EVIDENCE FOR *IN VITRO* AND *IN VIVO* MOLECULAR "DARWINIAN" SELECTION

by

GERALD H. RANK

B.S.A.H. University of Manitoba, 1963

M.Sc. University of Manitoba, 1964

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Department of Botany

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

Molecular "Darwinian" selection is initially defined as the generation of a variant molecule, within a population of self-replicating molecules, that becomes the predominant molecular form in descendent populations by virtue of its replicative superiority.

The molecular biology of the replication of *Qβ in vivo* is presented prior to providing evidence for molecular "Darwinian" selection of Qβ-RNA *in vitro*. Critical experiments undertaken to prove that *Qβ-replicase* is capable of catalyzing the synthesis of molecular replicas of primer Qβ-RNA *in vitro* are described. This information is followed by a description of the experiments utilized to demonstrate the occurrence of molecular "Darwinian" selection of Qβ-RNA under different selective environments. Molecular and phenotypic properties of mutant RNA molecules are discussed.

After an account of the expectations of molecular "Darwinian" selection *in vivo*: *suppressitivity, gross alterations of DNA base composition in micro-organisms,* and the *von Magnus effect* are presented as evidence for molecular "Darwinian" selection *in vivo*.

Suppressive respiratory-deficiency in *Saccharomyces cerevisiae* is taken as a model system for the investigation of suppressitivity. A literature review of the data generated by genetic and cytological analyses of suppressitivity was found to be accommodated by the hypothesis of molecular "Darwinian" selection *in vivo*. In addition, the author's genetic analyses of: (i) mosaic colonies, (ii) the effect of the cytoplasmically-inherited suppressive-factor on a cytoplasmically-inherited erythromycin-resistant marker, and (iii) high and low suppressitivity, were interpreted as evidence

for the hypothesis. Biochemical evidence is presented that indicates that the suppressive-factor is an abnormal mitochondrial DNA with a replicative superiority to normal mitochondrial DNA.

A brief discussion of the possible influence of molecular "Darwinian" selection on pre and post cellular evolution is presented

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IN VITRO EVIDENCE FOR MOLECULAR "DARWINIAN" SELECTION

"Darwinian" selection at the population level is believed to be a major factor contributing to species evolution. Two basic postulates embodied in the concept of "Darwinian" selection are (i) the generation within a population of a variant form that has a reproductive advantage over other members of the population and (ii) the reproductive differential which favours the variant also enables it to become the predominant form in descendent populations.

"Darwinian" selection at the molecular level will thus be defined in this essay as the generation of a variant molecule, within a population of self-replicating molecules, that becomes the predominant molecular form in descendent populations by virtue of its replicative superiority. Self-replication is a characteristic observed only in two molecular species: RNA and DNA. These molecules are capable of forming self-replicas from precursor nucleoside triphosphates in the presence of suitable polymerases and cations. Thus molecular self-replication is defined as the catalyst dependent production of exact molecular replicas.

Molecular "Darwinian" selection *in vitro* was first reported by Mills, Peterson and Spiegelman (1967) for the RNA extracted from *Q*β, a small single stranded RNA bacteriophage. Several useful reviews covering various aspects of the RNA bacteriophages have been published (August et al., 1968; Erickson and Franklin, 1966; Erickson, 1968; Lodish, 1968; Shapiro and August, 1966; Spiegelman and Haruna, 1966; Spiegelman et al., 1968; Weissman and Ochoa, 1967; Weissman, Feix and Slor, 1968; Zinder, 1965). *Q*β is representative of a group of closely related single stranded RNA phage (Lodish, 1968; Zinder, 1965) that are capable in nature of infecting only

male strains (F^+, *Hfr* or *F'*) of *E.coli*, since the phage attaches only to the male pili. Nevertheless infection of spheroblasts of *E.coli* by Qβ-RNA achieved plating efficiencies of 10^{-6} to 10^{-7} infectious units per input strand of viral RNA (Spiegelman et al., 1965). Following a fifteen minute eclipse ten to twenty thousand phage particles are produced. Only two to fifty percent of the particles are capable of forming plaques. Viable particles can not be separated from non-viable particles (Cooper and Zinder, 1963). Distinct serological properties can be used to identify Qβ from other similar RNA coliphages such as *MS-2* (Overly et al., 1966).

Merely three kinds of macromolecules have been demonstrated in these coliphages: single-stranded RNA with a molecular weight of 1.1×10^6; a major coat protein (lacking histidine) composed of 129 amino acids for a total molecular weight of 14,000; and a minor coat protein, the A protein, with a molecular weight of 35,000. A mature phage particle is composed of one RNA molecule, 180 major coat molecules and one A protein molecule (Erickson, 1968). Infective viral particles have been assembled *in vitro* from these three constituents (Roberts and Steitz, 1967).

In addition to functioning as a template for self-replication, single-stranded viral RNA is employed as a messenger in the synthesis of viral-specific proteins. Complementation tests of conditional lethal mutants (amber and temperature sensitive) have defined three viral RNA cistrons (Weissman and Ochoa, 1967; Lodish 1968; Zinder, 1965). Acrylamide gel electrophoresis of protein synthesized by the viral RNA genome *in vivo* and *in vitro* also identified three coliphage specific proteins: the histidine deficient major coat protein; the A protein; and a RNA polymerase used in the self-replication of viral RNA (Erikson, 1968).

Isolation and purification of RNA polymerase was attempted in order to elucidate the enigma of self-replication of viral RNA. Initially, RNA polymerases competent in the *in vitro* synthesis of viral RNA lacked the rigorous properties necessary to prove they were the bona fide *in vivo* polymerases. RNA *synthetase*, isolated from *E.coli* infected with *MS-2*, was dependent upon the presence of all four nucleoside triphosphates for maximal RNA synthesis (Weissmann et al., 1964). A requirement for added template could not be shown. Since RNase treatment caused an irreversible inactivation of RNA *synthetase* it appears that an indigenous template was associated with the enzyme. Viral RNA *polymerase*, which was purified 100 fold from *f2*-infected *E.coli* (August et al., 1965), utilized a variety of RNAs such as *f2*, *TMV* and soluble or ribosomal RNA as template. The RNA synthesized was similar in base composition to the complement of added RNA. Because synthesis discontinued soon after the complement was made, the enzyme seems to be a component responsible only for the synthesis of a strand complementary in base composition to the added primer.

Haruna, Nozu, Ohtaka and Spiegelman (1963) achieved the first successful demonstration of extensive replication of viral RNA *in vitro*. RNA *replicase*, isolated from *MS-2* infected *E.coli* by negative protamine fractionation and DEAE column chromatography, was capable of protracted synthesis of *MS-2*-RNA. Maximal replicase activity was dependent upon the addition of ribonucleoside triphosphates (CTP, UTP, GTP, ATP), magnesium chloride and *MS-2*-RNA. RNA synthesis was not observed when *MS-2*-RNA was substituted by other RNA primers. A similar dependence upon homologous template was subsequently demonstrated for *Qβ replicase* (Haruna and Speigelman, 1965 (a)). Neither *MS-2*-RNA, ribosomal RNA, tRNA, *satellite tobacco necrosis viral*-RNA (STNV-RNA) or DNA caused an appreciable synthesis of RNA in the presence of *Qβ-replicase*. Similarly, 17S and 7S fragments of *Qβ*-RNA were found to stimulate RNA

synthesis to only 4% of the rate observed for intact 28S Qβ-RNA (Haruna and Spiegelman, 1965 (b)). Such template specificity would seem necessary to ensure viral RNA replication in a host cell replete with heterologous RNA molecules and is convincing evidence that the purified *replicase* is a bona fide RNA polymerase *in vivo*.

Detailed examination of molecular replication *in vitro* requires the technical competence to ascertain the qualitative and quantitative properties of any product synthesized as well as a rigorous demonstration of the chemical purity of all added components. Of the components present in a standard reaction mixture (Haruna and Spiegelman, 1965 (a)) of volume 0.25 ml (tris HCl ph 7.4, magnesium chloride, CTP, ATP, UTP, GTP, RNA template and *replicase*) only the purity of the *replicase* required special consideration. Although Qβ-*replicase* was sufficiently purified after DEAE chromatography (Haruna and Spiegelman, 1965 (a)) to demonstrate a synthetic requirement for added primer, *replicase* preparations still contained 6.5×10^{11} sphaeroblast particle forming units per milligram of enzyme. Equilibrium banding in CsCl followed by zonal centrifugation in linear gradients of sucrose, resulted in an enzyme purity of less than fifty sphaeroblast particle forming units per milligram of *replicase*, without any reduction in the synthetic activities of Qβ-*replicase* (Pace and Spiegelman, 1966 (a)). Standard sedimentation techniques and polyacrylamide gel electrophoresis were used to determine the size and molecular weight of RNA synthesized. Identification of forms of RNA involved in the *in vitro* synthesis was greatly facilitated by the use of polyacrylamide gel electrophoresis (Bishop et al., 1967; Mills et al., 1967). P^{32} incorporation, from a radioactive ribonucleoside triphosphate precursor, into a trichloroacetic acid insoluble product was used as a quantitative assay of RNA synthesis.

Further evidence of the purity of *Qβ-replicase* was provided by the low level of template required for saturation of the enzyme (one γ of RNA to forty γ of enzyme). The amount of RNA synthesized after two hours of template saturation equalled five times the amount of added template; synthesis continued for another three hours (Haruna and Spiegelman, 1965 (a)). The autocatalytic synthesis of RNA prior to saturation of the enzyme indicated that RNA synthesized *in vitro* was itself capable of acting as template for *Qβ-replicase* (Haruna and Spiegelman, 1965 (c)). Virtually any amount of RNA could be synthesized if sufficient precursors and *replicase* were supplied. At 37°C thirty nucleotides were estimated to be incorporated per second into a growing strand. A mature strand of 3300 nucleotides would require 110 seconds to complete synthesis at this rate (Feix, Pollet and Weissmann, 1968). The synthesized RNA had the same sedimentation coefficient (S_{20},w=28) and base composition(U/A=1.33) (Haruna and Spiegelman, 1966; Mills, Peterson and Spiegelman, 1967) as *Qβ*-RNA.

Similarities in size and base composition of product RNA are only suggestive of exact copying of the template provided. Subtle changes in base composition would not be observed by these techniques. Although sequence determination of the first 175 nucleotides from the 5' terminus of *Qβ*-RNA has been reported (Billeter et al., 1969), the complete base sequence of *Qβ*-RNA has not yet been determined. A biological definition of precise replication is provided by maintenance of all of the functional properties of the original molecule. Preservation of biological function by *Qβ*-RNA synthesized *in vitro* was shown by the ability of product RNA to infect sphaeroblasts. An increase in RNA synthesized was paralleled by an increase in sphaeroblast particle forming units; for example, a 75 fold increase in RNA synthesized resulted in a 35 fold increase in particle forming units (Spiegelman, Haruna, Holland, Beaudreau and Mills, 1965). Furthermore, synthesized RNA was diluted

by sequential transfer into successive tubes containing the standard reaction mixture (minus template) until the eighth tube contained less than one strand of input primer. Yet the infectious units per sphaeroblast assay remained as high as in the first tube. The dilution procedure was continued to the fifteenth tube with the maintenance of infectious units synthesized. Clearly, RNA synthesized *in vitro* is itself replicated accurately to the extent of maintaining its biological function.

Although the previously mentioned properties of *replicase*: complete dependence on homologous template; prolonged RNA synthesis (more than five hours); synthesis of infectious viral RNA indistinguishable in size and base composition from added templete; saturation at low levels of template; autocatalytic synthesis at sub-saturation concentration; parallel increase in RNA synthesis and infectious units; and synthesis of infectious RNA after the original template had been removed by dilution, are all consistent with the conjecture that *replicase* functions only as a catalyst, they do not prove that RNA is the instructive agent in the replication process. For example, *Qβ-replicase* could contain a minute contaminant whose replication is activated by added RNA. Proof that RNA is the instructive agent was obtained experimentally by providing normal *replicase* (isolated from *E.coli* infected with non-mutant *Qβ*) with two alternative templates: viral RNA extracted from normal *Qβ*; and RNA isolated from a temperature sensitive mutant which scored a relative efficiency of plating of 2.5×10^{-2} at 41°C (compared to 100 at 34°C) on a spheroblast assay. As expected, RNA synthesized from *replicase* primed with normal Qβ-RNA resulted in the same relative efficiency of plating at both temperatures, whereas the product from temperature-sensitive RNA primed *replicase* maintained the parental phenotype of differential spheroblast infectivity at 34 and 41°C (Pace and Spiegelman, 1966 (b)). Thus RNA was the instructive agent.

In view of the abundant supporting evidence for the original report of extensive RNA replication *in vitro* (Haruna, Nozu, Ohtaka and Spiegelman, 1963) it is surprising that the exact composition of *replicase* remains controversial. Centrifugation for 36 hours in a sucrose gradient resolved *Qβ-replicase* into two components: a "light" component of molecular weight 80,000; and a heavy component of molecular weight 130,000 (Eikhorn and Spiegelman, 1967) capable of acting as a poly-G-polymerase. Neither component catalyzed the synthesis of *Q*β-RNA alone, but in combination they achieved normal *replicase* activity. Subsequently the "light" component was isolated from uninfected cells (Eikhom, Stockley and Spiegelman, 1968); it is therefore not coded for by the viral genome. In other hands *Qβ-replicase* has shown a different fractionation. Two small components, a heat stable and partially protease resistant 3S component (Factor I), and a heat and pepsin sensitive 1.5S component (Factor II), both isolated from uninfected cells, were required for activation of the "polymerase" catalyzed synthesis of *Q*β-RNA (August, 1969; August et al., 1968; Shapiro, Franze de Fernandez and August, 1968). The "Factors" of August et al. did not substitute for the "light" component of Spiegelman et al. (Eikhom, Stockley and Spiegelman, 1968). Experiments with temperature-sensitive (*ts*) mutants of a similar single-stranded RNA coliphage, *f2*, also support the concept that several molecular components are required for extensive RNA synthesis. Mutant *ts-6* continued synthesis of viral RNA for 3 minutes after a shift to the non-permissive temperature but was unable to synthesize RNA complementary to the viral template (Lodish and Zinder, 1966). This *in vivo* experiment indicates the presence of at least two components: (i) a host component (dependent upon the viral component) and (ii) a viral component coded for by the *ts-6* cistron. Both components are necessary for sustained RNA synthesis.

Qβ-replicase, whatever its molecular structure, is unquestionably capable of catalyzing the prolonged synthesis of Qβ-RNA *in vitro*. The reaction starts with an RNA template and at termination has produced more of the same; therefore, all intermediary steps must be passed through during the synthesis. A temporal examination of molecular forms intervening between template and product enabled an elucidation of the mechanism of synthesis.

In addition to Qβ-RNA the following molecular forms were isolated from the reaction mixture catalyzed by *Qβ-replicase*: (i) 28S "minus" strands (the molecular complement of 28SQβ-RNA extracted from viral particles- i.e., "minus" strands have an U/A ratio of 0.75 whereas "plus" strands (viral Qβ-RNA) have an U/A ratio of 1.33); (ii) completely double-stranded RNA referred to as replicating form (RF) or Hofschneider structure (HS); and (iii) a structure consisting of double-stranded RNA with one or more attached single-stranded RNA molecules of varying length referred to as replicative intermediate (RI) or the Franklin structure (FS)[1].

Isolation of "minus" strands was achieved by taking advantage of their ability to anneal with Qβ-RNA ("plus" strands). Denatured double-stranded RNA was reannealed with a large *excess* of *fragmented* plus strands. Cellulose chromatography then separated double-stranded RNA from excess single stranded "plus" fragments. Heat denaturation, to separate "plus" fragments from full "minus" strands, was followed by velocity centrifugation which isolated "minus" strands in a pure form (Pollet, Knolle, and Weissman, 1967).

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[1] FS sediments as a broad band in contrast to HS which is observed only as a 13S structure. However, on treatment with ribonuclease all FS has a sedimentation coefficient of 13S presumably because nascent partially-completed single stranded RNA chains are removed. The 13S form has the physical and chemical properties expected of a double-stranded viral RNA. Since a labelled RNA precursor can be chased from FS into single-stranded viral RNA, the FS is often referred to as a replicative intermediate whereas double-stranded RNA without attached chains is referred to as a replicative form (Franklin, 1966).

"Minus" strands were characterized by being converted into an RNase-resistant (double-stranded) form by "plus" strands (Pollet, Knolle and Weissmann, 1967) and by having a base composition complementary to *Q*β-RNA (Feix, Pollet and Weissmann, 1968). "Minus" strands were not able to infect spheroblasts; presumably because *replicase* is coded for by the "plus" strand (Pollet, Knolle and Weissmann, 1967). *Q*β-*replicase* catalyzed *in vitro* RNA synthesis when "minus" strands were added as template (Feix, Pollet and Weissmann, 1968; Mills, Bishop and Spiegelman, 1968; Weissmann, Feix, Slor and Pollet, 1967). Biochemical analysis of synthesized RNA was dependent on the development of the double isotope specific dilution assay - an efficient quantitative assay for "plus" and "minus" strands (Weissmann, Feix, Slor and Pollet, 1967). For this assay H^3 labelled product RNA was mixed with (i) a small quantity of P^{32} labelled viral "plus" strands as a control, (ii) an excess of unlabelled double-stranded *Q*β-RNA, and (iii) increasing amounts of unlabelled *Q*β-RNA. The mixture was heated (120°C for 3 minutes) and then cooled (60 minutes at 80°C) prior to determining RNase-resistant P^{32} and H^3-radioactivity. In this procedure H^3-labelled "minus" strands are protected from RNase activity by the addition of *Q*β-RNA. From a plot of the fraction of P^{32} and H^3 RNase resistant radioactivity at different concentrations of unlabelled *Q*β-RNA, the relative amounts of "plus" and "minus" strands in the synthesized H^3-RNA can be determined (Weissmann, Feix, Slor and Pollet, 1967). The "minus" primed reaction[1] yielded an initially high value for product "plus" strands which steadily declined in value (i.e., 74% "minus" strands at 2.5 minutes, to 21% at 10 minutes and 11.5% at 25 minutes) (Weissmann, Feix, Slor and Pollet, 1967). In contrast the "plus" primed reaction assayed approximately 10% "minus"

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[1] In fact denatured double-stranded RNA was used as a primer. However, the reaction products were sufficiently dissimilar to the "plus" primed reaction that the synthesis observed was attributed to the template activity of "minus" strands.

strands throughout the reaction. After 40 minutes of incubation the product was 91% "plus" and 9% "minus" regardless of whether "plus" or "minus" strands were used as primer. Two minutes after the addition of purified non-infectious *Qβ*-"minus" strands, 1×10^6 infectious units had been synthesized. Four minutes after the addition of "minus" primer, three times the input RNA had been synthesized for a total of 2.7×10^6 infectious units (Feix, Pollet and Weissmann, 1968). Although RNA had been synthesized no increase in infectious units was observed for six minutes with a "plus" primed reaction; after thirty minutes of synthesis the "plus" primed reaction resulted in a sixteen fold increase in infectious units whereas the "minus" primed reaction assayed a thirty-two fold increase in spheroblast infectivity (Feix, Pollet and Weissmann, 1968). Thus it appears that "minus" strands result in the immediate synthesis of "plus" strands whereas primer "plus" strands first direct the synthesis of noninfectious "minus" strands which are subsequently utilized as a template for "plus" strand synthesis.

In addition to the lack of increase in infectious units during the first six minutes, Mills, Pace and Spiegelman (1966) also reported a *loss* of infectious units during the early synthesis of a reaction primed with "plus" strands. A maximum loss of 75% of infectious units contributed by the "plus" strand template was observed at four minutes. H^3-template at four minutes was associated with P^{32}-product in an uninfectious molecular form with a sedimentation coefficient of 15S. Heating the 15S form resulted in infectivity, which suggested that a double-stranded RNA was involved in the replication of infectious 28S *Qβ*-RNA. Additional evidence of the double-stranded nature of the 15S form was provided by its partial resistance to ribonuclease.

Further analysis of intervening molecular forms was achieved by acylamide gel electrophoresis of the reaction mixture (Bishop, Claybrook, Pace and Spiegelman, 1967). Acrylamide gel electrophoresis provided an

efficient quantitative analysis of *Q*β-RNA, as well as of the HS (Franche and Hofsneider, 1966) and the FS (Franklin, 1966). The loss of infectivity in the early synthetic period was found to be quantitatively correlated with *template* involvement in the HS and FS (Bishop, Claybrook, Pace and Spiegelman, 1967). One minute after the initiation of synthesis some of the P^{32}-*Q*β-RNA template was associated with the HS. P^{32}-*Q*β-RNA primer appeared in the FS one-half minute later and at the end of two minutes more P^{32}-template was associated with FS than with HS. The ratio of P^{32}-FS to P^{32}-HS did not change appreciably in the interval from three to five minutes. P^{32}-template in HS and FS was accompanied by a loss of free P^{32}-template so that by three minutes 69% of P^{32}-primer was associated with HS and FS, consistent with the earlier report (Mills, Pace and Spiegelman, 1966) of a 75% loss in template infectivity at four minutes. Supporting evidence for intermediate molecular complexes involved in the *in vitro* synthesis of *Q*β-RNA was provided by following α P^{32} UTP labelled *product* in the early stages of a reaction primed with H^3-template (Pace, Bishop and Spiegelman, 1967). The earliest detectable P^{32}-product was found in the HS region at one minute (associated with a small fraction of the H^3 template); this was followed by incorporation into the FS one minute later. P^{32}-product *Q*β-RNA was observed subsequent to the appearance of label in the HS and FS. The salient features of these experiments are that: primer RNA first becomes part of a noninfectious RNA complex; all of the first product synthesized is associated with the initiating template in the HS region; subsequent to the formation of HS structures template and product are found in the FS; and infectious *Q*β-RNA is synthesized only after FS structures are observed. These observations indicate that HS and FS are sequential template-containing intermediates in the synthesis of

Qβ-product and that the FS generates new Qβ-RNA strands[1]. Additional proof of such a mechanism was provided by "pulse-chase" experiments (Pace, Bishop and Spiegelman, 1968). During a period of active Qβ-RNA synthesis UTP^{32} was incorporated into FS for one minute followed by a cold chase for ten minutes. As expected, the pulsed radioactivity found in the FS was subsequently chased into mature Qβ-RNA. Thus the FS appears to be the immediate physical precursor to Qβ-RNA.

Incorporation of β, γ-P^{32} labelled ATP, UTP, CTP and GTP into product RNA was utilized to show that both "plus" and "minus" strands are synthesized in a 5' to 3' direction by copying the complementary template from the 3' to the 5' end (August et al., 1968; Mills, Bishop and Spiegelman, 1968; Spiegelman et al., 1968). When reaction mixtures primed with "plus" or "minus" strands were supplied with the four β, γ-P^{32} labelled ribonucleoside triphosphates only β, γ-P^{32}-GTP was incorporated into complete or incomplete strands. A 5' to 3' synthesis predicts that only the unique 5' terminal ribonucleoside triphosphate would retain its β and γ phosphate since the β and γ phosphates of other ribonucleoside triphosphates would be cleaved when adjacent bases are joined. In contrast, incomplete product strands of a 3' to 5' synthesis would contain all ribonucleoside triphosphates.

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[1] Weissman et al. (1968) provide evidence that double-stranded RNA is an artifact of extraction and that HS and FS are not the actual replicating complexes. Support of their view comes from the observation that neither HS nor FS can prime Qβ-*replicase* without prior heat denaturation (Spiegelman et al., 1968). Weissmann et al. (1968) propose that template and product are held together by relicase and/or a few hydrogen-bonded regions during replication and are converted into a double-helical structure by extraction with phenol or detergents. However, such an interpretation is not at variance with the above postulated mechanism of Qβ-RNA replication by forming a "minus" strand complementary to the original template; the "minus" strand subsequently is utilized as a template for the formation of a "plus" strand.

Hence the observation of only β, γ-P^{32}-GTP being incorporated indicates that "plus" and "minus" strands are synthesized in a 5' to 3' direction and that the 3' end of "plus" and "minus" strands utilized by *Qβ-replicase* end in cytosine.

To recapitulate, an *in vitro* system capable of extensive replication of *Q*β-RNA has been developed as well as the necessary technology for the qualitative and quantitative analysis of product RNA. Synthesis, shown to be dependent upon the addition of intact homologous template, produced RNA with the same base composition and sedimentation properties as the added template. Maintenance of the function of all three *Q*β-RNA cistrons was indicated by the ability of product RNA to infect host spheroblasts. Extensive serial dilution of synthesized RNA revealed that product RNA was itself able to act as a template. Proof that RNA was the instructive agent in the replicative process was provided by the demonstration that the product of a reaction primed with mutant RNA maintained the mutant phenotype. In addition to *Q*β-RNA, HS, FS and "minus" strands were shown to be involved in the replicative process. When primed with a "plus" strand *Qβ-replicase* synthesizes a complementary "minus" strand which is itself subsequently used as a template for the synthesis of "plus" strands. Synthesis of both "plus" and "minus" strands proceeds in the 5' to 3' direction.

