It is perhaps worth noting in this connection that hexoses appear to be near the critical size for penetration into the three-dimensional structure of proteins (Giles and McKay, 1962) and thus a specific interaction cannot be ruled out.

Pekar and Frank (1972) have pointed out that on the basis of their calculated association constants for zinc-free insulin, the ratio of insulin monomer to dimer at the insulin concentration levels in serum would range from 26,000:1 (under glucose challenge) to 80,000:1 (fasting) providing strong evidence that the monomer is the active form of insulin. If the glucose effect demonstrated in the present work operates at physiological concentrations these ratios (particularly the lower one) would be even higher.

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Sequence Homology between Mitochondrial DNA and Nuclear DNA in the Yeast, *Saccharomyces cerevisiae*†

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ABSTRACT: Sequence homology between mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in a haploid, respiratory sufficient strain of the yeast, *Saccharomyces cerevisiae*, has been investigated by means of DNA-DNA and DNA-cRNA filter hybridization. [^{3}H]nDNA and mtDNA, both highly purified by CsCl density gradient centrifugation, were hybridized at low temperature with formamide for 48 hr. The resulting mtDNA-nDNA hybrids were examined for the fidelity of the hybrid formed (absence of nonhomologous AT or mismatched sequences) by thermal dissociation. The method was checked by comparison with *Clostridium* DNA-nDNA hybrids. Hybridization results with either high or low molecular weight nDNA, after correction for nonhomologous hybridized sequences, indicate that approximately two mtDNA genome equivalents of homology are present in the nuclear genome. This homology was shown not to result from cross contaminating DNA sequences. *In vitro* complementary RNA (cRNA), templated from highly purified mtDNA, was also hybridized with CsCl fractionated, high molecular weight nDNA in an independent assay. Identical amounts of homology were detected. Hybridization to gradients containing sheared nDNA resulted in a shift in buoyant density of homologous sequences. These results show that significant amounts of mtDNA is homologous with nDNA and that this mtDNA is inserted within nDNA.

Mitochondrial inheritance and transmission is generally assumed to be regulated by an extranuclear cytoplasmic system. The possibility of a nuclear control acting either independently or in concordance with a cytoplasmic system has not been ruled out, however. Wilkie has proposed (Wilkie, 1963, 1970) that in yeast a "master copy" of mtDNA,[1] associated with the nucleus, might have some role in mitochondrial biogenesis. Genetic evidence has yet to substan-

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[1] Abbreviations used are: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; cRNA, *in vitro* synthesized complementary mitochondrial RNA; SDS, sodium dodecyl sulfate; PVP, polyvinylpyrrolidone; SSC, 0.15 M NaCl-0.015 M sodium citrate.

tiate this hypothesis, although recent studies with ethidium bromide mutagenesis on petite induction and inhibition of mtDNA synthesis in yeast suggest that some form of mitochondrial master template might be operative in mtDNA regeneration (Luha *et al.*, 1971; Whittaker *et al.*, 1972).

One means of determining if a master copy exists is to assay for mtDNA–nDNA sequence homology by molecular hybridization techniques. Attempts to detect molecular homologies between the two genomes in yeast thus far have indicated little homology between mtDNA and nDNA (Michaelis *et al.*, 1972; Cohen *et al.*, 1970; Fukuhara, 1970). In all of these studies the amounts of homology between the two genomes were quantified by DNA–RNA hybridization techniques using either *in vivo* synthesized mtRNA or cRNA synthesized from mtDNA templates with *E. coli* RNA polymerase. Because both of these RNA preparations are likely to represent a small fraction of the total mitochondrial genome, transcribed under specific conditions, these results are at best only semiquantitative and might be expected to lead to underestimates.

In order to assess homology between the two genomes a number of requirements must be fulfilled: DNA–DNA hybridization is necessary to ensure that full complements of both genomes are involved in the hybrid; both mtDNA and nDNA preparations used for hybridization must be free of contamination with the other genome; the fidelity of hybridization must be assured by denaturation analysis (McCarthy, 1967); and the distribution of hybridized sequences should be determined to exclude possible hybridization of nonhomologous or mismatched sequences.

We have reexamined the question of sequence homology between the mitochondrial and nuclear genomes of a haploid, respiratory sufficient strain of the yeast, *Saccharomyces cerevisiae*. Both DNA–DNA and DNA–cRNA hybridization experiments demonstrate that significant homology between the two genomes does exist. CsCl gradient hybridization analyses indicate that this homology represents nucleotide sequences integrated within the nuclear genomes and that it does not result from contamination of nDNA preparations with mtDNA.

Materials and Methods

Strains. S. cerevisiae D-243-2B-R_1, (ρ^+,P_7) (designated R_1), a haploid, respiratory sufficient strain, auxotrophic for adenine, was obtained from Dr. P. P. Slonimski. mtDNA in this strain comprises 16% of total cellular DNA. Buoyant densities in neutral CsCl of nuclear and mtDNA are 1.698 and 1.683 g/cm^3, respectively. Additional information concerning the geneology of this strain can be found in Bernardi *et al.* (1970).

Growth and Labeling of Cells. Cells used for the preparation of unlabeled mtDNA and nDNA were grown in 2% galactose, 2% Bactopeptone, 1% yeast extract, and 10 μg/ml of adenine. Growth was at room temperature for 36 hr in aerated 3- or 6-l. glass bottles. Radioactive labeling of cells was carried out in 200 ml of medium containing 2% galactose, 1% Difco yeast nitrogen base with amino acids (filter sterilized), 10 μCi/ml of [^{3}H]adenine (3.3 Ci/mmol), and 2 μg/ml of cold adenine. When the culture approached stationary phase of growth, measured by turbidity at 600 mμ, an additional 10 μg/ml of cold adenine was added and the cells were harvested one generation later during logarithmic growth. Over 90% of the isotope was incorporated.

