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Evolutionary Stability of the Histone Genes of Sea Urchins†

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ABSTRACT: Hybridization of sea urchin histone mRNA and filter-bound DNA has been used to estimate the base-sequence divergence of histone genes in evolution. Saturation hybridization experiments indicate that approximately 1000 copies of each histone gene are present in DNA isolated from sea urchin sperm. Thermal stabilities of the hybrids show that there is little if any heterogeneity in these repeated genes, *i.e.*, genes for a particular histone appear to be identical or very nearly so. Divergence among histone genes from sea urchins and other species was studied by hybridization of ^{3}H-labeled 9–12S sea urchin RNA with filter-bound DNA from various organisms. Thermal stability measurements and RNase susceptibility were used to estimate nucleotide substitutions. Some of the histone genes, like their protein gene products, are extremely conserved in evolution. Sea urchin 9–12S mRNA forms stable hybrids with numerous invertebrate DNAs as well as DNA from vertebrates and a higher plant. Comparisons of nucleotide substitutions with amino acid replacements in the histone proteins suggest that low levels of third position changes leading to synonymous codons have occurred. However, these substitutions have not accumulated at the rate predicted if these substitutions were strictly neutral. This suggests that selection pressure operates at the level of nucleic acids as well as on the proteins for which they code.

Early embryogenesis in echinoderms is characterized by a period of rapid DNA synthesis and cell division (see Hinegardner, 1967). The rate of protein synthesis increases rapidly upon fertilization (Hultin, 1961) as revealed by amino acid incorporation associated with polyribosomes (Rinaldi and Monroy, 1969). Small polysomes predominate during cleavage stages, although several size classes are present by blastulation (Nemer, 1972). As might be expected in rapidly dividing cells, a large fraction of the small polysomes are involved in the synthesis of nuclear and chromosomal proteins. Kedes *et al.* (1969) have estimated that approximately 50% of the proteins synthesized during cleavage accumulate in the nucleus and about half of these appear to be histones. Labeling patterns of the nascent polypeptide chains of small polysomes showed incorporation of large amounts of lysine or arginine and trace amounts of tryptophan, typical of the amino acid composition of histones (Nemer and Lindsay, 1969; Kedes *et al.*, 1969). More recent experiments have shown that during *in vitro* incubation of small polysomes, amino acid chain elongation occurs and identifiable histones are synthesized (Moav and Nemer, 1971).

Pulse-labeled RNA from these small polysomes contains a predominant 9S species which has many of the properties predicted for histone mRNA (Kedes and Gross, 1969). Recent reports which described 9S RNA-dependent *in vitro* translation of histones have unquestionably identified histone mRNA as a component of the 9S fraction (Gross *et al.*, 1973). Similar experiments have also been reported for 9S HeLa cell RNA (Jacobs-Lorena *et al.*, 1972). The *in vitro* translation products include proteins which correspond to HeLa cell histones in electrophoretic mobility and tryptic peptide patterns (Breindl and Gallwitz, 1973).

The 9–12S fraction of sea urchin RNA also displays characteristic hybridization properties (Kedes and Birnstiel, 1971; Weinberg *et al.*, 1972; McCarthy and Farquhar, 1972). A major fraction hybridizes with reiterated DNA (Kedes and Birnstiel, 1971) and cross-reacts with heterologous DNA (Weinberg *et al.*, 1972; McCarthy and Farquhar, 1972). Each

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peak resulting from fractionation of the same RNA on acrylamide gels exhibits these hybridization properties, consistent with the probability that they correspond to discrete classes of histone mRNAs. The size, base composition, and preliminary base sequence determinations of these discrete RNA peaks correspond to those predictable from known histone amino acid sequences (Grunstein *et al.*, 1973). Despite this evidence it has not yet been unequivocally demonstrated that the hybridization characteristics and messenger activity are attributes of the same RNA molecules.

Isolation and identification of histone mRNA not only provides a tool for the study of the synthesis and function of specific messages in development, but can also be used to probe the evolution of specific genes. Hybridization techniques have been used extensively to determine the average rates of base sequence change in total DNA from bacteria, plants, and animals.

Analysis of evolutionary divergence rates of specific genes such as those mentioned above together with comparative amino acid sequence data for numerous proteins have shown that there are large variations in the rates of divergence of different genes (see Dayhoff and McLaughlin, 1969). The fact that many base changes result in synonymous codons suggest that base substitutions in a particular gene could accumulate more rapidly than amino acid changes in the protein for which it codes (Kimura, 1968; King and Jukes, 1969). Estimates of these "neutral mutations" can be determined by comparing divergence rates of genes and their corresponding proteins. Histone genes and proteins are especially well suited for these comparisons because of the highly conserved nature of some of the proteins. Since the amino acid substitution rate is extremely low in histones III (Candido and Dixon, 1972) and IV (Smith *et al.*, 1970), very few base changes result in amino acid changes. Consequently, interspecies divergence in base sequence of these conserved histone genes can be used to estimate the accumulation of synonymous codon changes.

Materials and Methods

Sea Urchin Embryo Culture. Strongylocentrotus purpuratus embryos were cultured by procedures similar to those described by Whiteley *et al.* (1966). Eggs and sperm were obtained by injection of 2–3 ml of 0.55 M KCl into the coelomic cavity. The eggs were washed with Millipore-filtered sea water until 95% fertilization was obtained in a test sample. Eggs from several females were fertilized separately with sperm from a single male: cultures which gave >95% fertilization were combined when large numbers of embryos were needed. Development in the combined cultures used showed synchrony similar to cultures of eggs from single females. Embryos were cultured at 9° in Millipore-filtered sea water containing 250 μg/ml of streptomycin and 100 μg/ml of penicillin. Aeration was provided by gentle bubbling of air into cultures in separatory funnels or by magnetic stirring of cultures in large beakers. These culture conditions produced morulae in 12 hr, hatched blastulae in 40 hr, and gastrulae in 72 hr.

Isolation of DNA. Invertebrate DNAs were prepared from sperm or excised gonadal tissue by treatment with sodium dodecyl sulfate and Pronase as described by Lambert and Laird (1971). Liver or whole embryos were the source of vertebrate DNAs. The tissue was processed by a modification of the procedure of Marmur (1961) as described by Church and McCarthy (1968). DNA samples were treated with pancreatic RNase (Worthington) which had been heated at 80° for 10 min to inactivate trace amounts of DNase. RNase incubations were carried out at 37° for 15 min in 1 × SSC (0.15 M NaCl–0.05 M sodium citrate) with 10 μg/ml of RNase and approximately 1 mg/ml of DNA.

Preparations of Polyribosomes. S. purpuratus morula stage embryos (12 hr at 9°) were used to prepare polysomes for the isolation of mRNA. Ten milliliters of packed embryos was washed once and resuspended in 100 ml of Millipore-filtered sea water containing above-described antibiotics. The cultures were placed on a reciprocal shaker and aerated for 1.5 hr; 1 mCi of [^{3}H]uridine was added if pulse-labeled RNA was to be prepared. Cell homogenates were prepared by a modification of the method by Iverson and Cohen (1969) as previously described (McCarthy and Farquhar, 1972).

The 1.5-hr aeration period was an absolute requirement for the isolation of intact polysomes. When this step was omitted polysome profiles showed predominantly mono-, di-, and trisomes with little or no material in the larger polysome regions of the gradient. Dissociation of polysomes has been shown to occur in HeLa cells under "stress conditions" such as elevated temperatures (McCormick and Penman, 1969) or deprivation of essential amino acids (Vaughan *et al.*, 1971). The polyribosome complexes re-form when the cells are returned to normal growth conditions. A similar situation probably exists when sea urchin embryos are exposed to lengthy periods of low oxygen tension such as during the preparation period necessary for harvesting large volume cultures. The aeration period likely serves as a "recovery period" in which polysomes reassociate. Since the aeration cultures are concentrated they can be harvested rapidly. Because of the necessity of minimizing the preparation time, it was not possible to harvest cultures larger than 100 ml at a time. However, once the postmitochondrial supernatant is prepared, polysomes are stable for long periods at 4° (Iverson and Cohen, 1969). Therefore large amounts of material could be prepared by processing individual 100-ml cultures to the postmitochondrial supernatant stage and holding this at 4° until enough material had been accumulated for preparative centrifugation.

Isolation of Histone mRNA. SUCROSE GRADIENT FRACTIONATION. Pulse-labeled 9–12S RNA fractions containing histone mRNA sequences were isolated from either small polysome regions of sucrose gradients or from total polysomes which had been collected by centrifugation. RNA was purified by a slight modification of procedures described by Shearer and McCarthy (1967). Small polysome fractions were concentrated from sucrose gradients by precipitation in 70% ethanol. Ethanol precipitates or polysome pellets were resuspended in 70% ethanol. Ethanol precipitates or polysome pellets were resuspended in 10 mM $MgCl_2$–10 mM Tris (pH 7.5) and DNase (Worthington) was added to a final concentration of 5 μg/ml. After incubation at 37° for 1 min the nuclease digestion was terminated by the addition of SD buffer (0.02 M sodium acetate (pH 5.2)–2 M LiCl–0.5% sodium dodecyl sulfate). In several instances RNA preparations were frozen at this point. RNA was extracted with SD-saturated redistilled phenol at 37° for 10 min; the phenol–aqueous phase interface material was reextracted at 45° for 10 min. These extracts were combined and treated with chloroform–octanol (24:1) at room temperature. RNA was concentrated either by precipitation with 70% ethanol or by dialysis against distilled water followed by lyophilization. Although ethanol precipitation requires less time the yield of pulse-labeled material is only about 80%. Essentially all of the radioactivity is recovered after dialysis and lyophilization.

Histone mRNA fractions were separated on 5–20% sucrose gradients in 0.01 M Tris, 0.01 M NaCl, 0.5% sodium dodecyl sulfate (pH 7.5), 1 mM EDTA (Chamberlain and Metz, 1972), or on similar gradients without sodium dodecyl sulfate. Sucrose gradients containing sodium dodecyl sulfate were centrifuged at 20°. Initial centrifugation conditions for sodium dodecyl sulfate gradients in a Spinco SW-40 rotor were determined experimentally. Conditions were chosen which would provide maximum separation of 4–5S RNA from the 9–12S mRNA and still display the 18S rRNA as a peak near the bottom of the gradient. Centrifugation at 38,000 rpm for 13.5 hr at 20° met these criteria and could be used to fractionate 3–4 mg of RNA from 10 ml of packed morulae on six 13-ml gradients containing 5–20% sucrose in sodium dodecyl sulfate buffer. Once the desired pattern was determined similar sucrose gradient profiles of polysomal RNA could be obtained under different conditions by use of conversion tables calculated by McEwen (1967). For example, RNA from preparative polysome isolations (7–15 mg) was separated on six 38-ml 5–20% sucrose by centrifugation at 26,000 rpm for 26 hr in a Spinco SW27 rotor at 20°. RNA from the 9–12S region of the gradient was dialyzed and lyophilized. After resuspending in distilled water, sodium dodecyl sulfate was partially removed by precipitation with KCl at 4°. Since sodium dodecyl sulfate was not completely removed from RNA prepared in this way, polysomal RNA was also separated on gradients without detergent. These gradients were centrifuged at 5° in a SW40 rotor at 38,000 rpm for 20 hr.

RNA which was separated by the above methods (±sodium dodecyl sulfate) contained contaminating material which adsorbed at 230 nm. This material is probably derived from dialysis tubing and concentrated during lyophilization. Although treatment of dialysis tubing with 1 mM EDTA, 1% $NaHCO_3$ at 100° for 15 min did not prevent such contamination, this material could be removed by gel filtration. RNA is excluded from G-50 or G-75 Sephadex columns while the 230-nm-adsorbing material is retarded.

Preparative gel electrophoresis. ^{3}H-pulse-labeled polysomal RNA was also fractionated on preparative polyacrylamide gels. Gel polymerization and electrophoresis were carried out according to methods of Loening (1967) with modifications for preparative gels (Hagen, 1973). RNA samples in 0.2 ml of the running buffer 0.04 M Tris–0.02 M sodium acetate–2 mM EDTA (pH 7.8), containing 10% sucrose were applied to the top of a 2.5% polyacrylamide gel. Electrophoresis was carried out at 17.5 mA at room temperature. RNA was eluted from the end of the gel by pumping running buffer through the elution chamber at 0.1 ml/min. A_{260} was monitored by an LKB Unicord II UV Absorbtimeter connected to an LKB recorder. The effluent was collected in 1-ml fractions in a Gilson Microfractionator; aliquots of fractions were monitored for radioactivity in 10 ml of toluene–Omnifluor; Triton X-100 (2:1) in a scintillation counter.

RNA–DNA Filter Hybridization. DNA filters were prepared by the method of Gillespie and Spiegelman (1965) with modifications described by Denhardt (1966) and McCarthy and McConaughy (1968). Filters with a diameter of 6.5 mm contained 10–18 μg of DNA. The amount of DNA retained on the filters after high-temperature incubations was determined by the A_{260} values after extraction of the filter-bound DNA with 1 N HCl at 100° for 15 min (Vincent *et al.*, 1969).

Filter hybridization reactions were incubated for 16–18 hr, at 60 or 67° in 0.2 ml of 2 × SSC (Church and McCarthy, 1968) in 40 × 100 mm specimen vials, which had been treated with Siliclad (Clay-Adams) according to the manufacturers instructions. Approximately 0.1 ml of mineral oil was layered on the surface of the liquid to prevent evaporation during the incubation period. The reaction was terminated by washing the filter(s) from each vial (see below) twice in 2 ml of 2 × SSC at the incubation temperature. Filters were skewered on insect pins, dried under a heat lamp and counted in 10 ml of toluene–Omnifluor in a Packard scintillation counter. Background counts were determined for each scintillation *via* used in counting filters containing less than 50 cpm; average background counts were used for filters with more than 50 cpm bound.

Although Church and McCarthy (1968) determined 0.2 ml to be the optimum volume for reactions containing one DNA filter per vial, it was found that 0.2-ml volume was sufficient to cover as many as five filters per vial. Hybridization reactions with multiple filters per vial were used to increase the DNA:RNA ratios and thereby increase the percentage of input radioactivity bound to the filters. Incubation of several filters in one vial does not give equal amounts of reaction on each filter, probably due to unequal contact of the filter-bound DNA with the RNA in solution. For this reason comparisons of individual filters from a single reaction vial are invalid; however the total amount of radioactivity bound to all of the filters is experimentally reproducible.

Thermal Stability of RNA–DNA Filter Hybrids. The thermal stability of [^{3}H]RNA–DNA hybrids was determined by measuring the radioactivity released from the filters at increasing temperatures. Filters were immersed in 2 ml of 1 × SSC at the desired temperature in a Haake circulating water bath. After 5 min the filters were transferred to another tube of buffer and the temperature was increased by 5°. The amount of radioactivity released at each temperature was determined by counting each 2-ml sample in 10 ml of toluene–Omnifluor scintillant containing 1.7 ml of Biosolv (Beckman).

At least 100 cpm were used to determine each melting profile. In the event that 100 cpm were not bound in individual filter reactions, multiple filters were used to measure thermal stabilities of the hybrids. The counting efficiency in the scintillant containing Biosolv is about 1.3 times that of the filters in toluene–Omnifluor alone; therefore 130% was considered to represent complete recovery of radioactivity.

Relative thermal stabilities were determined by measuring the T_m of the various hybrids. This is defined as the temperature at which 50% of the radioactivity is released from the DNA filters.

Results

Analysis of Total Pulse-Labeled DNA from Small Polysomes. Hybridization of polysomal RNA from sea urchin morulae with DNA from distantly related species was used as an assay for conserved RNA in this fraction. Histone mRNA might be expected to reflect the conserved amino acid sequences found in some of the histone proteins. However the accumulation of a large number of neutral substitutions could result in considerable differences in base sequence even in genes for highly conserved proteins in divergent species.

Total [^{3}H]RNA from small polysomes were hybridized with mouse and pea DNA filters to determine if any RNA sequences in this fraction were sufficiently conserved to hybridize with DNAs from distantly related species. The amount of hybridization with mouse and pea DNAs although low, was significantly above background. This indicated that a highly conserved RNA species, probably histone mRNA, was present in pulse-labeled RNA from small polysomes. Reaction

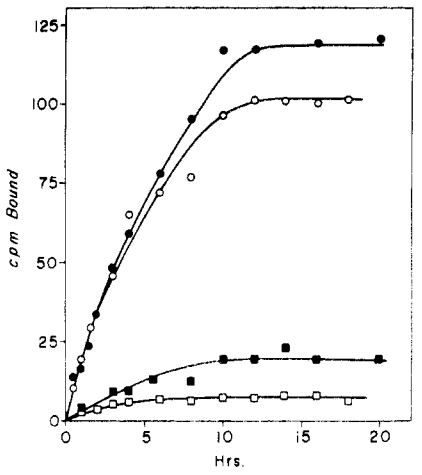

FIGURE 1: Kinetics of polysomal RNA–DNA hybrid formation. Total [^{3}H]uridine pulse-labeled RNA from small polysomes (4–7 ribosomes) was incubated with filter-bound sea urchin DNA (12 μg/filter) or mouse DNA (11 μg/filter) in 2 $\times$ SSC at 60 or 67°. Input radioactivity was 1000 cpm/vial. Filters were removed at the indicated times, suspended in 2 ml of 2 $\times$ SSC at the incubation temperature, agitated, and immediately transferred to a second 2-ml aliquot of 2 $\times$ SSC where they were held for 5 min. RNA–DNA hybrids were assayed by scintillation counting in toluene–Omnifluor. Data are shown for sea urchin DNA filters incubated at 60° (●) and 67° (○) and for mouse filters at 60° (■) and 67° (□).

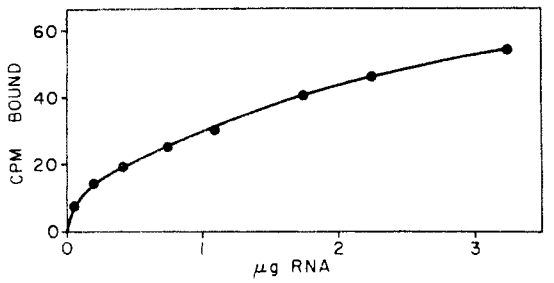

FIGURE 3: Saturation hybridization of 9–12S mRNA. DNA filters containing 18 μg of sea urchin sperm DNA were incubated with the indicated amounts of [^{3}H]uridine pulse-labeled 9–12S mRNA (900 cpm/μg). All incubations were in 0.2 ml of 2 $\times$ SSC at 67° for 16 hr. The reaction was terminated by two 5-min washes in 2 ml of the incubation buffer at 67°.

kinetics for polysomal [^{3}H]RNA and sea urchin and mouse DNAs are shown in Figure 1. The reaction is essentially complete in 10 hr. The relatively rapid reaction at the low nucleic acid concentrations used in these filter hybridization reactions suggested that this conserved RNA species was encoded by multiple DNA sequences (Kedes and Birnstiel, 1971). Unique or single copy RNA transcripts will not react with DNA under these same conditions (Church and McCarthy, 1968; Melli *et al.*, 1971).

Fractionation of Polysomal RNA. Since pulse-labeled polysomal RNA appeared to contain conserved histone mRNA sequences, further purification of these sequences was attempted using sucrose gradients (McCarthy and Farquhar, 1972) and preparative gel electrophoresis (Figure 2). The usual tRNA and rRNA species are present in large amounts; smaller quantities of 9–12S and 20S material are detectable by radioactivity. Although the preparative polyacrylamide gel technique yields well separated [^{3}H]RNA, the efficacy of the method is limited by the fact that quantities greater than 1 mg cannot be fractionated on these gels and some degradation of RNA often occurs. On the other hand, sucrose gradients could be used for fractionating very large quantities (15 mg) of polysomal RNA. Smaller SW40 gradients (McCarthy and Farquhar, 1972) were used routinely to separate polysomal RNA from 10 ml of embryo cultures; when preparative procedures were used to prepare polysomes, larger SW27 gradients (Farquhar, 1972) were utilized in RNA fractionation.

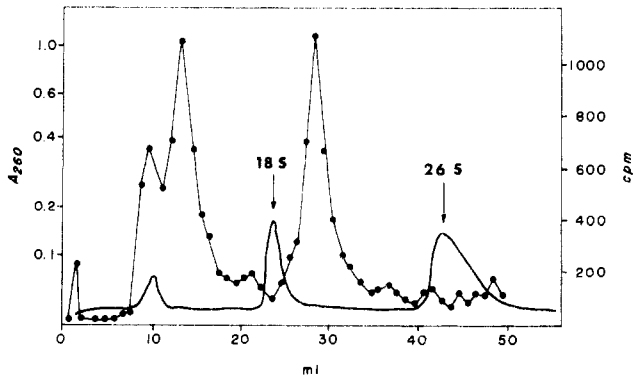

FIGURE 2: Polyacrylamide gel fractionation of polysomal RNA. ^{3}H pulse-labeled polysomal RNA (180 μg 1.5 $\times$ 10^4 cpm) was applied to a 2.5% polyacrylamide gel as described in the Materials and Methods section. Electrophoresis was carried out at pH 7.8 at 17.5 mA at room temperature. A_{260} is plotted as the heavy solid line; radioactivity in the 1-ml samples is described by the points connected by the light line (●).

The 9–12S mRNA isolated by these procedures was 97–99% alkali labile and 85–90% RNase digestible under hydrolysis conditions described in Materials and Methods. Hybrids formed at low RNA : DNA ratios (0.02 or less) with several preparations of 9–12S RNA and *S. purpuratus* sperm DNA had T_m values of 83–84° in 1 $\times$ SSC.

The 20S species, also reported by others (Kedes and Birnstiel, 1971; Hynes *et al.*, 1972), is apparently derived from small polysomal RNA, since it was present in similar quantities in RNA preparations from small polysomes and total polysomes. It was digested by alkali or RNase to the same extent as the 9–12S mRNA but formed hybrids with a slightly lower T_m; hybrids with *S. purpuratus* DNA had a T_m of 80° in 1 $\times$ SSC. Several experiments suggested that the 20S RNA might contain aggregated 9S histone mRNA. Unlabeled 9S RNA decreased the binding of ^{3}H-labeled 20S RNA in hybridization competition experiments, ^{3}H-labeled 20S RNA and ^{3}H-labeled 9S RNA form hybrids with DNAs of divergent species which exhibited similar T_m values. Furthermore, after dialysis and dilution the 20S material redistributes as 9S and 20S peaks on sucrose gradients (Farquhar, 1972).

Multiplicity of Histone Genes. The 9–12S RNA from small polysomes was hybridized with DNA filters in order to obtain an estimate of the fraction of the genome complementary to this group of RNA molecules. The saturation hybridization curve is shown in Figure 3. These data are replotted in double-reciprocal form (Figure 4A) and as a Scatchard plot (Figure 4B). Both reciprocal plots suggest more than one component, but the Scatchard plot allows a more complete analysis of the data. The bounded form of the Scatchard plot indicates the possible extent of reaction; therefore, it is possible to determine whether data points lie across a broad range of the saturation curve or fall at one of the extremes. These possibilities cannot be distinguished in the open form double-reciprocal plot. In addition linear amounts of RNA inputs yield linear data points in the Scatchard plot, but not in the double-reciprocal plot. This allows a more precise extrapola-

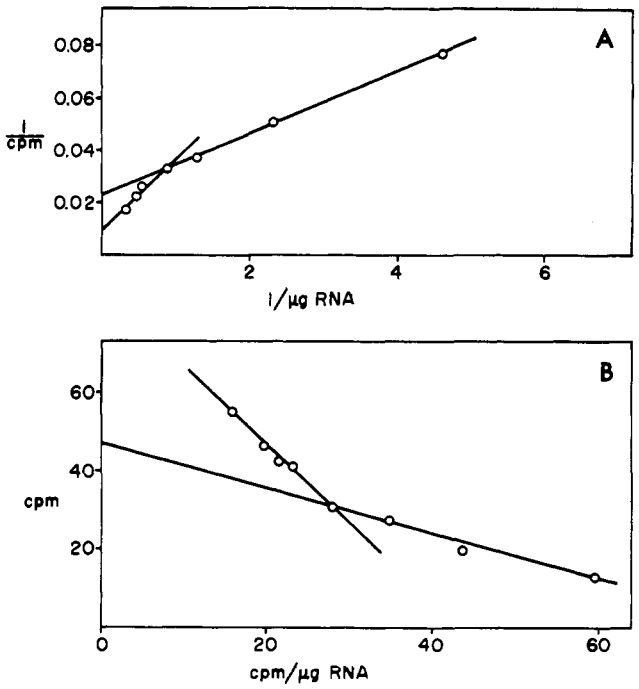

FIGURE 4: Reciprocal plots of saturation hybridization data. Data from Figure 3 are replotted as double-reciprocal (A) or Scatchard (B) plots.

tion of components which react at low RNA:DNA ratios. Saturation values can be determined from both plots from the abcissa intercept value; however, the Scatchard plot has the advantage of a second intercept (ordinate) which is proportional to the association constant of the hybrid. The Scatchard plot suggests that the 9–12S mRNA contains at least two populations of RNA molecules: (1) a rapidly reacting fraction of repeated sequences which has a relatively high association constant and (2) a more slowly reacting component with a lower association constant. Further evidence for heterogeneity in the 9–12S mRNA is given in Figure 5, which shows the thermal stabilities of the hybrids formed at various RNA:DNA ratios. Hybrids formed at low RNA:DNA ratios have high thermal stabilities; as more RNA reacts at higher RNA:DNA ratios the thermal stability decreases. The thermal stability measurements correlate well with characteristics determined by the Scatchard plot; *i.e.*, the rapidly reacting hybrids with high association constants contain more thermostable well-matched sequences than the more slowly reacting fraction which has a lower association constant.

