Comparison of the Mitochondrial Ribonucleic Acid from a Wild-Type Grande and a Cytoplasmic Petite Yeast by Ribonucleic Acid–Deoxyribonucleic Acid Hybridization†

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ABSTRACT: Mitochondrial RNA (mt-RNA) preparations of a wild-type grande strain and a spontaneously mutated cytoplasmic petite strain of yeast, *Saccharomyces cerevisiae*, have been studied by hybridization to mitochondrial DNA (mt-DNA). Increasing amounts of ^{3}H-labeled mt-RNAs were hybridized to filter bound grande and petite mt-DNA until a saturation level was reached. The saturation level with grande [^{3}H]mt-RNA was 15% for the grande mt-DNA and 7–8% for the petite mt-DNA. The maximal level of binding of the petite [^{3}H]mt-RNA was 13% with the grande mt-DNA and 26% with the petite mt-DNA. Hybridization competition studies showed that the grande and petite mt-RNAs each contained sequences not present in the other. The ^{3}H-labeled mt-RNA–mt-DNA hybrids were thermally denatured to construct melting curves. Grande [^{3}H]mt-RNA hybridized to grande mt-DNA had a T_m of 76.7°, whereas the petite [^{3}H]mt-RNA–petite mt-DNA hybrid had a T_m of 68.2°. The T_m's of the heterohybrids, *i.e.*, grande [^{3}H]mt-RNA–petite mt-DNA, and petite [^{3}H]mt-RNA–grande mt-DNA, were 72.1 and 70.0°, respectively. These results indicate that grande and petite mitochondria each contain RNA transcripts of mt-DNA which are not present in the other.

It is now clearly established that mitochondria contain RNAs that are distinct from those found in the cytoplasm. Mitochondrial ribosomal RNA and transfer RNA have been shown to differ in physical and hybridization properties from their counterparts in the cytoplasm (see Borst and Grivell, 1971; Borst, 1972; and Casey *et al.*, 1972, for reviews). Mitochondrial RNAs hybridize specifically with and presumably are transcripts of mt-DNA.[1]

The mt-DNA of cytoplasmic petite (respiratory deficient) mutants in yeast are greatly modified, as indicated by altered buoyant density and base composition, and by DNA–DNA hybridization and renaturation kinetic studies (Mounolou *et al.*, 1966; Bernardi *et al.*, 1968; Mehrotra and Mahler, 1968; Carnevali *et al.*, 1969; Bernardi *et al.*, 1970; Fauman, 1970; Cohen *et al.*, 1972; Hollenberg *et al.*, 1972a,b; Fauman and Rabinowitz, 1972). Examination of the mt-RNA of petites may help to characterize the changes in petite mt-DNA.

In an earlier study, Wintersberger and Viehauser (1968) found no mt-RNA in a petite strain and could detect no hybridization between grande mitochondrial ribosomal RNA and petite mt-DNA. Fukuhara *et al.* (1969), however, showed that total RNA extracted from a different petite strain did indeed contain RNA that hybridized with both petite and grande mt-DNA. Petite mt-RNA hybridized more efficiently to its own mt-DNA than to the grande mt-DNA, and conversely, grande mt-RNA hybridized to a greater extent to the grande mt-DNA than to the petite mt-DNA.

This paper represents a more detailed study by hybridization techniques of mt-RNA isolated from a petite and a grande yeast strain. The data indicated that mitochondria from a grande and petite yeast strain each contain RNA sequences not present in the other mt-DNAs.

Material and Methods

Yeast Strains and Growth. The two chromosomal isogenic strains of yeast used are subclones from the original haploid strains D243-2B-R1 and D243-2B-R1/6 obtained from the laboratory of Dr. P. P. Slonimski (Centre de Génétique Moléculaire du C.N.R.S., 91 Gif-sur-Yvette, France). D243-2B-R1 is a grande and D243-2B-R1/6 is a spontaneously mutated cytoplasmic petite strain. The strains have been characterized previously (Mounolou *et al.*, 1966; Fukuhara *et al.*, 1969; Cohen *et al.*, 1972). Cultures were grown according to the methods described earlier (Casey *et al.*, 1972).

Preparation of Mitochondria. Mitochondria from grande and petite were isolated as previously described (Rabinowitz *et al.*, 1969).

