and AUCG was the same at 48 and 72 hr, within experimental error.

Discussion

In this study, we have investigated the ability of free and synthetase-bound $tRNA^{Ile}$ to bind complementary oligonucleotides. Attention was focused on the anticodon loop, the dihydrouridine loop, and the 3′-ACCA end of the molecule. With several other tRNAs, numerous investigators have shown that the TψC loop is buried or shielded (Brostoff and Ingram, 1967; Yoshida and Ukita, 1968; Litt, 1969; O. C. Uhlenbeck, 1972)[1] and consequently this region was not carefully explored in our studies. Considerably more thorough investigations of oligonucleotide binding to *free* tRNA are published elsewhere (Uhlenbeck *et al.*, 1970; O. C. Uhlenbeck, 1972).[1]

The results given in Table I show that a strong specific association occurs between the codon triplet AUC and the anticodon sequence GAU of the $tRNA^{Ile}$. The marked specificity is evidenced by the fact that a mere change of the C (of AUC) to A, U, or G eliminates the strong binding. In view of the strong association of AUC, it is somewhat surprising that AUU does not bind more strongly than $K = 1200\ M^{-1}$, the upper limit suggested by two experiments. Söll *et al.* (1965) observed stimulation of the binding of $tRNA^{Ile}$ to ribosomes by both AUU and AUC, although AUC appeared to be the more effective of the two.

All four AUCN tetramers bond more strongly than AUC, with AUCA and AUCG being the strongest. In the case of the perfectly complementary tetramer AUCA, the association constant is about 15- to 20-fold greater than that of AUC. A large enhancement in going from the complementary trimer to the complementary tetramer has also been observed in the hybridization of oligomers to the anticodon sequence of other specific tRNAs (Uhlenbeck *et al.*, 1970; O. C. Uhlenbeck, 1972).[1] Although AUCA binds very strongly, the sequence isomer AUAC does not bind at all. However, it is surprising that AUCG hybridizes as strongly as AUCA. This result may be rationalized by the possibility of a G-U "wobble" interaction (Crick, 1966) of the G-terminated tetramer with the U on the 5′ side of the anticodon. The fact that AUCC or AUCU bind more strongly than AUC, although less than AUCA or AUCG, is not as easily explained, However, enhanced binding of complementary triplets by

the addition of a noncomplementary fourth base has been observed in other systems (see Lewis, 1971, for discussion).

Oligonucleotides tabulated in Table III are complementary to several different regions of $tRNA^{Ile}$. The tetramer UCAG complements the CUGA sequence, which is shifted by one base to the 5′ side of the anticodon sequence. This oligomer binds with an association constant of 6400, which is considerably less than the binding of the complementary tetramer to the anticodon sequence itself. Similar asymmetry in the binding to the anticodon loop has been observed with other tRNAs (Uhlenbeck *et al.*, 1970; O. C. Uhlenbeck, 1972).[1]

In the case of $tRNA_f^{Met}$ and $tRNA^{Tyr}$, complementary oligonucleotides bind to sections of the dihydrouridine (D) loop which do not contain a D (O. C. Uhlenbeck, 1972).[1] However, in the more abundant species of $tRNA^{Ile}$, this loop contains three D residues (Yarus and Barrel, 1971) spaced in such a way as to prevent formation of an uninterrupted sequence of base pairs of greater than two units (because of their nonplanarity, the D residues cannot participate in Watson–Crick base pairs). Thus, it is not surprising that oligomers such as CCAC and ACCU do not bind to $tRNA^{Ile}$.

The fact that CCAC does not strongly hybridize to $tRNA^{Ile}$ is of interest from another standpoint. This sequence also occurs in the arm of the TψC loop, in the cloverleaf structure (see Figure 1). Hence, the lack of strong binding of CCAC confirms previous observations that the putative double-stranded regions of the cloverleaf structure cannot hybridize complementary oligonucleotides (Uhlenbeck *et al.*, 1970; O. C. Ulhenbeck, 1972).[1] Further indirect evidence for the cloverleaf secondary structure is thus provided.