Spheroplast Formation and DNA Isolation. Spheroplasts were prepared by a modification of methods reported by Duell *et al.* (1964) and Mehrotra and Mahler (1968). Log phase cells were harvested and washed with distilled water by 10-min centrifugations at 4000*g* and 20° in a Sorvall RC2-B centrifuge. The cells were suspended in 2.5 volumes of 0.14 M 2-β-mercaptoethanol–0.14 M EDTA (pH 8.0) and incubated for 0.5 hr at 33° with gentle agitation. After centrifugation, they were resuspended in one volume of 1.8 M sorbitol and one-third volume of 1.0 M citrate–phosphate buffer, pH 5.8, was added; 1 ml of glusulase was added per 10 g of cells and incubation was continued for 1 hr at 33° with gentle agitation. Spheroplast formation was monitored by observing the decrease in turbidity produced by a 1/100 dilution of the sample in distilled water. Cell lysis was usually greater than 80% after 1 hr of incubation. Spheroplasts were washed twice in cold 1.8 M sorbitol prior to lysis.

nDNA was isolated from spheroplasts or purified nuclei. Nuclei were prepared according to a modification of the method used by Rozijn and Tonino (1964). Spheroplasts were suspended in 4 volumes of 0.02% Triton X-100 (w/v) and 4% PVP buffer (4% PVP-40, 0.001 M $MgCl_2$, 0.02 M KPO_4, pH 6.5) and broken in a glass homogenizer fitted with a motor driven Teflon pestle. The homogenate was immediately diluted with an equal volume of 0.6 M sucrose and 4% PVP buffer and centrifuged at 3000*g* for 7 min to pellet nuclei. The nuclear pellet was resuspended in 0.6 M sucrose and 4% PVP buffer and centrifuged a second time. The pellet was then suspended in 13 ml of 0.6 M sucrose and 4% PVP buffer and layered on top of a sucrose gradient containing 5.0 ml of 2.3 M sucrose in 8% PVP, 10.0 ml of 2.0 M sucrose in 4% PVP, and 10.0 ml of 1.5 M sucrose in 4% PVP. Centrifugation was in a Beckman SW 27 rotor for 2 hr at 24,500 rpm and 5°. The bulk of the nuclei banded at the interphase between the 2.0 and 2.3 M sucrose layers. Electron microscopy of these preparations revealed only a small amount of mitochondrial contamination associated with the nuclear membranes.

Nuclear pellets or spheroplasts were lysed in 3 volumes of 0.15 M NaCl, 0.1 M EDTA (pH 8.0), and 2% SDS and deproteinized with chloroform–isoamyl alcohol (24:1), and the nucleic acids were precipitated with 2 volumes of 95% ethanol at −20° for 2 hr or more. The precipitate was suspended in 1 X SSC, and treated consecutively with pancreatic RNase (50 μg/ml), T_1 RNase (10 units/ml), and Pronase (50 μg/ml), all at 37° for 0.5 hr. The sample was again deproteinized, precipitated with ethanol, and suspended in a small volume of 0.1 X SSC.

mtDNA was prepared from isolated mitochondria according to a modification of the method used by Hollenberg *et al.* (1970). Spheroplast preparations were suspended in an equal volume of 0.35 M sucrose, 0.1% bovine serum albumin (fraction V), and 1 mM EDTA (pH 7.6) and homogenized at 5° in a Waring Blendor with a rheostat setting at 35/100 for 15 sec. Four volumes of buffer were added and the suspension was homogenized at a rheostat setting of 100/100 for 20 sec. The homogenate was repeatedly centrifuged at 4000*g* for 10-min intervals until a pellet was no longer obtained and then at 20,000*g* for 20 min to pellet mitochondria. The pellet was suspended in 0.35 M sucrose and 0.005 M $MgCl_2$ containing 200 μg/ml of DNase and incubated for 1 hr at 5° with occasional shaking to remove contaminating nDNA. The mitochondria were then washed twice in 0.35 M sucrose and 0.1 M EDTA (pH 8.0) and DNA was extracted by the same procedure used for nDNA.

Analytical CsCl density gradient centrifugation of mtDNA extracted by this procedure showed no detectable nDNA contamination.

DNA concentrations were estimated spectrophotometrically, assuming 1.0 OD 260 mμ units to be equal to 50 μg/ml. Specific activities were determined by spotting aliquots of purified DNA on Millipore filters followed by liquid scintillation counting in a 2,5-diphenyloxazole, 1,4-bis[2-(5-phenyloxazolyl)]benzene, and toluene cocktail.

Preparative CsCl Density Gradient Centrifugation. Nuclear and mtDNA preparations were both freed of contaminating DNA by two cycles of CsCl gradient centrifugation. Gradients were prepared by the addition of 3.5 ml of DNA to 4.4 g of solid CsCl and adjusted to a density of 1.690 g/ml. Gradients were overlaid with mineral oil and centrifuged at 33,000 rpm for 65 hr at 20° in a Spinco angle 65 rotor. Gradients were collected from the bottom with a 20 gauge needle in 12-drop fractions.

Figure 1a and b shows OD profiles of nuclear and mtDNA after two cycles of CsCl centrifugation. No cross contamination is visible. Further proof of purity was demonstrated in a reconstruction experiment (Figures 1c and d), in which [^{3}H]mtDNA, previously purified by two cycles of CsCl centrifugation, was mixed with DNA extracted from spheroplast lysates. After centrifugation, tubes containing nDNA were removed and purified by another centrifugation after which the radioactivity in the nuclear region of the gradient was used as an estimate of the amount of mtDNA contaminating the nDNA. After one cycle of centrifugation, only 1% of the mtDNA was found in the nuclear fraction. After two cycles of centrifugation it was reduced to 0.12%.

High molecular weight mtDNA (average molecular weight of 1×10^7) was selected for the reconstruction experiments to avoid the possibility that the mtDNA, already having been centrifuged twice in CsCl, might have lost mtDNA sequences which would be expected to band with and contaminate the nuclear region. The high molecular weight should preclude such a loss and the 0.12% is believed to be a valid estimate of contamination to be expected in normal preparations of nDNA.

Sonication of DNA and Molecular Weight Determination. One-milliliter samples of DNA were sonicated in 0.1 X SSC for 10 sec at 5 ° with a Sonifer Cell Disrupter 185 set at maximum voltage. This procedure yielded DNA with an average molecular weight of 500,000. Sedimentation rates and molecular weights were determined according to Studier (1965), using a Spinco Model E ultracentrifuge.