In theory many of the functions of *Q*β-RNA (such as information for the coat proteins and *replicase*) should be dispensable in the *in vitro* replicating system. In the test-tube environment all the components necessary for self-replication are provided so that many sequences coding for information necessary for *in vivo* replication should no longer be required. Maintenance of the ability to utilize *Qβ-replicase* as a catalyst would nevertheless appear to be an essential function of the molecule still required in the

in vitro system (unless an alternative method of replication was generated by some cryptic mechanism). Molecular "Darwinian" selection should be possible in such an *in vitro* system since a spontaneously generated variant molecule with a replicative superiority would be expected to become the predominant molecular species by virtue of its replicative superiority. Such considerations led Mills, Peterson and Spiegelman (1967) to design an experiment capable of answering the following question: "What will happen to the RNA molecules if the only demand made upon them is the Biblical injunction, *multiply*, with the biological proviso that they do so as rapidly as possible?"[1]

An experimental system capable of providing selection pressure for fast replicating variant molecules was achieved by a serial transfer experiment that selected newly synthesized product RNA (Mills, Peterson and Spiegelman, 1967). At the start of the experiment 0.2 ug[2] of *Q*β-RNA was added to 0.25 ml of a standard reaction mixture. After 20 minutes of synthesis a 20 λ sample was used to prime the second reaction, and so on for the first 13 reactions. This procedure was followed by 16 reactions for 15 minutes, 9 reactions for 10 minutes, 14 reactions for 7 minutes, and 23 reactions of 5 minutes duration for a total of 75 reactions. That molecular "Darwinian" selection had occurred was indicated by the cessation of synthesis of biologically functional RNA (as measured by a spheroblast assay) by the fifth transfer. Furthermore, the general trend towards faster replication (measured by the rate of incorporation of P^{32}-UTP) in later reaction mixtures was highlighted by a dramatically increased rate of synthesis in the 9th reaction mixture. In terms of completed molecules the single-stranded product of the 75th

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[1] Mills, Peterson and Spiegelman (1967), page 217.

[2] 0.2 ug of 28S *Q*β-RNA equals 1×10^{11} strands (Pace and Spiegelman, 1966).

transfer was shown to replicate fifteen times faster than *Q*β-RNA. The sedimentation coefficient of RNA from the main peak of the 9th reaction mixture was 20S instead of 28S expected for infectious *Q*β-RNA. By the 30th transfer the main peak was 15S; and by the 75th transfer it was 12S, indicating the evolution of a molecule with a lower molecular weight. Sedimentation anslysis of the 9th, 15th and 30th reaction mixtures also revealed complexes in minor peaks with HS and FS sedimentation coefficients. The minor peak of the 75th transfer (separated by acrylamide gel electrophoresis) was partially resistant to RNase, denoting the presence of a partially-double stranded structure in the reaction mixture. "Minus" strands isolated from the 75th reaction mixture were capable of priming *Qβ-replicase* (Mills, Bishop and Spiegelman, 1968). The presence of HS, FS and "minus" strands in the reaction mixtures suggests that variant RNA replicates by the same mechanism as *Q*β-RNA. The molecular weight of single-stranded RNA of the 75th transfer, referred to as *V-1*, was 1.7×10^5 daltons, which corresponds to a molecule 550 nucleotides long compared to the 3600 nucleotides present in a mature *Q*β-RNA molecule. In addition the base composition of *V-1* was increased 5 mole % in quanine but decreased in adenine and cytosine by 2.4 mole %. The conclusion seems inescapable. *V-1* is a variant self-replicating molecule with a replicative superiority over the molecular prototype *Q*β-RNA - unequivocal evidence of *in vitro* "Darwinian" selection at the molecular level.

In the selection of *V-1*, product RNA was diluted 12.5 fold at each transfer, and selection pressure for fast replicating molecules was maintained by shortening the time interval between transfers. Another isolate (*V-2*) was selected by maintaining the incubation time constant at 15 minutes but applying increasing dilutions of product RNA, thus creating an environment favorable for the selection of molecules capable of interacting with *Qβ-replicase* with an increased efficiency (Levisohn and Spiegelman, 1968).

The first tube received 0.07 ug of 28S *Q*β-RNA. Product RNA was diluted 125 fold for the next 5 transfers; 0.01 ml of a 1 x 10^6 dilution was used for the next 7 transfers; and 0.01 ml from 1 x 10^7 to 2.5 x 10^{10} dilutions were used to seed reaction mixtures in the last 5 transfers. Variant single-stranded RNA of the 17th transfer (*V-2*) had the same relative electrophoretic mobility on polyacrylamide gels thus indicating a molecular weight similar to *V-1*. Purified "plus" strands of *V-2* had a different base composition than *V-1* or *Q*β -RNA (Levisohn and Spiegelman, 1969). Sequence analysis of the 5' end of *V-2* "plus" strands indicate an octanucleotide ($ppp(Gp)_4(Ap)_2(CP)_2$) similar to *Q*β-RNA (Bishop, Mills and Spiegelman, 1968). However, *V-2* exhibited a striking phenotypic difference to *V-1* in the ability to prime *Q*β-*replicase* at low template concentrations (Levisohn and Spiegelman, 1968). Measurable RNA synthesis was observed after 15 minutes of priming a standard reaction mixture with 0.29 uuug[1] of *V-2* RNA. In contrast 300 times as much template *V-1* RNA was required to obtain a measurable synthesis in 15 minutes. In addition the doubling time of *V-2* was 0.403 minutes during expodential growth as compared to 0.456 minutes for *V-1*. Thus the artificial environment of low template concentration was successful in selecting a variant with an enhanced ability of interacting with *Q*β-*replicase*. Clearly, the different selective pressures used to select *V-1* and *V-2* selected different spontaneously arising variants, the variant selected being that molecule with a replicative superiority in the selective environment.

V-1 and *V-2* were selected under environmental conditions that fostered maximal RNA replication (i.e., standard reaction mixture at 38°C). Experiments were also designed (Levisohn and Spiegelman, 1969) to ascertain if superior replicating molecules could be selected in an environment that inhibits normal RNA replication. Suboptimal levels of precursor ribonucleoside triphosphate greatly inhibit the rate of RNA replication. For example; the

[1] 0.229 uuug equals one strand of *V-2* RNA (Levisohn and Spiegelman, 1968).

rate of synthesis of *V-2* was 25% and 5% of normal when incubated on two and one mu moles of CTP/0.125 ml of reaction mixture, respectively ("complete medium" contained 100 mu moles of CTP). *V-4* was isolated from the 10th transfer of a series of dilutions[1] in "medium" containing 2 mu moles of CTP/ 0.125 ml, the first tube being primed with *Q*β-RNA. *V-4* was then used to prime a series of reaction mixtures containing one mu mole of CTP; thirty serial dilutions[1] led to the isolation of *V-6*. The doubling times of *V-2*, *V-4* and *V-6* were 1.81, 1.41 and 1.16 during logarithmetic growth in limiting CTP (1 mu mole/0.125 ml), indicating that *V-4* and *V-6* are variant molecules with a replicative superiority in low levels of CTP. No detectable differences in base composition or molecular weight could be detected between *V-2* and *V-6*. Preliminary results indicate that *V-4* and *V-6* produce allosteric effects on the *replicase* that enables the enzyme-substrate complex to utilize CTP more efficiently at low CTP concentrations (Levisohn and Spiegelman, 1969).

Tubercidin (an analogue of adenosine) also inhibits the *in vitro* replication of RNA. The replication rate of *V-8* , a variant derived from *V-6* capable of enhanced replication on low levels of ATP, was inhibited four-fold when 30 mu moles of tubercidin was added to the reaction mixture. *V-9* isolated by serial dilution on tubercidin "medium" originally seeded with *V-8*, had a doubling time of 2 minutes in the presence of the inhibitor as compared to 4.1 minutes for *V-8*. In the absence of tubercidin both *V-9* and the molecular prototype (*V-8*) have the same doubling time - one minute (Levisohn and Spiegelman, 1969).

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[1] The initial dilution of 1.25×10^{4} was increased gradually to 1×10^{11} (Levisohn and Spiegelman, 1969).

In summary there is unequivocal evidence that molecular "Darwinian" selection occurs during the *in vitro* synthesis of Qβ-RNA. Imposition of a specific selection pressure resulted in the evolution of a specific molecular variant. The molecular variant isolated had the property of replicative superiority in the selective environment. In addition to achieving replicative superiority by discarding dispensible information, some variants were produced with no detectable differences in base composition or molecular length (i.e., *V-2* and *V-6*). Such mutants are at present differentiated only by their phenotypic expressions.

IN VIVO EVIDENCE FOR MOLECULAR "DARWINIAN" SELECTION

Introduction

The wide range of phenotypes exhibited by *Q*β-RNA when subjected to different environments (Mills, Peterson and Spiegelman, 1967; Levisohn and Spiegelman, 1968; Levisohn and Spiegelman, 1969) is truly remarkable. The number of different molecular variants possible would appear to be limited only by the ingenuity of the investigator to devise environments that provide a selective advantage for novel RNA molecules. Detection of molecular "Darwinian" selection *in vitro* was dependent upon the development of a system capable of unlimited synthesis of a specific molecule as well as the advanced technology capable of detecting any variant molecules generated in the system. These necessary requirements have been achieved only for *Q*β-RNA *in vitro* and one wonders if the phenomenon is a general one.

It is the purpose of the author to provide evidence for molecular "Darwinian" selection *in vivo* in the remainder of this essay. Before embarking upon this adventure, it would be well to reflect briefly upon the observations predicted if molecular "Darwinian" selection occurred *in vivo*.

Consider the localization of a self-replicating molecule in nature. A given self-replicating molecule occupies a very specific part of the total space inhabited by all the self-replicating molecules of a given biological-system.[1] For example, mapping of a chromosomal gene indicates a specific and constant location in the chromosome relative to other genes; and DNA of

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[1] A biological-system in this essay will be defined as the totality of biological structures and precursors necessary to achieve sustained replication of the self-replicating molecules contained in the biological-system.

organelles such as mitochondria and chloroplasts is limited to these organelles and is not found in areas such as the nucleus which are occupied by other self-replicating molecules. The space occupied by a self-replicating molecule may vary with the functional state (replication, transmission, transcription or translation) of the molecule. However, each self-replicating molecule occupies a distinctly unique space when functioning in a biological-system; the totality of space occupied by a self-replicating molecule in all of its functional states will be referred to as a *molecular-niche* in the remainder of this essay. Implicit in the concept of a molecular niche is the existence of control mechanism(s) that limit the number of replicas of a given self-replicating molecule.

By definition molecular "Darwinian" selection requires the appearance of a variant self-replicating molecule with a replicative superiority over the molecular prototype in a given environment. As many replicas of the variant molecule as possible would be produced in a given biological-system. However, unlimited replication of a variant molecule (or the molecular prototype) would be incompatible with survival (continued replication) of the molecule. The quantitative upper limit of variant molecules would be expected to be controlled by the same mechanisms that control the quantity of normal molecules. For example, if the percentage of normal mitochondrial DNA is ten percent of the total DNA, any abnormal mitochondrial DNA would not be expected to greatly exceed ten per cent of the total.

The replicative superiority of the variant molecule would have the potential of becoming the sole molecular species in the niche occupied by the molecular prototype. Although the tendency would be for the variant molecule

to become the sole species occupying the molecular niche, certain aspects of biological systems would allow for the maintenance of the normal molecule. Firstly, self-replicating molecules of a biological system are compartmentalized into units and subunits of varying size and complexity such as cells, mitochondria, chloroplasts, nuclei, and protein coats in the case of viruses. In the reproduction of these units chance molecular segregation from a common pool of normal and variant molecules could result in units containing only normal molecules. Such units could then co-exist with units containing only abnormal molecules if there were no transfer of molecules between units (assuming that molecular composition has no effect on the replication rate of the units). Secondly, a biological system is not static but rather is in a constant state of flux. The molecular environment of a self-replicating molecule can change greatly in response to external and internal stimuli. Implicit in the hypothesis of "Darwinian" selection is the dependence of replicative superiority on environment. A molecule with a replicative superiority in one environment may have a replicative inferiority in another. Consider a spontaneously-generated mutant viral molecule that has arisen two thirds of the way through the infectious cycle. Further assume that the mutant viral molecule has a replicative superiority only in the molecular environment provided in the host cell in the latter one-third of the infectious cycle. Coinfection of a host cell by a variant and a normal viral molecule would initially favor replication of the latter molecule, whereas the final one-third of the infectious cycle would favor the replication of the variant molecule. Similarly, a variant chloroplast DNA molecule may have a replicative superiority over the molecular prototype only under optimal levels of the precursor deoxyribonucleoside triphosphates. At a certain stage in the cell cycle such as nuclear DNA synthesis, these precursors may become limiting thereby conferring a replicative advantage to the normal cholroplast DNA. Thus the molecular environment may initially

favor the replication of a variant self-replicating molecule and be subsequently altered to favor the replication of the normal self-replicating molecule. (An important corollary in that molecular replication could change the environment in such a manner as to be sub-optimal for the replication of the causative molecule). A cyclic environmental fluctuation could in this manner prevent the variant self-replicating molecule from becoming the sole molecular species.

The location of a variant self-replicating molecule need not be restricted to the niche of the parental prototype as the variant may be successful in occupying the niche of other resident self-replicating molecules as well. The possibility also exists that a variant molecule is so altered in properties that it is unable to occupy any of the existing niches but is so successful in self-duplication that the biological-system supplies a *de novo* niche for the variant.

The effect of molecular "Darwinian" selection on the biological-system would depend greatly on the function of the self-replicating molecule in the biological-system. A broad classification of dispensable and indispensable self-replicating molecules can be made. Since the functional requirements of a biological-system vary greatly, a self-replicating molecule may be dispensable in one environment but not another. For example, the information required for sexual reproduction would not be required by an organism in an environment capable of supporting continued asexual reproduction. However, the vast majority of self-replicating molecules would be indespensable for the survival of the biological unit. Any incautious self-replicating molecule that sheds indespensable information in order to gain a replicative advantage would run the risk of self-elimination. Unless sufficient normal self-replicating molecules can be maintained by some mechanism as described above, the biological unit would become defunct and with it the variant molecule.

Multiplicity of units is one of the major characteristics of a biological system. Loss of a unit due to the generation of a variant self-replicating molecule would be very difficult to detect by current technology. Consider the demise of a cell (the largest biological unit) in a colony of bacterial cells (one of the smaller biological-systems). The loss created by cell lethality during the growth of a colony would be replaced by the progeny of other cells and pass undetected. *Thus the majority of "Darwinian" molecular selection in vivo could not be detected by modern technology and would be overlooked.*

On the other hand self-replicating molecules with a dispensable function would occasionally be expected to provide observable examples of molecular "Darwinian" selection *in vivo*. Minimal requirements for a rigorous demonstration of molecular "Darwinian" selection *in vivo* would require: (i) isolation of the variant self-replicating molecule and its molecular prototype in a chemically pure form; (ii) detection of differences in chemical composition between the variant and prototype molecule; and (iii) the *in vivo* demonstration of a replicative differential between the variant and prototype molecule. Technical difficulties would be expected to impede the demonstration of one or more of the three minimal requirements in many biological systems. For example, subtle changes in molecular composition, as exemplified by the change from *V-2* to *V-6* (Levisohn and Spiegelman, 1969), could not be detected biochemically. However, gross changes in molecular composition, such as the decrease in length and alteration in base composition of *V-1* from *Q*β-RNA (Mills, Peterson and Spiegelman, 1967), would be easily detected by current biochemical techniques.

Unless molecular "Darwinain" selection *in vivo* occurred with an unexpectedly high frequency, the detection of grossly variant self-replicating

molecules by the sole use of biochemical techniques would be laborious and most likely futile. As an aid to their detection the loss of an easily observable dispensable function, caused by a gross molecular alteration, would serve as a useful indicator.

Genetic analysis would be expected to provide an *in vivo* demonstration of any reproductive differential between differing variants and their prototype self-replicating molecule. Variform isolates of variant self-replicating molecules and their molecular prototype would be expected to have unique phenotypic expressions. Hybrid biological units containing alternative forms of a self-replicating molecule could be obtained from suitable crosses. The phenotypic expression of alternative molecular forms in progeny units would then form the basis of a genetic analysis.

Genetic analysis would be expected to provide evidence for the surprising prediction that progeny units, in many instances, would show the *complete and irreversible loss* of the parental self-replicating molecules with a replicative inferiority. It is important to emphasize the difference between this prediction and the usual dominance-recessive relationships found between genetic markers. Classical genetic analysis demonstrates that the lack of phenotypic expression of the recessive marker is not due to the irrevocable loss of the material basis for the recessive phenotype. Rather, the recessive marker can be shown to have been physically present, but unexpressed. The hypothesis of molecular "Darwinian" selection makes the diametrically opposite prediction that lack of phenotypic expression by a genetic marker is due to the irreversible *loss* of the material basis for the phenotypic expression.

Maintenance of both parental self-replicating molecules by environmental fluctuations, as mentioned earlier, would be expected in some biological

systems. Additionally, stable progeny units containing only one parental form would be expected to occur as the result of chance segregation of parental self-replicating molecules. The interaction of (i) environmental modification of differential replication and (ii) chance segregation, would elicit the unusual phenotypic expression of *continuous segregation.*

Genetic analysis would also be expected to provide a visible demonstration of a variant self-replicating molecule capable of occupying niche(s) in addition to that of the parental prototype. Alteration of the phenotypic expression of the parental prototype would act as a signal that the parental niche was occupied by the variant molecule. Subsequent to this cue other molecular niche(s) would be occupied by the variant molecule. Such a series of events would provide the marvel of *dependence of one phenotypic alteration upon the prior appearance of another.*

Simultaneous loss of more than one function would be an indication of a grossly variant molecule generated from a molecular prototype normally carrying information for more than one function. Phenotypically, such an alteration would appear to mimic a classical deletion. However, a variant molecule endowed with replicative superiority would have the additional property of suppressitivity.

Another genetic manifestation of molecular "Darwinian" selection *in vivo* would be the dependence of phenotypic expression on external environmental parameters. Alteration of the external environment could often be expected to vary the replicative differential of self-replicating molecules and thus alter some of their phenotypic properties.

Suppressitivity

Since the molecular basis of heredity was not firmly established until the mid-twentieth century, early evidence for molecular "Darwinian" selection *in vivo* was dependent upon genetic analysis. Thus, of the three aforementioned requirements for proof of molecular "Darwinian" selection *in vivo*, only evidence for the demonstration of a replicative differential between the variant and prototype molecule can be found in the early literature.

Genetic analysis revealed that the normal phenotype could be irreversibly lost from the progeny of a cross between a mutant form and the normal prototype. In such a cross the mutant form was said to be *suppressive* (Darlington, 1944) since, unlike the usual dominant-recessive relationship between markers, the alternative form (and thus its molecular basis) was either absent from, or irreversibly inactivated in, progeny cells. Although Darlington (1944) could not discern whether the material basis for the normal phenotype was absent or irreversibly inactivated in mutant progeny cells, he stated that suppressitivity implied that, "plasmagenes[1] have rates of reproduction which can be varied widely ..... and that their chemical equilibria must be subject in some degree to developmental as well as environmental conditions."[2] In other words, he considered the lack of phenotypic expression of the normal plasmagene to be due to the absence of its material basis in mutant progeny cells. The disappearance of the normal determinant was caused by the replicative superiority of the mutant determinant and furthermore, the replicative differential between the two determinants was environmentally influenced - an apt description of the expectations of molecular "Darwinian" selection *in vivo*!

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[1] The characteristics discussed were cytoplasmically-inherited; the term *plasmagene* referred to the hereditary material that was extranuclear and thus resided in the cytoplasm.

[2] Darlington (1944), page 166.

Suppressive cytoplasmically-inherited mutants are found in many species and affect many diverse characteristics such as: "killer" in *Paramecium aurelia*, variegated and pale in *Scolopendrium vulgare*, rogue (pointed leaves and curved pods) in peas (Darlington, 1944); red in *Aspergillus nidulans*, vegetative death in *Aspergillus glaucus*, and senescence in *Podospora anserina* (Jinks, 1964(a)); respiratory deficiency in *Saccharomyces cerevisiae* (Ephrussi, Hottinguer and Roman, 1955); *abn-1* and *abn-2* (reduced growth rate, absence of aerial hyphae and protoperithecia) in *Neurospora crassa* (Garnjobst, Wilson and Tatum, 1965); abnormal growth mutants such as *stp-A* in *Neurospora crassa* (Bertrand and Pittenger, 1969) ; and "killer" in *Saccharomyces cerevisiae* (Somers and Bevan, 1969). A striking example of the expression of suppressive plasmagenes in *Neurospora crassa* was provided by the continuous growth experiments of Bertrand and Pittenger (1969). In one experiment hyphal tips with abnormal growth characteristics were selected after 110 days of continuous growth in special tubes designed for continuous hyphal propagation. The growth mutant (and others selected in a similar manner) was characterized by (i) pleiotrophy (affecting growth, conidial viability, female fertility and causing mitochondrial abnormalities such as cytochrome $a+a_3$ and b deficiencies) and (ii) a suppressive cytoplasmic-inheritance (when analyzed by the heterokaryon test).

Unfortunately, little is known of the molecular and/or cytological basis of most of the suppressive cytoplasmically-inherited markers studied. Consider a mutant and the corresponding normal marker (cytoplasmically-inherited) and their respective phenotypes. Using the most generalized classification, the mutant marker is *genetically* defined as being suppressive if its phenotype is not expressed in progeny cells issued from the hybrid cell formed by crossing the mutant and normal strain. However, lack of expression of a normal marker does not necessarily imply that the molecular-

niche of the normal molecular prototype has been occupied by a mutant molecule with a replicative superiority. Other plausible mechanisms are capable of accounting for the lack of the normal phenotypic expression in progeny cells such as: the irreversible inactivation of the normal molecular prototype; prezygotic elimination of the molecular prototype by the lack of contribution of plasmagenes by the smaller male gamete, as exemplified by the maternal inheritance of *stp-A* in *Neurospora crassa* (Bertrand and Pittenger, 1969); postzygotic destruction of the molecular prototype typified by the maintenance of plasmagenes contributed only by the *mt+* mating type in *Chlamydomonas reinhardi*, even though both gametes contribute equally to the zygote (Gillham, 1969); and chance somatic segregation resulting in the accidental exclusion of the molecular prototype from progeny cells (Jinks, 1964 (b)). Genetic analysis without knowledge of the molecular and cytological basis of the suppressive phenotype cannot demonstrate unequivocally that molecular "Darwinian" selection, rather than inactivation, prezygotic elimination, postzygotic destruction, or chance segregation, is the underlying mechanism causing suppressitivity. Fortunately, detailed genetical, molecular and cytological information is available for the suppressive cytoplasmically-inherited respiratory-deficient mutant in *Saccharomyces cerevisiae*. In the belief that the thorough investigation of a phenomenon in one species is more informative than a superficial examination of many species, the remainder of this section will concentrate on presenting evidence that suppressive respiratory-deficiency in *S. cerevisiae* is the phenotypic expression of molecular "Darwinian" selection *in vivo*. Information gleaned from critical experiments in other species will be mentioned when relevant.

Cytoplasmically-inherited respiratory-deficient mutants ([*rho*-])[1] in *S. cerevisiae* arise spontaneously with a minimum frequency of 1%. These mutants are characterized by the absence of cytochromes $a+a_3$, b and c_1 (Ephrussi, 1953; Borst and Kroon, 1969). [*rho*-] strains are unable to utilize non-fermentable energy sources such as glycerol but can derive sufficient energy for growth from glycolytic metabolism of a fermentable energy source such as glucose; thus any self-replicating molecule carrying information required for functional mitochondria is dispensable in *S. cerevisiae*, thereby allowing for the possibility of molecular "Darwinian" selection *in vivo* without resultant cell lethality. All spontaneous arising [*rho*-] cells are initially suppressive ([*rho-(s)*]) (author, unpublished results); however, [*rho-(s)*] can segregate to give a stable [*rho*-] strain with zero suppressitivity ([*rho-(n)*]) (Ephrussi, Hottinguer and Roman, 1955; author, unpublished results[2]). Zygote colonies of the [*rho*+] by [*rho-(n)*] cross are all [*rho*+], whereas a variable number (5-99%) of those from the cross [*rho-(s)*]by [*rho*+] are [*rho(-)*]. The % suppressitivity of a given [*rho-(s)*] strain is defined as the % of [*rho*-] zygote colonies produced when crossed to a [*rho*+] strain. Hence a [*rho*-] strain that produces 50% [*rho*-] zygote colonies when crossed to a [*rho*+] strain is defined as being 50% suppressive ([*rho-(50%s)*]. The hereditary component in [*rho-(s)*] cells that is responsible for the phenotypic expression of suppressitivity is referred to as the *suppressive factor (SF)* (Ephrussi, Jakob and Grandchamp, 1966). Extending this rationale there is a normal hereditary component in a [*rho*+] cell, referred to as the normal factor (*NF*), which can "mutate" to give rise to the *SF*. Ephrussi and

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[1] Genetic nomenclature of *S. cerevisiae* used in this essay follows the protocol outlined by the Yeast Genetics Supplement to the Microbial Bulletin No.31 (von. Borstel, 1969). In this system a marker enclosed by brackets ([]) is cytoplasmically inherited.