Purification of Mitochondrial and Nuclear DNA. DNA was prepared from grande and petite mitochondria that had been digested with DNase by the hydroxylapatite method as described previously (Fauman, 1970; Casey *et al.*, 1972). Nuclear DNA was first isolated on hydroxylapatite and then purified in a CsCl density gradient to remove any mt-DNA contamination. To prepare the CsCl gradient, 4.3750 g of pure CsCl was dissolved in 3.4 ml of DNA solution. The refractive index was adjusted to 1.3990 and the samples were centrifuged for 33 hr at 43,000 rpm in the 65 rotor of an L2-65B Spinco ultracentrifuge at 25°. The nuclear DNA fractions were pooled, dialyzed against 1 × SSC (0.15 M NaCl–0.015 M Na_3-citrate), and used for hybridization.

The purity of the grande and petite mt-DNA preparations was checked by CsCl isopycnic centrifugation in the Beckman Model E analytical ultracentrifuge. No contamination by

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[1] Abbreviations used are: mt-DNA, mitochondrial DNA; mt-RNA, mitochondrial RNA; Me_2POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; POP, 2,5-diphenyloxazole; SSC, standard saline citrate, 0.15 M NaCl–0.015 M Na_3-citrate.

nuclear DNA was detected even when cells were overloaded (5 μg of mt-DNA), indicating that the mt-DNA preparations were at least 95% pure.

Extraction and Purification of RNA. RNA was extracted from mitochondrial pellets by a procedure modified from the method of Kirby (1968). The pellets were suspended in 5–10 vol of 0.15 M NaOAc–0.1 M NaCl–2% sodium dodecyl sulfate, 0.1% in macaloid (Baroid Division, National Lead Co.); an equal volume of 6% 4-aminosalicylate was added followed by 2 vol of a phenol–cresol mixture consisting of 500 g of phenol, 70 ml of *m*-cresol (distilled two times), 55 ml of distilled water, and 0.5 g of 8-hydroxyquinoline (Eastman Organic Chemicals). The mixture was extracted at 4° for 20 min and then centrifuged at 20,000*g* for 10 min. The aqueous phase was removed and made 2.0% in NaCl. An equal volume of phenol–cresol was added and the mixture was again extracted for 15 min. After centrifugation, 2.5 vol of 100% ethanol was added to the aqueous phase and it was stored in the freezer for several hours. The precipitated RNA was centrifuged at 5000*g* for 5 min and the supernatant drained off. The resultant RNA pellet was washed three times with 95% ethanol to remove the residual phenol and cresol.

The mitochondrial RNA preparation was digested with DNase to remove possible contamination with mt-DNA. The mt-RNA was dissolved in 2.0 ml of buffer consisting of 0.05 M NaCl–0.01 M $MgCl_2$–0.01 M Tris, pH 7.2, and 40 μg of DNase (Worthington, RNase free) for 30 min at 37°. The mitochondrial RNA was then extracted with phenol–cresol and again precipitated with ethanol as described above. The RNA precipitate was collected by centrifugation and washed three times with 95% ethanol.

Transfer RNA was removed from the RNA preparations used for hybridization by the method of Kirby (1968). The RNA pellet was extracted three times with 0.5 ml of cold 3.0 M NaOAc buffer, pH 6.0, per milligram of RNA. The undissolved RNA was centrifuged after each extraction and the supernatant containing the transfer RNA discarded. Three extractions removed 15–20% of the total mt-RNA.

Hybridization. Nitrocellulose filters were loaded with DNA according to the method of Gillespie and Spiegelman (1965). The DNA in 1 × SSC was denatured with 0.2 ml of 1.0 N NaOH in 1.0 ml of DNA solution for 20 min at room temperature. The solution was cooled to 4°, neutralized with cold 1.0 N HCl, adjusted to 4 × SSC, and loaded on filters. The filters were dried at room temperature for 1 hr and then at 60° overnight. mt-DNA (1 μg) was loaded on each filter.

The microhybridization technique described previously (Fauman, 1970; Fauman and Rabinowitz, 1972) was used for the competition studies. mt-DNA filters and incubation solution containing labeled denatured mt-DNA were sealed between two pieces of parafilm and the entire microhybridization unit was immersed in a water bath at 35°. Mitochondrial RNA hybridization saturation curves were obtained by macrohybridization in a total volume of 0.5 ml in glass scintillation vials. Filters containing 1.0 μg of mt-DNA were incubated for 24 hr at 35° in RNA solutions containing 2 × SSC and 36% formamide (Matheson, Coleman and Bell). At the end of the incubation the filters were washed in 1 l. of 2 × SSC and incubated in RNase solution for 1 hr at 35°, in 1.0 ml of 2 × SSC containing 20 μg of pancreatic RNase (Calbiochem, boiled 10 min), and 10 units of T_1RNase (Calbiochem). At the end of the RNase digestion the filters were washed on both sides with 50 ml of 2 × SSC, dried, and counted in toluene base scintillation fluid containing 5.0 g of PPO and 0.1 g of Me_2POPOP per liter of toluene.