The remaining oligomers in Table II are trinucleotides which complement various sequences in areas which are not hydrogen bonded in the cloverleaf secondary structure. None of these binds significantly except for GGU. This triplet doubtless binds to the ACC sequence of the ACCA end of the molecule (O. C. Uhlenbeck, 1972).[1] This sequence is common to most tRNAs (Zachau, 1969a,b), and indeed many species appear to bind GGU very strongly (O. C. Uhlenbeck, 1972).[1,4] The large association constant of GGU results from the presence of two G·C pairs as well as from the fact that by binding it extends the double-stranded region of the amino acid acceptor arm by joining to the existing helix with an U-A stack.

We now turn to a consideration of the binding of oligonucleotides to IRS-bound $tRNA^{Ile}$. The question arises, of course, as to whether or not the $tRNA^{Ile}$–synthetase complex at 0° in 0.5–1 M NaCl is an accurate representative of the complex at more physiological conditions, *e.g.*, 37° and 0.1 M NaCl. This is a difficult question to answer, but some helpful data are available. The association constant between IRS and $tRNA^{Ile}$ is of the order of 10^7 M^{-1} in 10 mM Mg^{2+}, 0.1 M NaCl, ~70 mM phosphate (pH 5.5), 17°. Under the same conditions, but with 1 M NaCl instead of 0.1 M, the association constant decreases to the order of 10^6 M^{-1}.[5] This implies that high salt reduces the free energy of association by ~1.5 kcal $mole^{-1}$ out of a total free-energy change of ~10.5 kcal $mole^{-1}$. Moreover, the ability of IRS at relatively low concentrations to eliminate oligonucleotide binding to $tRNA^{Ile}$ (Figure 2 and Table III) certainly implies it binds strongly to $tRNA^{Ile}$ at 0° in 0.5–1 M NaCl. Since, therefore, a strong association does exist in high salt, it is reasonable to conclude that the major parts of the interaction between enzyme and $tRNA^{Ile}$ are preserved.

Figure 2 shows that addition of IRS reduces the binding between GGU and $tRNA^{Ile}$ until somewhat greater than stoichiometric amounts of enzyme have been added. At this point, the binding of GGU is completely abolished. The values of R given in Figure 2 apply to $(tRNA)^{Ile} \approx 12$ μM. Since roughly stoichiometric quantities of enzyme are capable of reducing R to about unity, the IRS-$tRNA^{Ile}$ association constant must be at least 10^6 M^{-1} under these conditions.

Similar results are found for the effect of IRS on the binding of AUCA and of AUCG to the anticodon sequence (see Table III); when added in sufficient amount, IRS eliminates binding of complementary tetranucleotides to the anticodon sequence of $tRNA^{Ile}$. *These results thus suggest that part or all of the ACC sequence at the 3′ terminus, and of the anticodon section, are shielded in the $tRNA^{Ile}$–IRS complex.* An alternative explanation is that one or both of these regions is merely distorted, but not shielded, so that it can no longer easily hybridize its complementary oligomer. This possibility is viewed somewhat less likely since some residual binding to the complexed $tRNA^{Ile}$ might still be possible, but to the contrary, none is observed.

The finding that the ACC of the 3′ terminus is blocked was expected since the enzyme attaches the amino acid to this end of the molecule. However, the simultaneous shielding of the anticodon section is somewhat surprising in view of the evidence that it is presumably quite removed from the CCA end of the molecule. For example, fluorescence energy-transfer experiments of Beardsley and Cantor (1970) suggest that the distance from the anticodon to the CCA terminus is not less than 40 Å. If this is true, then the enzyme–tRNA interaction encompasses regions which are quite far apart indeed. Interestingly enough, this notion is implicit in the suggestion of Mirzabekov *et al.* (1969) who offer that the synthetase recognition loci on $tRNA^{Val}$ (yeast) involve two bases on the anticodon loop and the first two bases at the 5′ end of the molecule. Of course, in the present studies no claim can be made as to the location of the actual recognition site.

[4] O. C. Uhlenbeck, unpublished material.

[5] S. Lam and P. R. Schimmel, to be published.