[2] From 0.5 to 2% of the cells of a [*rho-(s)*] strain are [*rho-(n)*]. [*rho-(n)*] strains have not been observed to give rise to [*rho-(s)*] strains.

Grandchamp (1965) used clonal studies to show that the degree of suppressitivity was transmissable by individual cells and was not the result of a [*rho*-] strain being a mixture of [*rho-(s)*] and [*rho-(n)*] cells.[1] [*rho*-] diploid cells cannot undergo meiosis in contrast to the sporulating ability of a heteroplasmic cell containing both the *NF* and the *SF* (Ephrussi, Hottinguer and Roman, 1955). Tetrad analysis of diploid heteroplasmic cells indicated that suppressitivity was cytoplasmically-inherited since all possible tetrad ratios were observed for [*rho*+]: [*rho*-] (i.e., 4:0, 3:1, 2:2, 1:3 and 0:4) (Rank and Person, appendix A). Furthermore, cells of the haploid [*rho*-] ascospore colonies were suppressive, revealing that lack of expression of the [*rho*+] phenotype was not due to the presence of a dominant nuclear gene coding for suppressitivity (Rank and Person, appendix A).

Explanation of different degrees of suppressitivity by the hypothesis of prezygotic elimination, postzygotic destruction, or chance somatic segregation requires the implausible assumption that different rates of elimination, destruction, or chance segregation are caused by different suppressive factors. Equally difficult to explain is the fact that both [*rho*+] and [*rho*-] zygote colonies are produced from a cross between a [*rho-(s)*] and a [*rho*+] strain. [*rho*+] zygote colonies contain an active, unaltered *NF* since they have a normal respiratory function, and [*rho*-] zygote colonies have been shown to contain an unaltered *SF* since cells of the [*rho*-] zygote colonies have a suppressitivity similar to the [*rho-(s)*] parental strain (Rank, appendix C). Micromanipulation in glycerol of the immediate progeny of zygotes from various crosses also provided evidence that the *SF* does not immediately destroy the *NF*, but rather eliminates it from the cell over a period of

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[1] This observation is correct for only 99 to 95% of cells of a [*rho-(s)*] strain since 1 to 5% of the cells from such a line were shown to have a greatly reduced suppressitivity (Rank, appendix C).

several cell divisions. (Ephrussi, Jakob and Grandchamp, 1966). Under aerobic growth conditions for parental cultures, zygotes of the crosses [*rho*+] by [*rho*+] and [*rho*+] by [*rho-(n)*] produced 17 buds in glycerol, [*rho-(n)*] by [*rho-(n)*] zygotes produced none, and [*rho-(s)*] by [*rho*+] zygotes produced a decreasing number of buds as the suppressitivity of the *SF* increased (i.e., 12.2 buds for 13% suppressitivity and 5.7 buds for 97% suppressitivity). The inability of [*rho-(n)*] by [*rho-(n)*] zygotes to form buds in glycerol indicates the necessity of the *NF* for bud formation in glycerol. Presumably the number of buds formed by a [*rho*+] by [*rho-(s)*] zygote is a measure of the time required to convert the zygote into a [*rho-(s)*] cell. Cessation of cell division by storage of [*rho*+] by [*rho-(s)*] zygotes at 30°C for 24 hours in 8.5% NaCl did not reduce the number of buds formed when these zygotes were transferred to glycerol containing medium; this indicates that cell division is required for elimination of the *SF*. Under anaerobic growth conditions, using glucose as an energy source, cytochrome b and $a+a_3$ are no longer measurable, presumably because O_2 is required as an inducer for the translation and/or transcription of the *NF*. A [*rho*+] strain was grown under anaerobic conditions for 30 generations, thus diluting out any preformed respiratory enzymes coded for by the *NF*, prior to crossing to a [*rho-(s)*] strain in glycerol. If the *SF* acts by destroying the *NF* zygotes formed from such a cross should not bud, since the [*rho*+] parental strain cannot contribute any respiratory capacity to the zygote. The zygotes produced the same number of buds with a given *SF* as when the [*rho*+] strain was grown under aerobic conditions. These results indicate that the *SF* does not immediately destroy or inactivate the *NF*, but rather eliminates the *NF* after a period of several cell divisions, consistent with the hypothesis of molecular "Darwinian" selection *in vivo*.

These observations suggest that the *NF* is unaltered and not lost by postzygotic destruction or prezygotic elimination; rather, the rate of loss from progeny cells is dependent upon the qualitative nature of the *SF*. This suggestion is accommodated by the hypothesis of molecular "Darwinian" selection as *SF*s from high suppressitivity strains would be equated with molecular variants with a faster replicative rate (Rank and Person, appendix A). That is, the *SF* of a 90% suppressive strain would replicate faster than the *SF* of a 70% suppressive strain, and so on. Whether a progeny cell becomes [*rho*+] or [*rho*−] would depend on the replicative differential between the variant molecule (*SF*) and its molecular prototype (*NF*) as well as the possibility of chance segregation resulting in a cell containing only one molecular species. The greater the replicative advantage of the *SF* the greater the number of [*rho*−] zygote colonies produced by crossing a [*rho*+] and [*rho*-*(s)*] strain. However, the rate of reduction to one molecular species (per progeny cell) by chance somatic segregation alone would be expected to be the same for qualitatively different suppressive factors.

A visual demonstration of the replicative differential between the *SF* and the *NF*, interacting with chance segregation of the *SF* or *NF* into progeny cells, is provided by mosaic colonies with a [*rho*−] base and a [*rho*+] apex (Ephrussi, Jakob and Grandchamp, 1966; Rank and Person, appendix A). Individual cells (ascospores) isolated by micromanipulation developed into mosaic colonies, providing proof that a mosaic colony originated from a single cell (Rank and Person, appendix A). The [*rho*−] base of spontaneously arising mosaic colonies was shown to be suppressive thus indicating that the *SF* was a causative agent in producing the colony (Rank and Person, appendix A). Since cells of the colony apex contained the *NF* and basal cells contained the spontaneously generated *SF*, it was suggested that a mosaic colony arose from

a heteroplasmic cell containing both the *NF* and the *SF* (Rank and Person, appendix A). A heteroplasmic cell (produced by crossing [*rho-(s)*] and [*rho*(+)]) was shown by micromanipulation studies to give rise to [*rho*+] and [*rho*-] cells as well as heteroplasmic daughter cells (Ephrussi, Jakob and Grandchamp, 1966). However, the progeny of many heteroplasmic cells would be expected to be predominately [*rho*-] cells due to the replicative superiority of the *SF*. Such [*rho*-] progeny cells would metabolize glucose via glycolysis and there would be no selection favouring the growth of any [*rho*+] cell produced by chance segregation of only *NF*s into a progeny cell. In later stages of growth, ethyl alcohol produced via glycolysis, would become the sole energy source and only cells containing the *NF* would then be capable of growth and reproduction. Any [*rho*+] cell produced by chance segregation would then divide rapidly, in contrast with the surrounding bulk of [*rho*-] cells, thus producing a mosaic colony (Rank and Person, appendix A). It is worth noting that the hypotheses of inactivation, prezygotic elimination, or post-zygotic destruction of the *NF* are incapable of accounting for the occurrence of mosaic colonies in a [*rho*+] by [*rho-(s)*] cross (Ephrussi, Jakob and Grandchamp, 1966).

Selection pressure was able to maintain heteroplasmic cells as shown by the maintenance of a high frequency of mosaic colonies in some cell lines. Selection for [*rho*+] or [*rho*-] colonies within these unstable lines greatly decreased or eliminated mosaic colonies (Rank and Person, appendix A). Thus under suitable environmental conditions heteroplasmic cells can be propagated that show the property of continuous segregation. Continuous segregation is of widespread occurrence in other species where suppressitivity has been demonstrated (Jinks, 1964(a); Jinks, (1964(b)). As mentioned previously, continuous segregation is expected if environmental fluctuations alternatively favour the replication of the variant (*SF*) and molecular prototype (*NF*).

Somatic segregation from a heteroplasmic cell, containing the *SF* and the *NF*, would produce progeny cells containing only the *NF* ([*rho*+]) or the *SF* ([*rho-(s)*]) Continuous segregation argues against prezygotic elimination or postzygotic destruction since both the *SF* and *NF* are maintained in the cell indefinitely unless one of the factors is excluded from a progeny cell by somatic segregation. Also the *NF* is not altered by the *SF* since [*rho*+] progeny cells are produced with a functional *NF*. If the *SF* arose by the process of molecular "Darwinian" selection, its phenotypic expression would be expected to exhibit environmental dependence since environmental dependence of replicative superiority is an underlying assumption in the process. A remarkable environmental modification of the phenotypic expression of a given *SF* was demonstrated by subjecting zygotes obtained from crossing [*rho*+] and [*rho-(s)*] lines to different temperatures and energy sources (Ephrussi, Jakob and Grandchamp, 1966). In particular, it was found that lowering the temperature from 30° to 18°C and substituting glycerol for acetate (in media containing 0.13% glucose to support the growth of [*rho*-] colonies) significantly lowered the suppressitivity of a *SF*. Since the frequency of [*rho*+] zygote colonies in the control crosses [*rho*+] by [*rho*+], and [*rho*+] by [*rho-(n)*] was not affected by growth on glycerol-glucose, the increased frequency of [*rho*-] zygote colonies in the cross [*rho*+] by [*rho-(s)*] when plated on glycerol-glucose medium or incubated at a lower temperature, must be due to the presence of the *SF* in these latter zygotes. Because both lower temperature and acetate decreased the rate of cell division, but only the former increased the % suppressitivity, cell division rate per se does not influence suppressitivity. [*rho*-] zygote colonies obtained from the crosses spread on glycerol-glucose and acetate-glucose were placed in glycerol liquid for three weeks to determine whether the additional [*rho*-] colonies formed on glycerol-glucose contained a small fraction of [*rho*+] cells that were not

expressed; the resultant lack of growth by all [*rho*-] colonies demonstrated that [*rho*-] colonies were composed of only [*rho*-] cells. Ephrussi, Jakob and Grandchamp (1966) concluded from this experiment that different environmental conditions can result in the different evolution of a zygote originally containing the same number of *NF*s and *SF*s.

In addition to [*rho*-] mutants, many chromosomally-inherited recessive mutants (*pet*)[1] have been reported (Beck, Mattoon, Hawthorne and Sherman, 1968; Miyake, 1968; Morita and Mifuchi, 1970; Negrotti and Wilkie, 1968; Parker and Mattoon, 1969; Sherman and Ephrussi, 1962; Sherman, 1963) which affect different aspects of mitochondrial function. Sherman and Ephrussi (1962) observed that a given *pet*;[*rho*+] strain did not become suppressive unless the *NF* had mutated to produce a *pet*;[*rho*-] strain which proved that respiratory deficiency does not, in itself, cause suppressitivity. However, an interesting observation was that *pet* mutants had a variable ability to maintain the *NF* (Sherman and Ephrussi, 1962; Sherman, 1963). For example, 55.0%, 99.9%, 100.0%, 40.0% and 1.0% of cells from ascospore colonies (derived from Pet/pet heterozygotes) of *pet 1, pet 2, pet 3, pet 4 and pet 5* were [*rho*-] (Sherman, 1963). The suppressitivity of [*rho*-] isolates of *pet 3* was determined and found to be similar to the suppressitivity of naturally arising [*rho*-] isolates in wild type strains. Haploid ascospores (isolated from asci of a *Pet 3/pet 3*;[*rho*+] diploid strain) of genotype *pet 3* were shown to maintain the *NF* for 5 to 25 generations (Sherman, 1963) before becoming *pet 3*;[*rho-(s)*]. As with [*rho*+] by [*rho-(s)*] zygotes (conversion measured by the inability to bud in glycerol (Ephrussi, Jakob and Grandchamp, 1966)), the conversion from [*rho*+] to [*rho-(s)*] by *pet 3* takes place over

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[1] A *pet* mutant is defined by von Borstel (1969) as any nuclear mutant unable to utilize nonfermentable carbon sources for growth. Other nuclear mutants that affect mitochondrial function but give partial growth on non-fermentable energy sources are known - i.e., cytochrome-c mutants (Sherman,1964).

several cell generations. The remarkable ability of *pet 3* to induce the *SF* is at present inexplicable, although Sherman (1963) suggests that such loci are involved in the retention or synthesis of the NF. Whatever the mechanism of induction, the possible involvement of nuclear genes in the generation of the *SF* is clearly established. This possibility appears to have been overlooked by Negrotti and Wilkie (1968), who reported a recessive nuclear mutant (*gi*) that they believed induced [*rho*+] to become [*rho*−] under conditions of anoxia or glucose repression. Cells of this strain on glucose medium (previously grown on a non-fermentable energy source) grew into mosaic colonies consisting of a [*rho*−] base and a [*rho*+] apex. The mosaic colonies were explained by suggesting that the basal *gi* cells were converted to [*rho*−] under the influence of glucose repression. However, a random spore analysis from a *Gi/gi* hybrid revealed a 11:52 ratio of *gi* (mosaic):*Gi* colonies, instead of the expected 1:1 ratio. It is difficult to agree with the authors that such a deviation could be explained by differential viability of normal spores. Rather the *gi* mutant could be thought of as a *pet* mutant with a partial ability (11/31.5 of the [*rho*+] ascospores) to induce the *SF*; the mosaic colonies observed could be the result of the replicative differential between the *SF* and *NF* interacting with chance segregation of the *NF* or *SF*, as explained above (Rank and Person, appendix A).

Although the above discussion of suppressitivity relied solely on information gleaned from genetic analyses, suppressitivity was not found to be accommodated by the hypotheses of prezygotic elimination, postzygotic destruction, or inactivation of the *NF*. In particular these hypotheses did not account for: the spontaneous generation of *SFs* with different degrees of suppressitivity; the recovery of an unaltered *SF* or *NF* in some of the progeny cells issued from a [*rho*+] by [*rho*−*(s)*] zygote; the demonstration that the *SF* converts a [*rho*+] by [*rho*−*(s)*] zygote to [*rho*−] only after a period of cell

division, irrespective of the presence of the translated product of the *NF* from the [*rho*+] strain; the existence of a heteroplasmic cell capable of continuous segregation; the presence of a mosaic colony composed of a [*rho-(s)*] base and an erumpent [*rho*+] apex; the environmental modification of the suppressiveness of a given *SF*; and the demonstration of nuclear mutants capable of generating the *SF*. Since the aetiology of suppressitivity is unknown, and since *all* the above observations are compatible with the hypothesis of molecular "Darwinian" selection *in vivo*, these observations were taken as being indicative that the *SF* is a spontaneously-generated variant of the *NF* and furthermore, that replicative superiority of the *SF* enables it to occupy the molecular-niche of the *NF*.

Since genetic evidence supports the view that the *SF* is a fast replicating variant of the *NF*, it becomes of great interest to ascertain the molecular and cytological bases of these factors. Because the *SF* resulted in respiratory deficiency, and because the process of respiration occurred in the mitochondrion, it is not surprising that the mitochondria of [*rho*+] and [*rho*-] strains differed in many aspects. In addition to lacking cytochromes $a+a_3$, b and c_1, the mitochondria of [*rho*-] differed from the mitochondria of [*rho*+] strains by: being deficient in a mitochondrial structural protein component (Tuppy and Swetly, 1968; Kuzela, Smigan and Kovac, 1969); containing an ATPase insensitive to oligomycin (Kuzela, Smigan and Kovan, 1969); being unable to incorporate amino acids *in vitro* (Kuzela; Smigan and Kovac, 1969); and containing an aberrant inner membrane (Federman and Avers, 1967; Yotsuyanagi, 1962). The simultaneous loss and/or alteration of many functions suggests that the *NF* has undergone a profound change similar perhaps to the gross change in base composition and molecular weight of *V-1*, as compared to Qβ-RNA (Mills, Peterson and Spiegelman, 1967).

Glucose concentrations in excess of 0.09% resulted in a diminution of well developed cristae in *S. cerevisiae* (reviewed by Marchant and Smith, 1968). Nevertheless, mitochondria from [*rho*-] cells in late log phase, a period of negligible glucose repression, differed from mitochondria in [*rho*+] or [*rho*+];*pet* cells by lacking well-defined cristae (Yotsuyangi, 1962). Federman and Avers (1967) observed two types of non-cristate mitochondrial profiles in [*rho-(s)*] stains: (i) mitochondria with the inner membrane immediately adjacent to the outer membrane throughout its entire contour length and (ii), mitochondria with the inner membrane arranged as concentric loops parallel to the major axis. Mitochondria of [*rho*+] strains, characterized by a few well-developed cristae arranged at random to the major axis, were not observed in [*rho-(s)*] strains. There was an average of 5.2 non-cristate mitochondrial profiles per section for [*rho-(s)*] cells (approximately 20 mitochondria per cell), and 9.5 cristate mitochondria per section in [*rho*+] cells (approximately 50 mitochondria per cell). All areas of the zygotic cell, formed by a broad connecting bridge between a [*rho*+] and [*rho*-] cell, contained cristate and non-cristate mitochondria, thus providing cytological evidence that pre-zygotic elimination at the mitochondrial level does not occur in this species. Similarly, the presence of cytochrome-oxidase positive mitochondria (from the [*rho*+] parent) and cytochrome-oxidase negative mitochondria (from the [*rho-(s)*] parent) in the first diploid zygotic bud argues against the occurrence of postzygotic destruction at the mitochondrial level. A random sample of [*rho*+] progeny cells from a [*rho*+] by [*rho-(s)*] cross demonstrated that non-cristate profiles decreased from 38% in the zygote to as low as 3.2% after one week of growth on agar slants. In contrast, [*rho*-] progeny cells descended from a [*rho*+] by [*rho-(s)*] zygote, contained 100% non-cristate profiles at the end of one week of growth. These results indicate that the emergence of [*rho*+] and [*rho-(s)*] progeny cells,

from a [*rho*+] by [*rho-(s)*] zygote, is paralleled by an increase in cristate and non-cristate mitochondria respectively.

Thus [*rho*-] progeny cells, descendent from a [*rho*+] by [*rho-(s)*] zygote, show a parallel increase in the genetically defined *SF* and the cytologically and biochemically defined abnormal mitochondria. (Both the *SF* and the mitochondria were contributed by the [*rho-(s)*] parental strain). These results suggest that the *NF* (a hereditary component required for normal mitochondrial function) has been altered to form the *SF*, the alteration resulting in a grossly aberrant mitochondrion and the phenotypic expression of suppressitivity. Indeed, in *Neurospora crassa* the micro-injection into normal hyphae of mitochondria isolated from a suppressive, cytoplasmically-inherited, mitochondrion - deficient mutant (*abn-1*) resulted in suppression of the normal phenotype and the pleiotrophic phenotypic expression of (*abn-1*). (Diacumakos, Garnjobst and Tatum, 1965).

Support for the view that mitochondria contain a self-replicating molecule (the *NF*) that codes for some of the mitochondrial components can be found in recent biochemical and genetic analyses of the mitochondrion. The chloramphenicol-sensitive incorporation of amino acids into the structural protein of the inner mitochondrial membrane by a mitochondrial fraction isolated from *S.cerevisiae* is indicative of a protein synthesizing system distinct from that of the cytoplasm (Yang and Criddle, 1969). A mitochondrial protein synthesizing system, separate from that of the cytoplasm, had previously been indicated by the interference with mitochondrial, but not cytoplasmic, protein synthesis by chloramphenicol and erythromycin (Linnane, 1968). In contrast, cycloheximide interfered with the cytoplasmic protein synthesizing system, but had no effect on mitochondrial protein synthesis. Inasmuch as erythromycin resistant (Linnane, Lamb, Christodoulou and Lukins, 1968) and

chloramphenicol resistant (author, unpublished results) strains were shown to be cytoplasmically-inherited, the *NF* of the mitochondrion is clearly implicated as the most probable source of information for these antibiotic resistance markers. Linnane, Lamb, Christodoulou and Lukins (1968) suggest that an altered mitochondrial ribosomal component forms the molecular basis for erythromycin resistance. Other evidence for the *possible* utilization of information of the *NF* in mitochondrial ribosomes is their deviation from cytoplasmic ribosomes in (i) sedimentation properties of the ribosomal subunits and ribosomal RNA (Wintersberger and Viehhauser, 1968) and (ii), the chromatographic properties of ribosomal proteins (Kunzel, 1969). Species of tRNA and aminoacyl-tRNA synthetases, found exclusively in the mitochondrion, are also indicative of an independent mitochondrial protein synthesizing system (Buck and Nass, 1969). However, cytoplasmic-inheritance by mutants of these mitochondrial components is required to eliminate the possibility of their being transcribed by nuclear DNA, translated on cytoplasmic ribosomes, and subsequently transported into the mitochondrion, as has been demonstrated for cytochrome-c in *Saccharomyces cerevisiae* (Mattoon and Sherman, 1966).

In addition to evidence for a unique mitochondrial protein synthesizing system, the discovery of mitochondrial DNA (MDNA) (for reviews on MDNA see Borst and Kroon (1969); Nass (1969); Slater et al. (1968) and Roodyn and Wilkie (1968)) and intramitochondrial components for its replication and transcription have greatly strengthened the concept of partial genetic autonomy of the mitochondrion. Incorporation of deoxyribonucleoside triphosphates into acid insoluble material, by isolated mitochondria of *S.cerevisiae*, with the expected Tm and buoyant density of MDNA, denotes an organelle with the necessary components for self-replication of MDNA (Wintersberger, 1968). Furthermore, a MDNA polymerase of molecular weight

150,000, extracted from the mitochondria of [*rho*+] and [*rho*-] strains (Wintersberger and Wintersberger, 1970), differed from nuclear DNA polymerase in (i) DEAE-cellulose elution characteristics, (ii) optiminal magnesium requirements and (iii) effectiveness of different DNA templates in promoting synthesis. A unique MDNA polymerase has also been described for rat liver mitochondria (Meyer and Simpson, 1968). Another essential component required for partial genetic autonomy, a DNA dependent-RNA polymerase, has been isolated from several species (reviewed by Neubert, Helge and Merker, 1968). Hybridization of RNA fractions with MDNA has been utilized to determine the extent of MDNA transcription. Buck and Nass (1969) demonstrated that specific mitochondrial tRNAs hybridized with MDNA but not with nuclear DNA.23S and 16S ribosomal RNA isolated from the mitochondria of a [*rho*+] strain of *S.cerevisiae* showed sequence complementarity only with MDNA (Wintersberger and Viehhauser, 1968), although they were unable to extract ribosomal RNA from the mitochondria of a [*rho*-] strain. Fukuhara, Faures and Genin (1969), when employing RNA extracted from whole cells of [*rho*+] and [*rho-(n)*] strains, observed twice as much hybridization with homologous crosses ([*rho*+]RNA on [*rho*+]MDNA, and [*rho-(n)*]RNA on [*rho-(n)*]MDNA) as with heterologous crosses ([*rho*+]RNA on [*rho-(n)*]MDNA, and [*rho-(n)*]RNA on [*rho*+]MDNA). RNA fractions purified by dehybridization-rehybridization with homologous DNA did not hybridize with nuclear DNA. The demonstration of MDNA-hybridizable RNA in whole cell extracts (Fukuhara, Faures and Genin, 1969), but not in isolated mitochondria (Wintersberger and Viehhauser, 1968) from [*rho*-] strains, indicates that RNA transcripts of MDNA may be transported to the cytoplasm and translated on cytoplasmic ribosomes. This possibility was also indicated by the continued synthesis of mitochondrial components in the presence of antibiotics that inhibit mitochondrial protein synthesis (Davey, Yu and Linnane, 1969; Kuntzel, 1969). An additional property of the MDNA of [*rho*+] and [*rho*-] strains of

S. cerevisiae is the inhibition of its replication *in vivo* by high glucose concentrations and anaerobosis (Rabinowitz, Getz, Casey and Swift, 1969).

In summary, the partial genetic autonomy of the mitochondrion was demonstrated by: the association of abnormal mitochondrial structure and function with the cytoplasmically-inherited *SF*; the alteration of the normal phenotype by injection of mitochondria from an abnormal strain; the demonstration of a mitochondrial protein synthesis with unique components one of which (erythromycin resistance) was cytoplasmically inherited; the presence of MDNA and unique mitochondrial components for its transcription and replication; and the isolation of RNA capable of hybridizing to MDNA, but not to nuclear DNA.

As MDNA is the only self-replicating molecule isolated from the mitochondrion, it must be *assumed that MDNA is the NF. A necessary corollary to this fundamental assumption is that the SF is an abnormal MDNA with a replicative superiority over normal MDNA, the replicative superiority of the abnormal MDNA potentially enabling it to occupy the molecular-niche of normal MDNA.* That normal and abnormal MDNA are the material basis for the *NF* and *SF* will remain an assumption until experimental procedures are developed for the transformation of [*rho*-] cells by MDNA. However, one indication of the validity of this assumption is demonstrated by a cross between "poky" - a mitochondrial mutant of *N.crassa* with MDNA densities of 1.698 and 1.720 g/cm^3, and *N.sitophila* with a MDNA density of 1.692 g/cm^3 (Reich and Luck, 1966). As expected, all ascospore colonies from this cross were "poky" since "poky" was utilized as the protoperithecial parent. MDNA isolated from these cultures had a density of 1.698 and 1.702g/cm^3; MDNA of density 1.692g/cm^3 from the conidial parent was absent. Thus there was a perfect correlation between the MDNA detected, and the phenotype previously associated with such a DNA. Clearly, MDNA appears to form the material basis for cytoplasmically-

inherited mitochondrial markers.