An estimate for the number of histone genes can be obtained by extrapolation of the saturation kinetics of the early reacting component of the 9–12S RNA as shown in Figure 4. Saturation occurs at an RNA:DNA ratio of 2.7×10^{-3}. Assuming transcription of only one DNA strand, this indicates that approximately 0.5% of the sea urchin DNA codes for histones. The average repeat frequency for genes for each of the five histone classes may be estimated at 1100 when the following assumptions are made: (1) the *S. purpuratus* haploid genome size is 4.5×10^{11} daltons; (2) the five major histone fractions which are synthesized at the morulae stage have equal numbers of genes; and (3) 9–12S mRNA contains messages for the small histones (IIb_1, IIb_2, III, and IV) as well as the larger histone I with an average histone mRNA molecular weight of 200,000. The latter assumption is based on molecular weight determinations for 9S and 12S RNA from sea urchin embryos (Slater and Spiegelman, 1970) and mouse

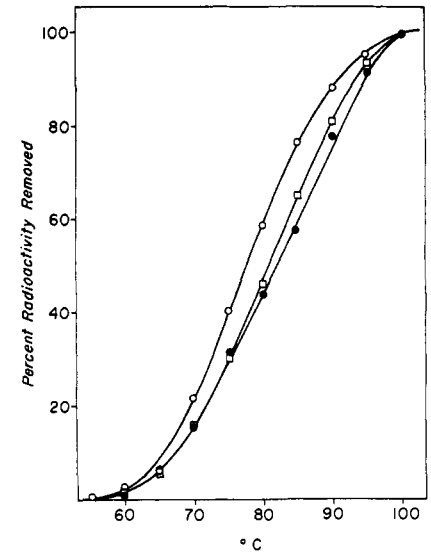

FIGURE 5: Thermal stabilities of hybrids formed at different RNA:DNA ratios. Hybrids formed with 18-μg filter bound sea urchin sperm DNA and increasing amounts of [^{3}H]uridine pulse-labeled 9–12S mRNA were dissociated by washing the filters in 2 ml of 1 × SSC at increasing temperatures. (●) RNA:DNA ratio of 0.01, T_m = 83.5°; (□), RNA:DNA ratio of 0.05, T_m = 81°; (○) RNA:DNA ratio of 0.15, T_m = 78°.

erythrocytes (Williamson *et al.*, 1971) and estimates of the minimum size mRNA needed for the various histones according to the molecular weight of the proteins.

Thermal stability and RNase resistance of hybrids formed at low RNA:DNA ratios were used to measure the extent of complementarity among the repeated histone genes. A comparison of the hybrids formed with homologous DNA (*S. purpuratus*) and DNA from a closely related sea urchin (*Strongylocentrotus drobachiensis*) is shown in Figure 6. Less than 5% of the hybrid is RNase sensitive in the homologous reaction and the T_m is 83° with or without enzyme treatment. However, some divergence in histone genes is detectable in the heterologous reaction (Weinberg *et al.*, 1972). Approximately 20% of the hybrid radioactivity is RNase sensitive.

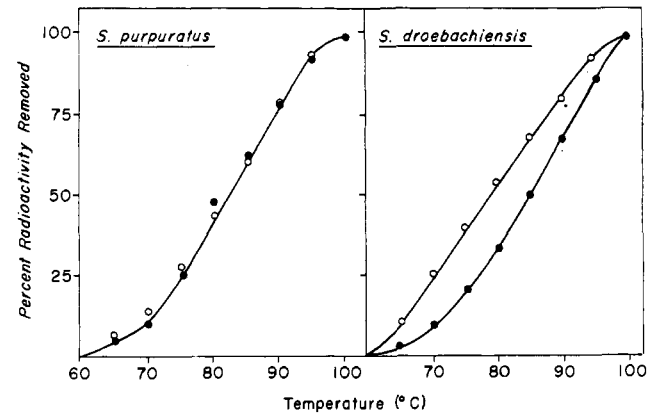

FIGURE 6: Thermal stabilities of homologous and heterologous hybrids after RNase treatment. Hybrids were formed by incubation of 1 μg of ^{3}H pulse-labeled 9–12S mRNA (900 cpm/μg) with 90 μg of filter bound *S. purpuratus* or *S. droebachiensis* sperm DNA in 0.2 ml of 2 × SSC at 67° for 16 hr. After washing in the incubation buffer, the filters were incubated at 37° for 20 min in 2 × SSC containing 10 μg/ml of RNase. Control filters were incubated under the same conditions in 2 × SSC alone. Thermal stabilities were measured in 1 × SSC for *S. purpuratus* hybrids: (●) RNase treated, T_m = 83°; (○) untreated, T_m = 83°; and *S. droebachiensis* hybrids: (●) RNase treated, T_m = 85°; (○) untreated, T_m = 80°.

TABLE I: Reaction of Histone mRNA with Various DNAs in 2 × SSC.[a]

DNA Source	Reaction Temperature 60° % cpm in Hybrid	60° T_m	67° % cpm in Hybrid	67° T_m
Echinoderms				
S. purpuratus (sea urchin)	16.9	77.0	13.6	83.5
Pisaster ochraceus (starfish)	3.2	70.5	1.9	80.0
Cucumaria miniata (sea cucumber)	2.2	70.0	4.1	77.0
Other invertebrates				
Aquorea aquorea (jellyfish)	3.5	69.0	2.6	75.0
Pododesmus macroshisma (oyster)	3.4	69.0		
Cancer magister (crab)	7.9	70.0		
Saccoglossus willipensis (acorn worm)	4.5	69.5	2.7	74.5
Vertebrates				
Oncorhynehus sp. (salmon)	3.1	69.0		
Xenopus laevis (toad)	1.1	69.0		
Gallus domestica (chicken)	1.8	67.5		
Mus musculus (mouse)	1.1	67.0	0.7	72.5
Plant				
Pisum sativum (pea)	2.0	67.5		
Bacterium				
Bacillus subtilis	0.3		0.02	

[a] Experimental details are described in legend to Figure 7.

Untreated hybrids have a T_m of 80°; enzymatic digestion of mismatched regions results in hybrids with a T_m of 85°. The fact that RNase digestion produces a heterologous hybrid with a slightly higher T_m (85°) than the homologous hybrid (83°) is probably due to experimental error; however, it is also possible that the effect is real and reflects increased conservatism among high G + C regions of the DNA.

Kedes and Birnstiel (1971) have estimated a G + C content of 51–53% for histone mRNA. The theoretical T_m for a perfectly matched 52% G + C RNA–DNA hybrid is 85° (Marmur and Doty, 1962; Church and McCarthy, 1970). The average T_m for homologous hybrids in several experiments at low RNA:DNA ratios and high stringency (67° and 2 × SSC) is 83.5°. Although these hybridization conditions favor the formation of high G + C RNA–DNA hybrids, the T_m is probably a minimum value due to some reaction of the heterogeneous RNA molecules contaminating the histone mRNA. Therefore, the exact number of base substitutions within a family of histone genes cannot be determined, but the small T_m difference between the theoretical (85°) and the experimental (83.5°) values suggests that the repeated genes for a particular histone must be very nearly identical (Weinberg *et al.*, 1972).

Evolution of Histone Genes. The 9–12S mRNA from small polysomes was hybridized to filter-bound DNA from a number of widely divergent species to assay for conserved sequences in this fraction and to estimate the rate of nucleotide substitutions in the hybridizable RNA. Stable hybrids were formed with all eukaryotic DNAs tested. The results of hybridization with DNA from several species are summarized in Table I. The amounts of hybrid formed show large variations due to the fact that the genome size and probably the numbers of genes coding for histones vary widely among the organisms tested. Initial hybridization reactions were carried out at relatively low stringency conditions (60°, 2 × SSC) in order to maximize the amounts of reaction with DNAs from divergent species. Melting profiles of hybrids formed under these conditions with invertebrate DNAs and vertebrate DNAs are shown in Figure 7A. The thermal stabilities of the hybrids formed at 60° in 2 × SSC are remarkably similar (Table I); substantially lower T_m's are seen only with DNAs from those species most distantly related to the sea urchin, *i.e.*, the higher vertebrates and a higher plant.

Although the less stringent conditions (60°, 2 × SSC) allow higher amounts of reaction with divergent species and are, therefore, quite useful in testing a wide variety of organisms, these conditions are not optimal for well-paired hybrids of high G + C content. Since the 9–12S mRNA contains a population of RNA molecules which form hybrids of low thermal stabilities (Figure 5) the T_m of the homologous hybrid probably reflects mismatching of these heterogeneous RNA molecules rather than an accurate measure of the thermal stability of the histone mRNA hybrids. Under conditions of higher stringency it is possible to maximize the histone mRNA hybridization, since the rate of reaction of high G + C hybrids increases with temperature (Straus and Bonner, 1972). At the same time the increased stringency minimizes the reaction of the heterogeneous RNA, since hybrids with a high degree of mismatching are unstable under these conditions (McCarthy and Farquhar, 1972).

Comparison of the thermal stabilities of hybrids formed at 60 and 67° (Table I) shows that more stringent conditions tend to amplify the differences in the various heterologous reactions. A lower degree of hybridization under these conditions is probably a result of two factors (1) the most highly mismatched sequences are not stable at the higher temperature and (2) the rate of reaction of sequences containing mispaired bases is decreased (Sutton and McCallum, 1971; McCarthy and Farquhar, 1972; Hutton and Wetmur, 1973). Melting profiles of hybrids formed at 67° in 2 × SSC are shown in Figure 7B.

Hybridization at 60° (Table I) suggests that sea urchin histone genes are equally divergent from those of other echinoderms, other invertebrates and lower vertebrates. However, the 67° reactions (Figure 7B and Table I) show significant differences in the thermal stabilities of hybrids formed with DNAs from these groups of animals. The ΔT_m of approximately 3–6° among echinoderms and 9° between sea urchin and jellyfish or acorn worm DNA indicates that detectable divergence has occurred within the echinoderms and between echinoderms and other invertebrate phyla.

Hybridization of 9–12S mRNA from *S. purpuratus* with DNAs of other sea urchins is shown in Table II. Equal amounts of reaction (per microgram of DNA) occur with DNAs from the *Stronglyocentrotus* species, although the decreased T_m in the heterologous hybrid indicates interspecies divergence. Less hybrid formation is seen with *Lytechinus pictus* DNA, but the thermal stability of the hybrid is the

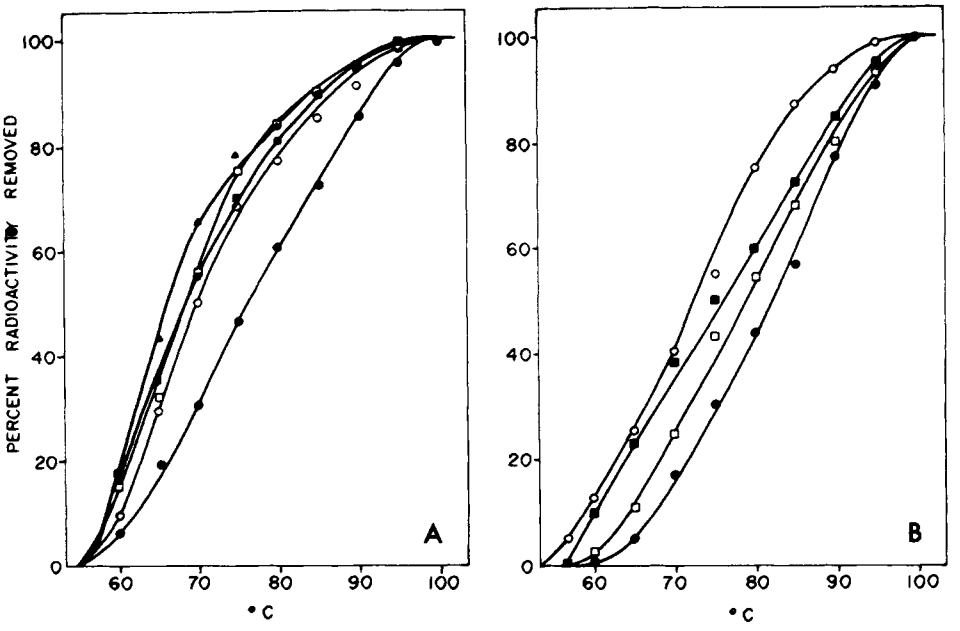

FIGURE 7: (A) Thermal dissociation profiles of hybrids formed by sea urchin histone mRNA with heterologous DNA at 60° in 2 × SSC. Filters containing 80–100 μg of DNA were incubated with 1.6 μg of 9–12S [^{3}H]RNA (1100 cpm) in 0.2 ml of 2 × SSC at 60° for 16 hr. Thermal stabilities were measured in 1 × SSC for hybrids formed with (●) sea urchin DNA, (○) starfish DNA, (□) jellyfish DNA, (■) salmon DNA, and (▲) mouse DNA. (B) Thermal dissociaton profiles of histone mRNA–DNA hybrids formed at high stringency. Experimental conditions were as described for (A) except that all incubations were at 67°. Melting profiles of hybrids formed with (●) sea urchin DNA, (□) starfish DNA, (■) jellyfish DNA, and (○) mouse DNA are shown.

same as that formed with *S. droebachiensis* DNA. A possible explanation is that the histone genes in *L. pictus* and *S. droebachiensis* are equally divergent from *S. purpuratus*, but *L. pictus* has fewer histone genes than members of the genus *Stronglyocentrotus*. However, it is more probable that different histone mRNAs are hybridized in the two species. Since amino acid sequences are not equally conserved in all of the histone classes (see references in Table III), the ΔT_m between the *S. droebachiensis–S. purpuratus* pair probably represents the average divergence of all the histones in closely related sea urchins, while the ΔT_m in the *L. pictus–S. droebachiensis* DNA–RNA hybrids could reflect divergence of only the more conserved histones in more distantly related sea urchins.