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Stimulation of Cell-Free Polypeptide Synthesis by a Protein Fraction Extracted from Chick Oviduct Polyribosomes*

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ABSTRACT: Estrogen induces synthesis of the specific protein ovalbumin in chick oviduct. We have previously demonstrated completion of ovalbumin chains *in vitro* on oviduct polyribosomes isolated from estrogen-treated chicks. The present experiments were undertaken in an attempt to establish a cell-free ribosomal system capable of *de novo* synthesis of ovalbumin. Extraction of oviduct polyribosomes with sucrose, EDTA, dithiothrietol, and KCl yields a soluble fraction (AvF) which is capable of stimulating both rate and extent of protein synthesis on washed ribosomes by 6- to 10-fold. This stimulation occurs in the presence of saturating amounts of aminoacyl synthetases and elongation factors and is observed whether protein synthesis is directed by endogenous (natural) or exogenous synthetic (polyuridylic acid) mRNA. The AvF-enhanced synthesis of protein is dependent upon time, number of ribosomes, and amount of AvF fraction. A protein nature is suggested for AvF factors since they are inactivated by heat (60°), Pronase, or *N*-ethylmaleimide but not by RNase. Protein synthesis is stimulated by AvF subsequent to aminoacylation of tRNA since stimulation occurs using both [^{14}C]-Val and [^{14}C]Leu-tRNA as labeled substrate. Furthermore, addition of AvF results in a distinct lowering of the Mg^{2+} concentration required for optimal protein synthesis. Again this "Mg^{2+} shift" occurs both with natural message using [^{14}C]Val or [^{14}C]Leu-tRNA and with polyuridylic acid using [^{14}C]Phe-tRNA. Stimulation of polyphenylalanine synthesis and lowering of Mg^{2+} optimum occur in the presence of saturating amounts of partially purified T_1 and T_2 indicating that AvF is distinct from elongation factors. Finally, as a method for identifying and separating a specific protein product of the AvF-stimulated reaction, peptides synthesized and released *in vitro* were subjected to antiovalbumin affinity chromatography. This procedure demonstrated that in the presence of AvF, 14% of the released peptides was immunologically similar to ovalbumin.

Administration of estrogen to the immature chick results in both morphologic differentiation and biochemical specialization of the oviduct (O'Malley *et al.*, 1969). Following cellular differentiation one of the new cell types (the tubular gland cell) produces the specific protein ovalbumin (O'Malley *et al.*, 1969; Kohler *et al.*, 1968). Moreover, once differentiation has occurred, production of this protein is regulated by estrogen and this regulation appears to occur at the level of nuclear transcription (O'Malley *et al.*, 1969; O'Malley and McGuire, 1968a,b).

We have recently demonstrated that estrogen administration results in the assembly of oviduct polyribosomes as well as an increase in their biosynthetic activity assayed in a cell-free protein-synthesizing system (Means *et al.*, 1971). This polyribosomal system is capable of synthesizing immunologically identifiable ovalbumin *in vitro* (Means and O'Malley, 1971). However, it is possible that the immunologically competent protein product represents only completion and release of previously existing nascent chains. Consequently we have begun to further define the cell-free system in order to prepare cellular materials capable of initiating and completing ovalbumin molecules entirely *in vitro*. It is anticipated that such a system would be capable of translating exogenous mRNA and, therefore, might be useful in assaying the unique products of estrogen directed transcription.

Miller *et al.* (1967) were the first to report a cell-free system derived from rabbit reticulocytes which was capable of *de novo* globin synthesis. This system has subsequently been shown to contain initiation factors and to be able to translate exogenous mRNA (Prichard *et al.*, 1970; Nienhuis *et al.*, 1971). Therefore, we undertook to develop a similar system from the oviduct. This report documents the isolation of an avian protein fraction (AvF) from oviduct polyribosomes which stimulate both total protein synthesis and ovalbumin synthesis *in vitro*. The data are consistent with the AvF-dependent stimulation of chain initiation.

Experimental Section

Preparation of Oviduct Ribosomes and AvF Factors. Female Rhode Island Red chicks (7 days old) were given daily sub-

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