A compendium of the recorded properties of MDNA of various strains of *S.cerevisiae* is presented in Table 1 in order to facilitate the comparison of MDNA found in [*rho*+] and [*rho*-] strains. At the outset, it must be stressed that confidence limits of % suppressitivity were not recorded for any of the [*rho*-] strains reported in Table 1. Also in many instances the % suppressitivity of a strain was not determined but simply indicated as being [*rho*-]. This is unfortunate since Rank (appendix C) has shown that there is a low frequency of cells in a given [*rho*-] strain that deviates greatly from the mean % suppressitivity. Unless sufficient cells of a [*rho*-] strain have been crossed to determine the statistical validity of % suppressitivity, a recording could be in error; for example, a strain classified as [*rho-(n)*] could in fact be a [*rho-(s)*] strain with low suppressitivity.

MDNA from [*rho*+] strains exhibited the following characteristics: a modal native density of 1.684g/cm^3 (see Table 1); an increase in buoyant density of approximately 0.011g/cm^3 when denatured by heating followed with rapid cooling (Tewari et al., 1966; Mehrotra et al., 1968; Moustacchi et al., 1966; Sinclair et al., 1967; Shapiro et al., 1968; Bernadi, Faures, Piperno and Slonimski, 1970); a thermal transition midpoint (T_m) of approximately 74^{o}C (Tewari et al., 1966; Mehrotra et al., 1968; Bak et al., 1969; Bernadi, Faures, Piperno and Slonimski, 1970); a molecular weight of 2x10^7 as calculated from its sedimentation coefficient (Tewari et al., 1966); a base composition of approximately 17% GC when determined directly by chromatographic analysis (Tewari et al., 1966; Mehrotra et al., 1968; Bernardi, Faures, Piperno and Slonimski, 1970); and a base composition of approximately 23% GC as calculated from its buoyant density or 13%GC when calculated using the thermal transition midpoint (Tewari et al., 1966; Bak et al, 1969; Bernardi,

TABLE I

Properties of mitochondria DNA of different strains of *Saccharomyces cerevisiae*

References	Genetic Strain	Buoyant Density ($g\ cm^{-3}$) Native	Buoyant Density ($g\ cm^{-3}$) Denatured	T_m (°C)	Base Composition (%GC) Direct Analysis	From Density	From T_m	From ORD	% Circular Molecules	Modal Length (μ) Circular	Modal Length (μ) Linear
A	[*rho*+]	1.684	1.693	75.0	21.0	19.0	14.0	-	-	-	-
B	[*rho*+]	1.687	-	-	-	-	-	-	-	-	-
"	[*rho*+];*pet7*	1.687	-	-	-	-	-	-	-	-	-
"	[*rho-(n)*][1];*pet7*	1.683	-	-	-	-	-	-	-	-	-
"	[*rho-(95%s)*][2],*pet7*	1.695	-	-	-	-	-	-	-	-	-
C	[*rho*+]	1.683	1.698	-	-	-	-	-	-	-	-
"	[*rho*+];*pet*	1.683	-	-	-	-	-	-	-	-	-
"	[*rho-*][3]	-	-	-	-	-	-	-	-	-	-
D	[*rho*+]	1.685	-	-	-	-	-	-	-	-	-
"	[*rho-*][4]	-	-	-	-	-	-	-	-	-	-
E	[*rho*+]	1.682	1.697	-	-	-	-	-	-	-	<5.0[5]
F	[*rho*+]	1.683	1.698	-	-	-	-	-	3.0	4.5	4.5
"	[*rho-*][6]	-	-	-	-	-	-	-	-	-	-
G	[*rho*+]	1.683	-	-	-	-	-	-	25.0	1.5	4.1
H	[*rho*+]	-	-	-	-	-	-	-	-	-	5.0[7]
I	[*rho*+]	1.684	-	-	-	-	-	-	50.0	4.5 to 5.5	5.0
"	[*rho-*]	1.681	-	-	-	-	-	-	8.0	1.2 to 2.5	3.0
J	[*rho-*][8]	1.680	-	-	-	-	-	-	38.0	0.5	2.0
K	[*rho*+]	1.685	-	-	-	-	-	-	-	-	-
"	[*rho-*]	1.672	-	-	3.8	-	-	-	17.0	0.5	0.5

continued:-

TABLE I - continued.

References	Genetic Strain	Buoyant Density ($g\ cm^{-3}$) Native	Buoyant Density ($g\ cm^{-3}$) Denatured	T_m (°C)	Base Composition (%GC) Direct Analysis	Base Composition (%GC) From Density	Base Composition (%GC) From T_m	Base Composition (%GC) From ORD	% Circular Molecules	Modal Length (u) Circular	Modal Length (u) Linear
L	[*rho*+]	1.683±.0006	1.692	72.5	17.0	-	-	-	-	-	-
"	[*rho*-*(n)*][9]	1.681	-	72.5	-	-	-	-	-	-	-
"	[*rho*-*(10%s)*]	1.676	1.679	66.5	-	-	-	-	-	-	-
"	[*rho*-*(50%s)*]	1.676	1.677	66.5	4.0	-	-	-	-	-	-
M	[*rho*+]	1.685	-	-	-	-	-	-	-	-	-
"	[*rho*-]	1.682	-	-	-	-	-	-	-	-	-
N	[*rho*+][10]	1.685	-	-	-	-	-	-	-	-	-
"	[*rho*+];*pet7*[10]	1.685	-	-	-	-	-	-	-	-	-
"	[*rho*-*(n)*];*pet7*[10]	1.682	-	-	-	-	-	-	-	-	-
"	[*rho*-*(n)*]	1.682	-	-	-	-	-	-	-	-	-
O	[*rho*+]	1.684	-	74.5	-	24.5	12.7	-	-	-	-
P	[*rho*-*(s)*][11]	1.678	-	-	-	-	-	-	-	-	-
"	*34*-[*rho*-*(s)*][12]	1.672	-	-	-	-	-	-	-	-	-
"	*33*-[*rho*-*(s)*][11]	1.677	-	-	-	-	-	-	-	-	-
"	[*rho*-*(s)*][13]	1.676	-	-	-	-	-	-	-	-	-
"	[*rho*-*(s)*][13]	1.672	-	-	-	-	-	-	-	-	-
"	*16*-[*rho*+]	1.684	-	-	-	-	-	-	-	-	-
"	[*rho*+][14]	1.683	-	-	-	-	-	-	-	-	-
"	[*rho*-][15]	1.674	-	-	-	-	-	-	-	-	-
"	[*rho*-][15]	1.678	-	-	-	-	-	-	-	-	-
"	[*rho*-][15]	1.671	-	-	-	-	-	-	-	-	-
"	[*rho*-][16]	1.675	-	-	-	-	-	-	-	-	-
"	[*rho*-][16]	1.675	-	-	-	-	-	-	-	-	-

continued:-

TABLE I - continued.

References	Genetic Strain	Buoyant Density (g cm^{-3}) Native	Buoyant Density (g cm^{-3}) Denatured	T_m (°C)	Base Composition (%GC) Direct Analysis	Base Composition (%GC) From Density	Base Composition (%GC) From T_m	Base Composition (%GC) From ORD	% Circular Molecules	Modal Length (μ) Circular	Modal Length (μ) Linear
Q	[*rho*+]	1.683	1.694	74.7	17.4	23.5	13.2	30.7[17]	-	-	-
"	[*rho-(95%s)*];*pet7*	1.683	1.685	73.4	15.5	23.5	10.0	36.2[17]	-	-	-
"	[*rho-(78%s)*];*pet7*	1.683	1.684	73.5	15.6	23.5	10.2	38.3[17]	-	-	-
"	[*rho*+]	1.683	1.694	73.7	16.8	23.5	10.7	20.4[17]	-	-	-
"	[*rho-(95%s)*]	1.678	1.678	73.3	12.6	19.0	9.8	27.8[17]	-	-	-

[1] The degree of suppressiveness was very low (less than 5%).

[2] Mounolou et al. (1966) recorded that the degree of suppressiveness was very high (≥90%). Bernardi et al. (1970) recorded that the suppressitivity of this strain was 95% and the buoyant density of mitochondrial DNA was 1.683 g cm^{-3}.

[3] Mitochondrial DNA was not detectable in two [*rho*-] strains although nuclear (1.699g cm^{-3} and 1.706g cm^{-3}) DNAs were.

[4] Mitochondrial DNA was not detectable in two [*rho*-] strains although nuclear (1.700g cm^{-3} and 1.704g cm^{-3}) DNAs were.

[5] Modal frequency was not given but DNA molecules less than 5μ were believed to be fragments. A small frequency of circular molecules was believed to be the result of nuclear contimination.

[6] No mitochondrial DNA was detected when grown under conditions of glucose repression (8% glucose).

[7] A report on preliminary results suggested modal length was 5μ although larger molecules were believed possible.

[8] The same strain was used by Avers et al. (1968). Improved techniques enabled the detection of 0.5μ molecules in the later publication.

[9] This was reported to be a [*rho-(15%s)*] strain by Federman and Avers (1967).

[10] The same strains used by Mounolou et al. (1966).

[11] Isolated from a [*rho*+] strain, number *34*, after acriflavin treatment.

TABLE I - continued.

[12] Isolated from a [*rho*+] strain, number *34*, after UV treatment.

[13] Isolated from *33*-[*rho*-*(s)*] after further acriflavin treatment.

[14] A [*rho*+] strain from the cross *33*-[*rho*-*(s)*] by *16*-[*rho*+].

[15] A [*rho*-] strain from the cross *33*-[*rho*-*(s)*] by *16*-[*rho*+].

[16] A [*rho*-] strain from the cross *33*-[*rho*-*(s)*] by *16*-[*rho*+].

[17] These values are taken from Table 3 of Bernardi and Timasheff (1970).

A - Tewari et al. (1966).
B - Mounolou et al. (1966).
C - Moustacchi et al. (1966).
D - Corneo et al. (1966).
E - Sinclair et al. (1967).
F - Shapiro et al. (1968).
G - Guérineau et al. (1968) (a), (b).
H - Borst et al. (1968).
I - Avers et al. (1968).
J - Billheimer et al. (1969).
K - Bernardi et al. (1968).
L - Mehrotra et al. (1968).
M - Wintersberger et al. (1968).
N - Fukuhara et al. (1969).
O - Bak et al. (1969).
P - Carnevali et al. (1969).
Q - Bernardi, Faures, Piperno and Slonimski (1970).

Faures, Piperno and Slonimski, 1970). The deviation in base composition between direct analysis and calculations based on the buoyant density, thermal transition midpoint, or optical rotary dispersion (ORD) is believed to be due to the presence in yeast MDNA of sequences of alternating poly (dAT:dAT)[1] and non-alternating poly (dA:dT)[2] (Bernardi, Faures, Piperno and Slonimski, 1970; Bernardi and Timasheff, 1970). There is a large variation in the recorded frequency of linear and circular molecules extracted from [*rho*+] cells and their respective lengths. Sinclair et al., (1967) attributed the small frequency of circular molecules observed to nuclear contamination. Shapiro et al., (1968) observed circular molecules formed by hydrogen bonding of cohesive ends, as well as covalent superhelical circles, whereas only covalent circular molecules were reported by Guerineau et al. (1968(b)) and Avers et al. (1968). The discrepancy in results of different investigators is likely influenced by extraction procedures since Avers et al. (1968) obtained different frequencies of circular molecules with the same strain when alternative extraction protocols were used. In general, however, the results suggest a basic length of linear and circular molecules of 5u with larger molecules composed of multiples of the basic unit, as observed for other species (Nass, 1969). Nuclear mutations (*pet*) that resulted in respiratory deficiency, but maintained the *NF*, did not affect any of the physical and chemical characteristics of MDNA (Mounolou et al., 1966; Moustacchi et al., 1966; Fukuhara et al., 1969; Bernardi, Faures, Piperno and Slonimski, 1970).

As expected by the hypothesis of molecular "Darwinian" selection, the MDNA of [*rho*-] strains differs from that of [*rho*+] strains. *Firstly*, the buoyant

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[1] Poly (dAT:dAT) refers to a copolymer of deoxyadenylic and deoxythymidlic acid in alternate sequence.

[2] Poly (dA:dT) refers to a copolymer consisting of one strand of deoxyodenylic acid and one strand of deoxythymidlic acid.

density is lower than, or equal to, that recorded for [*rho*+] MDNA (see Table 1) - with one exception. MDNA of the exceptional strain was originally reported by Mounolou et al. (1966) to have a buoyant density of 1.695g/cm^3 whereas a subsequent analysis by Bernardi, Faures, Piperno and Slonimski (1970) indicated a density of 1.683g/cm^3 for MDNA of the same strain. In view of its consistency with other recorded values, the latter density (1.683g/cm^3) is more likely to be the correct one. Large alterations in buoyant density (i.e., 1.672g/cm^3 (Bernardi et al.,1968), 1.676 g/cm^3 (Mehrotra and Mahler, 1968), 1.671g/cm^3 (Carnevali et al., 1969) and 1.678g/cm^3 (Bernardi, Faures, Piperno and Slonimski, 1970)) of [*rho-s*] MDNA, as compared to [*rho*+] MDNA, suggests a gross alteration in the molecular prototype, similar perhaps to the modification of *Q*β-RNA that resulted in *V-1*. MDNA isolated from suppressive strains with the same buoyant density (i.e., 1.683 g/cm^3 (Bernardi, Faures, Piperno and Slonimiski, 1970)) as normal MDNA indicates a more subtle alteration of the molecular prototype, parallel perhaps to the difference between variants *V-2* and *V-6* of *Q*β-RNA (Levisohn and Spiegelman, 1969). Other characteristics of the MDNA to be discussed later indicate differences between the MDNA of these [*rho-s*] and [*rho*+] strains. Failure to extract MDNA from [*rho*-] strains was reported by Moustacchi et al. (1966), Corneo et al. (1966) and Shapiro et al. (1968). Although the high glucose concentration (8%) used by Shapiro et al. (1968) may have suppressed MDNA synthesis (Robinowitz, 1969), Moustacchi et al. (1966) avoided this possibility by isolating MDNA from cells grown under conditions of negligible glucose repression. Since the suppressitivity of these strains was not recorded it is tempting to assume that they were [*rho-(n)*] strains and that such strains do not contain MDNA. The lack of suppressitivity of such strains would then be explainable on the basis of having lost both the variant and molecular prototype; in crosses to [*rho*+] strains, the [*rho*+] phenotype would then be suppressive to the [*rho-(n)*] phenotype, as observed.

If such an assumption is correct, the [*rho-(n)*] strains purported to contain MDNA (Mounolou et al., 1966; Mehrotra et al., 1968; Fukuhara et al., 1969) should have been classified as [*rho-(s)*] strains with a low suppressitivity. In fact, one of the [*rho-(n)*] strains reported to contain MDNA (Mehrotra et al., 1968) was previously reported by Federman and Avers (1967) to be [*rho-(15%s)*]. The inability of [*rho-(n)*] strains to give rise to [*rho-(s)*] strains (Ephrussi, Hottinguer and Roman, 1955; author, unpublished results) is also consistent with the loss of MDNA from [*rho-(n)*] strains. In any event, the buoyant density of MDNA from some [*rho-(s)*] strains deviates greatly from that of [*rho+*] strains. Carnevali et al. (1969) made the important observation that the buoyant density of MDNA from [*rho-*] progeny cells descendent from a [*rho+*] by [*rho-(s)*] zygote was similar to the buoyant density of the [*rho-(s)*] parent. Thus the *SF* (abnormal MDNA) of the suppressive parent appears to be transmitted to [*rho-*] progeny cells issued from a [*rho+*] by [*rho-(s)*] zygote; this observation was confirmed by the elegant genetic analysis of Rank (appendix C).

Secondly, the density of denatured MDNA of [*rho-(s)*] strains does not increase significantly over the native buoyant density (Mehrotra and Mahler, 1968; Bernardi, Faures, Piperno and Slonimski, 1970) in contrast to an increase of 0.011g/cm^3 for denatured MDNA from [*rho+*] strains. Bernardi, Faures, Piperno and Slonimski (1970) suggest that a relative enrichment of poly (dA:dT) and/or poly (dAT:dAT) has occurrrd in the [*rho-*] mitochondrial DNA resulting in an enhanced ability of such MDNA to renature. (An increase in buoyant density upon denaturing is observed for other DNAs.) Perhaps this small alteration is capable of producing a variant molecule with a replicative superiority since two of these [*rho-(s)*] strains had a buoyant density similar to the [*rho+*] parental strain.

Thirdly, the T_m of MDNA from some [*rho-s*] strains was 66.5°C (Mehrotra and Mahler, 1968) instead of 74°C expected for [*rho+*] MDNA. Since the T_m of poly (dAT:dAT) is 65°C whereas the T_m of poly (dA:dT) is 73°C (Bernardi, Faures, Piperno and Slonimski, 1970), the MDNA of these strains is likely the result of a large increase in sequences of poly (dAT:dAT). This was confirmed by an analysis of the base composition to be discussed later. MDNA from other [*rho-(s)*] strains gave a less dramatic change in T_m - i.e., 73.4, 73.7 and 73.3°C (Bernardi, Faures, Piperno and Slonimski, 1970). However, a detailed analysis of the ultraviolet melting curves of the MDNA from these strains revealed that they differed from the corresponding [*rho+*] MDNA by (i) having a negligible residual hyperchromicity when fast-cooled after heating to 100°C and (ii), having a multimodal melting curve. The lack of residual hyperchromicity after fast-cooling is indicative of a return to the double-stranded state which was interpreted as indicative of a relative increase in poly (dA:dT) and/or poly (dAT:dAT) over the molecular prototype. Analysis of the differential melting curves of these [*rho-(s)*] MDNAs revealed that they contained two main components, one melting at approximately 73.0°C, and the other at approximately 77.0°C, in contrast to the broad thermal transition of [*rho+*] MDNA with only one major component melting at 71.5 to 72.5°C. Hence the melting characteristics of [*rho-*] MDNA supports the data from buoyant densities of at least two types of aberrant MDNA: (i) abnormal MDNA with a gross alteration in base composition (i.e., poly (dAT:dAT)) and (ii), abnormal MDNA with a more subtle, but nevertheless dramatic, change caused by a smaller increase in poly (dA:dT) and/or poly (dAT:dAT).

Fourthly, altered base compositions of [*rho-*] MDNA were found by direct chromatographic methods. In contrast to the 17% GC found in [*rho+*], 3.8% GC (Bernardi et al., 1968), 4.0% GC (Mehrotra and Mahler, 1968), and 15.5% GC, 15.6% GC, or 12.6% GC (Bernardi, Faures, Piperno and Slonimski, 1970) were

found for [*rho*-] MDNA. Such dramatic changes in base composition are clearly consistent with a large alteration of the molecular prototype. The base compositions as calculated from buoyant densities, T_m, and ORD are not reliable enough to discuss the difference between [*rho*+] and [*rho*-] MDNA since these methods are not reliable with DNA that has a high AT content.

Fifthly, Bernardi and Timasheff (1970) compared the optical rotary dispersion and circular dichroism spectra of [*rho*+] and [*rho*-] MDNA with that of poly (dAT:dAT) and poly (dA:dT). The spectra of both [*rho*+] and [*rho*-] MDNA displayed characteristics of poly (dAT:dAT) and poly (dA:dT) indicating that both MDNAs contained such sequences. However, the spectra of the MDNA from [*rho*-] strains indicated an increase in poly (dA:dT) and poly (dAT:dAT) sequences over that of [*rho*+] strains.

Lastly, the length of MDNA in [*rho*-] strains, although variable, appears to be shorter than [*rho*+] MDNA. Of particular interest is the high frequency of small circles, 0.5u in contour length, reported by Billheimer and Avers (1969). Previous investigations with this same strain (Avers et al., 1968) did not reveal such a high frequency of small circular molecules due to a limitation in the techniques used. The 0.5u molecules would be expected to have a replicative superiority over the molecular prototype (5u) suggesting an obvious mechanism of *in vivo* molecular "Darwinian" selection, as was observed *in vitro* for variants of *Q*β-RNA.

Thus the expectation that a [*rho-(s)*] strain should have an abnormal MDNA is supported by the native and denatured buoyant density, melting characteristics, base composition, ORD spectra and molecular length of such MDNA. A consideration of the number of copies of MDNA per yeast cell also supports the concept that the [*rho-(s)*] state is achieved by molecular "Darwinian" selection *in vivo*. Osmotically lysed mitochondria of a [*rho*+]

strain revealed that a mitochondrion could contain as many as 8 MDNA molecules (Avers et al., 1968). Similarly, it was calculated (Billheimer and Avers, 1969) that mitochondria from a [*rho*-] strain contain as much MDNA as mitochondria from a [*rho*+] strain. These results are in accordance with the report of 2-6 MDNA molecules per mitochondrion observed in higher organisms (Nass, 1969). Renaturation kinetics of chick liver MDNA indicates that the information content of MDNA is compatible with a molecular length of 5μ (Borst, Van Bruggen and Ruttenberg, 1968). Therefore, the minimum amount of MDNA per mitochondrion is estimated at two 5μ molecules of similar base sequence. An average of 50 mitochondria per [*rho*+] cell (Billheimer and Avers, 1969) gives a minimum estimate of 100 MDNA molecules per yeast cell. *A priori* expectations are that classical mutants would not be expressed in such a highly polyploid system. Yet [*rho-(s)*] cells occur with a minimum frequency of 1.0% in [*rho*+] lines. These facts alone suggest that the most likely mechanism of generating a [*rho-(s)*] cell is through the generation of an abnormal MDNA with a replicative superiority over normal MDNA.

Although several authors (Slonimski, 1968; Carnevali et al., 1969; Borst and Kroon, 1969; Rank and Person, appendix A) have suggested that suppressitivity may be caused by the replicative superiority of abnormal MDNA, the classical explanation of suppressitivity would be to assume that abnormal MDNA coded for an aberrant protein that causes the phenotypic expression of suppressitivity. A minimum function of such an aberrant protein would be to cause normal MDNA to become abnormal MDNA, since irreversible [*rho*-] progeny cells from a [*rho*+] by [*rho-(s)*] cross contain the *SF* (Carnevali et al., 1969; Rank, appendix C). This hypothesis requires that translates of MDNA coded for by one mitochondrion can be transported to other mitochondria. Since [*rho*-] mitochondria do not support protein synthesis (Kuzela et al., 1969) the translation of mitochondrial transcripts would have to occur on cytoplasmic

ribosomes prior to being transported into other mitochondria. (Support for this possibility is found in the work of Kuntzel (1969) and Davey et al. (1969). The major difficulty of such a hypothesis is that MDNA from some [*rho-(s)*] strains is composed of 96% poly (dAT:dAT). At the most this abnormal MDNA could be expected to code for a few functional proteins (Fukuhara et al., 1969) as portions of MDNA containing only A and T in alternate sequence would code for a non-functional tyrosine-isoleucine polypeptide. Consider a [*rho+*] cell with 100 functional MDNA molecules. To produce a [*rho-(s)*] cell a hypothetical mutation in one MDNA molecule would then have to be translated. The translated product would have to cause the abnormal replication of sufficient MDNA molecules to produce a progeny cell containing all MDNA composed of 96% poly (dAT:dAT) - yet still maintaining the base sequence of the codon responsible for the aberrant protein. It seems inconceivable that drastic alteration of the molecular prototype to a 96% poly (dAT:dAT) would always occur without altering the codon for the hypothetical aberrant protein! Also the high frequency of classical mutations required to produce 1.0% naturally arising [*rho-(s)*] cells does not seem probable. Experimental evidence against this hypothesis was also provided by demonstrating that the *SF* of a strain that had eliminated the genetic information on MDNA for erythromycin resistance (and thus coding for an aberrant protein by the above hypothesis), did not immediately inactivate the MDNA information for erythromycin resistance when crossed to another [*rho-(s)*] strain that still carried the cytoplasmic information for erythromycin resistance (Rank, appendix B).

Two specific predictions of the hypothesis of molecular "Darwinian" selection tested by genetic analysis were found to be upheld. *Firstly*, the hypothesis predicts that a genetic marker on MDNA would be eliminated by the generation of a *SF*, since the fast replicating variant would be expected

to occupy the molecular-niche of normal MDNA. Accordingly, the expression of a cytoplasmically-inherited, erythromycin-resistant ([ER^r]) marker was studied with and without the generation of a *SF* in a [ER^r]; [*rho*+] strain (Rank, appendix B). It was found that (i), the loss of the [ER^r] marker was dependent upon the generation of a *SF* and (ii), with the generation of the *SF*, an unstable state was produced for the [ER^r] marker. [ER^r]; [*rho-(s)*] strains constantly segregated to give rise to strains without the [ER^r] marker. These results were explained by assuming that the *SF* was an abnormal fast-replicating MDNA which occupied the molecular-niche for MDNA after a period of many cell divisions. The unstable [ER^r];[*rho-(s)*] state represents a heteroplasm containing normal (information for ER^r) and abnormal MDNA (the *SF*). Segregation of strains from the unstable state that have lost the [ER^r] factor would be the end result of an interaction between the replicative differential of the two types of MDNA and chance segregation to produce a cell containing only one of the heteroplasmic components. Essentially the same observations were reported by Gingold et al. (1969) with a different aetiological interpretation. *Secondly*, the hypothesis predicts that MDNA of a high suppressitivity strain has a replicative advantage over the MDNA of a low suppressitivity strain. One high and several low suppressitivity strains were defined by crossing to a common $\alpha/_{\alpha}$[*rho*+] diploid. The high and low suppressitivity strains were then crossed to bring the *SF*s of these strains into a common cytoplasm. The resultant diploid (formed by crossing the low by high suppressitivity strain) was then crossed to the original $\alpha/_{\alpha}$[*rho*+] diploid. The suppressitivity of the hybrid diploid was that expected if the *SF* of the high suppressitivity haploid parent had eliminated the *SF* from the low suppressitivity parent by virtue of its replicative superiority (Rank, appendix C).