In vitro Synthesis of Complementary Mitochondrial RNA. Highly radioactive cRNA, complementary to mtDNA, was prepared with fraction V of *Escherichia coli* RNA polymerase prepared according to Berg *et al.* (1971). Transcription was carried out in 0.25 ml of 40 mM Tris (pH 7.9), 150 mM KCl, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 70 mM EDTA, 6 mM 2-β-mercaptoethanol, and 5×10^{-9} mol each of [^{3}H]ATP (9.5 Ci/mmol), [^{3}H]CTP (28.4 Ci/mmol), [^{3}H]UTP (22.2 Ci/mmol), and unlabeled GTP. The reaction mixture contained 10 μg of template DNA and 10 units of polymerase. After 2 hr at 37°, 100 μg/ml of pancreatic DNase was added and the mixture was incubated at 37° for 15 min. The solution was cooled to 5° and 0.25 ml of 0.1 M sodium acetate (pH 5.0) and 5 μg/ml of poly(vinyl sulfate) were added. cRNA was isolated by adding SDS to 2% and extracting the mixture with phenol, followed by purification on a Sephadex G-50 column equilibrated and eluted with

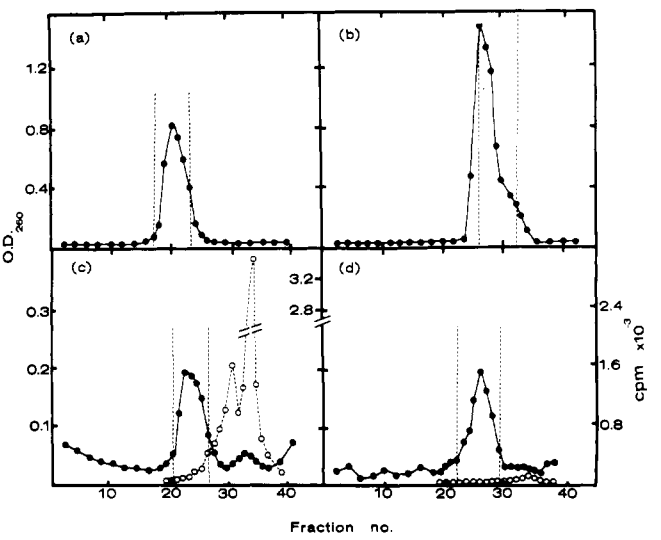

FIGURE 1: CsCl density gradient centrifugation of nDNA and mtDNA: (a) nDNA after two CsCl centrifugations; (b) mtDNA after two CsCl centrifugations. The fractions between the hatched lines were collected and represent purified DNA. (c) and (d), a reconstruction experiment determining the amount of mtDNA contamination of nDNA. In this experiment, 0.24 μg of [^{3}H]mtDNA (13,000 cpm), purified by two CsCl centrifugations, was mixed with DNA extracted from spheroplast lysates. The nDNA was then purified by CsCl centrifugation. The amount of [^{3}H]mtDNA in each fraction was determined by scintillation counting after the addition of 100 μg of calf thymus DNA and an equal volume of cold 10% trichloroacetic acid to each aliquot. Precipitates were collected on filters, dried, and counted as described in Materials and Methods. (c), first CsCl centrifugation; the fractions between the hatched lines, representing nDNA and containing 1.05% mtDNA contaminate, were pooled and centrifuged a second time. (d) nDNA after two CsCl centrifugations, mtDNA contamination was reduced to 0.12%. (●—●) optical density; (○ - - ○) [^{3}H] mtDNA.

0.1 X SSC. From the known base composition of mtDNA and the specific activity of nucleotide precursors used, the cRNA prepared by this method was estimated to have a specific activity of 5.3×10^7 cpm/μg.

Hybridization. Alkali denatured DNA, diluted to less than 1 μg/ml in 4 X SSC, was fixed to Millipore filters (HAWG) by gravity filtration and dried for 12–15 hr at 70°. The filters were then preincubated in 2 X SSC, 0.02% PVP-360, bovine serum albumin (fraction V), and ficoll for 5 hr at 60° according to Denhardt (1966). Preincubation of filters reduced background counts for DNA–DNA hybridization to 0.4% and for DNA–cRNA hybridizations to 0.01%. Heat denatured DNA or heat denatured cRNA was hybridized according to McConaughy *et al.* (1969) in 2.0 ml (4.0 ml for gradient hybridization) of 2 X SSC and 25% formamide for 48 hr at 37°. DNA–DNA hybrids were washed in 5 ml of 2 X SSC for 20 min at 37°, 2 hr in 2 X SSC at room temperature with mild agitation, and then on both sides with 20 ml of 2 X SSC by rapid suction filtration. DNA–cRNA hybrids were digested with pancreatic and T_1 RNase in 1 X SSC and washed by the same procedure used for DNA–DNA hybrid filters. Radioactivity was determined by liquid scintillation spectrophotometry.

Comparison of the low temperature formamide method with hybridization at 60° for 20 hr showed the formamide method to result in 20–30% greater hybridization due to the longer annealing time. Thermal dissociation profiles of both type hybrids gave T_m's of 75–77°, in agreement with the value of 75.2° for native mtDNA reported by Gordon and Rabinowitz (1973).

TABLE I: mtDNA-[^{3}H]nDNA Hybridization.[a]

Expt No.	DNA on Filter	cpm Bound	Corr for Dissociation at 37° (%)[b]	Hybridization (%)	mtDNA Equiv[c]	T_m (°C)
1	mtDNA (8 μg)	863	31	0.74	1.9	73
	Clostridium DNA (10 μg)	347				
2	mtDNA (8 μg)	889	22	0.86	2.2	75
	Clostridium DNA (10 μg)	341				
3	mtDNA (3.3 μg)	760	21	0.52	2.6	75.2
	mtDNA (3.3 μg)	710	(21)[d]	0.49		
	Clostridium DNA (10 μg)	250				

[a] All hybridizations were performed as described in Materials and Methods. Expt 1 had an input of 80,000 cpm (2.1 μg) of [^{3}H]nDNA, sonicated to an average molecular weight of 500,000. Expt 2 input was 80,000 cpm, unsonicated [^{3}H]nDNA. Expt 3 input was 114,000 cpm (3.0 μg), sonicated [^{3}H]nDNA. All values are corrected for 30–40 cpm bound to blank filters. [b] Described in text. [c] One mitochondrial genome equivalent is equal to 0.38% hybridization per haploid nuclear genome, assuming a mitochondrial genome size of 5×10^7 daltons (Hollenberg *et al.*, 1970) and a nuclear genome size of 1.3×10^{10} daltons (Ogur *et al.*, 1952). [d] Estimate: dissociation at 37° not determined experimentally.