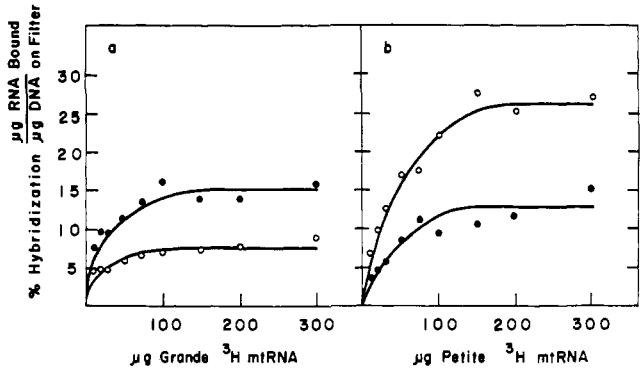

FIGURE 1: Hybridization saturation of grande [^{3}H]mt-RNA and petite [^{3}H]mt-RNA. Separate filters, containing 1.0 μg of grande mt-DNA (●) and 1.0 μg of petite mt-DNA (○), were added to vials containing increasing amounts of grande [^{3}H]mt-RNA (a) or petite [^{3}H]mt-RNA (b) in 0.5 ml of 2 × SSC and 36% formamide and incubated at 35° for 24 hr. The hybrids were treated with RNase, washed, dried, and counted. The specific radioactivity of the grande mt-RNA was 1570 cpm/μg; that of petite mt-RNA was 2000 cpm/μg. Filter blanks without DNA or with *E. coli* DNA varied from less than 0.5 to 9% of the counts bound to mt-DNA, and have been subtracted.

Hybrid Melting Curves. The thermal stability of the RNA–DNA hybrids bound to filters was measured. After hybridization the filters were washed, dried, and incubated successively in vials with 1.0 ml of 1 × SSC for 5 min at temperature increments of 5°. Triton scintillation fluid was added to each vial and the [^{3}H]DNA released at each temperature counted in the Packard Tricarb scintillation counter.

Isotope Labeling. To obtain ^{3}H-labeled mt-RNA, yeast was grown overnight in 700 ml of YPG medium containing 2.5 mCi of [8-^{3}H]adenine (12–22 Ci/mmol; Schwarz BioResearch, Orangeburg, N. Y.). Cytoplasmic ribosomal RNA labeled with ^{32}P was obtained by growth overnight in 700 ml of medium containing 2.0 mCi of ^{32}P (Mallinckrodt Nuclear).

Results

Hybridization Studies. RNA HYBRIDIZATION SATURATION CURVES. Hybridizations of grande and petite mt-RNAs to grande and petite mt-DNAs are shown in Figure 1a,b. The hybridization of grande [^{3}H]mt-RNA to grande mt-DNA is referred to as grande mt-RNA *iso*hybridization, whereas the hybridization of the grande labeled mt-RNA to petite mt-DNA is denoted as grande mt-RNA *hetero*hybridization. The same convention is followed for the hybridization of petite [^{3}H]mt-RNA to grande and petite mt-DNA, forming a petite mt-RNA heterohybrid and petite mt-RNA isohybrid, respectively. Labeled mt-RNA was added in increasing amounts in each hybridization series until a maximal level of hybridization was reached. The saturation value obtained was considered to represent a measure of the fraction of the mt-DNA that was homologous to the mt-RNA tested.