Gross Alterations of DNA Base Composition in Micro-organisms.

Minimal requirements for the demonstration of molecular "Darwinian" selection *in vivo* (i.e., identification of the variant and prototype molecule and *in vivo* evidence of the variant's replicative superiority) has been observed for [*rho-(s)*] strains of *S. cerevisiae*. One of the striking characteristics of some of the variant MDNA molecules identified was the alteration in base composition from 17% GC to 4% GC (Bernardi et al., 1968; Mehrotra et al., 1968). This molecular alteration confers a replicative advantage upon the variant molecule. A gross alteration in base composition is, in itself, suggestive of molecular "Darwinian" selection *in vivo* since classical mutations are not usually caused by such an alteration. A point mutation would result only in the substitution of a single base pair and would not produce a detectable alteration in base composition. Similarly, a large deletion would not be expected to change the base composition since there is a random distribution of bases along the length of a DNA or RNA molecule, leading to the expectation that a deletion would result in a simultaneous decrease in GC and AT pairs without changing the overall base composition. Any large alterations in overall base composition can then be taken as tentative evidence for molecular "Darwinian" selection *in vivo* since the phenomenon is consistent with such a hypothesis. Indeed it is difficult to visualize a mechanism other than molecular "Darwinian" selection that is capable of explaining gross alterations of DNA base composition *in vivo*.

Copper sulfate was used to induce a small colony mutant (*SC-22*) of *Bacillus subtilis* with a DNA base composition of 64.8%GC, in contrast to the parental (*B. subtilis 168*) DNA composition of 41.9% GC (Weed, 1963). Subsequent work (Duc-Nguyen and Weed, 1964; Kelley and Weed, 1965) resulted in the isolation of two additional mutants: (i) *4G* - a mutant isolated from

B. subtilis 168 containing 55.0% GC and (ii), *4G-SC* - a segregant that arose as a fast-growing sector from *4G* and which had a DNA composed of 71%GC. Unfortunately appropriate genetic markers were not utilized for proving unequivocally that strains *SC-22, 4G* and *4G-SC* were not the result of contamination of the parental culture; however, a large colony revertent of *SC-22* maintained the auxotrophic marker of the parental strain and was transformed to prototrophy by DNA from a prototropic strain (Weed, 1963). Using unselective methods, Gause (1968) isolated two similar mutants from 5-fluorouracil treated *B. subtilis 168*, which had a DNA GC content of 64.0 and 62.9%. Small colony mutants isolated after 5-fluorouracil treatment of *B. subtilis 168* gave a differential growth response to different levels of tetracycline since mutants with an altered DNA base composition were 32 times more sensitive to tetracycline than small colony mutants with a normal base composition. Using small colony size and tetracyline sensitivity as an indication of base alteration, Gause (1968) isolated eleven mutants with a DNA composed of from 37.8 to 39.5%GC in contrast to the parental composition of 41.9%GC. Hence both a large increase or decrease in %GC can occur in the overall DNA base composition of *B. subtilis*. Although the mutants with an altered DNA base composition from *B. subtilis 168* did not have sufficient genetic markers to eliminate the possibility of contamination, the large number of mutants isolated by different investigators indicates that the mutants observed were likely variants of *B. sublitis 168* rather than contaminants.

De Ley (1964) isolated a mutant with a DNA base composition 3.7% higher in GC content than the parental streptomycin-resistant strain of *Agrobacterium tumefaciens*. The mutant was not believed to be a contaminant because: (i), both the mutant and parental strains were resistant to streptomycin at the same concentration and (ii), the agglutination liter with rabbit anti-

serum was the same for both strains.

A strain of *Bordetella pertussis* was shown by Bacon, Overend, Lloyd and Peacoke (1966) to have a DNA composition of 57.5%GC in contrast to the expected 67.6%GC. The base compositions as determined by chromatographic and sedimentation properties were identical. They were unable to eliminate the possibility of contamination since the mutant and normal strains were obtained as separate cultures from different laboratories.

A selective method was also devised for the isolation of mutants with a DNA base composition different from the 32.4%GC expected for *Staphylococcus aureus* (Gause, 1968). Advantage was taken of the inhibition of normal staphylococci by 5-fluoro-2-deoxyuridine which allowed for an enrichment of mutants with an altered base composition. Although genetic markers were not utilized, the mutants were shown to have the same antigenic properties as the parental strain (Gause, 1966; Gause, 1968). Ultraviolet irradiation and 5-fluoro-2-deoxyuridine enrichment led to the isolation of eight mutants with a grossly altered base composition (i.e., 69.5%GC to 70.2%GC; Gause, 1968). Another indication of the base alteration was the increased sensitivity of the mutants to substances which specifically interfere with mucleic acids such as mitomycin C, trypaflavine, streptonigrin and daunomycin. Hybridization studies between DNA isolated from the normal and mutant strains indicated virtually no hybridization between the mutant and normal DNA. However, there was partial or total hybridization (31.0 to 100.0%) between the DNA of different mutants with an increased GC content. The lack of hybridization between mutant and parental DNA is unexpected in view of their reported similarity in antigenic properties. The large increase in GC content (approximately 38.0%) is perhaps indicative that a *de novo* niche has been created for one or more variant molecules.

Bacterium paracoli 5099 is the most extensively investigated organism for the generation of mutants with abnormal base compositions (Gause, Dudnik, Laiko and Netyksa, 1967). Small yellowish mutants of this strain with an increased sensitivity to trypaflavine were found to be invariably correlated with a distorted DNA base composition. The mutants were found to show cross-agglutination to rabbit anti-serum induced by the parental culture, although sufficient genetic markers were not utilized to eliminate the possibility of the mutants being contaminants. The induction of mutants was achieved by both exposure to ultraviolet irradiation and 5-fluorouracil. Fifty small, yellowish, trypaflavine-sensitive mutants had a GC content of 67.0 to 71.0% in contrast to the parental DNA composition of 48.0%GC. As was the case for DNA base composition mutants of *S. aureus* (Gause, 1968), the mutants of *B. paracoli 5099* had an increased sensitivity to inhibitors of DNA synthesis such as streptonigrin and mitomycin C (Gause et al., 1967). A remarkable similarity to the loss of cytoplasmically-inherited erythromycin resistance in *S. cerevisiae* (Rank, appendix B) by the generation of a molecular variant (the *SF*) was observed for a kanamycin-resistant strain of *B. paracoli 5099*. All mutants of this strain with an altered DNA base composition were no longer resistant to kanamycin! Perhaps as with *S. cerevisiae* the molecular prototype that carries the information for antibiotic resistance is eliminated from the cell by the variant molecule.

It is worth noting that most of the mutants of different species with an altered base composition have defective respiratory systems and appear to have undergone a profound change in metabolism. These properties led Gause (1966) to consider such mutants as models for cancer cells. If the altered metabolism is caused by the altered DNA base composition of these cells, the hypothesis of molecular "Darwinian" selection serves as a useful model to explain the primary cause of some types of cancer.

In summary, a wide variety of species has been reported to give rise to variants with an abnormal base composition. Although the possibility of contamination has not been completely eliminated, the numerous reports of such mutants lend credence to their genesis from parental strains. Further research on genetically well-defined parental strains is required to eliminate the possibility of genesis by contamination. Genetic analysis of the mutants is also necessary to establish the occurrence of replicative superiority of the variant molecule *in vivo*. In any event, the aetiology of a grossly altered base composition is presently unknown and molecular "Darwinian" selection is a plausible hypothesis capable of explaining this phenomenon.

The von Magnus Effect

Certain aspects of viral multiplication *in vivo* are similar to the *in vitro* system used by Spiegelman to demonstrate molecular "Darwinian" selection. Consider the population of molecules generated by a single viral molecule injected into a host cell. All the information of the viral genome would be required for the production of viral progeny. However, a spontaneously generated variant molecule with a decreased information content could be replicated in this system if the variant maintained those characteristics required for replication, since the information required for replication would be coded for by the molecular prototype and the host cell genome. Thus in the *in vivo* system the molecular prototype interacts with the host cell to provide an effective biological-system[1], whereas *in vitro*, the biological-system is provided by the investigator. In the latter system replication can proceed if all the molecules are similarly variant, but *in vivo* replication requires sufficient copies of the prototype genome to provide an effective biological-system. If the variant molecule had a replicative advantage over the molecular prototype, the variant would be expected to increase in number relative to the molecular prototype, in a given environment. Excessive replication of the variant could alter the environment (i.e., by lowering a precursor below a threshold value which limited variant but not prototype replication) in such a manner that the molecular prototype would be preferentially replicated. Such a variant will be referred to as a *dependent* variant. Replication of the molecular prototype could then proceed at the expense of the dependent variant thus creating an environment (i.e., by raising the precursor level above the

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[1] See footnote on p.19 of this thesis.

threshold level that limits replication of the variant) that favored variant replication. In this manner alternative environmental modification by variant and prototype replication could achieve an equilibrium which maintains both molecules at a certain level. Alternatively, the variants replicative advantage over the molecular prototype may be unaffected by the environmental modification caused by its own replication; such a variant will be referred to as a *refractory* variant. A refractory variant would be self-eliminating in a system of continuous replication. However, in the biological-system utilized there is an upper limit, imposed by viral maturation and cell lysis, to the amount of replication. Hence even with a refractory variant there is a possibility of maintaining the molecular prototype. Whether the variant is dependent or refractory it could be incorporated into a viral particle in the same manner as the molecular prototype. Although a variant progeny particle would then be able to inject its variant genome into a host cell it would not be expected to produce progeny since, by definition, it has lost essential genetic information in order to achieve a replicative advantage. Multiple infection with dissimilar variants that have dispensed with information on different areas of the genome could be expected to yield infective and non-infective progeny if genetic recombination and complementation occurs. Similarly, coinfection of a host cell with a variant and molecular prototype would be expected to yield infective and non-infective progeny particles.

Clearly, if viral molecules undergo molecular "Darwinian" selection *in vivo* the above mentioned deliberations lead to the expectation that (i) defective viral particles will be formed that are incapable of directing viral multiplication at a multiplicity of infection (m.o.i.) less than or equal to one and (ii), that a defective viral particle contains a defective genome that can interfere with normal viral reproduction.

The first well documented observations that can be interpreted as evidence for "Darwinian" selection of viral molecules *in vivo* were reported by von Magnus (1954). *Influenza* virus was conveniently cultured by injecting viral seed into the allantoic cavity of the chick embryo. The ability of this RNA virus to agglutinate red blood cells was utilized as a convenient method for determining the number of viral particles. It was found that the maximum infectivity-hemagglutinin ratio (I:A ratio) obtainable was 10^6. An I:A ratio of 10^6 was observed for the viral progeny of an egg infected with a greatly diluted viral seed (m.o.i. less than 1). However, von Magnus found that the I:A ratio of recently harvested viral progeny could be greatly lowered by using undiluted progeny from the allantoic cavity as seed for starting a new cycle of infection. Starting with seed with a maximum I:A ratio of 10^6 a series of eggs was infected with the undiluted viral progeny (m.o.i.> 25) of the preceeding egg. There was a gradual decrease in the I:A ratio with increasing undiluted passage until by the third undiluted passage the I:A ratio was approximately 10^2. In other words, by simply using undiluted progeny at a high m.o.i., the infectivity of hemaglutinin particles was decreased to one ten thousandth of that observed for progeny produced from eggs seeded with a low m.o.i. The maximum decrease in the I:A ratio was achieved by the third undiluted passage. The reduction in infective titer by using undiluted viral progeny as seed for successive infections (serial undiluted passage) is referred to as the *von Magnus effect*.

The *von Magnus effect* is consistent with the expectations of the hypothesis of "Darwinian" selection of viral RNA *in vivo* since this hypothesis predicts that variant molecules are spontaneously generated which have a replicative superiority over the molecular prototype. Variant molecules, formed during the infectious cycle of a cell originally infected with one prototype molecule, would be incorporated into viral particles prior to cell lysis.

Undiluted passage of viral progeny would result in infection of a host cell with both variant and prototype molecules; the replicative superiority of the variant molecule would result in a relative increase in variant molecules over the molecular prototype with increasing undiluted passage. The lower limit to the relative frequency of defective molecules (and thus defective particles) would be determined by (i) the extent of the dependence of the variant molecule on the presence of the complete genome for information required for viral multiplication and (ii), the dependent or refractory nature of the variant molecules.

Virus particles from undiluted passage were shown to be similar to virus particules from diluted passage in surface properties by: (i) similar adsorption and elution rates from chicken red blood cells (von Magnus, 1954); (ii) similar agglutination rates with guinea pig and chicken red blood cells (von Magnus, 1954); (iii) similar ability of dilute suspensions of formal-inactivated particles to immunize mice (von Magnus, 1954) and; (iv) carrying the same ether-soluble (s) antigen (Lief and Henle, 1956). Also electron micrographs of infective and defective viral particles revealed no detectable differences in size and shape (von Magnus, 1954). However, the defective particles differed from high infectivity particles in sedimentation properties. Virus particles from dilute passage consisted mainly of a homogeneous group with a sedimentation constant of 750S and a minor hetere-genous fraction with an average sedimentation constant of 500S. With increasing undiluted passage the slow sedimenting component gradually replaced the homogeneous 750S fraction until an average sedimentation constant of 535S (with a range of 430S to 650S) was attained (von Magnus, 1954). As the viral particles were similar in size, shape, and surface properties, but differed in sedimentation properties, the defective slow sedimenting particles would be expected to have a decreased density. The hypothesis of molecular

"Darwinian" selection suggests that the decrease in density may be the result of a decreased nucleic acid content of the defective particle, similar perhaps to the decreased length observed for variants of Qβ-RNA *in vitro*. Decreased size would confer a replicative advantage to the variant molecule by simply requiring less time to complete replication. These expectations were confirmed by a comparison of the density and RNA content of viral particles harvested from allantoic cavities infected with viral particles from diluted and undiluted passage. Viral particles from diluted seed had a density of $1.23 g/cm^3$, whereas progeny particles from undiluted seed had an uneven distribution centered around a lower buoyant density (Duesberg, 1968). An analysis revealed that the RNA of *von Magnus* virus contained $3 \pm 1.5\%$ of the total incorporated P^{32} compared with $11 \pm 2.0\%$ for progeny virus issued from dilute seed. Polyacrylamide gel electrophoresis of RNA from particles of dilute passage displayed that the normal influenza viral particles probably contains 5 physically distinct RNA components. Small RNA components, not detected in particles from diluted passage, were isolated from viral particles of a third undiluted passage. Larger RNA components similar to those from diluted passage virions were also observed for *von Magnus* particles. These small RNA molecules may represent variant molecules with a replicative superiority to the molecular prototype. Unfortunately virus particles from the third undiluted passage were not separated into different fractions on the basis of their density. It is of interest to determine if the particles of lighter density contained only small RNA molecules as the large RNA molecules observed in *von Magnus* particles may have been contributed by the low frequency of normal particles present in lysates from undiluted passage. Nevertheless the relative frequency of small molecules increased as the I:A ratio decreased as expected by the hypothesis of "Darwinian" selection.

Further support for the hypothesis of "Darwinian" selection is found in the observation that the infectivity of undiluted passage particles can be increased to the maximum (I:A ratio of 10^6) by one diluted viral passage. Under these conditions it is expected that defective particles will not replicate since the host cell is not coinfected with a normal helper virus. On the other hand, cells infected with normal viral particles will produce primarily normal progeny, since any defective molecules generated would not undergo enough rounds of replication in one passage to greatly increase in frequency. Competition experiments also support the hypothesis since defective particles from undiluted passage are capable of interfering with the reproduction of normal infective viral particles. Eggs inoculated with undiluted passage particles were subsequently inoculated with large amounts of diluted passage particles at different time intervals. Multiplication of the second seed was suppressed by the undiluted passage particles. Furthermore as the time of inoculation with diluted seed increased there was an increased production of defective viral particles until the inoculation at 18 hours showed virtually no increase in the production of normal viral particles (von Magnus, 1954).

The general occurrence of defective viral particles was indicated by the reference of von Magnus to defective particles observed in lysates of *Newcastle, fowl plague* and *turnip yellow mosaic* virus by other investigators (von Magnus, 1954). A well documented and interesting account of defective particles in *rift valley fever virus* was reported by Mims (1956). Maximum titres of 10^9 were observed when serum from mice was diluted 10^{-8} prior to inoculation. Undiluted passage reduced the infective titre to $10^{4.2}$. As with *influenza* virus the infective titre could be returned to the maximum level by one diluted passage. Competitive ability of defective particles was demonstrated by injecting undiluted passage virions into mice previously

infected with diluted serum. The infective titre of progeny virions was reduced drastically (to $10^{5.5}$) by the addition of undiluted passage virions to the extent that some mice survived the infection. Mice able to survive such an infection nevertheless developed specific neutralizing antibodies. The unusual reduction in infectivity upon undiluted passage led Mims to remark "It should not be forgotten how artificial the circumstances are when incomplete virus is produced. By means of a syringe or pipette virus is confronted with a situation it has never before encountered. Nature's inocula, whether by insect bite or infective droplet, are usually much smaller, and it is not surprizing that the highly efficient system of virus growth which has always followed such inocula should break down under unprecedented laboratory circumstances."[1]

As with *rift valley fever virus*, a fraction of mouse fibroblast cells infected with *western equine encephalomyelitis* virus survived the infection and gave rise to vigorous cell cultures (Chambers, 1957). That defective viral genomes could be produced in this virus was shown by the ability of undiluted passage to produce the *von Magnus effect*. Of the cells that survived the infection some continued to produce virus for many months; eventually these cells gave rise to stable cells that were no longer capable of producing virus particles. Cells that were still able to produce virions were resistant to infection by particles from diluted passage. Resistant cells could be heteroplasmic for normal and dependent variant viral molecules. The dependent nature of the variant would allow for the maintenance of both the variant and molecular prototype in the host cell. Presumably the concentration of prototype molecules is not sufficient to

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[1] From Mims (1956) p.142.

cause cell lethality. Resistance to lethal infection by diluted passage particles is explained by the model of "Darwinian" selection, since resident variant molecules would competitively interfere with the replication of newly introduced prototype molecules. Similarly, daughter cells that have lost the ability to produce viral particles could be the result of (i) chance segregation of a cell lacking the molecular prototype or (ii), the spontaneous generation of refractory variants.

A thorough examination of the host's ability to survive following infection was reported by Lehmann-Grube et al. (1969). Cells infected with *lymphocytic choriomeningitis* initially produce infective progeny without cell damage. After continued cell multiplication the virus particles produced were not infective, an indication of the *von Magnus effect*. Cells containing defective particles interfered with the replication of normal virions applied as a secondary inoculation. Also as with *western equine encephalomyelitis* virus, segregation of infected cell cultures produced lineages completely free of virus. As previously discussed these observations are consistent with the hypothesis of molecular "Darwinian" selection *in vivo*. An excellent summary of the role of defective particles in the aetiology of viral disease was recently published by Huang and Baltimore (1970).

Undiluted passage of *vesicular stomatitis virus* through chick embryo cell monolayers also resulted in the *von Magnus* effect (Cooper and Bellett, 1959). In addition, the defective particles were shown (i) to interfere with the replication of normal particles and (ii), to produce maximum infection titers after one dilute passage. As expected by the hypothesis of "Darwinian" selection the defective particles had a reduced sedimentation coefficient of 25 ± 2.5S as compared to 42 ± 2.4S for normal particles (Huang and Wagner, 1966). Furthermore, the molecular weight of RNA isolated from defective particles

was calculated from sedimentation coefficients to be 1.3×10^6 as compared to 4.0×10^6 for particles from diluted passage. Replicative superiority of small molecular weight RNA present in defective particles may form the material basis for the *von Magnus effect* in this virus.

The oligonucleotide pattern of defective particles of *fowl plague* virus (an RNA virus that undergoes the *von Magnus effect*) was shown to be similar to that of dilute passage particles (Rott and Scholtissek, 1963). This indicates that the RNA contained in the defective particle was not derived from the host cell. P^{32} incorporation into RNA of *von Magnus* virions was one third that of infective particles.

A DNA virus, *simian virus 40*, produced the *von Magnus effect* upon undiluted passage through African green monkey cells (Uchida, Watanabe and Kato, 1966). As expected, the defective viral form was shown by competition experiments to interfere with the replication of normal viral particles. Two types of defective particles were defined on the basis of their ability to induce the formation of the T and V antigens (Uchida, Yoshiike, Watanabe, and Furuno, 1968); the average buoyant density of these defective particles was lower than that of infective particles. A further indication of the maintenance of some biological information by the defective particles was their apparent ability to induce tumor formation in hamsters (Uchida and Watanabe, 1968). DNA extracted from light defective particles was found to have an average contour length of $1.50 \pm .12\mu$ in comparison to $1.76 \pm .09\mu$ for DNA extracted from infective dilute passage particles. DNA extracted from both dilute and undilute passage particles was circular (Yoshike, 1968); which supports the assumption that the shorter variant molecules were generated as fast replicating variants of the molecular prototype.

Another DNA virus, *polyoma*, displayed the *von Magnus* effect upon undiluted passage (m.o.i. was 10 to 100) through mouse embryo cells (Blackstein et al., 1969). A comparison was made between the major DNA component (I, = a closed supercoiled circular helix) of lysates from diluted and undiluted serial passage. In contrast to one major peak (I*a*) for diluted passage virions, velocity sedimentation analysis revealed the presence of two slower sedimenting peaks (I*c* and I*d*) in virions from undiluted serial passage. The hypothesis of "Darwinian" selection predicts that the *von Magnus effect* is caused by components I*c* and I*d*. Support for this hypothesis is suggested by the following characteristics of I*c* and I*d*: (i) I*c* and I*d* were unable to infect cells and produce plaques in contrast to the plague producing ability of I*a* - consistent with the concept that the variant molecules have dispensed with biological information in order to gain a replicative advantage; (ii) components I*a*, I*c* and I*d* had the same random coil configuration, and conformation state (tightly compact "knots" of DNA) - consistent with the proposal that I*c* and I*d* have evolved from I*a* (preliminary results with hybridization experiments indicate that I*c* and I*d* have sequences in common with I*a*) and (iii), components I*c* and I*d* had a mean contour length of $1.12 \pm 0.09\mu$ and $0.75 \pm 0.08\mu$ in contrast to a length of $1.53 \pm 0.07\mu$ for I*a* - consistent with the assumption that the variant molecules have a replicative superiority by virtue of their shorter length.

CONCLUDING REMARKS

In addition to offering an explanation for some of my own observations, the hypothesis of molecular "Darwinian" selection *in vivo*, provided an attractive explanation of the *von Magnus effect*, and accounted for the occurrence of *grossly altered DNA base compositions* in some micro-organisms. It was mentioned that the function of most self-replicating molecules is indispensable, and that the observation of molecular "Darwinian" selection of an indespensabe molecule would appear to be impossible in the light of current technology. However, there is an unexplained phenomenon, *lethal sectoring* (James, 1967), which is suggestive of "Darwinian" selection of an indispensable molecule. *Lethal sectoring* refers to the production of progeny cells that are incapable of giving rise to viable progeny. The hypothesis of molecular "Darwinian" selection would accomodate this observation by assuming the existance of a bimolecular state for the niche of an indispensable molecule. Production of a daughter cell with only the variant molecule occupying the niche would result in cell lethality. Although such an explanation is highly conjectural, lines that have the property of *continuous segregation of lethal sectoring* have been observed - an expectation of the hypothesis.

Many intriguing questions are raised by the hypothesis of molecular "Darwinian" selection. What is the mechanism that determines the number and location of molecular niches? What determines the number of molecules per niche? What significance does the hypothesis have in pre and post-cellular evolution?