Thermal Dissociation of Hybrids. Thermal dissociation of filter-bound hybrids was performed in vials containing 1 ml of 1 X SSC. Sealed vials were placed in a water jacketed sealed beaker, fitted with a stirring bar, through which temperature controlled 25% glycerol was circulated. The amounts of radioactivity released were determined at 5° intervals, with 5 min of equilibration time at each temperature. Each filter was washed with 1 ml of buffer at the same temperature and then transferred to another vial containing 1 ml of 1 X SSC at the next higher temperature. The dissociation and wash buffers were combined and 100 μg/ml of calf thymus DNA was added, followed by 2 ml of cold 10% trichloroacetic acid. After 0.5 hr the precipitate was collected on Millipore filters and radioactivity was determined as described above.

Chemicals. All radioactive chemicals were purchased from New England Nuclear Corp. Pancreatic and T_1 RNase were from Sigma Chemical Co. Electrophoretically pure DNase was obtained from Worthington. Pronase was from Calbiochem and glusulase from Endo Laboratories. Optical grade CsCl was purchased from Harshaw Chemical Co. Yeast extracts, Bactopeptone, and yeast nitrogen base with amino acids was obtained from Difco Laboratories.

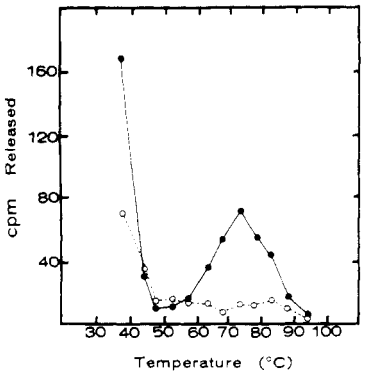

FIGURE 2: Thermal dissociation analysis of mtDNA-[^{3}H]nDNA hybrid and *Clostridium* DNA-[^{3}H]nDNA hybrid. Hybridization and dissociation conditions are described in Materials and Methods and Table I. (●—●) mtDNA-[^{3}H]nDNA hybrid; (○ - - ○) *Clostridium* DNA-[^{3}H]nDNA hybrid.

Results

mtDNA-nDNA Hybridization and Thermal Dissociation Analysis of Hybrids. Sequence homology between the mitochondrial and nuclear genomes was determined by DNA-DNA hybridization. Table I presents the results of three different assays. In each experiment nDNA in formamide, at 37°, was annealed with mtDNA on filters, for 48 hr. A *Clostridium* DNA filter was included in each experiment to provide a base level of hybridization with a nonhomologous high A + T genome. In all three experiments, there was a significantly larger amount of hybridization with mtDNA than with the bacterial control (cpm bound column). Rehybridization of the reaction mixture with fresh DNA containing filters resulted in only 10% as much hybrid as with fresh solutions, indicating essentially complete removal of all mtDNA homologous segments.

Experiment 1 differs from experiment 2 in that the nDNA in solution was sonicated to avoid any possibility that unhybridized free ends might have been involved. The size of nDNA does not appear to affect the results significantly. In experiment 3, two filters, each with one-half the amount of mtDNA, were placed together in the same vial with nDNA. The amount of hybridization was not significantly different from results obtained when all of the mtDNA was on one filter.

After hybridization, the above filters were subjected to thermal dissociation analysis to determine the nature of hybrid formed (Figure 2). As expected, most of the counts bound to *Clostridium* filters dissociated at low temperature; about 40–50% dissociated around 37° and 70% dissociated below 65°. About 30% of the counts on mtDNA filters also dissociated at 37°. The remainder of the counts on mtDNA filters, about 70%, dissociated in the range of 65–85°. In Figure 3 the counts dissociating at different temperatures are plotted as a standard thermal denaturation profile. The T_m values range from 73.0 to 75.2°, values which are in good agreement with our own controls and with values reported for native mtDNA by others (Gordon and Rabinowitz, 1973). A nDNA-[^{3}H]nDNA hybrid, formed under the same conditions as the mtDNA-[^{3}H]nDNA hybrids, dissociated with a T_m of 82.1°. Taken together, the dissociation data show that a relatively small

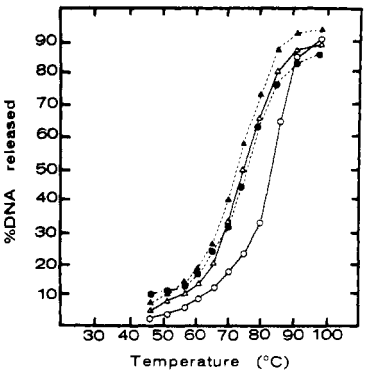

FIGURE 3: Thermal dissociation profiles of mtDNA-[^{3}H]nDNA hybrids (Table I) and an nDNA-[^{3}H]nDNA hybrid. Hybridization and dissociation conditions are described in Materials and Methods and Table I. The nDNA-[^{3}H]nDNA hybrid was formed in formamide at the same temperature as the mtDNA-[^{3}H]nDNA hybrids. (▲ - - ▲ - -, —Δ—Δ—, - - ● - - ●) mtDNA-[^{3}H]nDNA hybrids with respective T_m's of 73.0, 75.0, and 75.2°; (O—O) nDNA-[^{3}H]nDNA hybrid, T_m of 82.1°.

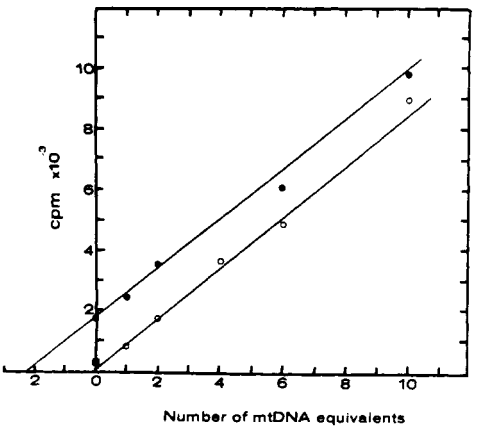

FIGURE 4: Quantitation of nDNA homology by mitochondrial cRNA hybridization. One set of filters contained 0.019, 0.038, 0.076, 0.114, and 0.19 μg of mtDNA and no nDNA. The other set contained the same quantities of mtDNA and each filter also had 5 μg of nDNA. Since 0.019 μg of mtDNA is worth 1 mtDNA equiv in 5 μg of nDNA, each set contained 1, 2, 4, 6, and 10 mtDNA equiv. A *Clostridium* DNA filter containing 5 μg was included as a control. All filters were hybridized together in 4.0 ml with a cRNA input of 1.6×10^6 cpm. All filters are corrected for counts bound to blank filters. nDNA filters were corrected for 20% contamination of the counts/min bound after subtracting the counts/min bound to equivalent mtDNA. (O) mtDNA filters; (●) nDNA filters with added mtDNA; (□) *Clostridium* DNA filter.

amount of the nDNA which hybridizes to mtDNA filters is unstable, probably nonspecific binding to the same kind of A + T sequences found in the nonhomologous bacterial DNA. The major portion, however, is annealed as hybrids equivalent in stability to native mtDNA.