Figure 1a shows the hybridization of grande [^{3}H]mt-RNA to 1.0 μg of grande and petite mt-DNAs on separate nitrocellulose filters. Hybridization values were corrected for nonspecific binding to blank filters containing no DNA or *E. coli* DNA that represented 2–7% of the [^{3}H]mt-RNA bound to filters containing homologous mt-DNA. Saturation of both mt-DNAs occurs at an input of about 100 μg of grande [^{3}H]mt-RNA. The saturation level ((micrograms of RNA bound/micrograms of DNA) × 100) for the grande mt-RNA

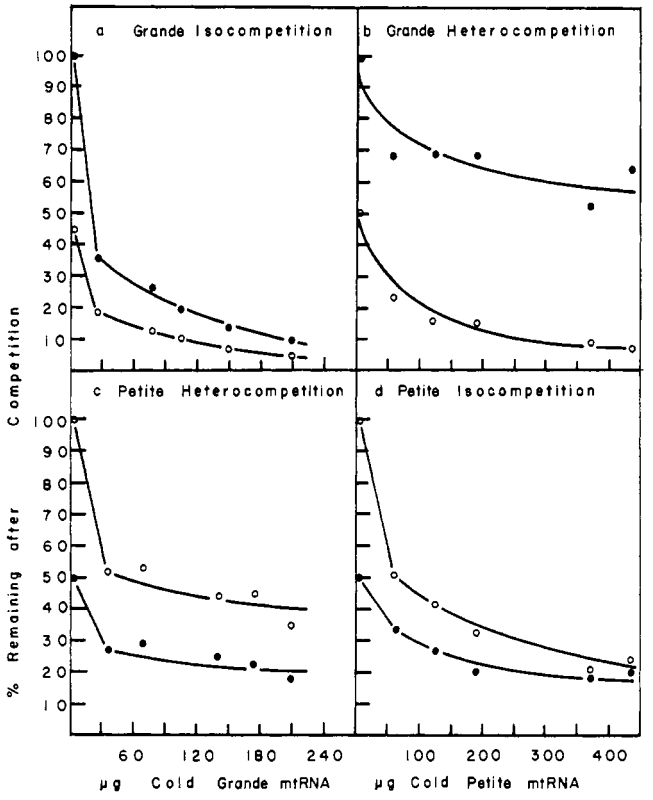

FIGURE 2: Hybridization competition studies of grande and petite mitochondrial ribosomal RNA. Ten micrograms of grande [^{3}H]mt-RNA hybridized to separate filters containing 1.0 μg of grande mt-DNA (●) and 1.0 μg of petite mt-DNA (○) was competed with increasing amounts of cold grande mt-RNA (a) or cold petite mt-RNA (b). The level of hybridization obtained in the isohybridization without competitor is taken as 100%. The specific activity of grande [^{3}H]mt-RNA in (a) was 1740 cpm/μg of RNA, and 193 cpm was bound to grande mt-DNA. The specific radioactivity of grande [^{3}H]mt-RNA in (b) was 1570 cpm/μg of RNA, and 130 cpm was bound to grande mt-DNA in the absence of competitor. Eight micrograms of petite [^{3}H]mt-RNA hybridized to separate filters containing 1.0 μg of grande mt-DNA (●) and 1.0 μg of petite mt-DNA (○) was competed with increasing amounts of cold grande mt-RNA (c). The specific radioactivity of petite [^{3}H]mt-RNA was 2200 cpm/μg of RNA, and 210 cpm was bound to petite mt-DNA in the absence of competitor. Thirty micrograms of petite [^{3}H]mt-RNA hybridized to separate filters containing 1.0 μg of grande mt-RNA (●) and 1.0 μg of petite mt-DNA (○) was competed with increasing amounts of cold petite mt-RNA (d). The specific radioactivity of petite mt-RNA was 1710 cpm/μg of RNA and 190 cpm was bound to petite mt-DNA in the absence of competitor.