A naive belief that the complex molecular structure of living organisms is the end result of the obeyance of fundamental physical and chemical properties of molecules (i.e., the doctrine of vitalism is not required for

an explanation of life), leads one to look for answers to these questions by considering the effect that molecular "Darwinian" selection would have on the evolution of a self-replicating molecule. (There seems little doubt that living organisms evolved from a system consisting of a self-replicating molecule and the necessary components required for its replication). As pre-cellular evolution would be expected to precede the appearance of living organisms, consider the evolution of a molecule in an extracellular-site. Prolonged replication of the molecular prototype would modify the immediate replicative environment thus creating a secondary environment within the extracellular-site. Spontaneously generated variant molecules would be produced during the replication of the molecular prototype. Any such variants that had replicative superiority in the secondary environment would be selected for, thus initiating molecular heterogeneity in the extracellular-site. Replication of the prototype and variant molecule(s) would create a tertiary environment that would then select for spontaneously generated variants with a replicative advantage in such an environment. Continuation of this process could result in a tremendously large population of different variant molecules all capable of subsequent evolution. Secondary niches would become occupied by variant molecules capable of replication in adjacent space (closer to the periphery of the extracellular-site) incapable of supporting the replication of the original molecular prototype. Molecules within such *de novo* niches would also undergo molecular "Darwinian" selection eventually creating tertiary niches, and so on.

Environmental conditions, at the periphery of the extracellular-site, may become so stringent that only co-ordinated, interdependent molecular-niches capable of modifying the peripheral environment, would be capable of continued replication. For example, the molecular product of a niche may result in the necessary modification of a precursor before it can be utilized

by the molecules of other niches. Some of the latter niches may in turn alter the pH of the peripheral environment thus maximizing the environment for molecular replication within niche(s) capable of precursor modification. An increasingly sophisticated interdependence between niches could result in a structure that was increasingly independent of the environment of the extra-cellular-site, until a multi-molecular structure akin to a simple cell is formed. (The multi-molecular structure would require the additional property of self-duplication in order to participate in further evolutionary changes.)

At the level of a simple cell the location of molecular-niches would be most accurately defined in relation to each other. For example, product dependent niches would be expected to he located proximal to the source of their requirement. The minimum number of molecular-niches would be that required to act as a co-ordinated replicative structure capable of partial independence from the external environment. However, molecular-niches that make no direct contribution to the fitness of the cell could be maintained since molecular "Darwinian" selection can occur independently of selection pressure placed upon the cell. For example, [*rho-(s)*] strains of *S. cerevisiae* are spontaneously produced from [*rho*+] strains grown in glycerol. Thus the niche for mitochondrial DNA is occupied by a variant molecule that places the cell at a selective disadvantage. The *relative independence* of molecular "Darwinian" selection from selection pressure at the cell level also allows for niches containing large numbers of molecules of a particular unique sequence.

Recent biochemical investigations have revealed the unexpected phenomenon that much of the DNA of higher organisms is present in many copies (Britten and Kohne,1967). They suggest that such repetitious DNA is the result of saltatory replications of a particular DNA sequence. Although Britten and

Kohne (1967) do not suggest the mechanism of such saltations, the end result - i.e., many copies of a particular nucleotide sequence, is consistent with the idea that saltations represent the creation of a *de novo* niche for a variant self-replicating molecule. They suggest that repititious DNA acts as a reservoir of information that is used subsequent to translocation and mutation. Perhaps repetitious DNA is another example of molecular "Darwinian" selection *in vivo* and this phenomenonis a basic source of information for evolution at the cellular and species level.

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APPENDIX A - Reversion of spontaneously arising respiratory deficiency in *Saccharomyces cerevisiae*.

REVERSION OF SPONTANEOUSLY ARISING RESPIRATORY DEFICIENCY IN *SACCHAROMYCES CEREVISIAE*

GERALD H. RANK AND CLAYTON PERSON

Department of Botany,
The University of British Columbia, Vancouver, British Columbia

Reversion of naturally-arising cytoplasmically-inherited respiratory deficiency in *Saccharomyces cerevisiae* was indicated by the occurrence of colonies with a respiratory sufficient apex arising from a respiratory deficient base. The basal respiratory deficient cells were shown to contain the suppressive factor. It was suggested that genetic information for the suppressive factor resided in abnormal mitochondrial DNA and that mosaic colonies arose from a heteroplasmic cell containing both normal and abnormal mitochondrial DNA.

Introduction

Spontaneously-arising, cytoplasmically-inherited respiratory deficient (RD) mutants in *S. cerevisiae* do not have cytochromes *a* and *b* and are unable to grow on non-fermentable energy sources such as glycerol. Crosses of RD to respiratory-sufficient (RS) strains define two types of cytoplasmically-inherited respiratory deficiency referred to as the neutral and suppressive petite (Ephrussi, Hottinguer and Roman, 1955). Zygote colonies of the RS by neutral petite cross are all RS whereas a variable number (1-99%) of those from the cross RS by suppressive petite are RD. A suppressive petite that produces 100% RD zygote colonies when crossed to RS has not been observed. The majority of naturally-arising petites are suppressive (Sherman and Ephrussi, 1962). Ephrussi and Grandchamp (1965) used clonal studies to show that the degree of suppressitivity was transmissable by individual cells and did not result from heterogeneity in cell populations. The biochemical and cytological nature of the genetic element that codes for suppressitivity, referred to as the suppressive factor (SF), is unknown. Neither the spontaneously-arising neutral nor the suppressive petite has been observed to revert to the RS condition.

Ephrussi, Jacob and Grandchamp (1966) postulated that zygotes and their vegetative progeny from the cross RS by suppressive petite can exist in an intermediate state, l'état prémutationnel (PS), which can give rise to progeny cells that are RS or RD. They obtained genetic evidence which indicated that the suppressive factor (SF), received from the RD strain, competitively interferes with the replication of the cytoplasmically inherited normal factor (NF), required for the synthesis of respiratory enzymes, which is received from the RS strain. The interaction between the SF and the NF in zygotic cells and their vegetative progeny often results in the development of mosaic colonies. These colonies, which have a RD base and a RS apex, are referred to as petites abcédés (PA). It is inferred that reversion from RD to RS has occurred in some of the cells of the developing colony.

Since the majority of naturally-arising petites from RS strains are suppressive one would expect also to find naturally-arising mosaic colonies similar to the petites abcédés. This report discusses the occurrence and possible origin of such colonies.

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Materials and Methods

Genetic Strains

Strain GR1 was developed from cultures provided by Dr. C. Hawthorne. The genotype of GR1 is as follows: *D/D*, *α/a*, *ad₂/ad₂*; ρ^+. In this formula *D* represents a gene for homothallism which, when present in haploid ascospores, brings about a directed mutation of the mating allele (α to *a* or the reverse), thus converting haploid cells to homozygous diploids (Hawthorne, 1963). Gene *ad₂* is for adenine dependency; with this mutant gene present a colony grown on complete medium is red if it is RS, and white if it is RD (Tavlitski, 1951; Sarachek, 1958; Silver and Eaton, 1969). The symbol ρ^+ denotes the normal cytoplasmic factor (NF) which is needed for respiratory sufficiency. A ρ^- strain is RD.

A second strain, designated *abi₅*, was also in this work; it is a haploid strain which is auxotrophic for histidine.

When strains GR1 and *abi₅* are crossed on minimal medium the vast majority of resulting colonies are triploid (*α/α/a*). However, a few diploid (*α/a*) colonies are also produced. Because these diploid colonies can grow on minimal medium, and because the individual ascospores can give rise to normal 1:1 segregations for nuclear markers, it is apparent that they have received only a single nucleus from strain GR1.

Media and Culture Methods

Minimal medium was composed of 0.67% Difco yeast nitrogen base, 2% glucose and 2% agar. Adenine hydrochloride and L-histidine HCl were added to minimal medium at concentrations of 10 mg/liter and 100 mg/liter respectively for determining genotypes in tetrad analyses.

Complete medium contained 1.0% yeast extract, 2.0% bactopeptone, 2.0% glucose with 2.0% agar added for solid media. Sporulation medium contained 0.1% glucose, 0.25% yeast extract and 1.0% potassium acetate.

All cultures were incubated at 30°C.

Tetrazolium agar (TZ) contained 0.1% triphenyl tetrazolium chloride and 1.5% agar dissolved in .067 M phosphate buffer. Tetrazolium (TZ) agar was cooled to 45°C and then poured over colonies. Three hours after treatment RS colonies or sectors stained red while RD colonies or sectors remained white (Ogur, 1957).

Tetrad Analysis

Sporulated cells were digested with glusulase for 10 minutes, washed once with water and dissected with the aid of a De Fonbrune micromanipulator.

Determination of percentage suppressitivity: Strains GR1; ρ^- and *abi₅*; ρ^+ were grown to the end of log phase. One-ml samples were then taken from each of the two cultures and mixed together in a test tube. The mixture was then incubated for 4½ hr at 30°C and plated at appropriate dilutions onto minimal medium. (With this procedure, 0.1 ml of a 10^{-3} dilution of the mixture gives rise to approximately 100 colonies.) After 4 days of growth the zygote colonies were stained with TZ to determine the percentage of suppressitivity. All cultures were checked for the frequency of back mutation, and the frequency of ρ^- cells in the *abi₅* culture was also determined by staining plated colonies with TZ.

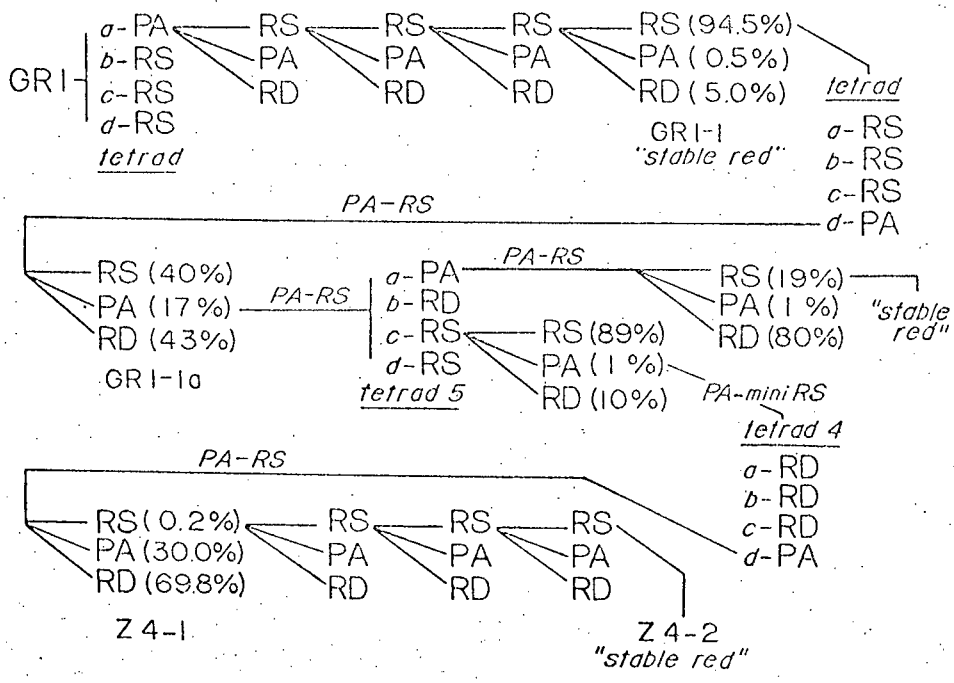


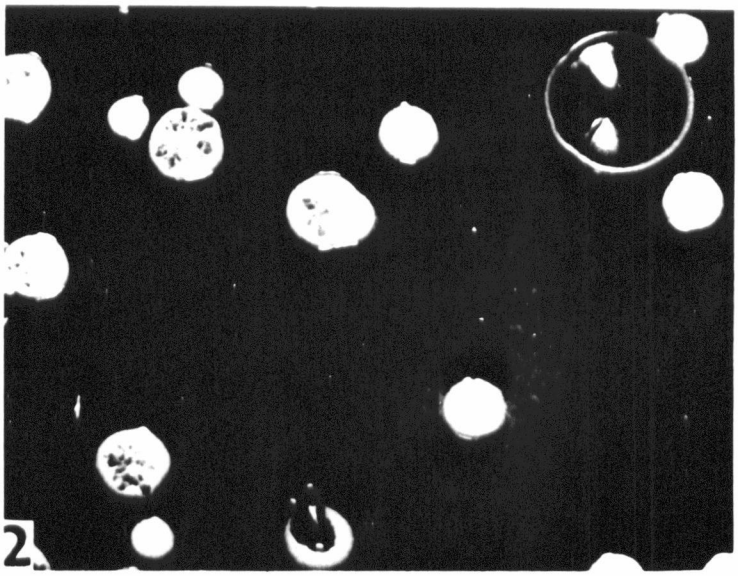
Fig. 1. Somatic segregation of GR1. For explanation see text.

Results

Isolates of GR1 gave rise to variable numbers of red (RS), white (RD) and mosaic colonies. The mosaic colonies, which were morphologically similar to the petites abcédés described by Ephrussi *et al.* in 1966, are referred to in this paper as PA colonies. As is shown in Fig. 2, the basal part of the PA colony is white; the sector appears as an erumpent red area rising from the white basal part of the colony. For convenience in later discussion the red (RS) and white (RD) areas of the PA colonies will be referred to as PA-RS and PA-RD areas, respectively. The relative dimensions of the red (PA-RS) and white (PA-RD) areas varied from one PA colony to another, but in nearly all cases the PA-RS area protruded noticeably from the remainder of the colony. It appears that the PA-RS represents a fast-growing area that has arisen in a colony that was initially white (RD). This interpretation is supported by the fact that non-mosaic RD colonies grow more slowly than RS colonies.

A second kind of mosaic colony was also observed. These occurred at a very low frequency and were distinguished by the fact that the red and white sectors were wedge-shaped. These are believed to have descended from two or more closely-adjacent parental cells; this type of colony can be easily distinguished from a PA colony.

Fig. 2. Somatic segregation during vegetative growth of an islate of GR1. The dark colony or sector is red (RS) and the light colony or sector is white (RD). The RS area of the PA colony in the lower central position is more prominant than the RS areas of other PA colonies. Fig. 3-5. Tetrad analysis of isolates of GR1: 3. Five tetrads showing all possible phenotypic ratios for RS vs RD. 4. Tetrads showing low meiotic stability and the occurrence of a PA ascospore colony. 5. Tetrads from a PA mini-RS colony where meiotic stability was 0.0%. Fig. 6. A PA mini-RS colony and two RD colonies.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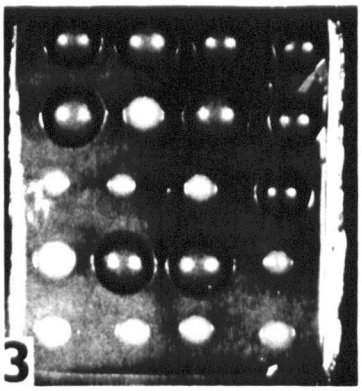
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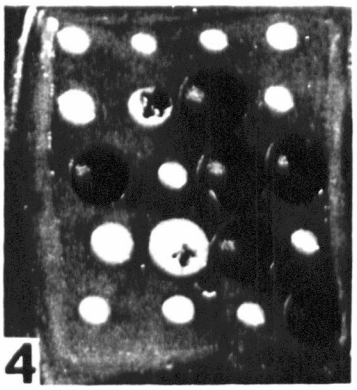
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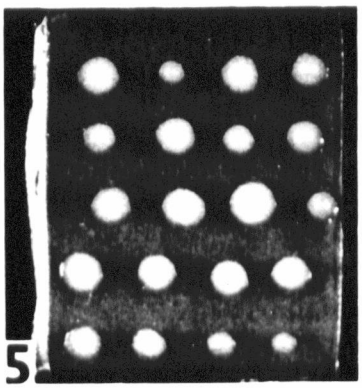
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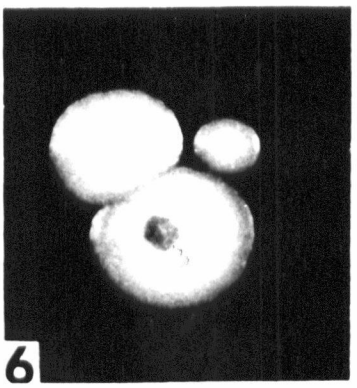
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Stability in Vegetative Lines

Of the three colony types obtainable from GR1 only one was entirely stable, producing only one type of colony when sampled, grown to stationary phase and spread onto complete agar; these colonies were classified as RD. Samples were also taken from the PA-RD areas of 35 PA colonies and similarly treated; 32 of these produced only white colonies whereas the remaining 3 produced a small percentage of red colonies. The PA colony type, which involves an apparent reversion to RS of cytoplasmically-inherited RD, is observed only occasionally among the white colonies.

The morphology of individual PA colonies suggests that the original cell line which gave rise to the colony was not entirely stable, and that the event leading to development of the PA-RS area involved a change from RD to RS in at least one cell of the developing colony. The fact that red (RS) colonies, when these are diluted and spread onto complete medium, characteristically produce a few white colonies can be taken as evidence that the reverse event, involving a cellular change from RS to RD, also occurs. However, this event, when it takes place during development of a red colony, does not lead to mosaicism, presumably because of the slower growth-rate of RD cells. These observations are similar to those recorded by Ephrussi *et al.* (1966) in their studies of zygote colonies produced in crosses of an RS by a suppressive petite strain. It was on the basis of these studies that Ephrussi *et al.* (1966) postulated the existence of the premutational state (PS) cell, which represents a cellular condition intermediate, or transitional, between RS and RD. Ephrussi *et al.* (1966) postulated that the PA colony developed from a PS cell. The natural occurrence of PA colonies during vegetative growth of GR1 indicate that such a mechanism is also responsible for the production of naturally-arising RD in *S. cerevisiae*.

Stability within Selected Lines

GR1 is a 'stable red' line (i.e. it produces, on transfer, more than 90% RS colonies). Large differences in the relative proportions of the three colony types (RS, PA and RD) were found among the various sublines that were established from GR1. The proportions differed not only between established sublines but also at different steps during establishment of a single subline. Both these points are illustrated in Fig. 1, which traces the lineage from GR1 to Z4-2. In the first step of this lineage a sample was taken from a PA ascospore colony, grown to stationary phase in complete liquid medium, then diluted and spread onto complete agar. Three repetitions of this procedure produced the stable red line GR1-1. One of the RS colonies was then put through meiosis and, among the resulting tetrads, one of them produced one PA and three RS ascospore colonies. The PA-RS area of the PA ascospore colony was the source of GR1-1a, which produced only 40% RS colonies. The PA-RS area of a PA colony, produced by GR1-1a, yielded a tetrad (No. 5) which gave one PA, one RD and two RS colonies. The RS area of the PA ascospore colony (5a), when sampled, diluted in water and spread on complete medium, produced only 19% RS colonies. However, one of these RS colonies, when similarly sampled, produced a stable red line. The RS ascospore colony (5c) when diluted in water and spread onto complete agar yielded a line containing 89% RS (i.e. almost as high as a stable red).

Figure 1 also shows that the percentage of RS colonies was reduced a second time, from 89 to 0.2% (as shown by strain Z4-1) before being brought back to the stable red level (> 90% RS) of Z4-2. It should be noted also that tetrad 4 was derived from a vegetative PA colony in which the PA-RS area was very small, amounting to ca. 1/50 - 1/20 the total area of the colony (see Fig. 6). This distinctive type of colony occurs only occasionally; for convenience in discussion the RS area of this type of colony will be referred to as the PA-mini RS area.

Figure 1 represents only a part of the full range of variability that actually occurs. From our observations there appears to be a continuum in the relative frequencies of RS, PA and RD colonies derivable from any given RS or PA culture. The only stable lines are those that are RD.

Percentage suppressitivity and cytoplasmic inheritance of RD: GR1 is a homozygous diploid since it originated from a haploid ascospore containing the homothallic gene *D*. Recessive nuclear genes for RD, arising through mutation during vegetative growth, would be masked in the diploid by the corresponding non-mutant allele. Although dominant nuclear genes for RD have not been reported a mutation of this kind in GR1 would be expected to give 100% RD zygote colonies when GR1; ρ^- is crossed with αbi_s; ρ^+. Neutral and suppressive petites would produce 100% RS and 1 - 99% RS zygote colonies, respectively, in the cross with αbi_s; ρ^+. In addition, the suppressive petite should produce some PA zygote colonies which would not be expected either from the neutral petite or through nuclear mutations.

The suppressitivity of various PA-RD and white isolates obtained from the cross between GR1; ρ^- and αbi_s; ρ^+ was determined by the method outlined. The percentage of suppressitivity was defined as (% PA colonies) + (% RD colonies) − (% ρ^- colonies in αbi_s).

Results of several experiments are recorded in Table I. All RD isolates produced the complementation pattern expected of a cytoplasmically inherited suppressive petite.

Low ascospore survival interfered with tetrad analysis of the triploid from the cross GR1; ρ^- by αbi_s; ρ^+ which was carried out to verify the cytoplasmic inheritance of RD. However, five diploid hybrids entered in Table II were obtained, apparently due to the presence of a low frequency of haploid cells in some cultures of GR1; ρ^-. 2:2 segregation for ad_7 and bi_s with their normal alleles indicated the hybrids were diploid.

All four ascospore colonies of 25 of the 32 tetrads analyzed were RS, as would be expected of cytoplasmic inheritance of RD. The frequency of RD colonies in the other six tetrads was that expected from vegetative segregation. One tetrad produced 2RS:1RD: 1 PA colony. Segregation of the homothallic gene was followed in this tetrad and it was found that the PA colony was haploid.

Thus copulation of a RD and RS cell is not necessary for the formation of a PA ascospore colony.

TABLE I

% of red, white and PA zygote colonies obtained from crossing GR1; ρ^- isolates with $a\,his_8$; ρ^+ and staining 4-day-old colonies with TZ

Source of ρ^- colony	% RD colonies	% RS colonies	% PA colonies	% ρ^- in $a\ his_8$	% Suppressitivity
Ascospore PA-RD	94.6	1.6	3.8	2.8	95.6
Ascospore ,,	21.8	70.1	8.1	2.1	27.8
Ascospore ,,	82.9	5.5	12.6	2.1	92.4
Ascospore ,,	90.9	5.5	3.6	2.1	91.4
Ascospore ,,	58.4	29.2	12.4	2.1	68.7
Ascospore ,,	89.5	4.1	6.4	2.1	93.8
Ascospore white	62.2	26.8	12.0	0.2	74.0
Ascospore ,,	63.1	10.4	26.5	0.2	89.4
Ascospore ,,	78.8	3.9	17.3	0.2	95.9
Ascospore ,,	79.9	3.3	16.8	0.2	96.5
Ascospore ,,	57.3	15.6	27.1	0.2	84.2
Vegetative PA-RD	50.7	10.8	38.5	0.2	89.0
Vegetative ,,	55.1	14.2	30.7	0.2	85.6
Vegetative ,,	35.1	15.3	49.6	0.2	84.5
Vegetative ,,	44.0	31.1	24.9	0.2	68.7
Vegetative ,,	14.6	40.0	45.4	0.2	59.8
Vegetative white	63.5	15.6	20.9	0.2	84.2
Vegetative ,,	7.8	83.1	9.1	0.2	16.7
Vegetative ,,	29.4	55.5	15.1	0.2	44.3
Vegetative ,,	36.0	43.1	20.9	0.2	56.7
Vegetative ,,	58.8	30.1	11.1	0.2	69.7

TABLE II

Tetrad analysis of GR1; ρ^- crosses to $a\ his_8$; ρ^+

Source of ρ^- colony from GR1	No. of complete tetrads analyzed	No. of tetrads 2:2 segn. for ad_2 & his_8	No. of tetrads 4 : 0 RS : RD	No. of tetrads 4 : 1 RS : RD
Ascospore white	9	9	6	3
Ascospore white	7	7	5	2
Ascospore white	3	3	2*	0
Ascospore PA-RD	7	7	6	1
Ascospore PA-RD	6	6	6	0

*Additional tetrad produced 2 RS : 1 RD : 1 PA colonies.

Meiotic Instability

Since RD strains of *S. cerevisiae* are unable to go through meiosis, stable white and PA-RD cultures placed into sporulation medium were unable to sporulate. Ephrussi, Hottinguer and Roman (1955) found that zygotes, formed by crossing RS and suppressive petite cells, produced a high frequency of RD ascospores when forced to go through meiosis without cell division. Limited observations of Sherman (1964) indicated that cultures that produced a high percentage of ρ^- cells also formed a high percentage of ρ^- ascospore colonies.

The indicated presence of the SF in RD cells and its inferred presence in PS cells predicts that cultures with a high frequency of PA and white colonies should have a high percentage of ρ^- ascospore colonies since a low meiotic stability is expected if an active SF is present. The somatic and meiotic stabilities of several red and PA-RS colonies are recorded in Table III. Somatic stability refers to the colonies formed during vegetative growth from which the recorded colony was sampled and transferred into sporulation medium. Cell division in sporulation medium could be controlled by altering the concentration of cells placed into sporulation medium.

Meiotic stability was defined as the percentage of ascospore colonies that were RS. A PA colony was classified as being RS. Figures 3 and 4 show that all possible phenotypic ratios were observed as expected of non-mendelian inheritance.