Discussion

Even though complete comparative amino acid sequence data for all of the major histone fractions is not available, partial sequences and chemical analyses suggest that the various histones have accumulated amino acid replacements at different rates in evolution (Table III). The five major histones can be classed in three groups on the basis of rates of evolutionary divergence: (1) low rate of amino acid substitution, histones III and IV; (2) intermediate rate of substitution, histones IIb_1 and IIb_2; and (3) rapid substitution rate, histone I (Panyim *et al.*, 1971; Oliver and Chalkley, 1972).

Histone IV contains fewer amino acid substitutions among divergent species than any other protein for which complete sequence data is available. Smith *et al.* (1970) have shown that there are only two amino acid differences in this protein isolated from peas and cattle. Histone III exhibits an amino acid substitution rate similar to histone IV; tryptic peptides of this protein from peas and cattle are indistinguishable (Fambrough and Bonner, 1968) and the first 25 residues of trout histone III (Candido and Dixon, 1972) are identical with those of the analogous calf thymus fraction (Olsen *et al.*, 1972).

A higher rate of divergence exists in histones IIb_2. Amino acid sequence determinations reveal a deletion of two residues and substitutions at three other positions when the first 22 residues of trout histone IIb_2 (Candido and Dixon, 1972) are compared with the corresponding region of calf thymus histone IIb_2 (Iwai *et al.*, 1970). Comparative sequence data for histones IIb_1 are not available, although a partial sequence of the calf thymus protein has been reported (Olsen *et al.*, 1972). However, polyacrylamide gel electropherograms of histones from numerous species suggest that histones IIb_1 and IIb_2 have accumulated amino acid substitutions at similar rates (Panyim *et al.*, 1971).

The very lysine-rich histone fraction, histone I, contains proteins which are the least conserved of all the histones. Heterogeneity within this fraction from a single tissue as well

TABLE II: Hybridization of *S. purpuratus* 9–12S mRNA with DNAs of Other Sea Urchins.[a]

DNA Source	DNA (μg)	% Input Radioactivity Bound	T_m
S. purpuratus	56	7.7	83.0
S. droebachiensis	66.5	9.3	80.0
L. pictus	62.5	3.5	80.5

[a] ^{3}H pulse-labeled 9–12S mRNA (1.8 μg, 900 cpm/μg) was incubated with filter-bound DNA as indicated in 2 × SSC at 67°. Thermal stabilities were measured in 1 × SSC as described in Materials and Methods.

TABLE III: Evolutionary Divergence in Histones.

Histone	Amino Acid Residues[a]	Species Compared	Method	Amino Acid Substitutions	Min % Base Substitutions
I (f_1)	*Ca.* 212	Cow Rabbit[b]	Partial sequence	$^{18}/_{73}$, two deletions	9
		Two rabbit fractions[b]	Partial sequence	$^{6}/_{74}$, two deletions	7
IIb_1 (f_{2a2})	136–157	Several vertebrates[c] and *Drosophila*[d]	Polyacrylamide gel electrophoresis	Similar to $11b_2$	
IIb_2 (f_{2b})	125	Cow[e] Trout[f]	Partial sequence	$^{3}/_{22}$, two deletions	13
III (f_3)	130–145	Cow[g] Trout[f]	Partial sequence	$^{0}/_{25}$	0
IV (f_{2a1})	102	Cow Pea[h]	Complete sequence	$^{2}/_{102}$	0.6

[a] Total residues in calf thymus histones from review by DeLange and Smith (1971). [b] Rall and Cole (1970) in review by DeLange and Smith (1971). [c] Panyim *et al.* (1971). [d] Oliver and Chalkley (1972). [e] Iwai *et al.* (1970). [f] Candido and Dixon (1972). [g] Olsen *et al.* (1972). [h] Smith *et al.* (1970). [i] The minimum percent base substitutions in the genes for the various histones are calculated from the available amino acid sequence data. Each amino acid substitution is assumed to represent a single base change. Deletions are considered as three base changes.

as numerous amino acid replacements in analogous proteins from closely related species are seen in histone I (Rall and Cole, 1970; Cohen and Gotchel, 1971).

The amino acid sequences of the more conserved histones (IIb_1, IIb_2, III, and IV) appear to have been fixed early in eukaryotic evolution. Although it is possible that these proteins arose from duplication and divergence of a single gene, only minimal sequence homology appears among the various histones of present day species (Olsen *et al.*, 1972; Candido and Dixon, 1972). Divergence could have occurred before the evolution of the ancestral eukaryotic species; however, it appears that convergent evolution (Kerkut, 1962) may best explain the few similarities which exist among the conserved histones.

The extent of base substitutions among histone genes of divergent species can be approximated from the amount of hybridizable RNA and the thermal stabilities of the histone mRNA–DNA hybrids. Studies of the properties of chemically altered DNA sequences which contain non-Watson–Crick base pairs indicate that mismatching lowers the hybrid thermal stability approximately 1.6° for each 1% altered base pairs (Ullman and McCarthy, 1973; Hutton and Wetmur, 1973). Obviously thermal stability measurements can only be used to quantitate base substitutions in those hybrids which are sufficiently well matched to form hybrids under the conditions employed. Well-matched histone mRNA–DNA hybrids have a T_m of approximately 84° in 1 × SSC; therefore, reaction conditions of 67° and 2 × SSC, equivalent to 62° and 1 × SSC (Schilkraut and Lifson, 1965), would allow hybrids to form only if they contained less than 15% mismatched bases. Under less stringent conditions, such as 60° and 2 × SSC, hybrids of similar G + C contents with nearly 20% mismatched bases would be stable. Estimates of the extents of base substitutions in the messages for the various histones have been calculated from the amino acid substitutions in the corresponding proteins (Table III). These calculations suggest that while all of the *S. purpuratus* histone messages react with DNA from a closely related sea urchin, *S. droebachiensis*, under stringent conditions only the messages for the most conserved histones (III and IV) would form stable hybrids with DNA from distantly related invertebrates or vertebrates.

