RNA CODEWORDS AND PROTEIN SYNTHESIS, II. NUCLEOTIDE SEQUENCE OF A VALINE RNA CODEWORD

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Recent studies demonstrate that synthetic polynucleotides, such as poly U, induce with specificity the binding of C^{14}-amino-acyl-sRNA to ribosomes.[1-4] Characteristics of binding in the absence of poly U also have been reported.[5] C^{14}-Phe-sRNA binding induced by poly U is thought to represent an early step in protein synthesis before peptide bond formation; however, the precise nature of the interaction has not been clarified.

To establish the sequence of nucleotides in RNA codewords, we devised a rapid, sensitive method for measuring C^{14}-amino-acyl-sRNA binding to ribosomes, and have investigated both characteristics of binding and the minimum oligonucleotide chain length required to direct such binding.[6] pUpUpU, pApApA, and pCpCpC directed the binding of Phe-, Lys-, and Pro-sRNA, respectively, whereas dinucleotides had no effect. Trinucleotides with 5′-terminal phosphate were more active than those with no terminal phosphate, whereas trinucleotides with 2′-(3′)-terminal phosphate were inactive.[6]

In this report, trinucleotides of known sequence were used to direct sRNA binding. GpUpU, but not its sequence isomers, UpGpU and UpUpG, was shown to induce Val-sRNA binding to ribosomes. These data indicate that the nucleotide sequence of an RNA codeword for valine is GpUpU.

Materials and Methods.—Analyses of poly- and oligonucleotides: (*a*) Paper electrophoresis was performed on Whatman 54 or 3 MM paper in 0.05 *M* NH_4COOH buffer, pH 2.7, at 80 v/cm for 0.5 hr with authentic reference markers. If a marker was not available, the expected mobility was calculated.[7] (*b*) Descending paper chromatography was performed at room temperature with Whatman 3 MM paper and the following solvents: (*A*) conc. NH_4OH-N-propanol-H_2O, 10/55/35, v/v; (*B*) 40 gm $(NH_4)_2SO_4$ dissolved in 100 ml 0.1 *M* sodium phosphate, pH 7.0.[8] Bands were visualized under UV light. (*c*) Ultraviolet absorption measurements were made in a Zeiss spectrophotometer, and spectra were obtained in a Cary recording spectrophotometer The absorbancy of eluates from paper was read against blank paper eluates. (*d*) The base ratio of the poly UG preparation (D-132) was 0.74/0.26 (U/G). The base ratio of UpUpG, UpGpU, and GpUpU preparations was determined by incubating 2.0 A^{260} units with 3.5×10^{-3} units of T_2 RNase[9] in 0.02 ml of 0.5 *M* ammonium acetate, pH 4.5, for 2.5 hr at 45°. The digestion products were separated by paper electrophoresis, eluted, and the absorbancy of each at appropriate λ_{max} was determined.

Preparation and separation of oligonucleotides: UpU was characterized as described.[6] UpUpG and UpG were obtained by incubating 3×10^3 A^{260} units of poly UG with 1.3×10^3 units of T_1-RNase (Sankyo Co., Ltd., Tokyo) in 4.7 ml of 0.1 *M* NH_4HCO_3 at 37° for 6 hr.[10] The reaction mixture was lyophilized, dissolved in 0.1 *M* $(NH_4)_2\ CO_3$, and terminal phosphates were removed by incubation with *E. coli* alkaline phosphatase, free of diesterase, as described by Heppel *et al.*[11] Oligonucleotide fractions were separated by paper chromatography with solvent *A* for 18 hr. Ultraviolet-absorbing bands with mobilities of UpG and UpUpG were eluted with H_2O, lyophilyzed, and purified separately by electrophoresis.