The most striking feature of the data in Table III is the low meiotic stability of cultures with a high percentage of white and PA colonies. Red and PA colonies of a given culture have a similar meiotic stability which indicates the

TABLE III

Somatic stability and meiotic stability of different isolates of GR1

Cultures	Type of RS colony sporulated	Somatic stability			Cell division in sporulation medium	Number of ascospore colonies analysed	Meiotic stability % RS ascospore colonies
		% RS	% RD	% PA			
GR1-1	RS	94.5	5.0	0.5	+	94	99.9
S1	PA	5.0	85.0	10.0	+	65	5.0
XVIIb	PA	0.0	95.9	4.1	+	40	0.0
F2	PA	95.0	5.0	0.0	+	67	80.0
B3	PA	85.0	15.0	0.0	+	49	81.0
GR1-1a	PA	40.0	43.0	17.0	+	37	62.0
GR1-1a	PA	40.0	43.0	17.0	+	23	34.0
GR1-1a	RS	40.0	43.0	17.0	+	22	50.0
B2	PA	34.0	45.0	21.0	+	29	19.0
B2	PA	34.0	45.0	21.0	+	44	14.0
B2	RS	34.0	45.0	21.0	+	27	29.0
5a	RS	19.0	80.0	1.0	−	35	21.0
5a	RS	19.0	80.0	1.0	−	34	11.0
5a	PA	19.0	80.0	1.0	−	35	40.0
5a	PA	19.0	80.0	1.0	−	39	18.0
5c	RS	89.0	10.0	1.0	−	34	80.0
5c	RS	89.0	10.0	1.0	−	18	83.0
5c	RS	89.0	10.0	1.0	−	42	93.0
5c	PA	89.0	10.0	1.0	−	55	92.0
5c	PA	89.0	10.0	1.0	−	39	79.0
5c	PA mini RS	89.0	10.0	1.0	−	48	73.0
5c	PA mini RS	89.0	10.0	1.0	−	71	32.0
5c	PA mini RS	89.0	10.0	1.0	+*	65	74.0
Z4	PA mini RS	——	——	—**	−	57	2.0
Z4	PA mini RS	——	——	—**	+*	38	3.0
Z4-3	RS	83.2	16.5	0.3	−	51	70.0
Z4-3	RS	83.2	16.5	0.3	−	35	40.0
Z4-3	RS	83.2	16.5	0.3	−	57	53.0
Z4-3	RS	83.2	16.5	0.3	−	59	42.0
Z4-3	PA mini RS	83.2	16.5	0.3	−	71	3.0
Z4-3	PA mini RS	83.2	16.5	0.3	+*	57	0.0

*A loopfull was taken from the preceding culture and placed into 3 ml of sporulation medium.
**Somatic stability was not recorded but meiotic stability of previous cell generation was 79%.

presence of the SF in red colonies as well as in PA colonies. The differences in meiotic stability between cultures indicate the presence of a qualitatively different SF. Failure to find a quantitative difference in the meiotic stability between RS and PA colonies of a given culture indicates that cells of the PA-RS sector are similar to cells of the red colony.

A PA-mini RS (Fig. 6) of a given culture could be expected to have a different meiotic stability if the cell composition of the PA-mini RS differed from the RS or normal PA-RS due to (i) limited growth caused by lack of cell nutrient, or (ii) the occurrence of a qualitatively different SF. Low meiotic stability (Fig. 5) due to limited growth of PA-mini RS (Fig. 6) could be overcome by allowing growth to occur in the sporulation medium.

As seen in Table III one PA-mini RS of 5c gave a meiotic stability (32%) much lower than the RS and PA colonies of this culture. The meiotic stability increased to 74% if the cells were allowed to grow in sporulation medium prior to the actual sporulation.

In contrast, the meiotic stabilities of PA-mini RS colonies of cultures Z4 and Z4-3 were not increased after growth in sporulation medium. The low meiotic stability of these colonies is possibly due to the spontaneous occurrence of a SF qualitatively different from that which normally occurs in PA cells of these cultures.

Discussion

An hypothesis to explain the results obtained must accommodate the following observations: (i) the appearance of three types of colonies (RS, PA and RD) whose relative frequencies can differ markedly in different selected lines; (ii) the stability of the RD lines; (iii) the great range in PA colony morphology; (iv) the suppressitivity of PA-RD and low meiotic stability of lines with a high percentage of PA and RD colonies; (v) similar meiotic stability of cells from PA and RS colonies from the same culture; (vi) the infrequent occurrence of PA-mini RS colonies with an unexpectedly low meiotic stability.

We accept the Ephrussi *et al.* (1966) hypothesis that cells can exist in any one of three cell states (RS, PS or RD). As depicted in Fig. 7, we visualize that the PS cell arises from the RS cell by the spontaneous event (represented by RS $\xrightarrow{a}$ PS in Fig. 7) in which the cytoplasmically-inherited SF is generated. Differences between rates of replication of the SF and the NF (Ephrussi *et al.*, 1966) form the basis for the PS cell to 'mutate' to either RS or RD. Domination by the NF results in the conversion of the PS cell to an RS cell (PS $\xrightarrow{b}$ RS) whereas domination by the SF converts the PS cell to an RD cell (PS $\xrightarrow{c}$ RD). The RD cell is stable since it lacks, and is unable to generate, the NF. All three cell states are capable of self-duplication.

The postulated existence of the three cell types (RS, PS and RD) is an inference based on the existence of the three colony types (RS, PA and RD). Thus, a PA colony is presumed to have arisen from a parental PS cell whose cytoplasm contained both the SF and the NF. Cells descendant from the PS cell may be RS, PA or RD, depending on (i) the relative rates of interconversion (represented by a, b and c) between the three cell types, and (ii) the relative growth rates (K_1, K_2 and K_3) of the three different types after

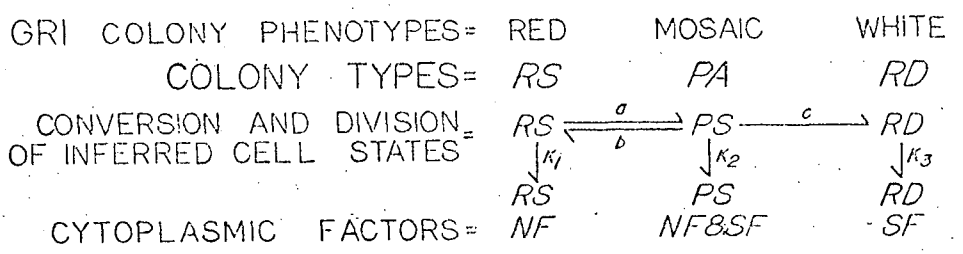

Fig. 7. Colonies of GR1 observed and the postulated conversions between cell states as caused by the presence of cytoplasmic factors. The letters *a*, *b* and *c* refer to the relative rates of interconversion between the three cell states and K_1, K_2 and K_3 refer to the relative growth rates of RS, PS and RD cells, respectively.

the conversion has taken place. The size of the PA-RS area of a PA colony is thus dependent on when, during PA colony development, the (PS $\xrightarrow{b}$ RS) event takes place, as well as on the extent to which the newly arisen RS sector is able to outgrow the cells which surround it ($K_1 > K_2$ or K_3).

The ability of the SF to dominate the NF is dependent upon (i) the qualitative nature of the SF, and (ii) environmental factors, such as energy source and temperature (Ephrussi *et al.*, 1966). Since the PS cell is capable of self duplication and does not immediately 'mutate' to RS or RD, it follows that selection for cells in the PS state (i.e. by sampling a PA-mini RS area) will result in an enrichment of PS and RD cells in the population. Alternatively, selection from RS colonies would be expected to lead to a decrease in PS and RD cells.

Suppressitivity and meiotic instability are caused by the SF (Ephrussi *et al.*, 1955). Since lines with a high percentage of PA and RD colonies are expected to have an active SF, it follows that such lines should have a low meiotic stability. A vigorous PA-RS sector would be expected to have a meiotic stability similar to that of an RS colony of the same culture since the PA-RS sector is composed mainly of RS cells. The poor growth of a PA-mini RS sector suggests that the cell population contains few, if any, RS cells, but rather contains mainly PA and RD cells. Cells from such a population would be expected to have an active SF and thus a low meiotic stability.

Reports in the literature describing a colony morphology similar to the PA reported here (Skovsted, 1956; Nagai, 1961) indicate that the phenomenon is not restricted to GR1. PA colonies have been observed in all strains of *S. cerevisiae* used by the authors.

In seeking a molecular mechanism to explain the three observed colony phenotypes (RS, PA and RD) it is necessary to consider the possible nature of the SF. Mitochondrial DNA (MDNA) appears to be the best biochemical candidate for the NF (Avers, Billheimer, Hoffman and Pauli, 1968; Wintersberger and Viehauser, 1968). MDNA from ρ^+ strains of *S. cerevisiae* has a base composition of 80% adenine plus thymine. Strains of *S. cerevisiae* containing the SF have been shown to have a MDNA consisting of a copolymer of deoxyadenylic and deoxythymidylic acid in alternate sequence (d(A-T)) with an upper limit of 4 mole % guanine plus cytosine (Mehrotra and Mahler, 1968; Bernardi, Carnevali, Nicolaieff, Piperno and Tecce, 1968). Carnevali, Morpurgo and Tecce (1969) have suggested the possibility that MDNA polymerase has a high probability of detachment from its MDNA template thereby

producing short d(A-T) enriched molecules which have replicative superiority that quickly dominates the MDNA population and thus causes the phenotype of suppressitivity. In this hypothesis, *the abnormal MDNA is the suppressive factor*. MDNA from RD mutants of *S. cerevisiae* were shown to have a contour length of 1.5 to 2.5 μ whereas RS strains contained MDNA with a contour length mainly of 4.5 to 5.5 μ (Avers, Billheimer, Hoffmann and Pauli, 1968). The greater replicative ability of d(A-T) is also supported by the report Okazaki and Kornberg, 1964) that *E. coli* and *B. subtilis* DNA polymerase have a reaction rate twenty times faster with d(A-T) as a primer than with native DNA as the primer.

If abnormal MDNA is the SF, as suggested by Carnevali *et al.* (1969), the PS would be initiated upon the production of one or more abnormal MDNA molecules. In more general terminology (Jinks, 1964), the PS state would represent a heteroplasmon containing two forms of a chondriogene. Alternative forms of the chondriogene, phenotypically expressed as RS and RD and genetically defined as the NF and SF, would have their molecular basis in normal and abnormal MDNA, respectively. The relatively greater rate of multiplication of abnormal MDNA in interaction with the replication of normal MDNA would provide a molecular basis for the competitive interaction between the NF and the SF. The qualitative difference in the SF, expressed phenotypically as different percentages of supressitivity, would have its genetic basis in different forms of the abnormal MDNA; perhaps the distribution of the 4% G plus C is important in this respect. Superimposed upon the interaction between the SF and the NF would be the phenomenon of chance distribution of the two types of mitochondria to produce an occasional daughter bud containing mitochondria with only normal MDNA, thereby producing an RS cell. A stable RD cell would contain only abnormal MDNA and the progeny of a PS cell would contain both normal and abnormal MDNA.

The progeny of a PS cell, which are predominantly RD cells, would initially metabolize glucose via glycolysis and there would be no selection favouring the growth of any RS cell (produced by chance cytoplasmic distribution and resulting in a cell containing only normal MDNA) during this time. In later stages of growth the ethyl alcohol, produced via glycolysis, would become the main energy source and functional mitochondria would be required for growth and reproduction. Any RS cell(s) produced by chance distribution would now divide rapidly, in contrast with the surrounding RD cells, to produce a PA colony. The RS cells of the PA colony would be expected to have a meiotic stability similar to RS colonies of the same line since it would contain the same number of normal mitochondria. Similar meiotic stabilities were observed (Table III).

The hypothesis that the abnormal MDNA may act as the SF by greater replicative ability is also able to provide an explanation for the low number of targets calculated in the induction of RD (Uchida, 1967; Slonimski, Perrodin and Croft, 1968). RD and RS mosaic colonies were observed following induction by UV (Sarachek, 1958; Pitman, 1959; Uchida, 1967), acriflavin (Ephrussi, 1951) and ethidium bromide (authors, unpublished results) which indicates that such induction may be due to the production of a SF which then interferes with the replication of the NF rather than a direct inactivation or destruction

of the NF as is commonly assumed. The suppressitivity of the d(A-T) MDNA would provide a basis for induction kinetics that requires a low number of targets.

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We wish to thank Dr. C. Hawthorne, Genetics Department, University of Seattle, for his kindness in providing certain yeast strains. One of us (G.R.) is a grateful recipient of the H. R. MacMillan Family Fellowship. We also wish to acknowledge financial support received from the National Research Council of Canada and from the Canada Department of Agriculture.

Résumé

Nous avons observé que des colonies de *Saccharomyces cerevisiae* pouvaient pallier des déficiences respiratories transmises génétiquement par le cytoplasme: cette réversion se traduit par l'apparition d'excroissances possédant des fonctions respiratoires normales sur une base déficiente. Le facteur suppressif a été mis en évidence dans les cellules à respiration déficiente. Nous suggérons que l'information génétique correspondant au facteur suppressif est contenue dans le DNA mitochondrique anormal et que les colonies mixtes sont filles d'une cellule hétéroplasmique contenant à la fois du DNA mitochondrique normal et anormal.

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APPENDIX B - Genetic evidence for "Darwinian" selection at the molecular level. I. The effect of the suppressive factor on cytoplasmically-inherited erythromycin-resistance in *Saccharomyces cerevisiae*.

GENETIC EVIDENCE FOR 'DARWINIAN' SELECTION AT THE MOLECULAR LEVEL. I. THE EFFECT OF THE SUPPRESSIVE FACTOR ON CYTOPLASMICALLY-INHERITED ERYTHROMYCIN-RESISTANCE IN *SACCHAROMYCES CEREVISIAE*

GERALD H. RANK

Department of Botany, The University of British Columbia, Vancouver, British Columbia

A study of the suppressive factor of *Saccharomyces cerevisiae* and its effects on the transmission of cytoplasmically-inherited erythromycin resistance showed that loss of erythromycin resistance was contingent on suppressitivity, and that strains carrying the suppressive factor continuously segregated progeny cells that lacked resistance. It was suggested that suppressitivity is due to the presence, in suppressive strains, of rapidly-replicating abnormal mitochondrial DNA, and that loss of erythromycin resistance (coded for by normal mitochondrial DNA) is due to the replicative superiority of abnormal mitochondrial DNA.

Introduction

Mills, Peterson and Spiegelman (1967) observed that an in vitro system for synthesizing Qβ-RNA spontaneously generated variant molecules. Selection for those variant molecules that were fast replicating resulted in the Qβ-RNA population being dominated by a molecular species that had lost 83% of its original genome and that replicated 15 times faster than the original RNA molecule. Biological activity of the Qβ-RNA was lost very early in the selection process thus raising the question as to whether such a process could occur in nature, where loss of essential genetic information by self-duplicating molecules, in order that they may replicate faster, would seem to be incompatible with the survival of an organism.

Mitochondrial DNA (MDNA) is a self-duplicating molecule that contains some of the information required for normal mitochondrial function (Wintersberger and Viehhauser, 1968). Functional MDNA, however, is not essential for survival of the facultative anaerobe *S. cerivisae;* this yeast can survive, without functional mitochondria, on energy derived from glycolysis. Thus 'Darwinian' selection of MDNA (the spontaneous generation of variant MDNA molecules and the domination of the MDNA population by those variant molecules that have a greater replicative ability) in *S. cerevisiae* need not be incompatible with survival of the organism.

Normal MDNA has a base composition of 80% adenine plus thymine whereas an abnormal MDNA (a copolymer of deoxythymidylic and deoxadenylic acid in alternate sequence (d (A-T)) with an upper limit of 4 mole % guanine plus cytosine) has been found in cytoplasmically-inherited respiratory-deficient strains of yeast that show the phenomenon of suppressitivity (Mehrotra and Mahler, 1968; Bernardi, Carnevali, Nicolaieff, Piperno and Tecce, 1968). Suppressive respiratory-deficient strains of *S. cerevisiae* produce variable numbers of respiratory-deficient zygote colonies when crossed to a normal respiratory-sufficient strain (Ephrussi, Hottinguer and Roman, 1955). The genetic

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determinant for suppressitivity, referred to as the suppressive factor (SF), was assumed to be abnormal MDNA (Carnevali, Morpurgo and Tecce, 1969; Rank and Person, 1969) and is believed to dominate the MDNA population by virtue of its greater replicative ability.

A cytoplasmically-inherited marker for erythromycin resistance, (the resistance being due to molecular alteration of the mitochondrial ribosome), is also believed to have its genetic basis in MDNA (Linnane, Lamb, Christodoulou and Lukins, 1968). If abnormal MDNA is able to become the sole molecular species by virtue of a replicative advantage, such abnormal MDNA should eliminate the information for erythromycin resistance believed to reside in normal MDNA. This report studies the relationship between the SF and the cytoplasmic inheritance of erythromycin resistance, and presents results that are consistent with the hypothesis that the replicative advantage of abnormal MDNA enables it to displace normal MDNA.

Materials and Methods

Strains

Haploid strains L410: *α ur his; ER*^{s}; ρ^{+} L411: *α ur his; ER*^{r}; ρ^{+} and D243-12A; *a ad*$_{1}$ *tr*$_{1}$*ly*$_{2}$*;ER*^{s}; ρ^{+} were obtained from Dr. H. B. Lukins of Monash University, Clayton, Victoria, Australia. Haploid strain M2: *a tr*$_{1}$*ad*$_{1}$*his;ER*^{r}; ρ^{+} was obtained from a tetrad analysis of a cross between L411 and D 243-12A. Diploid strain R37: *a/a ad*$_{1}$*/ad*$_{1}$ *leu*$_{1}$*/leu*$_{1}$*;ER*^{s} ρ^{+} was obtained from Dr. H. L. Roman of the Department of Genetics, Seattle, Washington. The symbols *ER*^{r} and *ER*^{s} refer to the cytoplasmically inherited marker for resistance and sensitivity to erythromycin-supplemented (1 mg/ml) glycerol medium. An *ER*^{r} strain that has lost its resistance to erythromycin will be designated *ER*$^{-}$. ρ^{+} is used to denote the cytoplasmically-inherited genetic information necessary for respiratory sufficiency commonly assumed to be MDNA. A ρ^{-} strain is respiratory deficient and thus unable to use glycerol as an energy source. The symbols *ur*, *his*, *ad*, *tr*, *ly* and *leu* refer to auxotrophic requirements for uracil, histidine, adenine, tryptophan, lysine and leucine respectively. Crosses between L410 (or L411) and D243-12A were plated onto 2% glucose minimal medium which supported only the growth of the prototrophic hybrid. Crosses between L411 and M2 were plated onto 2% glucose histidine-supplemented minimal medium which supported only the growth of the *α/a his/his* hybrid. Diploid strain R37 was crossed to an *α/a his/his* diploid by plating the copulation mixture of these two strains onto 2% glucose minimal media which allowed growth only of the prototrophic *a/a/a/α* tetraploid zygote.

Media

Minimal medium contained 0.6% Difco yeast nitrogen base, 2% glucose and 2% agar. For supplemented minimal media amino acids were added to a final concentration of 100 mg/l and purine and pyrimidine bases were added to a final concentration of 10 mg/l. Complete medium contained 1% yeast extract and 2% bactopeptone, with 2% agar added for solid medium. The energy source was either 2% glucose or 4% glycerol. Erythromycin glucoheptonate, obtained from Eli Lilly and Co., was millipored and added to complete medium (at 50°C) to a final concentration of 1 mg/ml. Sporulation medium contained 0.1% glucose, 0.25% yeast extract and 1.0% potassium

acetate. All cultures were incubated at 30°C. Tetrazolium agar contained 0.1% triphenyl tetrazolium chloride and 1.5% agar dissolved in 0.067 M phosphate buffer (Ogur, *et al.* 1957). Tetrad analyses and determination of per cent suppressitivity were carried out as described previously (Rank and Person, 1969).

Determination of the % Transmission of the ER^r Factor by ρ^- Isolates

Erythromycin does not interfere with the growth of *S. cerevisiae* when energy is obtained via glycolysis using glucose as the energy source. However, an erythromycin-sensitive strain will not grow in the presence of erythromycin when glycerol is used as the energy source. Since functional mitochondria are required to oxidize glycerol, the lack of growth on erythromycin-supplemented glycerol medium indicates that erythromycin interferes with mitochondrial function. Thus, an erythromycin-resistant strain of *S. cerevisiae* is defined as a strain that will grow on glycerol in the presence of erythromycin. The presence or absence of the ER^r factor in ρ^- isolates from an ER^r; ρ^+ strain cannot be determined directly since a ρ^- isolate is unable to use glycerol as an energy source. Therefore, the ability of a ρ^- strain to transmit the ER^r factor was used to determine whether or not the ER^r factor was present. This was done by crossing the ρ^- isolates to an ER^s; ρ^+ strain and scoring the ρ^+ hybrid progeny for erythromycin resistance which could only have come from the ρ^- strain.

Results

Selection for ER^-;ρ^+ from ER^r;ρ^+

1000 ρ^+ colonies from M2 and L411, growing on glucose complete medium, were replica-plated onto glycerol complete medium with and without erythromycin. All colonies isolated from M2 and L411 grew on both media which confirms the observation of Linnane, Saunders, Gingold and Lukins (1968) that ER^-; ρ^+ cells either do not occur or they occur at a very low frequency. This is in sharp contrast with the frequent occurrence (1-5 %) of ρ^- cells in strains M2 and L411.

Selection for ER–; ρ^- and ER^r; ρ^- from ER^r; ρ^+

ρ^- strains were selected at random from M2 and L411. ρ^+ colonies of strains M2 and L411 are red and buff, respectively; ρ^+ colonies of both strains are white. The ability of ρ^- strains, picked at random from M2 and L411, to transmit the ER^r factor was determined by patching 25 ρ^+ zygote colonies, obtained from crossing ρ^- strains to an ER^s; ρ^+ strain, onto complete glycerol and complete glycerol erythromycin media. Growth on both media indicated that the ρ^- strain contained ER^r whereas growth on only glycerol complete medium indicated the ρ^- strain was ER^-. Per cent suppressitivity of the ρ^- strains was determined in the usual manner by crossing them to a ρ^+ tester and staining the resulting zygote colonies with tetrazolium (Rank and Person, 1969).

The results are recorded in Table I where it is seen that all ρ^- strains were suppressive. Both M2 and L411 produced ER^-; ρ^- and ER^r; ρ^- cells although the relative frequency of ER^r; ρ^- cells was much higher for strain M2. ER^r; ρ^- strains varied greatly in their ability to transmit the ER^r factor.

TABLE I

Per cent transmission of ER^r, and % suppressitivity of spontaneously arising ρ^- strains

ρ^+ strain	ρ^- strain isolated from ρ^+ strain	% transmission of ER^r[1]	% suppressitivity[2]
M2	1	40	29.27
	2	34	28.32
	3	16	21.54
	4	40	16.42
	5	4	16.22
	6	0	79.10
	7	64	12.64
	8	38	24.14
	9	68	28.03
	10	12	17.17
	11	28	23.48
	12	16	21.60
L411	1	0	66.77
	2	0	68.24
	3	68	60.66
	4	0	43.49
	5	0	79.98
	6	0	64.61
	7	0	77.75
	8	0	84.44
	9	0	33.46
	10	0	33.15
	11	0	65.73
	12	0	68.50
	13	0	78.33
	14	56	54.45
	15	36	75.00
	16	0	47.15
	17	0	68.87
	18	4	73.65
	19	0	78.47
	20	36	73.94
	21	0	82.85

[1]Twenty-five ρ^+ zygote colonies obtained from crossing the ρ^- strain to an ER^s; ρ^+ tester strain were patched onto glycerol complete and to erythromycin-supplemented glycerol complete media. The % of colonies that grew on both media is recorded as the % transmission of ER^r.
[2]Approximately 100 zygote colonies obtained from crossing the ρ^- strain to a ρ^+ tester strain were scored for their respiratory capacity by staining with tetrazolium. % suppressitivity was defined as the % of respiratory deficient plus mosaic zygote colonies minus the % of ρ^- colonies produced by the ρ^+ tester strain.

Segregation of ER^-; ρ^- from ER^r; ρ^-

To determine whether the observed differences in per cent transmission of erythromycin resistance by ER^r; ρ^- strains were caused by a heterogeneous mixture of ER^-; ρ^- and ER^r; ρ^- cells, several isolates were taken from ER^r; ρ^- strains of M2 and L411 and crossed to an ER^s; ρ^- tester. As seen in Table II all three strains (L411(B), M2(D) and L411(E) produced stable ER^-; ρ^- isolates incapable of transmitting the ER^r factor. The varying levels of per cent transmission of ER^r appear to be the result of the continuous segregation of ER^r; ρ^- cells to produce ER^-; ρ^- cells. This is particularly striking in the case of strain L411(B) which originally transmitted the ER^r factor to 80% of the zygotes. All 10 isolates of this strain failed to transmit the ER^r factor.

TABLE II

Spontaneous segregation of ER^r; ρ^- to produce ER^-; ρ^-

Strain	Isolate from strain	% transmission of ER^r	% suppressitivity
*			
L411(B): ER^r; ρ^-	1	0	65.70
	2	0	54.54
	3	0	93.51
	4	0	71.39
	5	0	63.90
	6	0	67.47
	7	0	88.47
	8	0	61.01
	9	0	66.84
	10	0	71.16
**			
M2(D): ER^r; ρ^-	1	0	17.02
	2	36	18.24
	3	48	17.09
	4	60	14.29
	5	0	29.17
	6	5	28.28
	7	76	21.11
	8	0	13.75
	9	0	18.33
***			
L411(E): ER^r; ρ^-	1	68	70.48
	2	0	90.85
	3	84	82.96
	4	84	40.75
	5	76	30.89
	6	88	24.48
	7	24	78.41
	8	8	84.68
	9	72	75.83
	10	36	72.64

*% transmission of ER^r by L411(B) was 80% and % suppressitivity was 74.55.
**% transmission of ER^r by M2(D) was 64% and % suppressitivity was 20.60.
***% transmission of ER^r by L411(E) was 79% and % suppressitivity was 60.66.