To determine the per cent of nDNA homologous with mtDNA, it was necessary first to correct for the DNA which dissociated at 37° (column 3 in Table I). From the corrected percentage, the number of mtDNA equivalents per nuclear genome was found to be about 2.2 per haploid genome (column 4).

Mitochondrial [^{3}H]cRNA-Nuclear DNA Hybridization. High specific activity [^{3}H]cRNA, transcribed from mtDNA, was hybridized with nDNA on filters. Quantification of cRNA-nDNA homology required a preliminary determination of the amount of cRNA (radioactivity) in hybrids that represented an mtDNA equivalent. Accordingly, a control set of filters containing graduated amounts of pure mtDNA were prepared, in addition to a second set, with the same amounts of mtDNA plus a known amount of nDNA. All filters were exposed to the same cRNA hybridization mixture. The amount of cRNA which is equal to an mtDNA equivalent was determined from the slope of the pure mtDNA filter set (Figure 4). The difference between hybridization to this set, and the set of filters containing the nDNA, permitted calculation of the number of mtDNA equivalents in the nDNA used. After correction for 20% contaminating counts (see below), a value of 2.2 mtDNA equivalents per haploid nuclear genome was determined. Upon thermal dissociation, 13% of the bound counts in the [^{3}H]cRNA-nDNA hybrid were released at 37°. Subtraction of this 13% from the 2.2 mtDNA equivalents reduced the homology to 1.9 mtDNA equivalents per haploid genome, a value in good agreement to that determined by DNA-DNA hybridization analysis. The T_m's for these cRNA-DNA hybrids are 2-5° lower than for native mtDNA (Figure 5). There was also a 2-3° difference in melting temperature between nDNA-cRNA hybrids and mtDNA-cRNA hybrids. The reasons for these slight discrepancies are not known.

In a separate set of experiments, homology of cRNA with purified nDNA was determined with three different preparations of cRNA, synthesized from three different mtDNA templates. Results of these experiments are in Table II. The amount of cRNA equal to 1 mtDNA equivalent was determined from the radioactivity bound to filters containing pure mtDNA. Calculation of the number of equivalents of mtDNA homologous segments on nDNA filters were made after correcting for possible mtDNA contaminants (see below), and after subtraction of the counts which are released at 37°. An average of 2.4 mtDNA equiv was estimated to be present in a haploid nuclear genome. The variability in relative hybridization values among samples of cRNA probably reflects variability of *in vitro* transcription. This probably also accounts for T_m values ranging from 65 to 75°. A similar variability was reported by Michaelis *et al.* (1972).

cRNA transcribed from mtDNA was also hybridized with nDNA preparations which were centrifuged in CsCl to display a density profile of the DNA with which cRNA was hybridizing. In the first experiment, high molecular weight nDNA ($35-40 \times 10^6$) was prepared by layering purified

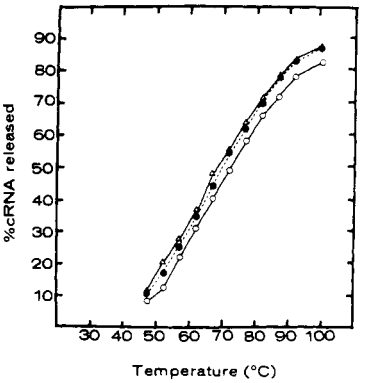

FIGURE 5: Thermal dissociation profiles of nDNA-cRNA and mtDNA-cRNA hybrids of Figure 4. Profiles have been corrected for dissociation at 37°. (O—O) mtDNA-cRNA hybrid, T_m = 72.0°; (Δ—Δ) nDNA-cRNA hybrid, T_m = 68.7°; (● - - ●) nDNA + 1 mtDNA equivalent-cRNA hybrid, T_m = 70.0°.

TABLE II: nDNA-[^{3}H]cRNA.[a]

Expt No.	DNA on Filter	cpm Bound	cpm Bound per μg of DNA	Corr for Dissociation at 37° (%)[b]	Rel Hybridization (%)	mtDNA Equiv
1a	mtDNA (9 μg)	40,274	4648		100	
	mtDNA (9 μg)	43,402				
	Clostridium DNA (10 μg)	323				
1b	nDNA (9.6 μg)	1,074	104	31 (31)[c]	1.5	3.9
	nDNA (9.6 μg)	940				
	Clostridium DNA (10 μg)	385				
2	mtDNA (9 μg)	80,655	8961		100	
	nDNA (9.6 μg)	782	81	26	0.67	1.7
	Clostridium DNA (10 μg)	25				
3	mtDNA (9 μg)	64,320	7146		100	
	nDNA (9.6 μg)	485	49	20	0.55	1.4
	Clostridium DNA (10 μg)	100				

[a] All hybridizations were performed as described in Materials and Methods. Each hybridization experiment was performed with [^{3}H]cRNA transcribed from a different mtDNA template. Hybridization of [^{3}H]cRNA to its own template is set at 100%. Input [^{3}H]cRNA; Expt 1 (a) and (b), 3.7×10^5 cpm; expt 2, 3.6×10^5 cpm; expt 3, 9.1×10^5 cpm. All values are corrected for 45–220 cpm bound to blank filters. cpm bound to nDNA filters have been corrected for 19% mtDNA contamination as described in text. [b] Described in text. [c] Estimate; dissociation at 37° not determined experimentally.

nuclei on top of CsCl gradients, followed by detergent lysis and centrifugation to equilibrium. Figure 6a shows that the DNA which hybridizes with cRNA is coincident with the density position of nDNA. cRNA also hybridized with a lower density region of the gradient containing contaminating mtDNA. The break in the radioactivity profile is a result of hybridizing each half of the gradient separately with different input amounts of [^{3}H]cRNA. Bulk DNA was hybridized separately from lighter regions of the gradient to preclude any possible release of contaminating mtDNA and entrapment of it by nDNA filters during hybridization. In a second experiment (Figure 6b), nDNA from the same nuclear sample was sheared by passing through a 30 gauge needle five times prior to centrifugation and hybridization with cRNA. The nDNA which was homologous with cRNA was shifted to a lighter region of the gradient after shearing. The interpretation of this result is that the cRNA homologous segments were larger than the length of DNA produced by shearing ($5–10 \times 10^5$) and since they have a density more like mtDNA than nDNA, they tended toward that density when released. In a third experiment, nDNA was carried through three cycles of centrifugation prior to hybridization (Figure 6c). DNA hybridizing with cRNA still bands with the nuclear peak, showing that unless DNA is reduced to a small size by shearing, the homologous segments remain with the bulk of nDNA.

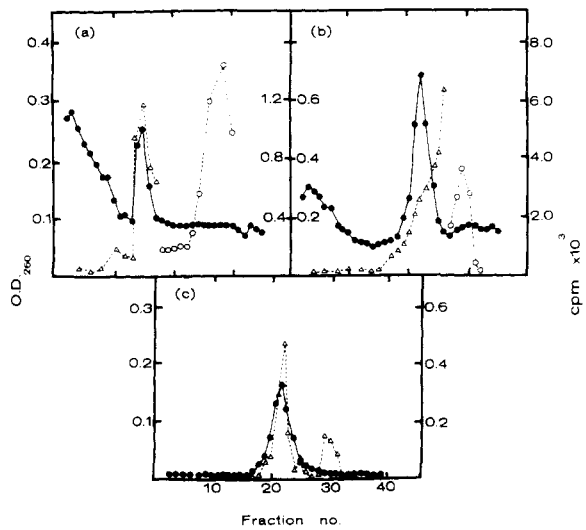

FIGURE 6: CsCl gradient analysis of the density of sequences of DNA homologous to mitochondrial [^{3}H]cRNA. A 1-ml sample of purified nuclei was divided into two equal parts, one layered directly on 2.9 ml of CsCl and lysed by the addition of 0.1 ml of 10% SDS. The second sample was lysed with 0.1 ml of 10% SDS and sheared through a 30 gauge needle five times before layering onto CsCl. After centrifugations, samples of the gradients were collected from the bottom with an 18 gauge needle, adjusted to 0.6 ml with water for optical density determination, and fixed to Millipore filters. After hybridization with [^{3}H]cRNA, as described in Materials and Methods, counts of each fraction were made. (a) unsheared high molecular weight DNA ($35–40 \times 10^6$); (b) sheared DNA; (c) nDNA, after three CsCl centrifugations and hybridized with [^{3}H]cRNA; 81% of the hybridized counts are coincident with nDNA. (●—●) optical density; (Δ - - Δ) (a) and (b), 5×10^5 cpm of [^{3}H]cRNA input; (○ - - ○) 1.2×10^5 cpm of [^{3}H]cRNA input; (Δ - - Δ) (c) 9×10^5 cpm of [^{3}H]cRNA input.

An additional test for possible entrapment of mtDNA within nDNA was to add mtDNA to high molecular DNA (*Xenopus laevis*) which was known to have no homology with the cRNA preparation. *Xenopus* DNA bands at the same buoyant density position as yeast nDNA. Figure 7a and b show that none of the mtDNA was trapped by bulk DNA; all of the cRNA hybridization was with the yeast mtDNA peak.

Although these centrifugation results show that cRNA hybridizing segments band discretely in the nuclear peak, and that there is very little mtDNA contamination of nDNA purified by CsCl centrifugation, Figure 6c nevertheless shows that there may still be some such contamination even after the third centrifugation. Data from several experiments, using nDNA isolated from either spheroplasts or purified nuclei, showed that this contamination was no more than 10–20%. Accordingly, to avoid overestimation of

mtDNA-nDNA homology, this per cent possible contamination was determined for the nDNA of all experiments and was subtacted from the counts bound to nDNA filters (Figure 4 and Table II) prior to estimating per cent homology, as mentioned above.

Discussion

Evidence derived from DNA-DNA hybridization and cRNA-DNA hybridization indicates that significant amounts of the yeast nuclear genome, about 0.75% of the genome or an amount equivalent to about 2 mtDNA copies, is homologous with the mitochondrial genome. A number of attempts have been made by others to see if such homology exists, and thus far the results have been either contradictory or inconclusive. Because of the important implications of such homology, as well as the caution dictated by the efforts reported in the literature, we have been especially conscious of the need for purity of assay solutions and confirmation of results by more than one technique. The minimal criteria for a convincing demonstration of homology are: (a) demonstrated purity of both mtDNA and nDNA preparations; (b) evidence that the molecular hybrids formed between nDNA and mtDNA exhibit the expected thermal stability; and (c) an indication that homology between the two genomes represents a significant portion, if not all of the mitochondrial genome.

The nDNAs used for all assays were prepared by two cycles of CsCl density gradient centrifugation. Reconstruction experiments with added [^{3}H]mtDNA (Figure 1c and d) confirmed the observation that contaminating mtDNA could account for very little of the homology observed (less than 10-20% from Figure 6c). The best evidence that contaminating mtDNA did not account for the homology was the fact that sequences of nDNA hybridizing with cRNA made from mtDNA banded as a discrete peak coincident with the nDNA peak and did not smear into the mtDNA region unless released by shearing.

Confidence in the purity of mtDNA was based on the following: (a) mtDNA was extracted from highly purified, DNase digested mitochondria; (b) no nDNA contamination of this DNA was ever detected by analytical ultracentrifugation; (c) mtDNA was subjected to two cycles of preparative centrifugation prior to use as a template for cRNA transcription to be absolutely sure of no nDNA contamination; and (d) thermal denaturation profiles of mtDNA-nDNA and cRNA-nDNA hybrids are sharp, similar to native mtDNA, and show no evidence of the shift toward a higher and broader melt that would be expected if cRNA had been transcribed from nDNA contaminating the template mtDNA. It is worth noting that, even if the template mtDNA were contaminated with the unlikely large amount of 1% nDNA, and even if this were transcribed with the same efficiency as mtDNA (unlikely in view of the results of Cato and Jones, 1972), less than one-half of the hybridized counts observed could be accounted for under our conditions of 10-20% hybridization efficiency.

The fidelity of base pairing between mtDNA and nDNA was examined by thermal denaturation profile analysis. The denaturation profiles of hybrids formed (Figure 3) were similar to those produced by native mtDNA and gave T_m's (73-75°) in close agreement with values reported for native mtDNA (Gordon and Rabinowitz, 1973). The 20-30% of nDNA counts on mtDNA filters which dissociated at 37° are considered to be nonspecific high A + T sequences or mismatched sequences. These counts were subtracted prior to calculating homology. As a test for nonspecific hybridization, *Clostridium* DNA was affixed to filters instead of mtDNA, and exposed to radioactive nDNA. As expected, the small number of counts which attached to filters were unstable and were released at 37°. Thermal melts of cRNA transcribed from mtDNA with nDNA gave profiles generally in agreement with the nDNA-mtDNA profiles. The reason for the 2-3° difference between the T_m of nDNA-cRNA hybrids and mtDNA-cRNA hybrids is not clear.

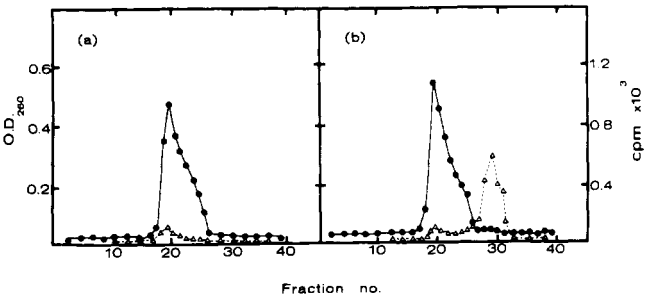

FIGURE 7: CsCl gradient hybridization analysis of *Xenopus laevis* DNA with yeast mtDNA added, followed by hybridization with [^{3}H]cRNA as described in Figure 4. (a) 75 μg of *X. laevis* DNA; (b) 75 μg of *X. laevis* DNA made 1.5% with yeast mtDNA. (●—●) optical density; (Δ - - Δ) 2.1 × 10^5 cpm of [^{3}H]cRNA input.

The experiments in which cRNA transcribed from mtDNA was hybridized with CsCl banded nDNA (Figures 6a and b) indicated that segments of nDNA which were homologous with mtDNA were integrated within the nuclear genome. Homologous sequences were associated with DNA having a density the same as bulk nDNA and thus banded with the bulk DNA. Shearing of nDNA prior to CsCl banding released these sequences so that they became skewed toward their own density position (Figure 6b). It is difficult, however, to estimate the size of these homologous sequences from the present experiments. The molecular weight determined for the DNA of Figure 6a is a minimal estimate, since the DNA is sized after gradient fractionation, a procedure which results in some DNA shearing. nDNA purified by two cycles of CsCl centrifugation and assayed by gradient hybridization (Figure 6c) has an average molecular weight of 1-2 × 10^7, determined for a number of preparations. Further speculation as to the size of integrated sequences will require a more detailed study of high molecular weight and sheared DNA.

Because of the sensitivity of cRNA assays, these cRNA-CsCl gradient nDNA experiments were examined quantitatively as an independent means of estimating the level of homology. Even small amounts of cross contamination would be obvious due to the widely separate banding positions of nDNA and mtDNA in yeast (Figure 6). Unfortunately, *E. coli* RNA polymerase transcribes only a limited portion of the entire mitochondrial genome (Tabak and Borst, 1970; Michaelis *et al.*, 1972; Dawid, 1972) and variability in the cRNA made might be expected. This may account for the variation seen in relative hybridization values obtained in nDNA-cRNA hybridization (Table II), as well as for differences in mtDNA-cRNA hybridization values. Variability in hybridization was also reported by Michaelis *et al.* (1972).

Despite the uncertainty in the cRNA transcripts, competition hybridization experiments show that *in vivo* synthesized mtRNA successfully competes for the cRNA-nDNA homologous sequences. These sequences must therefore be at least partially represented by RNA sequences tran-

scribed *in vivo*. They are thus not restricted to nontranscribed spacer or regulator sequences. The conditions of our experiment do not permit an estimation of the precentage of cRNA–nDNA homology that is homologous with *in vivo* transcripts. Preliminary results show about 16% competition when cold RNA is in great excess. Competition of the same RNA for hybridization of cRNA with mtDNA is somewhat greater, indicating that much of the *in vitro* transcripts are also sequences transcribed *in vivo*. RNA extracted from post-mitochondrial cytoplasm did not compete with cRNA for sites on either mtDNA or nDNA.

We do not believe the homologies reported here to be inconsistent with data presented in earlier reports where little or no homology between the two genomes was assumed. Fukuhara (1970) and Michaelis *et al.* (1972) reported homology values of 4–6 and 0.4–2.5%, respectively, but were reluctant to suggest that these results were meaningful. We agree with the authors that their data should be interpreted with caution, but disagree with their tentative conclusion that significant homology was absent. Hollenberg *et al.* (1970) report values suggesting less than 1/2 copies worth of homology. This value may be accurate for the *in vivo* mtRNA transcribed under the physiological conditions of growth and methods of RNA extraction they used and does not necessarily disagree with ours. The large differences in both mtDNA and nDNA genome sizes between yeast and vertebrates are such that we feel it is not useful to compare our results to those for chick mtDNA and nDNA (Tabak *et al.*, 1973) and *Xenopus laevis* mtDNA and nDNA (Dawid and Blackler, 1972).

This evidence which we present for nuclear–mitochondrial genome homology will attract the attention of those who are interested in the question of mitochondrial biogenesis and evolution. Evidence for a complete mtDNA genome, integrated within the nuclear genome, would certainly be of interest in these questions. We wish to make it clear that this is not what we are reporting. Rather, our data show that there is a portion of nDNA, which is homologous to mtDNA, inserted in the nuclear genome, and equivalent in amount to about two mtDNA molecules. Our data do not say that all of the segments of an mtDNA genome is involved. It does say that the hybridizing regions of nDNA are homologous with DNA having a buoyant density and thermal denaturation characteristics like mtDNA. Our data also offer little information regarding the size or arrangements of homologous segments of nDNA. The segments are sufficiently large that they are released to form a skewed peak when nDNA is sheared to $5–10 \times 10^5$ daltons size, but the upper size limit cannot be determined by our techniques. In fact, a complete mtDNA segment of 5×10^7 daltons would necessarily have been broken, with some portions having been released and removed by CsCl centrifugation, had it been inserted as a continuous segment. Also very large insertions would very likely have skewed the nuclear homology peak, a phenomenon which was not observed. We tentatively conclude that the segments are reasonably large, but not as large as a continuous mtDNA genome equivalent.

Our results thus do not show that a "master copy" of mtDNA is present in nDNA. Nevertheless, sufficient attention has been paid to the purity of the DNA preparations to eliminate all reasonable likelihood of cross contamination, as well as to the characterization of the mtDNA–nDNA hybrids produced, that serious attention must be paid to the significant amounts of mtDNA like segments we find to be present in the nuclear genome. A number of interesting questions might, therefore, be raised regarding the importance of these homologies to mitochondrial biogenesis and evolution. Do they represent evolutionary relics whose previous function has been assumed by cytoplasmic mtDNA? Do they represent homology of only certain segments of the mtDNA; sequences which may be held in common between the two genomes to regulate the mutual interaction of the two genomes? Or do they function as a master copy, as proposed by Wilkie (1963, 1970), necessary for control of mitochondrial biogenesis? Such a master gene need not be integrated during all phases of the life cycle nor need it necessarily be integrated as a continuous unit.

In view of the rapid evolutionary history of mitochondria, a history deduced from the great differences in mtDNA size, base composition, and transcriptional products among different organisms, it is difficult to imagine that retention of nonfunctional sequences of the magnitude we find in yeast. The limited number of competition experiments done do not support a conclusion of homologies being limited to nontranscribed regulatory or spacer sequences.

The theoretical importance of a master template is thus quite evident and of considerable interest. Though our results do not demonstrate a master template, the homology present must be considered in formulating any model of molecular interaction between the mitochondrial and nuclear genomes.

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The Isolation of Terminally Cross-Linked DNA and Kinetics of Venom Phosphodiesterase†

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ABSTRACT: The action of venom phosphodiesterase (exonuclease) on non-cross-linked and mitomycin C cross-linked *Escherichia coli* DNA fragments has been investigated. Kinetic studies reveal that the rate of phosphodiesterase catalyzed hydrolysis is primarily controlled by the cross-link density, and that added alkaline phosphatase and Mg^{2+} in the digest have a slight stimulatory effect on the reaction rate. Cross-links are shown to inhibit exonuclease hydrolysis, and limited digestion can be used to decrease the concentration of non-cross-linked fragments in randomly cross-linked DNA. Thermal denaturation profiles of partially digested DNA show that end effects in the helix-coil transition of low molecular weight cross-linked DNA are slight compared to the effects of cross-link density and nucleotide composition heterogeneity.

The thermal denaturation of duplex DNA has been the subject of much experimental and theoretical study. From this work, many features of DNA molecular structure have come to be recognized as determinants of the denaturation behavior. Specifically, melting is dependent upon (1) the nucleation of helical regions, (2) the differing thermal stability of A-T and G-C base pairs, (3) molecular weight, (4) the loop entropy, (5) slip degeneracy, (6) base sequence, (7) electrostatic effects, and (8) specific interactions with solvent and/or other solutes. Several of these factors have been investigated in detail from both theoretical and experimental sides. For instance, the molecular weight dependence of transition midpoints and breadths seems well understood (Crothers *et al.,* 1965); the linear dependence of midpoint temperatures on gross A-T composition is well known; the difference in breadths of transitions for homopolymers and heteropolymers is rationally ascribed to slip degeneracy. Other factors, such as (7) and (8) above, are less well understood, but at least are experimentally controlled to be constant.

From the biological standpoint, the ultimate aim of investigations on denaturation behavior is the deduction of base sequence information, of whatever kind, from melting profiles. However, this goal cannot be achieved unless and until the several influential aspects of DNA structure listed above have been quantified.

It is most probable that the major current difficulty confronting this interpretation results from the interplay between (4) loop entropy, (5) slip degeneracy, and (6) base sequence. This paper describes the procedures we have used to produce modified DNA of such structure that at least partial separation of these effects is achieved. The aim of the work is to produce relatively short and fractionated DNA fragments that are cross-linked at both ends, and which therefore cannot melt by unzipping. The sequence of operations, up to the fractionation, is depicted schematically in Figure 1. In a subsequent paper we will present the denaturation behavior of the fractions.

The procedure makes use of the known (Iyer and Szybalski, 1963, 1964; Szybalski and Iyer, 1964, 1967; Weissbach and Lisio, 1965) capability of the reduced form of mitomycin C to cross-link duplex DNA. Subsequently, the 3′ chain ends may be removed with venom phosphodiesterase (Laskowski, 1966; Razzell and Khorana, 1959; Richards and Laskowski, 1969) in the expectation that cross-links effectively impede more complete hydrolysis of the chains (Pricer and Weissbach, 1965; Sarkar, 1967). The data presented here further characterizes this inhibitory action of cross-links. In addition, we have investigated the stimulatory effect of phosphatase and Mg^{2+} on the exonuclease digestion. The kinetics of the digestions lead to a plausible conjecture concerning the mode of action of venom phosphodiesterase on duplex DNA.

Experimental Section

Materials. The DNA was isolated from strain B *Escherichia coli* cells by a modification of the method of Marmur (Marmur, 1961); the 2-propanol precipitation was omitted and a deproteinization by phenol extraction was added. The venom phosphodiesterase from *Crotalus adamanteus* and bacterial alkaline phosphatase were purchased from Worthington Biochemical Corp. The exonuclease assays were done by the method of Koerner and Sinsheimer (Koerner and Sinsheimer, 1957) using calcium bis(*p*-nitrophenyl) phosphate (0.12 mg/ml) as a substrate. Concentrations are expressed in units/ml where 1 unit is arbitrarily defined as the activity of enzyme in a 3-ml assay volume which produces a total increase of 0.30 absorbance unit in 30 min.

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