GpUpU and GpU were obtained by digesting poly UG with purified pork liver nuclease,[6] removing terminal phosphates, and separating fractions by paper chromatography and electrophoresis as described above. This procedure yielded a fraction containing UpG and GpU and also one containing GpUpU, UpGpU, and UpUpG. Paper chromatography with solvent *B* for

36 hr resolved each dinucleotide. The trinucleotide fraction was separated into two bands by chromatography with solvent *B*. The band nearest the origin contained GpUpU of high purity. It was eluted with H_2O, absorbed on acid-washed Norite, and eluted with 45% ethanol 0.5 *M* NH_4OH. GpUpU was purified again by chromatography with solvent *B*, eluted, and desalted as described.

A derivative of pancreatic RNase A was used by Dr. Merton Bernfield to catalyze the transfer of uridine-2',3'-cyclic phosphate to GpU.[12] UpGpU was purified from the reaction mixture by paper chromatography and electrophoresis as described for UpUpG.

Characterization of triplets: GpUpU, UpGpU, and UpUpG preparations were purified as described. Only one spot was observed after each was subjected to paper chromatography with solvents *A* or *B* and to paper electrophoresis. Base-ratio and sequence analyses established the purity, chain length, and base sequence of each triplet preparation. Base-ratio analyses were as follows: GpUpU, 1.2 U, 1.0 Up, 1.0 Gp; UpGpU, 1.0 U, 1.0 Up, .09 Gp; UpUpG, 2.1 Up, 1.0 G.

Base sequence was determined as follows: 2.0 A^{260} units of each trinucleotide was digested separately with T_1-RNase (as described earlier) and in other reactions, with 2.0 μg of chromatographically purified pancreatic RNase (Sigma) in 0.02 ml of 0.1 *M* $(NH_4)_2CO_3$, at 37° for 6 hr. Reaction products were separated by paper electrophoresis. Dinucleotide products were eluted, lyophilized, and digested with the RNase to which they were susceptible, and the products again were separated by electrophoresis. Nucleosides and nucleotides were identified by their electrophoretic mobility as well as by their spectra.

After digestion of GpUpU, UpGpU, and UpUpG with pancreatic RNase, only the following products were obtained: GpUp + U; Up + GpU; and Up + G, respectively. After digestion of GpUp and GpU with T_1-RNase, Gp + Up and Gp + U were obtained, respectively. T_1-RNase digestion of GpUpU, UpGpU, and UpUpG yielded Gp + UpU, UpGp + U, and UpUpG, respectively. Digestion of UpU, UpGp, and UpUpG with pancreatic RNase yielded Up + U, Up + Gp, and Up + G, respectively. No other UV-absorbing material was found. Since a 2% contaminant could have been detected, the purity of each trinucleotide was estimated to be >98%. GpUpU and UpGpU have not been purified previously.

Ribosomes, sRNA, and E. coli extracts: E. coli W 3100 sRNA was prepared from cells grown to late log phase in 0.5% nutrient broth, 1% glucose.[25] *E. coli* B sRNA was obtained from General Biochemicals, Inc. Each C^{14}-amino-acyl-sRNA was prepared in the presence of 19 C^{12}-amino acids as described elsewhere.[6] In additional experiments C^{14}-Leu-sRNA also was prepared in the absence of other amino acids. C^{14}-L-Val, C^{14}-L-Phe, and C^{14}-L-Leu, uniformly labeled with radioactivities of 293, 229, and 435 cpm/$\mu\mu$mole (Packard Corp. scintillation counter), respectively, were obtained from New England Nuclear Corp. Ribosomes for binding studies and *E. coli* extracts (DNase-treated, preincubated, S-30 fraction) for amino acid incorporation into protein studies were prepared as described elsewhere.[6, 13]

Assays: Each 50-μl reaction mixture contained 0.1 *M* tris-acetate, pH 7.2; 0.02 *M* magnesium acetate; 0.05 *M* KCl; and 1.0 A^{260} units of ribosomes unless otherwise specified. Incubations were at 24° for 20 min unless otherwise indicated. C^{14}-amino-acyl-sRNA binding to ribosomes was determined, as reported elsewhere,[6] by washing the ribosomes on Millipore filters. Ribosomes with bound sRNA remained on the filter.