Because of the redundancy of the genetic code, it is possible for nucleotide substitutions to accumulate in structural genes which will not result in amino acid substitutions in the corresponding protein. These synonymous codon changes have been called neutral mutations and are the basis for the theory of non-Darwinian evolution (King and Jukes, 1969). Simply stated, this theory suggests that third position codon changes which produce synonymous codons are immune to natural selection (*i.e.*, they are neither advantageous or harmful) and could accumulate at a rate similar to mutation rates. Kimura (1968) has calculated that out of 549 possible codon changes, 134 or 25% could lead to synonymous codons which would not result in amino acid changes. Assuming complete conservation of amino acid sequence, corresponding genes of two distantly related organisms could differ by as much as 17% if all possible synonymous codon changes accumulate (Kohne, 1970). In the case of histone IV the change in the mRNA base sequence expected from the amino acid substitutions would be approximately 0.5% for the pair cow–pea and similar levels of base substitution probably exist in the mRNAs for histone III (Table III). Since the other histone fractions show substantially higher rates of base substitutions even among species more closely related than higher plants and animals, the hybridization experiments with sea urchin histone mRNA and DNA of divergent species probably measure only base sequence homologies among the most conserved histones (III and IV). Thus the divergence of approximately 8% of the nucleotide sequences in the histone genes from the distantly related pair of species, sea urchin and mouse, suggests some 7.5% base substitutions attributable to third position changes (total nucleotide substitutions, 8%, less 0.5% calculated from amino acid changes in divergent species). Presumably the level of third position substitutions could approach the limiting value of 17% if a more distantly

related pair of species were considered. However, it should be emphasized that such a rate of accumulation of base substitutions is not as rapid as would be expected if these third position changes are strictly neutral.

Estimates of the rate of accumulation of base substitutions within the phylum *Echinodermata* also suggest low levels of neutral mutations. Within the genus *Stronglyocentrotus* only 2% of the bases differ in the histone genes of *S. purpuratus* and *S. droebachiensis*. Since the same amount of hybrid formation occurs in the homologous and heterologous reactions, this 2% difference probably represents an average divergence of all of the histone genes (Weinberg *et al.*, 1972). Undoubtedly some of these base changes have produced amino acid changes in the more rapidly evolving histones. However, even assuming that all of these changes are neutral, their rate of accumulation is still well below the average eukaryotic mutation rate of 10^{-5} mutations/gene per generation (Ohno, 1972). Speciation in the genus *Stronglyocentrotus* sea urchins probably occurred at least 30–40 million years ago (Moore, 1966); a base substitution rate of 2% in the histone genes would give a mutation rate of 4×10^{-7} mutations/gene per generation based on a single-stranded molecular weight of 200,000 daltons for an average gene and a generation time of 1 year. A similar estimate of the rate of divergence of sequence in 9S RNA between two other genera of sea urchins, *Psammechinus* and *Paracentrotus*, has been reported by Weinberg *et al.* (1972).

Comparisons of nucleotide sequences of the related RNA bacteriophages R17, MS2, and f2 also show less than the maximum number of third position substitutions (Robertson and Jeppeson, 1972). Coat proteins (400 amino acids) of the three viruses differ by one amino acid residue, while the nucleotide sequences which code for them differ in about 4% of the bases. However, these nucleotide substitutions which are neutral in the sense that they result in synonymous codons are definitely nonrandom; U-C transitions occur much more frequently than other substitutions. The partial nucleotide sequence analysis of the silk fibroin message provides additional evidence that nucleotides in the third position of synonymous codons to not occur randomly (Suzuki and Brown, 1972). Although some codon degeneracy exists in the mRNA, unequal distributions of synonymous codons were found for glycine, serine, and alanine, the principal amino acids in silk fibroin.

Nonrandom distribution of bases in DNA such as those discussed earlier have led to the concept of a DNA phenotype (Schaap, 1971). The high levels of double-stranded regions reported in RNA bacteriophage messengers (Sanger, 1971; Jeppeson *et al.*, 1970) and proposed for eukaryotic mRNA (White *et al.*, 1972) suggest that certain phenotypes (conformations) are also important in RNA structure and function. In other words, selection pressure probably acts on nucleic acids as well as their protein gene products (Ball, 1973). Third position changes, which may be neutral in their coding properties may be advantageous in terms of the conformation of DNA or transcribed RNA.

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Messenger Ribonucleic Acid Metabolism in Mammalian Mitochondria. Origin of Ethidium Bromide Resistant Poly(adenylic acid) Containing Ribonucleic Acid in Ehrlich Ascites Mitochondria†

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ABSTRACT: The patterns of inhibition of actinomycin D and ethidium bromide on both *in vivo* and *in vitro* RNA synthesis in Ehrlich ascites mitochondria have been studied. Both the *in vivo* and the *in vitro* labeled mitochondria contain high molecular weight RNA migrating between 3 and 28 S. Only the *in vivo* RNA but not the *in vitro* RNA contains RNase resistant filter bindable poly(A) sequences. The *in vitro* RNA synthesis is extremely sensitive to ethidium bromide, while the *in vivo* synthesis of mtRNA is less sensitive to this drug (55–60%). Actinomycin D, on the other hand, affects the *in vivo* RNA synthesis by about 35%, and has no effect on the *in vitro* system. The ethidium bromide resistant mtRNA contains an increased amount of poly(A) as against actinomycin D resistant RNA which is extremely low in poly(A) content. The ethidium bromide resistant RNA has a half-life of 35–40 min, which coincides with the half-life of *in vivo* labeled poly(A) in mitochondria. Actinomycin D resistant RNA and the *in vitro* RNA, on the other hand, decay more rapidly with a half-life of 10 min. On the basis of these results and also kinetics of ethidium bromide resistance and appearance of poly(A), it has been proposed that the ethidium bromide resistance observed in the *in vivo* experiments is probably because of the effect of nRNA synthesis and that poly(A) containing RNA found in mitochondria are of nuclear origin. This hypothesis was further strengthened by DNA–RNA hybridization experiments.

Extensive research during the last decade has unequivocally shown the presence of specific systems for the synthesis of DNA, RNA, and protein in mitochondria (Ashwell and Work, 1970). Since mitochondrial systems are dissimilar to nucleocytoplasmic systems, many scientists consider these organelles as partly autonomous (for references, see Ashwell and Work, 1970). Nevertheless, it is now widely accepted that mitochondrial biogenesis is a complex event involving the expression of both mitochondrial and nuclear genomes (Woodward *et al.*, 1970; Borst, 1972). Most of the mito-

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