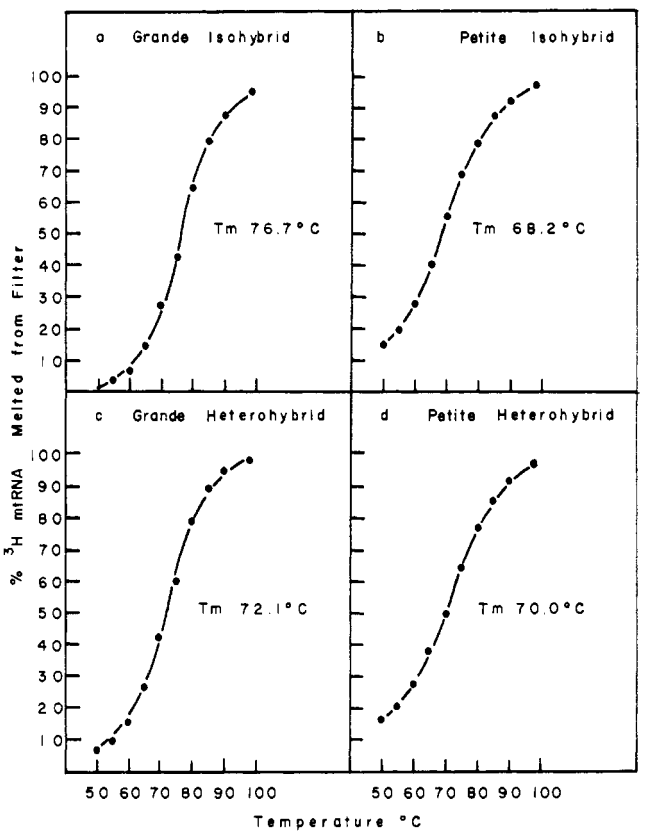

FIGURE 3: Thermal denaturation curves of mitochondrial RNA DNA hybrids. Hybrids of grande [^{3}H]mt-RNA to grande mt-DNA (a) or petite mt-DNA (c) and hybrids of petite [^{3}H]mt-RNA to petite mt-DNA (b) or grande mt-DNA (d) were melted as described in Materials and Methods. Initial radioactivity of the hybrids was for (a) grande isohybrid, 236 cpm, (b) petite isohybrid, 520 cpm, (c) grande heterohybrid, 120 cpm, and (d) petite heterohybrid, 262 cpm.

isohybrid is 15–16%, the value for the grande mt-RNA heterohybrid is 7–8%.

Figure 1b shows the hybridization of petite [^{3}H]mt-RNA to 1.0 μg of grande and petite mt-DNAs. Saturation of the two mt-DNA's with petite [^{3}H]mt-RNA is attained with an input of 160–200 μg of RNA. The petite mt-RNA isohybrid saturates at about 26–27%, and the petite mt-RNA heterohybrid at 12–13%. Thus, in hybridizations with both grande and petite [^{3}H]mt-RNA, the saturation levels of the heterohybrids are only about 50% of those of the isohybrids. It may also be noted that the petite isohybrid saturates at a considerably higher level than the grande isohybrid. Furthermore, the petite heterohybrid saturation level is almost as high as that of the grande mt-RNA isohybrid.

Competition Studies. Competition studies between labeled and unlabeled mt-RNAs are shown in Figure 2a–d. A convention similar to that used above to discuss hybridization experiments is used to describe the competition studies. Thus, the competition of grande [^{3}H]mt-RNA hybridization with unlabeled grande mt-RNA is called a grande mt-RNA isocompetition while competition of grande [^{3}H]mt-RNA hybridization with unlabeled petite mt-RNA is called a grande mt-RNA heterocompetition. Competition of petite [^{3}H]mt-RNA hybridization with unlabeled petite mt-RNA or grande mt-RNA is referred to as petite mt-RNA isocompetitions or heterocompetitions, respectively.

Figure 2a shows that, as expected, unlabeled grande mt-RNA almost completely competes with grande [^{3}H]mt-RNA in the hybridization to both mt-DNAs. The petite mt-RNA isocompetition in Figure 2d shows a similar pattern; however, the amount of unlabeled petite mt-RNA competitor needed is considerably greater than the amount of grande mt-RNA competitor required in the grande isocompetition (Figure 2a). The petite mt-RNA isocompetition is also not as complete as the grande mt-RNA isocompetition. These effects may be due to a lower concentration of petite mt-RNA in the mitochondrial RNA preparation which decreases the effective competing mt-RNA species.

The grande mt-RNA heterocompetitions are shown in Figure 3b. The hybridization of grande [^{3}H]mt-RNA to the petite mt-DNA is effectively competed out by unlabeled petite mt-RNA (lower curve), but the binding of grande [^{3}H]mt-RNA to grande mt-DNA is reduced by only 40–50% (upper curve). Thus it appears that the grande mt-RNA contains a

considerable number of sequences homologous to grande mt-DNA that are not present in the petite mt-RNA. Furthermore, those sequences in grande mt-RNA that are homologous with petite mt-DNA are effectively competed for by the petite mt-RNA.