C^{14}-amino acid incorporation into protein was determined as reported previously.[13]

Results.—Effect of GpUpU and Poly UG upon the binding of C^{14}-valine sRNA to ribosomes: The data of Figure 1 show that the binding of Val-sRNA to ribosomes is stimulated by the addition of GpUpU or poly UG and is dependent upon the concentration of either template. More Val-sRNA is bound to ribosomes in the presence of poly UG than in the presence of an equivalent amount of GpUpU. We have observed similar differences between the activity of pUpUpU and poly U.[6]

In Figure 2, the rates of binding at 0° and 24° are compared, and the specificity of GpUpU and poly UG for amino-acyl-sRNA is shown. At 0°, poly UG markedly stimulated Val- and Phe-sRNA binding to ribosomes, whereas GpUpU stimulated only Val-sRNA binding. At 24° the rates of binding directed by poly UG were higher than at 0°. Slightly higher binding in control reactions was observed

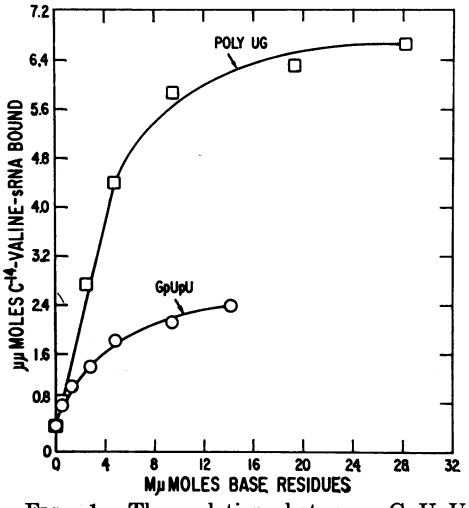

FIG. 1.—The relation between GpUpU (○) and poly UG (□) concentration and C^{14}-Val-sRNA binding to ribosomes. Each reaction mixture contained 2.0 A^{260} units of ribosomes and 18.9 μμmoles of C^{14}-Val attached to 0.44 A^{260} units of sRNA. Poly UG and GpUpU were added as specified.

(especially of Leu-sRNA). Under the conditions employed, poly UG had little activity in directing Leu-sRNA binding to ribosomes, compared with its ability to direct amino acid incorporation into protein.

Codeword specificity and the effect of (Mg^{++}) *concentration:* The data of Figure 3 demonstrate that poly UG stimulates binding of Val- and Phe-sRNA to ribosomes optimally at 0.02–0.03 *M* (Mg^{++}), whereas GpUpU directs binding of Val-sRNA optimally at higher concentrations. At higher (Mg^{++}) concentrations, a small and apparently nonspecific increase in Val-, Leu-, and Phe-sRNA binding was observed in control reaction mixtures (no addition). Tri- and polynucleotides induce specific sRNA binding. GpUpU directed only Val-sRNA binding, whereas UpGpU and UpUpG had no effect upon either Val-, Leu-, or Phe-sRNA. The addition of tri- or polynucleotides did not appreciably increase Leu-sRNA binding over that of control reactions within the range of (Mg^{++}) concentrations tested.

The specificity of di-, tri-, and polynucleotides in directing amino-acyl-sRNA binding is shown in Table 1. Poly UG directed Val- and Phe-sRNA binding, whereas poly U directed the binding only of Phe-sRNA. UpU, GpU, and UpG did not stimulate Val-, Phe-, or Leu-sRNA binding.

The effect of GpUpU was tested upon the binding of 17 other sRNA preparations, each with a different C^{14}-amino accepted (C^{14}-asparagine and C^{14}-glutamine-sRNA not tested). GpUpU was found to direct the binding only of Val-sRNA.

Amino acid incorporation into protein: Since poly UG had little effect upon Leu-sRNA binding to ribosomes, we investigated the ability of poly UG to direct

TABLE 1

CODEWORD SPECIFICITY

Expt.	Addition, mμmoles base residues	C^{14}-Amino-Acyl-sRNA Bound to Ribosomes, μμmoles C^{14}-Val-sRNA	C^{14}-Phe-sRNA	C^{14}-Leu-sRNA
1	None	0.38	0.22	0.37
	4.7 Poly U	0.23	4.73	0.22
	4.7 Poly UG	2.65	1.93	0.24
2	None	0.40	0.22	0.62
	4.7 GpUpU	1.11	0.27	0.56
	4.7 UpGpU	0.40	0.25	0.55
	4.7 UpUpG	0.37	0.25	0.44
3	None	0.18	0.22	0.69
	4.7 GpU	0.20	0.22	0.69
	4.7 UpG	0.19	0.21	0.71
	4.7 UpU	0.18	0.20	0.67

Oligonucleotide specificity in directing sRNA binding to ribosomes. Where indicated, 9.10 μmoles C^{14}-Val attached to 0.3 A^{260} units of sRNA, 17.0 μμmoles C^{14}-Phe attached to 0.7 A^{260} units of sRNA, or 21.0 μμmoles C^{14}-Leu attached to 1.5 A^{260} units of sRNA were added to reaction mixtures.

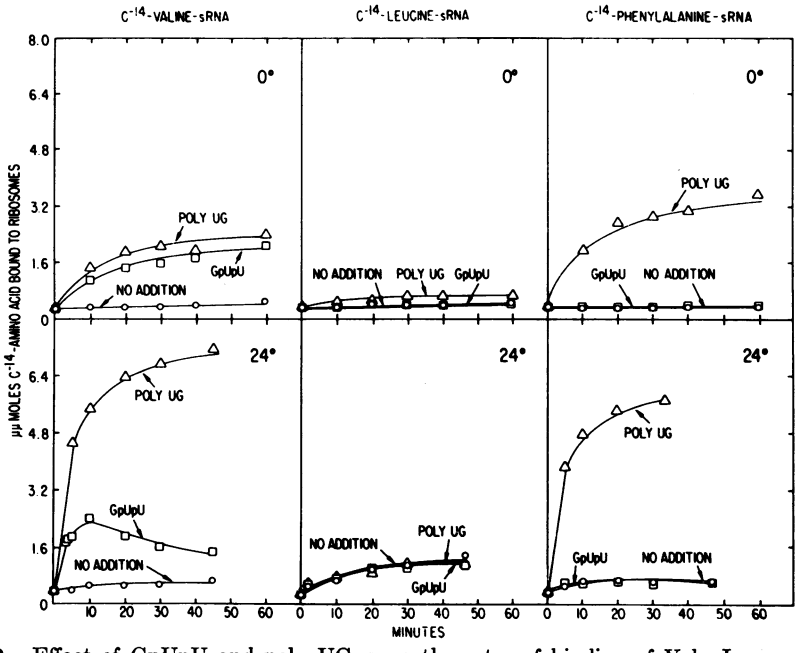

FIG. 2.—Effect of GpUpU and poly UG upon the rates of binding of Val-, Leu-, and Phe-sRNA to ribosomes at 0° and 24°. The symbols represent the addition of: (△) poly UG, 4.70 mμmoles of base residues; (□) GpUpU, 2.35 mμmoles of base residues; (○) no addition. Where indicated, 17 mμmoles of C^{14}-Val attached to 0.44 A^{260} units of sRNA, 8.5 μμmoles C^{14}-Phe attached to 1.5 A^{260} units sRNA, or 21.0 μμmoles of C^{14}-Leu attached to 1.5 A^{260} units of sRNA were added. Samples were incubated at the temperatures and for the times indicated.

amino acid incorporation into protein in *E. coli* extracts at 0° and 24° (also at 37° to make these data comparable to previously reported studies[14, 15]). As shown in Table 2, poly UG directed Phe, Val, and Leu into protein at 37° and 24°, and as reported previously,[14, 15] Leu incorporation almost equaled that of Val. Amino acid incorporation into protein was not detected at 0°.

Discussion.—Previously we have shown that UpUpU, ApApA, and pCpCpC specifically induce the binding to ribosomes of Phe-, Lys-, and Pro-sRNA, respectively. The present study demonstrates that the trinucleotide, GpUpU, can induce the binding of Val-sRNA to ribosomes, whereas sequence isomers such as UpGpU and UpUpG, and dinucleotides have no activity. When the specificity of poly UG was compared with that of GpUpU, the polynucleotide was found to direct both

TABLE 2

C^{14}-AMINO ACID INCORPORATION INTO PROTEIN

Temperature of incubation	Addition	Amino Acid Incorporated into Protein, mμmoles C^{14}-valine	C^{14}-phenylalanine	C^{14}-leucine
0°	None	<0.03	<0.03	<0.03
	Poly UG, 53 mμmoles	<0.03	<0.03	<0.03
24°	None	<0.03	0.04	0.04
	Poly UG, 53 mμmoles	0.70	1.57	0.67
37°	None	0.10	0.09	0.08
	Poly UG, 53 mμmoles	1.04	2.05	0.96

Each 0.1-ml reaction mixture contained 0.3 mg *E. coli* protein (DNase-treated, preincubated S-30 fraction[20]), 20 mμmoles of either C^{14}-Val, C^{14}-Phe, or C^{14}-Leu (4.8, 4.0, 4.0 μcuries/μmole, respectively), in addition to components previously described.[20] Reactions were incubated for 10, 20, or 60 min at 37°, 24°, or 0°, respectively.

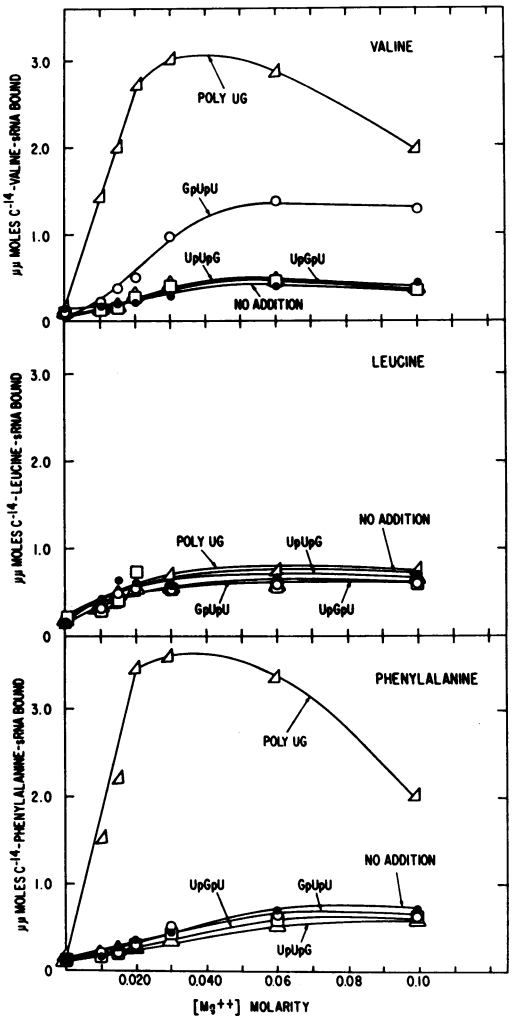

FIG. 3.—Relationship between magnesium acetate concentration and C^{14}-amino-acyl-sRNA binding to ribosomes. The symbols represent the addition of: (◿) poly UG, 4.70 mμmoles of base; (○) GpUpU, 2.35 mμmoles of base; (□) UpGpU, 2.35 mμmoles of base; (△) UpUpG, 2.35 mμmoles of base; (●) no addition. 9.10 μμmoles C^{14}-Val attached to 0.3 A^{260} units of sRNA, 25.0 μμmoles C^{14}-Leu attached to 1.5 A^{260} units of sRNA, or 10.0 μμmoles C^{14}-Phe attached to 0.7 A^{260} units of sRNA were added where indicated. The (Mg^{++}) concentration of each reaction mixture is shown on the abscissa. Samples were washed with buffer containing the appropriate concentration of (Mg^{++}).