The Effect of ER^-; ρ^- Cytoplasm on the Transmittance of ER^r by an ER^r; ρ^- Strain

To determine whether the ER^-; ρ^- cytoplasm contained a factor(s) capable of the immediate destruction or inactivation of the ER^r factor, haploid strains ER^-; ρ^- and ER^r; ρ^- (derived from L411 and M2 respectively) were first crossed on minimal histidine medium to bring such hypothetical factor(s) in a common cytoplasm with the ER^r factor. The resultant histidine-requiring ρ^- diploid was then crossed to R37 on minimal media and ρ^- tetraploid $a/a/a/\alpha$ colonies were spotted onto glycerol complete and glycerol erythromycin-supplemented complete media to score the transmission of the ER^r factor. The tetraploidy of the final cross ((L411) $\times$ (M2)) $\times$ (R37) was verified by tetrad analyses which produced tetraploid inheritance for the parental markers uracil, tryptophan and leucine auxotrophy. Five ER^-; ρ^- strains from L411 were crossed to a common ER^r; ρ^- strain from M2 (continuous segregation of ER^r; ρ^- to

produce ER^-; ρ^- necessitated the crossing of a sufficient number of ER^r; ρ^- cells to ensure that they were not all ER^-; ρ^-). Twenty diploid ρ^- colonies from each of the five crosses were then crossed to R37 and scored for transmittance of ER^r. Erythromycin resistance was transferred to R37 by all five of the ρ^- diploids formed by the cross between ER^-; ρ^- strains derived from L411 and the common M2 parent carrying the ER^r factor. Thus, it appears that the ER^-; ρ^- cytoplasm does not contain a factor(s) capable of the immediate destruction or inactivation of the ER^r factor.

Discussion

The somatic segregation observed for ER^r; ρ^+ strains is outlined in Fig. 1. Loss of the ER^r factor was observed only in those cells that had become ρ^-. ER^-; ρ^- cells were stable and did not revert to the ρ^+ or ER^r phenotype. Genetic analyses of the cytoplasm of ER^-; ρ^- strains indicated that they did not contain element(s) capable of the immediate destruction or inactivation of the ER^r factor. All ρ^- cells were suppressive and thus carried the SF.

The dependence of the ρ^- and ER^- phenotype upon the spontaneous generation of the SF implicates the SF as the causative agent in producing the somatic segregation observed. If it is granted that abnormal MDNA is the SF, the model of 'Darwinian' selection of MDNA adequately explains the results obtained. The model predicts that acquisition of an abnormal MDNA molecule(s) with replicative superiority potentiates a cell's progeny to contain abnormal MDNA as the only species of MDNA. However many cell divisions may occur before abnormal MDNA becomes the sole molecular species. Mosaic colonies consisting of a respiratory-deficient base and a respiratory-sufficient apex (Ephrussi, Jakob and Grandchamp, 1966; Rank and Person, 1969) indicated the presence of a heteroplasmic cell containing normal and abnormal MDNA. The heteroplasmic state could be maintained by selection pressure (Rank and Person, (1969), indicating that abnormal MDNA is not able to immediately dominate the cell.

Similarly, this model predicts that an ER^r; ρ^+ cell becomes an ER^r; ρ^- cell through the spontaneous generation of the SF, producing a cell heteroplasmic for normal (that MDNA which codes for erythromycin resistance) and abnor-

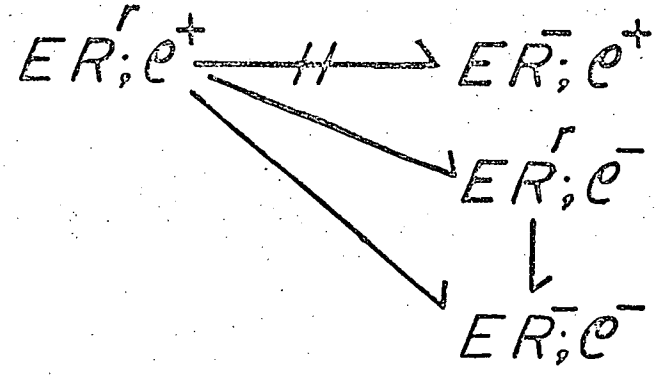

Fig. 1. Somatic segregation of the ER^r factor and its relationship to the SF (all ρ^- isolates contained the SF).

mal MDNA (the SF). That abnormal MDNA has to be generated in order to eliminate the ER^r factor is supported by the fact that ER^-; ρ^+ cells were not observed. The replicative advantage of abnormal MDNA potentiates an ER^r; ρ^- cell to produce progeny containing only abnormal MDNA, thus eliminating the genetic information for erythromycin resistance. The model of 'Darwinian' selection correctly predicts that ER^r; ρ^- cells would be unstable and constantly segregate to produce ER^-; ρ^- progeny containing only abnormal MDNA. All ER^-; ρ^- cells would be stable and unable to generate information for erythromycin resistance or respiratory sufficiency since this information is lost by the fast replicating abnormal MDNA.

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Résumé

La présente étude se rapport à l'effet du facteur suppressif de *Saccharomyces cerevisiae* sur la transmission cytoplasmique de la résistance à l'erythromycine. La disparition de la résistance à l'érythromycine est liée au facteur suppresif et les variétiés que le portent engendrent continuellement des cellules dépourvues de resistance. Nous suggérons que le facteur suppressif est dû à la présence de DNA mitochondrique anormal se dédoublant rapidement et que la disparition de résistance à l'érythromycine (portée par la DNA mitochondrique normal) provient de la plus grande capacité de dedoublement du DNA anormal.

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APPENDIX C – Genetic evidence for "Darwinian" selection at the molecular level. II. Genetic analysis of cytoplasmically-inherited high and low suppressitivity in *Saccharomyces cerevisiae*.

Genetic Evidence for "Darwinian" Selection at the Molecular Level: II Genetic Analysis of Cytoplasmically - Inherited High and Low Supressitivity in *Saccharomyces cervisiae*.

GERALD H. RANK.

Department of Botany, The University of British Columbia, Vancouver 8, British Columbia.

SUMMARY

A method was devised for the genetic analysis of cytoplasmically - inherited high and low suppressitivity in *S. cervisiae* thus enabling a test of the prediction, by the model of "Darwinian" selection of mitochondrial DNA, that abnormal mitochondrial DNA of a high suppressitivity strain has a replicative advantage over abnormal mitochondrial DNA of a low suppressitivity strain. Support for the model was indicated by the ability of the suppressive factor resident in the high suppressitivity strain to control the phenotypic expression of suppressitivity in zygotes formed by crossing a low and high suppressitivity strain.

INTRODUCTION

A model has been proposed (Carnevali, Morpurgo and Tecce, 1969; Rank and Person, 1969; Rank, 1970) to explain the suppressitivity (the suppression of the respiratory - sufficient (RS) phenotype in zygote colonies arising from conjugation between an RS and a respiratory - deficient (RD) cell) of cytoplasmically - inherited respiratory deficient strains of yeast. The cytoplasmically - inherited genetic element of the RD cell responsible for the conversion of an RS by RD zygote to an RD

colony is referred to as the suppressive factor. The proposed model assumes that spontaneously generated abnormal mitochondrial DNA is the suppressive factor and that such abnormal mitochondrial DNA has a replicative superiority over normal mitochondrial DNA.

A specific prediction of this model is that an RD strain exhibiting high suppressitivity (producing a low frequency of RS zygote colonies when crossed to an RS strain) would contain abnormal mitochondrial DNA with a replicative superiority over the abnormal mitochondrial DNA of an RD strain exhibiting low suppressitivity (producing a high frequency of RS zygote colonies when crossed to an RS strain). The following report describes a method for the genetic analysis of crosses between high and low suppressitivity strains which was used to test this prediction, and presents the results of studies in which the method was used. As expected, the phenotypic expression of suppressitivity in such crosses was controlled by the suppressive factor resident in the high suppressitivity strain.

MATERIALS and METHODS

A detailed description of strains, media and methods of genetic analyses is given in an earlier report (Rank, 1970).

Genetic Strains: Haploid strain L49: α ur his ; ρ^- was isolated as a ρ^- derivative of L411: α ur his ; ρ^+. Haploid strains M2-1, M2-2, M2-3 and M2-4 arose as spontaneously arising ρ^- isolates from M2: a tr_2 ad_1 his; ρ^+. Suppressitivity of all ρ^- strains (α, a, or a/α) was determined by crosses to the diploid strain R175: α/α leu_1/leu_1 ad_6/ad_6; ρ^+.

Determination of suppressitivity: The suppressitivity of a ρ^- strain

can be determined only by crossing it to a ρ^+ strain since suppressitivity is defined by the percentage of colonies resulting from such a cross that are RS. Haploid ρ^- strains of mating type α or a were first crossed to R175 (to produce an $\alpha/\alpha/\alpha$ or a/a/a triploid) to define high and low suppressitivity. L49, selected as the common high suppressitivity parent, was then crossed to four low suppressitivity strains derived from M2 thus producing an a/α his/his;ρ^- diploid. This ρ^- diploid was then crossed to R175 (to produce an a/α/α/α tetraploid) to determine which of the suppressive factors (the factor for high suppressitivity from L49 or the factor for low suppressitivity from the ρ^- isolate of M2) controlled the phenotypic expression of suppressitivity.

Variation of suppressitivity within any haploid or diploid ρ^- strain was determined by sampling 18 to 20 individual colonies from each strain and crossing them individually to R175. An average of 144 zygote colonies from each cross was then examined in order to determine the level of suppressitivity.

All cultures were shaken in 2% glucose - 1% yeast extract - 2% bacto-peptone medium at 30°C for 48 hours to a final concentration of 2×10^8 cells per ml before crossing. Samples of 0.5 ml were then taken from pairs of parental cultures and placed in a common tube for three hours at 30°C prior to spreading on selective media that allowed only the growth of zygotes. Tetrozolium staining of three - day - old zygote colonies determined their respiratory capacity.

Copulation mixtures of L49 and ρ^- isolates of M2 were plated on 2% glucose, histidine - supplemented minimal media which permitted only the growth of the a/α his/his;ρ^- zygote. Crosses $\alpha;\rho^-$ or a/$\alpha;\rho^-$ by R175

produced 50 to 200 zygote colonies when 0.05 ml of the undiluted copulation mixture was spread onto 2% glucose, minimal media. The copulation mixture of R175 by ρ- isolates of M2 yielded 50 to 200 zygote colonies when 0.02ml of a 1/10 dilution was spread on 2% glucose, minimal media.

RESULTS

Variation of suppressitivity within a ρ- line: As reported earlier (Rank and Person, 1969) all ρ- strains produced three types of colonies (RS, PA (colony with a RD base and a RS apex) and RD) when crossed to R175. The mean percentage and standard error of the mean of PA and RD zygote colonies from crosses of different ρ- lines to R175 are recorded in Table 1. It is clear that there is enough uniformity among isolates within a ρ- strain to allow the clear definition of high (Fig. 1) and low (Fig. 2) suppressitivity. For example, the Students "t" 95% confidence limits for the mean percentage RD colonies in the crosses L49, M2-1, M2-2 M2-3 and M2-4 by R175 are 61.13 $\pm$ 3.96, 24.04 $\pm$ 10.06, 11.61 $\pm$ 2.18, 13.26 $\pm$ 3.01 and 7.48 $\pm$ 1.58 respectively. However, it must be noted that most ρ- strains contain a low frequency (approximately 1 to 5%) of isolates that deviate greatly from the mean percentage of RS, PA and RD colonies. For example, the range in percentages of RD zygote colonies produced by isolates from L49, M2-1, M2-2, M2-3 and M2-4 was 35.00 to 75.00, 21.70 to 78.38, 2.48 to 21.05, 0.10 to 25.71 and 2.11 to 13.53, respectively. A similar variation in frequency of RS, PA and RD colonies was observed when isolates of diploid ρ- strains were crossed to R175 (see Fig 3 and 4). This serves to emphasize that genetic analysis of suppressitivity requires that suppressitivity be determined on an adequate

number of isolates of a ρ- strain to obtain a clear estimate of the variation within the ρ- strain.

Alteration of suppressitivity by ploidy level of the ρ- strain: Haploid ρ- cells were crossed with R175 in the original definition of high and low suppressitivity whereas diploid ρ- cells were crossed with R175 in the genetic analysis of high and low suppressitivity. Therefore it was necessary to determine if the suppressive factor, resident in haploid L49, produced an altered suppressitivity when present in an a/α; ρ- diploid. Diploid a/α his/his; ρ- colonies were isolated from the cross L49 by M2. The suppressive factor present in such a/α his/his; ρ- cells originated in L49 since M2 is a respiratory sufficient strain. As seen in Table 1 the suppressitivity of haploid cells (61.13% RD zygote colonies) is lower than that of diploid cells (88.08% RD zygote colonies) which contain the same suppressive factor. The presence of the suppressive factor of L49 in a diploid cell, in the genetic analysis of high and low suppressitivity, requires that 88.08 rather than 61.13 be taken as the expected percentage of RD zygote colonies for such an analysis.

Genetic analysis of high and low suppressitivity: Without exception the suppressitivity of the ρ- diploid (formed by the fusion of a high with a low suppressitivity cell) was that expected if the suppressive factor from the highly suppressive cell was regnant (see Table 1). For example, the percentage of RD zygote colonies was increased from 24.04 to 92.02, 11.61 to 82.22, 13.26 to 89.77 and 7.48 to 87.83 when the suppressive factor from L49 was added to the cells of strain M2-1, M2-2, M2-3 and M2.4, respectively. The high percentage of RD tetraploid zygote colonies (92.02, 82.22, 89.77 and 87.83) is adequately explained by the

presence in the diploid ρ- cell of the suppressive factor of L49 which produced 88.08% RD tetraploid zygote colonies.

DISCUSSION

The model of "Darwinian" selection of mitochondrial DNA, with abnormal mitochondrial DNA equated with the suppressive factor, adequately predicts the results observed. A ρ- strain exhibiting high suppressitivity is expected to have an abnormal mitochondrial DNA with a replicative superiority over the abnormal mitochondrial DNA of a low suppressitivity strain. When these two mitochondrial DNA molecules are brought together in a common hybrid cell, the faster replicating abnormal mitochondrial DNA from the high suppressitivity strain should become the predominant molecular species in the majority of progeny cells. Such hybrid cells should exhibit the phenotypic expression of high suppressitivity, as was observed (Table 1).

Variation of suppressitivity within a haploid or diploid ρ- strain reflects the ability of mitochondrial DNA with a replicative inferiority to be maintained in some cell lineages for many cell generations. Heteroplasmic cells, containing both normal and abnormal mitochondrial DNA, could be maintained by selection pressure, indicating that abnormal mitochondrial DNA may not immediately become the sole molecular species (Rank and Person, 1969). Similarly, the maintenance of normal mitochondrial DNA for many cell generations was indicated by the presence of ρ- cells with the cytoplasmically inherited factor for erythromycin resistance (Rank, 1970). Chance segregation of only low suppressitivity mitochondrial DNA into a daughter bud would explain the infrequent occurrence of isolates

with greatly reduced suppressitivity. Similarly, the spontaneous generation of new forms of abnormal mitochondrial DNA with a greater replicative superiority would explain the infrequent occurrence of isolates with an unexpectedly high suppressitivity.

Support for the model of "Darwinian" selection comes from the demonstration by Diacumakos, Garnjobst and Tatum (1965) that the microinjection of mitochondria from a cytoplasmically - inherited Neurospora mutant (abn -1), into a single hyphal compartment, resulted in the suppression of the normal phenotype and the pleiotrophic phenotypic expression of the mutant (abn -1). Although they were unable to identify the active component in the mitochondrial preparation, recent biochemical studies of S. cerevisiae support the premise that "Darwinian" selection can occur at the level of mitochondrial DNA rather than at the level of mitochondrion. Mitochondrial DNA synthesis independent of mitochondrial division was observed in anaerobically grown yeast cells (which lack the typical mitochondrial profiles) by Fukuhara (1967). In addition, the requirement for normal synthesis of abnormal mitochondrial DNA is indicated by the ability of mitochondrial DNA synthesis in a ρ^- strain to respond to oxygen induction and glucose repression (Rabinowitz, Getz, Casey and Swift, 1969).

As an alternative to "Darwinian" selection, the classical hypothesis to explain suppressitivity would be that suppressitivity is caused by the formation of an aberrant protein. Genetic information for such an abberant protein would have to reside in mitochondrial DNA since suppressitivity is cytoplasmically inherited. Failure to extract 23S and 16S RNA from ρ^- mitochondria (Wintersberger and Viehhauser, 1968),

and the inability of ρ- mitochondria to carry out protein synthesis (Kuzela, Smigan and Kovac, 1969) suggest that an aberrant protein could not be produced within the mitochondrion. However, Fukuhara, Faures and Genin (1969) extracted RNA from whole cells of a non - suppressive ρ- strain (neutral petite)[1] that hybridized with mitochondrial DNA, which suggest that transcripts of abnormal mitochondrial DNA may be translated on cytoplasmic ribosomes. Further evidence that mitochondrial DNA transcripts may be translated on cytoplasmic ribosomes is the failure of antibiotics, which interfere with mitochondrial protein synthesis, to disrupt the protein synthesizing ability of mitochondria extracted from cells cultured in the presence of the antibiotic (Davey, Yu and Linnane, 1969). A necessary function of any postulated aberrant protein is the interference with normal mitochondrial DNA synthesis, since ρ- progeny of ρ- hybrid cells formed by crossing a suppressive ρ- strain with a RS strain are suppressive and therefore contain abnormal mitochondrial DNA (96% adenine plus thymine and 4% guanine plus cytosine (d(A-T)) (Mehrotra and Mahler, 1968; Bernardi, Carnevali, Nicolaieff, Piperino and Tecce, 1968). However, the mitochondrial DNA cistron that codes for the postulated aberrant protein must be maintained in its postulated mutant form since conversion to a d(A-T) copolymer would limit any translated protein to a non functional tyrosine - isoleucine polypeptide. The restrictions of

[1]The model of "Darwinian" selection predicts that mitochondrial DNA of a neutral petite would not have a replicative advantage over normal mitochondrial DNA. This is supported by the calculation (Fukuhara, Faures and Genin, 1969) that the small change in buoyant density of DNA taken from a neutral petite (1.685 g/ml of ρ+ to 1.682 g/ml for ρ-) represents only a 3% decrease in GC content. Mehrotra and Mahler (1968) report a neutral petite with mitochondrial DNA of a similar buoyant density (1.681 g/ml) and a thermal transition midpoint similar to normal mitochondrial DNA.

the above mechanism as well as the necessary high frequency of the postulated mitochondrial DNA mutation for an aberrant protein (1% of cells in a ρ+ strain become ρ-) argue against the hypothesis that suppressitivity is achieved at the level of translation.

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Table 1

Percentage of PA[1] and RD zygote colonies produced by crossing ρ- isolates to R175.

CROSS	Mean % PA zygote colonies	Mean % RD zygote colonies	Ploidy of zygotes	% PA and RD in R175	No. of ρ- colonies crossed to R175	Average No. of zygote colonies counted per cross
L49 by R175	26.74 ± 0.91[3]	61.13 ± 1.89[3]	3n	1.96	20	203
(L49 by M2;ρ+)[2] " "	6.23 ± 0.55	88.08 ± 0.69	4n	3.98	20	172
M2-1 " "	19.46 ± 3.24	24.04 ± 4.81	3n	3.24	20	53
(L49 by M2-1) " "	3.47 ± 0.58	92.02 ± 3.24	4n	2.48	20	208
M2-2 " "	0.86 ± 0.29	11.61 ± 1.04	3n	1.01	20	58
(L49 by M2-2) " "	5.12 ± 0.63	82.22 ± 0.70	4n	2.28	20	88
M2-3 " "	7.59 ± 2.31	13.26 ± 1.44	3n	1.48	18	97
(L49 by M2-3) " "	7.11 ± 2.88	89.77 ± 4.14	4n	4.04	20	257
M2-4 " "	0.72 ± 0.15	7.48 ± 0.76	3n	3.22	20	171
(L49 by M2-4) " "	7.72 ± 0.91	87.83 ± 3.08	4n	6.33	19	137

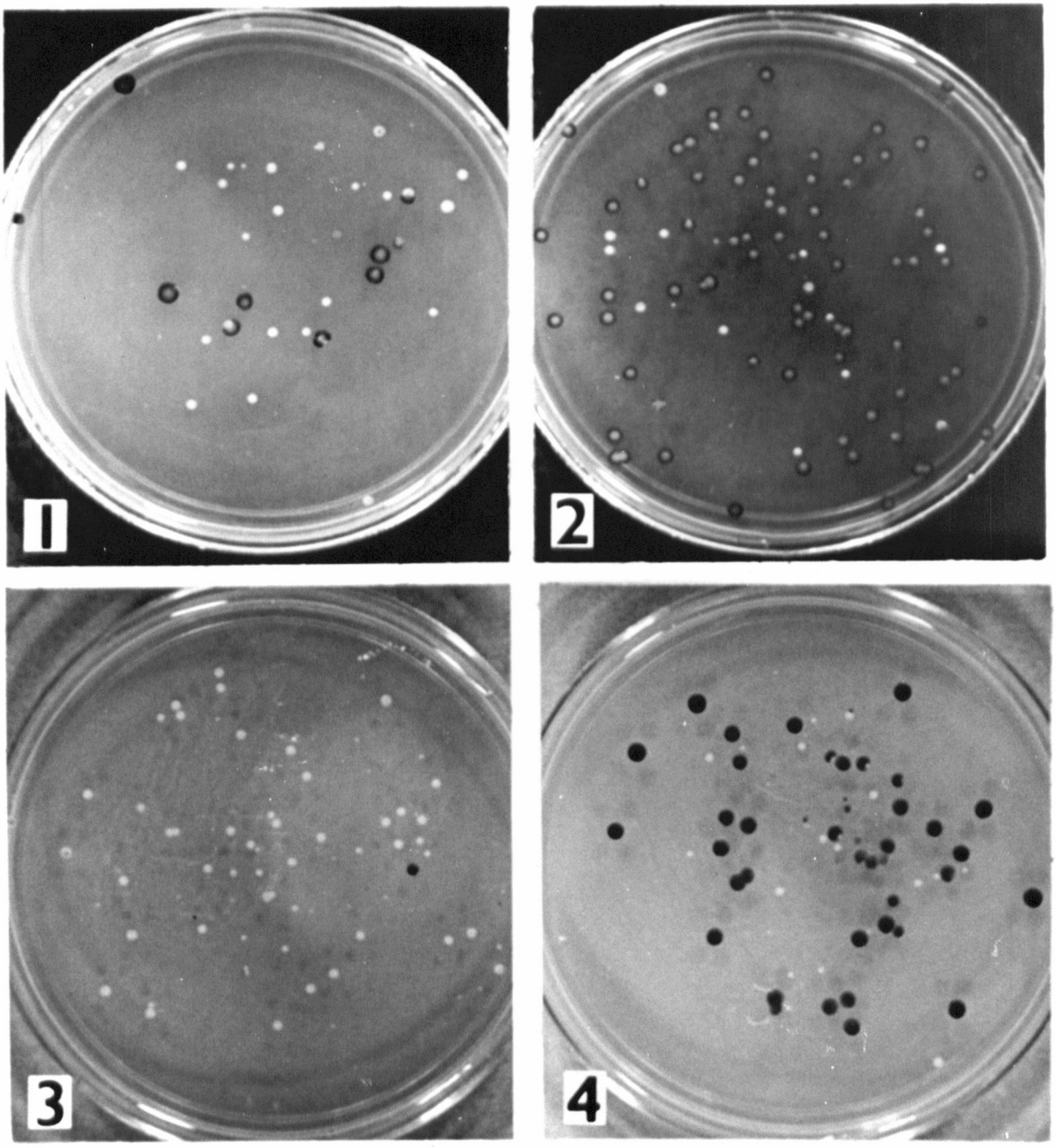
1 Mosaic colonies with a respiratory deficient base and a respiratory sufficient apex.
2 a/α, his/his; ρ- zygote colonies were selected and then crossed to R175.
3 Standard error of the mean.

Figure 1. Triploid ($\alpha/\alpha/\alpha$) zygote colonies from the cross L49 by R175. The dark area or sector is RS and the light area or sector is RD.

Figure 2. Triploid ($\underline{a}/\alpha/\alpha$) zygote colonies from the cross M2-3 by R175.

Figure 3. Tetraploid ($\underline{a}/\alpha/\alpha/\alpha$) zygote colonies from the most prevalent isolate of the cross (L49 by M2-3) by R175.

Figure 4. Tetraploid ($\underline{a}/\alpha/\alpha/\alpha$) zygote colonies from a rare isolate of the cross (L49 by M2-3) by R175.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