In the petite mt-RNA heterocompetition (Figure 2c), there is again a considerable fraction of petite [^{3}H]mt-RNA (about 40%) that is not competed for by grande mt-RNA in the hybridization with petite mt-DNA. Thus, petite mt-RNA appears to contain sequences not present in grande mt-RNA. It should also be noted that there is a residual 20% binding of [^{3}H]petite mt-RNA to grande mt-DNA after competition with grande mt-RNA (Figure 2c, lower curve). Petite mt-RNA therefore may contain transcripts that are homologous to grande mt-DNA, but are not present in the grande mt-RNA preparations.

Melting Curves of Hybrids. Figure 3a–d shows the melting curves of the grande and petite iso- and heterohybrids. The two isohybrids have T_m values which differ by approximately 8.5°. The grande mt-RNA isohybrid has a T_m of 76.7° (Figure 3a); the T_m of petite mt-RNA isohybrid is 68.2° (Figure 3b). The heterohybrids have intermediate T_m values. The grande mt-RNA heterohybrid has a T_m of 72.1° (Figure 3c) and the petite mt-RNA heterohybrid has a T_m of 70.0° (Figure 3d).

Discussion

The hybridization data described in this paper show that mitochondria of a spontaneously mutated cytoplasmic petite strain of yeast contain RNA of high molecular weight, which hybridizes to its own mt-DNA, and to mt-DNA of the wild-type grande yeast. The RNA preparations are undoubtedly heterogenous and contain a variety of RNAs. Polyacrylamide gel electrophoresis showed a polydisperse pattern of high molecular weight RNAs (results not shown). The hybridization saturation levels obtained—13–14% in the grande isohybridization and 25–26% in the petite isohybridization—are considerably higher than the 2.4% reported for purified yeast ribosomal RNA (Reijnders *et al.*, 1972).

The results of the hybridization saturation and hybridization competition studies show that the grande and petite mitochondrial RNA preparations each contain transcripts that are not present in the other. Saturation hybridization levels of grande mt-RNA to grande mt-DNA or of petite mt-RNA to petite mt-DNA (the isohybridizations) are about two times higher than those for the respective heterohybridizations. Furthermore, 40–50% of the hybridization of grande mt-RNA with grande mt-DNA is not competed for by petite mt-RNA. Similarly, grande mt-RNA incompletely competes for the hybridization of petite mt-RNA with petite mt-DNA.

The simplest explanation for the presence of sequences in petite and in grande mitochondrial RNA not represented in the other is that they reflect changes in mt-DNA. Thus, a gross sequence change in petite mt-DNA that is transcribed into RNA could explain the hybridization data.

An alternate explanation is that petite mt-DNA is a simple deletion of wild-type mt-DNA, but that a wider range of transcripts of mt-DNA is present in the petite mt-DNA preparations. The higher petite isohybrid saturation level (26% compared to 13% for the grande isohybrid) is consistent with this view. Also supporting a more extensive transcription of petite mt-DNA, or a greater recovery of transcripts, is the finding that grande mt-RNA competes incompletely with petite [^{3}H]mt-RNA for hybridization with grande mt-DNA.

The melting curves of the RNA–DNA hybrids support the specificity of our hybridization results. The differences in T_m obtained are consistent with the higher AT content of the petite mt-DNA and the higher A + U content of the petite mt-RNA. The fact that the T_m values of heterohybrids were not lower than those of isohybrids provides evidence against extensive widely scattered base sequence changes in petite mt-DNA. In that case heterohybrids with decreased stability and lower T_m would be expected. If base sequence changes are present in petite mt-RNA, they must be severe enough to prevent entirely the hybridization of the affected segment with grande mt-DNA.

Our results confirm and extend the findings previously reported by Fukuhara *et al.* (1969), who studied the hybridization of total cell RNA isolated from the same grande strain and a petite strain that was the source of the subclone used in our study. They also found the isohybridizations to be about two times greater than the heterohybridization, but their hybridization curves never reached saturation, and the maximum hybridization level observed was only 4%.

Filter DNA–DNA hybridization studies of the same strains studied here (Fauman, 1970; Fauman and Rabinowitz, 1972; Gordon and Rabinowitz, 1973) indicated large (30–50%) changes in the petite mt-DNA. The second-order renaturation rate of the petite mt-DNA was increased about twofold (Fauman and Rabinowitz, 1972), suggesting some loss of genetic complexity. Reiteration of a part of the DNA was also suggested by the presence of a rapid renaturing fraction of the petite DNA.

The results presented in this paper cannot differentiate between the possible alterations in petite mt-DNA. The data show, however, that a petite mutant may contain mitochondrial RNA species which are absent in wild-type yeast.