Phe- and Val-sRNA binding, but GpUpU induced the binding of only Val-sRNA. We conclude that the nucleotides of a valine codeword in mRNA are arranged in the sequence GpUpU. Since UpUpG does not replace GpUpU, recognition occurs with polarity.

As discussed elsewhere,[6] the ability of tri- but not dinucleotides to induce sRNA binding demonstrates directly that triplets may code for amino acids, and agrees with conclusions derived from both genetic[19] and biochemical studies.[20]

Studies of the incorporation of amino acids stimulated by various polynucleotides together with the data on amino acid replacements in mutants provide a basis for

assignment of codewords to amino acids.[16-18] Unfortunately, widespread degeneracy and the difficulty of assigning words which contain three different bases prevent unique assignments. Several self-consistent schemes are possible. The additional information provided by the definite knowledge of even a single codeword such as GpUpU provides a distinction among these different possibilities. The reassignments suggested to make GpUpU the codeword for valine consistent with the other data are listed in Table 3. For example, ApUpU was assigned to isoleucine on the basis of studies employing randomly ordered polynucleotides to direct amino acid incorporation into protein, and the HNO_2-induced replacement of isoleucine by valine in TMV coat protein.

TABLE 3

PREDICTED NUCLEOTIDE SEQUENCES OF RNA CODEWORDS

Amino acid	Sequence	Reference
Valine	GpUpU	Derived experimentally
Isoleucine	ApUpU	17, 18
	Ap (UA)	
Tyrosine	Up (AU)	
Alanine	GpCpC	16
	Gp (CA)	
	Gp (CU)	
Glutamic acid	GpApA*	16
	Gp(AU)	
Glycine	Gp (GU)	16
	Gp (GA)	
	Gp (GC)	
	Note: UpGpG is ruled out	
Arginine	ApGpA	16
	CpGpC	
	(C)Gp(A)	
Threonine	Ap(CA)	17, 18
	(ACC)	

* The assignment of GpApA to glutamic acid is based upon limited amino acid incorporation into protein data and is, therefore, tentative.[23, 24]

Nucleotides within parentheses have not been arranged in sequence. Amino acid replacements used for these predictions were found in *E. coli* by Yanofsky and co-workers[16] or were induced by HNO_2 in TMV by Wittmann and Wittmann-Liebold[17] and also by Tsugita.[18]

It should be noted that the predicted sequence for isoleucine, ApUpU, and also the GpUpU sequence found for valine do not agree with reported sequence determinations[21] which, on the basis of experiments with uncharacterized polynucleotides, assign the sequence ApUpU to tyrosine, tentatively assign GpUpU to cysteine, and derive the polarity of mRNA for protein synthesis.

Although our results show only marginal stimulation of Leu-sRNA binding by poly UG, we have consistently induced Leu- and also Cys-sRNA binding with poly UG when reactions were incubated at 0° and at a (Mg^{++}) concentration of 0.03 *M*. Leu-sRNA binding was readily induced by poly UC which confirms results reported by Kaji and Kaji.[3] Further studies with Leu- and Cys-sRNA are in progress.

We have shown that specific binding of sRNA can be induced at 0° and have described preliminary evidence which indicates that intact amino-acyl-sRNA can be eluted from ribosomes after incubation.[6] Although these data suggest that formation of peptide bonds is not required for binding to ribosomes, a requirement for the first transfer enzyme and GTP may exist (cf. refs. 1–4, and 6).

Since synonym codewords corresponding to one amino acid often differ in base composition by only one nucleotide,[20] it is possible that bases in common may

occupy identical positions within each triplet. A triplet code can be constructed wherein recognition between two out of three nucleotide pairs may, in some cases, suffice for coding; or, alternatively, a base at one position in the triplet may pair optionally with more than one base.[22-24] Ambiguous and synonymous codewords may involve such recognitions. The development of a rapid method for measuring sRNA binding to ribosomes and the use of trinucleotides of known sequence to direct such binding should provide a general method of great simplicity to test these possibilities and also to study the genetic function of other nucleotide sequences and interactions between specific codewords, sRNA, and ribosomes.

Summary.—The binding of C^{14}-valine-sRNA to ribosomes was directed both by the trinucleotide, GpUpU, and by poly UG, but not by UpGpU, UpUpG, or dinucleotides. GpUpU had no effect upon the binding to ribosomes of sRNA corresponding to 17 other amino acids. The nucleotide sequence GpUpU was proposed for a valine RNA codeword. The implications of these findings and predictions based on amino acid replacement data are discussed briefly.

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The following abbreviations are used: Val, valine; Phe, phenylalanine; Leu, leucine; p oly U, polyuridylic acid; poly C, polycytidylic acid; poly A, polyadenylic acid; poly UG, copolymer of uridylic and guanylic acids; TCA, trichloroacetic acid; sRNA, transfer RNA; mRNA, messenger RNA. For mono- and oligonucleotides of specific structure, the "p" to the left of a terminal nucleoside initial indicates a 5′-terminal phosphate; the "p" to the right, a 2′-(3′)-terminal phosphate. Internal phosphates of oligonucleotides are (3′,5′)-linkages.

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*STRUCTURAL HOMOZYGOSITY IN MARGINAL POPULATIONS OF NEARCTIC AND NEOTROPICAL SPECIES OF DROSOPHILA IN FLORIDA**

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Peninsular Florida is of special biogeographical interest because of its intermediate position astride two biotic realms, the Nearctic and the Neotropical. Thus, not only do many species with continental distributions in North America have their southern geographical margins there, but also a number of Neotropical forms enter Florida from the south and terminate within the peninsula. Two species endemic to the different realms may have distributions in which the marginal populations from different directions overlap, in some cases quite narrowly. In this paper, new data are presented on the genetic properties of the populations of several species of Drosophila from such a bilateral marginal area in central Florida. These data indicate that both of the principal species studied, *Drosophila robusta* (Nearctic realm) and *Drosophila acutilabella* (Neotropical realm), show the phenomenon of structural homozygosity at the margin, although each displays extensive chromosomal polymorphism in more central parts of its range to the north and south. The importance of such marginal homozygosity for theories of interdeme evolution and allopatric species formation has been extensively discussed.[1, 2]

Materials and Methods.—Adult flies were attracted by placing fermenting fruit and vegetable baits directly in the forest. Collecting large samples from marginal populations for quantitative study has proved extraordinarily difficult. Most Florida collections of *Drosophila robusta*, for example, have consisted of a few scattered individuals only.[3] Recent success in making a large collection of this species in central Florida appears to be associated with the selection of not only just the right forested area, but also the right season for making collections. In the latitude of St. Louis, in the center of its range, *D. robusta* shows its great spring flush of population numbers about June 15.[3] The comparable season in central Florida was judged to be about April 15; collections at about this time in 1964 yielded a highly satisfactory sample of this species (Table 1). Approximately 10% of the *robusta* captured were newly emerged.

The area selected for intensive study was a deciduous lowland swamp just east of the bridge over the St. Johns River near the small fishing village of Astor, Volusia Co., Florida. The river flows due north, and the biota along its banks receives considerable biogeographical influence from ecocommunities to the north. Away from the river, pine forest prevails. The major trees, in