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Correlation between Narrow-Banded Ultraviolet Spectra and Oxygen Equilibrium Functions in Native and Chemically Modified Human Hemoglobins†

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ABSTRACT: Correlation between narrow-banded ultraviolet spectra and oxygen equilibrium functions was studied by using human adult hemoglobin and its derivatives prepared by treating it with iodoacetamide, *N*-ethylmaleimide and carboxypeptidase A. Differential spectrum, *i.e.*, the first derivative of absorption spectrum, showed a fine structure with a maximum at 289.5 nm and two minima at 286.5 and 293.5 nm for all the hemoglobins at the position corresponding to the shoulder in the absorption spectrum. The magnitude of the fine structure in the differential spectrum for native oxyhemoglobin was twofold that for deoxy one. The magnitude of the fine structure for deoxy form and the association constant for the first oxygen molecule were increased by the chemical modifications of protein as well as by the stripping of 2,3-diphosphoglycerate, and there was a distinct correlation between the two quantities. Neither the magnitude of the fine structure for oxy form nor the association constant for the fourth oxygen molecule were affected by the chemical modifications or by 2,3-diphosphoglycerate. A fine structure also appeared around 290 nm in oxy *vs.* deoxy difference spectra of all the hemoglobins. The magnitude of the fine structure in difference spectra was closely related to the magnitude of cooperativity in oxygen binding which was measured by overall free energy of interaction among the oxygen binding sites or by Hill's coefficient. The less the cooperativity, the smaller the magnitude of the fine structure of the difference spectra whether 2,3-diphosphoglycerate was present or absent. These spectral changes cannot be explained only by heme contribution, indicating that there are contributions of aromatic chromophores. Although the aromatic groups responsible for the spectral changes cannot be explicitly identified, the present results are consistent with the idea that C3β tryptophans located at the $\alpha_1\beta_2$ contact are most probably involved. The data also suggest that the differential spectra around 290 nm are partial reflections of the conformational states the tetramer assumes in the oxy–deoxy reaction and that the oxy *vs.* deoxy difference spectra indicate the extent of the conformational changes which play key roles in the cooperative oxygen binding of hemoglobin.

In order to investigate the molecular mechanism for the allosteric effects in hemoglobin, *i.e.*, cooperative oxygen binding, Bohr effect, reciprocal binding of 2,3-diphosphoglycerate (P_2-glycerate[1]), etc., it is critically important to study the relationship between the allosteric ligand binding functions and conformational changes in the protein moiety induced by ligand binding.

Difference spectrum between oxy- and deoxyhemoglobins in the ultraviolet (uv) region from about 280 to 295 nm exhibits narrow-banded difference peaks which are superposed on the broader heme contribution and are attributable to perturbations of aromatic chromophores (Enoki and Tyuma, 1964; Briehl and Hobbs, 1970). If the perturbations are due to environmental changes around specific aromatic groups in the protein, it can be expected that the uv difference spectra reflect conformational changes in the protein and are related to the allosteric ligand binding functions of hemoglobin.

It has been found in the present study that the spectral changes in the uv region are closely related to the oxygen equilibrium functions.

Materials and Methods

Materials. Hemoglobin prepared from the blood of normal human adults (Hb A, native Hb) was freed from phosphates as described by Benesch *et al.* (1968). Three kinds of chemically modified hemoglobins were prepared from the stripped hemoglobin as follows. The reactive sulfhydryl groups of F9 (93) β-cysteines were blocked by the reaction with ten- and fivefold molar excesses of iodoacetamide and *N*-ethylmaleimide, respectively, in 0.1 M Bis-Tris buffer (pH 7.3) for 1 hr at 25° (Benesch and Bensch, 1961) and the resulting

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[1] Abbreviations used are: P_2-glycerate, 2,3-diphosphoglycerate; native Hb, Hb(AcAm), Hb(MalN), and Hb(CPase), human adult hemoglobin untreated and its derivatives prepared by treatment with iodoacetamide, *N*-ethylmaleimide, and carboxypeptidase A, respectively; *Y*, fractional saturation of hemoglobin with oxygen; *p*, oxygen pressure; P_{50}, oxygen pressure at the half-saturation; k_1 and k_4, intrinsic association constants for the first and fourth oxygen molecules, respectively; *n*, Hill's coefficient; ΔF_1, overall free energy of interaction among the oxygen binding sites; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol.