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MUTATION, RECOMBINATION, AND ANEUPLOIDY

M.L. Dixon
(Ph.D. Thesis)

September 1983

Donner Laboratory

Biology &
Medicine
Division

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A YEAST SCREENING SYSTEM FOR THE DETECTION OF MUTATION, RECOMBINATION, AND ANEUPLOIDY

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September 1983

ABSTRACT

Two diploid strains of the yeast Saccharomyces cerevisiae were constructed to detect a wide spectrum of genetic damage. Strain XD99 and its related strains can detect chromosome loss, important in the induction of teratogenesis, aneuploidy, and possibly carcinogenesis. Two positive selection techniques were developed which select for the simultaneous loss of both arms of chromosome X. Confirmation of chromosome loss using a centromere-linked marker was found to be essential for distinguishing chromosome loss from coincident crossing-over. In suspension tests para-fluorophenylalanine was found to induce chromosome loss whereas mitomycin C primarily induced coincident crossing-over.

The high selectivity of strain XD99 allowed the development of a spot test for chromosome loss. Methyl benzimidazole-2-yl-carbamate and ethyl methanesulfonate, found to induce chromosome loss in other yeast systems, induced chromosome loss in the spot test. Two carcinogens not tested for chromosome loss in yeast before, 4-nitroquinoline-N-oxide and N-methyl-N'-nitro-N-nitrosoguanidine, also induced chromosome loss. Chromosome X monosomics were found to be unstable and were restored to euploidy. This restoration of euploidy may have important implications regarding chromosome loss as a mechanism of

promotion.

Strain XD83 can detect multiple genetic changes:
nuclear frameshift and base pair substitution mutations,
nuclear mitotic crossing-over and gene conversion and
mitochondrial large deletions and forward point mutations.
The known carcinogens ethyl methanesulfonate, ICR-170,
N-methyl-N'-nitro-N-nitrosoguanidine, 4-nitroquinoline
-N-oxide, and ultraviolet light and the questionable
carcinogen, ethidium bromide, were tested. None of the
carcinogens were specific in their induced spectrum of
damage. All of the carcinogens induced multiple genetic
effects. Only ethidium bromide induced a highly specific
spectrum of damage: petite induction. This two strain
system may be used to screen agents for their genetic
toxicity. The variety of endpoints monitored may allow the
detection of certain carcinogens and other genetically
toxic agents which have escaped detection in other systems.
Furthermore, this system may be useful in the study of
possible mechanisms of carcinogenesis and aneuploidy.

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I. INTRODUCTION

Two diploid strains of the yeast <u>Saccharomyces</u> <u>cerevisiae</u> were constructed for screening genetically toxic agents. Many such DNA damaging agents are carcinogens (Ames et al., 1973a), some are teratogens (Connors, 1975), some induce aneuploidy (Griffiths, 1982), and some may even be involved in atherosclerosis (Benditt and Benditt, 1973) and aging (Burnet, 1974). Unfortunately, many of these agents are present in our environment. Short-term screening systems can help identify these DNA damaging agents and may help prevent the introduction of new agents.

To screen potentially genotoxic agents a number of screening systems are needed. Most short-term screening systems are being used to identify potential carcinogens. Although it appears that carcinogenesis is a multistep process and possibly a series of genetic changes may be involved, many of the existing screening systems can monitor only one or a few genetic endpoints. In addition, a eukaryotic system is required for monitoring chromosomal changes such as recombination and aneuploidy. The two yeast strains constructed for this study are able to screen for multiple genetic changes quickly and inexpensively. In addition, known carcinogens and promoters can be tested in this system to see if certain types of genetic damage are correlated with carcinogenicity. Furthermore, each type

of genetic damage studied here is significant and merits
monitoring in its own right.

A. Rationale for Short-Term Screening Systems

It has been estimated that 70 to 90% of all cancers are
environmentally related (Higginson, 1969). Although this
is probably not the percentage of cancer that is
preventable (Higginson, 1976; Higginson and Muir, 1979),
the identification of carcinogens in the environment may be
a step in reducing risk. Ames' group has found a 85 ± 5%
correlation between carcinogenicity in animal systems and
mutagenicity in a Salmonella/microsome assay using 300
chemicals (McCann et al., 1975a; McCann and Ames, 1976;
Ames and McCann, 1981). This high correlation supports the
somatic mutation theory of carcinogenesis (Boveri, 1929).
Since it is time consuming and costly to do mammalian tests
on each suspect chemical, this strong correlation also
suggests the utility of microbial systems for screening
possible carcinogens as mutagens (Ames, 1979).

In addition to a possible mutational component to
cancer, Benditt and Benditt (1973) have proposed that
atherosclerosis may also have a mutational origin. The
monoclonal nature of atherosclerotic plaques suggests that
these plaques may have originated from one mutated cell.
Benditt and Benditt have further proposed that chemical
mutagens may be carried through the blood by serum

lipoproteins to the smooth muscle cells of the arterial walls where they are released and cause mutations. Shu and Nichols (1979) have demonstrated the uptake of benzo(a)pyrene by human blood lipoproteins in vitro. Serum lipoproteins have been found to form complexes with some carcinogens (Avignan, 1959; Chalmers, 1955) and lipoprotein receptors have been found in several mammalian cell lines (Brown and Goldstein, 1975; Brown and Goldstein, 1976; Ho et al., 1976; Bierman et al., 1974).

Aside from the possible mutational component in cancer and heart disease, mutation per se can have serious consequences. Although accurate estimates are difficult to make, it appears that there is a strong genetic component in much of human disease. There are approximately 1000 different dominant and X-linked hereditary diseases in man (Vogel, 1979) including nine forms of hereditary neoplasms (Knudson et al., 1973). Bilateral retinoblastoma is an example of one such hereditary neoplasm (Knudson, 1971 and 1977). Additionally, there are over 1000 known recessive diseases (Vogel, 1979) including sickle cell anemia, cystic fibrosis, and Tay Sachs disease.

Mutations in germinal cells are heritable and can lead to an increase in the gene burden of the population. A mutation in a developing somatic cell is not heritable but may cause a developmental defect, teratogenesis, or possibly a neoplasm in that individual. In certain syndromes, such as Wilms' tumor-aniridia syndrome, the

coexistence of birth defects and cancer suggests a common
etiologic mechanism perhaps occurring in early gestation
(Knudson and Strong, 1972). Likewise certain germinal cell
defects correlate with a high incidence of cancer. A
notable example is the high incidence of leukemia in Down's
syndrome (Stewart et al., 1958).

Aneuploidy contributes significantly to embryonic,
fetal, and infant mortality (for a review see Hook, 1983)
and is responsible for a number of human cytogenetic
abnormalities including the well-known Down's syndrome.
Approximately 50% of spontaneous abortions and about 0.6%
of liveborns have chromosomal abnormalities, primarily
resulting from aneuploidy (for a review see Sankaranarayan,
1979). Figure 1 summarizes the estimated proportion of
aneuploidies per million human conceptions. Usually
aneuploidies involving the autosomal chromosomes have more
severe phenotypic effects than those involving sex
chromosomes: generally autosomal monosomies are lethal and
autosomal trisomies frequently involve severe abnormalities
whereas sex-chromosomal aneuploidies may have less severe
abnormalities (Sankaranarayanan, 1979).

The proportion of mitotic aneuploidy is difficult to
estimate. However, an early mitotic event might lead to
severe abnormalities and some cases of chromosomal
aneuploidy are mosaic and probably have arisen from mitotic
events (Sankaranarayanan, 1979). The possible
contribution of aneuploidy to carcinogenesis will be

```
                              1,000,000
                             CONCEPTIONS

              850,000                          150,000
             LIVEBIRTHS                    SPONT. ABORTIONS

     833,000              17,000               75,000
     CHILDREN        PERINATAL DEATHS       CHR. ABNORMAL

     5165
  CHR. ABNORMAL
                                                   39,000 TRISOMICS
                                                   (3,510 TRISOMY-21)

             1849 SEX CHR.       1427 MALES        13,500 45,X
             ANEUPLOIDS
                                 422 FEMALES       12,750 TRIPLOIDS

                                 42 PATAU'S SYNDR. (+D)   4,500 TETRAPLOIDS
             1183 AUT.
             TRISOMICS           100 EDWARD'S SYNDR. (+E)  5,250 OTHERS
                                 1041 DOWN'S SYNDR. (+G)

             758 BALANCED
             ROBERTSONIAN T

             758 BALANCED
             RECIPROCAL T

             117 INVERSIONS

             500 UNBALANCED
             STR. ABERRATIONS
```

Figure 1.   Estimated proportion of aneuploidy in humans (redrawn from
            Sankaranarayanan, 1979)

discussed in the next section. Although certain agents
which induce aneuploidy also induce cancer, the
relationship appears to be founded primarily on those
agents which induce mutations. Mutation, however, is not
the only nor probably the primary means of inducing
aneuploidy. For example, agents which disrupt the spindle
apparatus might induce aneuploidy but not mutation.

With the above considerations in mind, the
characteristics of a good screening system are that it is
fast, inexpensive, reliable, reproducible, easy to use,
well-characterized, and extrapolates well to humans.

B. Carcinogenesis as a Multi-Stage Process

Carcinogenesis appears to be a multi-stage process.
The simple two-stage model of carcinogenesis based on
studies in mouse skin, i.e., initiation followed by
promotion, has been given particular attention. Some of
the major current thoughts concerning this model and the
carcinogenic process in general are outlined below.

1. Initiation

The initiation step was proposed to be a mutation step
in Boveri's somatic mutation theory of cancer (Boveri,
1929). This theory had little experimental support in its
early days because mutagenicity and carcinogenicity were

not correlated for many agents (Burdette, 1955). However, support and interest arose after it was realized that metabolic activation is necessary for many carcinogens to act as mutagens (Miller and Miller, 1966 and 1971). It is known that many chemical carcinogens can cause DNA adducts through electrophilic interactions with nucleophilic centers in the DNA (Miller and Miller, 1971). As previously mentioned, the somatic mutation theory has gained further support and acceptance through the correlation seen between mutagenicity and carcinogenicity, most notably in the Salmonella/microsome assay (McCann et al., 1975a; McCann and Ames, 1976; Ames et al., 1975; Bridges, 1976). DNA damage and repair and its relationship to cancer has also provided support for this theory (for a review see Setlow, 1978).

The viral oncogene hypothesis originally proposed by Huebner and Todaro (Huebner and Todaro, 1969; Todaro and Huebner, 1972) now complements the somatic mutation theory of cancer. Virally induced carcinogenesis is known to occur in a number of mammals and is postulated to be involved with certain specific human cancers: Burkitt's lymphoma and nasopharyngeal carcinoma from the Epstein-Barr virus and hepatocellular carcinoma from hepatitis B virus (Temin, 1980). Nevertheless, viruses are not believed to be a major source of human cancer (Temin, 1980). Oncogenes, transforming or cancer genes, were first found in the retroviruses, formerly called RNA tumor viruses (for

reviews on retroviruses see Varmus, 1982 and Bishop, 1983).
Homologs of these retroviral oncogenes have been found in
vertebrate cells (Bishop, 1981; Cooper, 1982). Moreover,
recent evidence indicates that the retroviruses received
their oncogenes from vertebrates (Bishop, 1981, 1982)
rather than vice versa as originally believed. These
proto-oncogenes from normal vertebrate cells are highly
conserved in evolution (Spector et al., 1978; Bishop, 1981)
which suggests that they may have an important, normal
function(s) in the cell. Possibly activation of these
proto-oncogenes results in cancer or the proto-oncogene is
mutated to an oncogene.

Recently, in fact, Shih and Weinberg (1982) isolated a
DNA sequence from the EJ human bladder carcinoma line
capable of transforming NIH/3T3 mouse fibroblasts. No
major rearrangements were found between the transforming
genes and their normal cellular counterparts although these
oncogenes transformed cells whereas the normal genes did
not. Similarly, Goldfarb et al. (1982) isolated and made a
preliminary characterization of a human transforming gene
from a T24 bladder carcinoma cell line. Subsequent studies
have shown that the genetic lesion, a G-T transversion
localized to the Gly-12 codon, in the oncogene of the human
EJ bladder carcinoma cell line had no effect on levels of
expression of the oncogene (Tabin et al., 1982).
Sequencing of the proto-oncogene and the T24 human bladder
carcinoma oncogene has also shown a single base change, a

G-T transversion, between the transforming gene and the
non-transforming gene (Reddy et al., 1982). This single
base pair substitution of guanosine to thymidine results in
the incorporation of a valine in lieu of a glycine into the
twelfth amino acid residue of the T24 oncogene-encoded p21
protein. This single amino acid substitution seems
sufficient to confer transforming properties to the gene
product of the T24 human bladder carcinoma oncogene (Reddy
et al., 1982). Similarly, Taparowsky et al. (1982) have
found the activation of the T24 bladder carcinoma
transforming gene is linked to a single amino acid change
in the coding region of the gene. However, they also note
that in addition to low levels of an altered gene product
mediating transformation so does overproduction of the
normal gene product. Other studies have also found that
the normal gene acquires transforming activity when
physically linked to a high efficiency retroviral promoter
(Chang et. al., 1982).

This single base pair change in oncogenes furnishes
strong support for a somatic mutation theory of cancer in
which cellular proto-oncogenes are oncogenetically
activated by a carcinogen (Bauer, 1928; Burdette, 1955;
Burnet, 1978). Nevertheless, the evidence in oncogenes
that one mutagenic step may lead to transformation must be
reconciled with the multi-stage model of carcinogenesis and
the usual latency period seen with most cancers. Possibly,
the NIH/3T3 cells used in these studies are pre-neoplastic

and already may be only one or a few steps away from transformation. It appears that besides changes in the proto-oncogene additional events are necessary for the development of cancer.

An alternative to the somatic mutation theory states that DNA rearrangements are the initiating events in cancer. Radman et al. (1982) claim that all carcinogens that have been tested thoroughly have induced some kind of chromosomal rearrangement. Carcinogens, Radman et al. (1982) suggest, may create or reveal sites for recombination or may induce or activate cellular systems leading to an increase in recombination. These rearrangements may affect carcinogenesis by changing gene expression or by allowing the activation of cellular oncogenes. Cairns (1981) has suggested that human cancer may be the result of genetic transpositions involving possibly transposons. Klein (1981) further suggests that genetic transpositions may result in the increased expression of normal cellular oncogenes. This increased expression of the cellular oncogene may be due to recombination with a powerful retroviral promoter (see Neel et al., 1981 for a discussion of the promoter insertion model of carcinogenesis) or from chromosomal translocation to a highly active region of the cellular genome. Fahmy and Fahmy (1980) have found that carcinogens besides inducing mutation can alter gene expression above that expected for mutation which supports the aberrant

differentation theory of cancer.

A number of oncogenic recombinations have been reported. Recent evidence on the translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas suggests that chromosomal abnormalities may be regarded as mistakes in recombination occurring in regions of the genome which are characterized by high levels of physiologic recombination (Dalla-Favera, 1983). Shen-Ong et al. (1982) have reported on a novel myc oncogene RNA from abortive immunoglobulin-gene recombination in mouse plasmacytomas. The recombination did not, however, alter the level of myc RNA which suggested that DNA rearrangements have altered the myc oncogene product. A cellular oncogene has been found to be translocated to the Philadelphia chromosome in chronic myelocytic leukaemia in a reciprocal exchange (de Klein et al., 1982). Rechavi et al. (1982) have reported on the activation of a cellular c-mos oncogene by a DNA rearrangement in a mouse myeloma. Furthermore, a number of deletions associated with translocations have been linked to certain human cancers: familial renal cell carcinoma (Pathak et al., 1982), retinoblastoma (Strong et al., 1981), and Wilms' tumor (Kaneko et al., 1981). Yunis (1983) has recently reviewed the evidence from high-resolution banding techniques that shows most of the human cancers studied have characteristic chromosomal defects.

A large number of carcinogens have been tested in yeast and many have been found to be recombinogenic (Murthy, 1979; Simmon, 1979). Among those carcinogens that are recombinogenic in yeast are a number of carcinogens that did not show mutagenicity in the standard Salmonella/ microsome assay (McCann et al., 1975a): thioacetamide, natulan, auramine, safrole, and 1'-hydroxysafrole (Simmon, 1979). Other recombinogenic carcinogens include actinomycin D (Nestman et al., 1981), bleomycin (Moore, 1978; Hannan and Nasim, 1978), and mitomycin C (Holliday, 1964; Takahashi, 1974; this study). However, it should be noted that recently Levin et al. (1982a) have developed a new Salmonella tester strain which is capable of detecting oxidative mutagens including bleomycin and mitomycin C. The anti-metabolite, methotrexate, which is not carcinogenic but which has transformed cells in culture has also induced recombination in yeast (Haynes, personal communication; Simmon, 1979). However, three metal carcinogens tested by Simmon (1979) were not recombinogenic in yeast nor were most of twenty-four metal carcinogens mutagenic or recombinogenic when tested by Singh (1983).

2. Promotion

Boutwell (1974) has proposed that there are at least two parts to the promotion phase: conversion of the inititated cell and propagation of the converted, initiated

cell. Berenblum and Armuth (1981) have proposed using the terms derepression and promotion to describe these two parts of promotion. They also consider the possibility that there may be separate and independent kinds of promotion instead of consecutive stages of a single type of promotion. They discuss evidence for the promoting action operating at the regulator gene level, the cell surface membrane, and cytoplasmic changes. The phorbol esters which are the most studied of the promoters, have been found not to bind to DNA and are negative in the Salmonella/ microsome assay (McCann et al., 1975a). However, recently, it has been proposed that tumor promoters may actually affect DNA by generating activated oxygen compounds that in turn damage the DNA (see Marx, 1983 for a short discussion of this). Ames et al. (1982) also suggest that promotion may involve damage to the cell membrane by lipid peroxidation (Demopoulos et al., 1980).

The promotion step (or steps) has been postulated to be a recombination process by Kinsella and Radman (1978) and Nagasawa and Little (1979). Kinsella and Radman (1978) found that the classic tumor promoter 12-o-tetradecanoyl-phorbol 13-acetate (TPA) induces sister chromatid exchanges in Chinese hamster cells. They suggest that TPA might induce enzymes involved in genetic recombination which could lead to the expression of an induced recessive genetic or epigenetic chromosomal change, and thereby complete a process begun by initiators. However, Kunz et

al. (1980) tested three promoters in yeast and did not see a consistent increase in recombination. Likewise, Parry et al. (1981) found that six promoters which they tested did not induce recombination in yeast. However, one incomplete promoter, mezerein, did induce recombination.

Chromosome loss and nondisjunction can lead to the expression of recessive genes through hemizygosity and to phenotypic changes from chromosomal imbalance. Therefore, these events may bring about promotion (for a review see Ohno, 1974). Kinsella and Radman (1978) argue, however, that one would expect chromosome loss to be effective before or after initiation which is not consistent with the finding that initiation must precede promotion. However, it is likely that a change of a 2n (diploid) cell to a 2n-1 (monosome) cell would be followed by reversion to 2n or be deleterious. Several studies in yeast have found that there appears to be a restoration of the euploid state following chromosome loss (Bruenn and Mortimer, 1970; Parry and Zimmermann, 1976; Esposito et al., 1982; Malone et al., 1980; Wood, 1982; this study). Recently, Parry et al. (1981) observed that all seven of the promoters they tested induced chromosome loss in yeast whereas six non-promoters, four of which were phorbol analogues, did not induce chromosome loss.

Although the Warburg theory (Warburg, 1931 and 1956) that tumor cells are grossly impaired in respiration does not seem to be universally true (Weinhouse, 1982),

nevertheless, more subtle changes due to a heritable mitochondrial abnormality could be involved in the carcinogenic process (Egilsson et al., 1979; Hoberman, 1975). Many carcinogens induce changes in the mitochondrial genome (Egilsson et al, 1979). Metal salts which have been technically a problem in the standard Salmonella/microsome assay (McCann and Ames, 1977; Hollstein and McCann, 1979) and thioacetamide and thiourea which were negative in the same assay (McCann et al., 1975a) have been shown to act as mitochondrial mutagens in yeast (Egilsson et al., 1979; Lindegren and Lindegren, 1973; Putrament et al., 1975 and 1977). In yeast the petite phenotype is due to a respiratory deficiency which is usually caused by a drastic change from large deletions in the mitochondrial DNA (mtDNA). The deletion process is followed by a tandem duplication of the remaining mtDNA and an amplification which restores the original quantity, but not information content, of the mtDNA (for a review see Borst and Grivell, 1978).

Pall (1981) has proposed that carcinogenesis may be due to a tandem duplication of a proto-oncogene followed by repeated sister strand crossing-over. If such is the case, then these changes may be monitored as an increase in recombinational events or even possibly as petite induction considering the parallels just mentioned.

C. Yeast as a Screening System

1. Rationale

The yeast _Saccharomyces cerevisiae_ is a single-celled
eukaryote with many of the screening advantages of a
prokaryote. It is relatively fast growing, inexpensive to
culture, genetically well-characterized (see Mortimer and
Schild, 1980 for the genetic map), and technically easy to
manipulate. It is a eukaryote and, therefore, its genetic
material is organized as chromatin, the meiotic and mitotic
cycles are typical of eukaryotes, and the cytoplasm
contains eukaryotic organelles and cytoskeletal structure.
Yeast has the further advantage of being able to exist as
either a stable diploid or haploid which allows it to be
genetically manipulated and recessive mutations to be
assayed. In addition, changes at the chromosomal level
such as recombination and nondisjunction can be seen in
yeast. Some tolerance of aneuploidy has been found in
yeast. Triploids yield about 18% viable meiotic products
and nearly all of these should be aneuploid (Campbell et
al., 1981). Multiple disomy has been found by Parry and
Cox (1971) and monosomy for a number of chromosomes by
Bruenn and Mortimer (1970). However, the mitotic stability
of different yeast disomes from triploid meiosis varies
and the number of different disomic chromosomes has a
skewed distribution from the expected binomial distribution

with a low mean which suggests that yeast do not tolerate high multiple disomy (Campbell et al., 1981). Hilger and Mortimer (1980) isolated strains stably disomic for up to five chromosomes which Campbell et al. (1980) suggest may be close to an upper limit for aneuploid tolerance.

Yeast are extremely versatile for screening chemicals. Like the bacterial assay systems, they can be used with an in vitro hydroxylation system (Mayer, 1971 and 1972), a mammalian activation system (Kelly and Parry, 1983) and urine metabolites (Marquardt and Siebert, 1971; Siebert, 1973; Siebert and Simon, 1973; Siebert et al., 1979). Exponentially growing yeast also contain their own endogenous activation system (Wiseman and Lim, 1975a and b) and can activate many promutagens to ultimate mutagens without further addition of exogenous activation material (Callen and Philpot, 1977; Callen et al., 1980; King et al., 1982). An advantage of endogenous activation is that the metabolite only needs to cross the nuclear membrane to reach the nuclear DNA. This may be important in detecting highly reactive and, therefore, short-lived free radicals which are intermediates in many types of radiation damage to biological systems and appear to be involved in chemical mutagenesis, carcinogenesis, and aging (Greenstock, 1981; Ames et al., 1982).

Unlike bacteria, yeast contain mitochondria, but unlike mammalian cells, the mitochodrial genome of yeast is nonessential and damage to it can be readily seen on

selective media and by phenotypic changes. Small deletions as well as point mutations in the mitochondria can be monitored as well by the induction of drug resistance (Grivell et al., 1973). Additionally, yeast can be used in a variety of treatment conditions: liquid suspension tests, spot tests (Fink and Lowenstein, 1969; Kunz et al., 1980), and host-mediated assays (Frezza et al., 1979 and 1983; Fahrig, 1971, 1973, 1974, and 1975). Although yeast have a pH optimum of 5-6, they can be used over a pH range of 3 to 9 (ICPEMC, 1983). Complex mixtures have also been tested successfully in a yeast assay system (Gairola, 1982).

The sensitivity of the Salmonella/microsome assay (Ames, 1971) to certain carcinogens was extended by adding a number of improvements: the deep rough mutation to remove a lipopolysaccharide penetration barrier (Ames et al., 1973b), R factor plasmids to increase error-prone recombination repair (McCann et al., 1975b), and liver homogenates to approximate mammalian metabolism (Ames et al., 1973a). Yeast do not have a lipopolysaccharide layer and the major barrier is the cell membrane and not the rigid cell wall (ICPEMC, 1983). Extensive studies have been made of the repair pathways and mutants in yeast (see reviews by Game, 1983; Haynes and Kunz, 1981; Lemontt, 1980). Repair-deficient strains have been used to study the repair pathways involved in chemical mutagenesis (Haynes and Kunz, 1981; Prakash, 1974 and 1976) and to detect chemical carcinogens in a repair assay (Sharp and

Parry, 1981).

For statistical purposes, yeast can be used in large numbers so that small effects can be seen. The cost and time involved in animal tests make large numbers a problem and frequently animal tests have suffered in sensitivity for this reason. A number of carcinogens were first discovered to be mutagens in short-term screening systems before their carcinogenicity was established (see Ames, 1979 for a discussion of five examples).

Several helpful reviews have been written on mutagenesis in yeast and more specifically with using yeast for mutagen screening (Brusick and Mayer, 1973; Loprieno, 1978; Kilbey, 1975; Lawrence, 1982; Mortimer and Manney, 1971; Zimmermann, 1973b, 1975). Yeast can also be used to detect a broad spectrum of genetic changes and for both forward and reverse mutation studies. Because they can detect a wide variety of genetic damage, they can be used to see correlations between different types of genetic damage and carcinogenicity.

2. Current yeast screening systems

The most widely used and known screening strains in yeast are those developed by Zimmermann and collaborators (Table 1). The D3 and D5 strains detect mitotic crossing-over using strains heterozygous at the ADE2 locus which can give color sectored colonies following mitotic

TABLE 1

Strains Developed by Zimmermann and Collaborators

Strain	Genotype	Genetic activity detected	Reference
D3	**a** CYH^R ade2 his8 α cyh^S ADE2 HIS8	mitotic crossing-over	Zimmermann et al., 1966
D4	**a** + ade2-1 trp5-27 + α gal2 ade2-2 trp5-12 leu1	mitotic gene conversion	Zimmermann and Schwaier, 1967
D5	**a** ade2-40 + leu1 + MAL4 α ade2-119 trp1 + MAL1 +	mitotic crossing-over	Zimmermann, 1973a
D6	**a** ade3 leu1 trp5 cyh2 met13 ade2-40 α + + + + + +	chromosome loss	Parry and Zimmermann, 1976
D7	**a** ade2-40 trp5-12 ilv1-92 α ade2-119 trp5-27 ilv1-92	mitotic crossing-over mitotic gene conversion mutation	Zimmermann et al., 1975

crossing-over (Zimmermann, 1973a). The D4 strain detects gene conversion at two independent loci (Zimmermann and Schwaier, 1967). The D6 strain detects mitotic chromosome nondisjunction of chromosome VII using heterozygosity at a number of sites on the chromosome (Parry and Zimmermann, 1976). The D7 strain simultaneously detects mitotic crossing-over, gene conversion, and reverse mutation (Zimmermann et al., 1975). Three other strains have also been developed which are less frequently used. They are D45 (Sherman and Roman, 1963) and the derivative strain D47 (Zimmermann and Schwaier, 1967) for detecting gene conversion and D_9J_2 (Parry et al., 1979a and b) for detecting nondisjunction.

Forward mutation in yeast usually has been monitored using either induction of canavanine resistance or a change in the color of the yeast colony. Wild-type yeast are sensitive to the toxic arginine analogue, canavanine. Mutation at the CAN1 locus which codes for the arginine permease confers resistance to canavanine in haploid yeast (Grenson et al., 1966; Whelan et al., 1979).

Forward mutation at the adel or ade2 locus results in the accumulation of a red pigment which results in a color change to pink or red from the wild-type cream white color of the yeast. Reverse mutation can, of course, be seen by using mutants which are red and mutate back to the cream color either through a reverse mutation at the site or a forward mutation either in a suppressor gene or an earlier

gene in the adenine biosynthetic pathway. The adenine locus has been used for recombination studies as well.

Sherman and collaborators have also made use of forward and reverse mutation at the cytochrome c locus to study the mutagenic spectrum of various agents (Sherman et al., 1974; Prakash et al., 1974; Lawrence et al., 1974).

The haploid strain XV185-14C (Mehta and von Borstel, 1981) contains four ochre suppressible markers, one missense marker, and one probable frameshift marker. Although useful for reversion studies, this strain is not suitable for recombination or aneuploidy studies since it is a haploid.

Mortimer, Brustad, and Cormack (1965) developed a strain, X841, which is capable of detecting a number of genetic effects: mitotic crossing-over, gene conversion, and mutation. The multiple genetic endpoints allowed a comparison of the relative biological effectiveness for inducing mutation, recombination, and lethality of various radiations as a function of linear energy transfer.

Recently, Esposito et al. (1982) developed a strain LBL1, disomic for chromosome VII, which simultaneously detects changes in chromosome number, mitotic gene conversion, and mitotic intergenic recombination using a change in color and expression of cycloheximide resistance. Further analysis can be used to distinguish the events from one another.

Fogel and Mortimer (1969) constructed strain BZ34 which

they used to study meiotic gene conversion. This strain
has been used in a number of studies for screening
chemicals for gene conversion (Murthy and Sankaranarayanan,
1975, 1978 a and b; Sankaranarayanan and Murthy, 1979).
Sankaranarayanan and Murthy (1979) report that this strain
is comparable in ability to detect DNA-damaging agents to
that of strains D4 and D7 developed by Zimmermann.

Mutational assay systems have been highly sensitive and
accurate in detecting carcinogens as mutagens. Certain
carcinogens, however, are not readily detected by reverse
mutational systems, but can be detected in recombinational
assay systems or in forward mutational systems.

3. A new yeast screening system

Two diploid yeast strains were constructed in this
study to monitor a broad spectrum of genetic damage.
Strain XD83 was derived from strain BZ34 (see above
discussion) and strain MC329 (Culbertson et al., 1977 and
1980) which can detect frameshift mutations. Strain XD83
represents a combination of a number of assay systems in
one strain. It can detect nuclear frameshift and base pair
substitution mutations, nuclear mitotic crossing-over and
gene conversion, and mitochondrial large deletions and
forward point mutations. All of these genetic changes have
been suggested as possible steps in the carcinogenic
process. A small, select number of widely used genotoxic

agents were tested both in suspension tests and spot tests with this system. A good agreement was obtained between the two methods.

Chromosome loss and nondisjunction are important mechanisms in the generation of chromosomal aneuploidies and developmental defects and may be involved in carcinogenesis. Since certain agents which affect chromosomal segregation, such as spindle poisons, may have no effect on other types of genetic phenomena, it is important to have an assay system for detecting this type of damage.

Strain XD99 and its related strains, XD26 and XD72, can detect chromosome loss and nondisjunction. Two positive selection techniques were developed which allow for selection of putative monosomics. An advantage of this system is that simultaneous loss of both arms of chromosome X is selected for simultaneously thereby greatly increasing the selectivity of the system. This chromosome loss system may be used for both suspension and spot testing. Further confirmation of chromosome loss is done using ochre suppressible markers in the strain.

II. MATERIALS AND METHODS

A. Yeast Strains

Heterothallic strains of Saccharomyces cerevisiae were used in all strain constructions and genetic analyses. Except where noted, all strains were derived from the stocks of Robert K. Mortimer and came from the Yeast Genetic Stock Center. The nomenclature and symbols for genetic markers were in accordance with the rules compiled by Sherman (1981). Strain List I (Table 2) lists the parent strains used for constructing the strains used in the screening tests (Strain List II, Table 3). The genealogy of the constructed strains are shown in Figures 2 and 3.

B. Descriptions of Markers Employed

The descriptions below are primarily from a compilation by Broach (1981). Further descriptions are given in the RESULTS section for certain markers. The map distances are from Mortimer and Schild (1980).

ade2-1: adenine auxotrophy; red pigment accumulates in colony. Gene product: phosphoribosylaminoimidazole carboxylase. Map position: 15R. Nonsense mutation;

TABLE 2

Strain List I

Strain	Genotype	Source
BZ34	**a** his5-2 lys1-1 + arg4-4 + thr1 + trp5-48 + ura3 ade2-1 α his5-2 lys1-1 pet1 + arg4-17 + leu1-12 trp5-48 met1 + ade2-2	YGSC
MC329	α his4-519 leu2-3 ade2-18 canR	M.C.
X3424-5D	**a** ilv3-1 trp1-1 lys1-1 gal2	YGSC
S1780C	α ura2-1 his6 thr1 arg4 met1 hom3 gal2	YGSC
X2182-3D(C59)	**a** his5-2 lys1-1 can1-100 trp5-48 ade2-1 met1-1 leu1-12 SUP4	YGSC
X2316-3C	α his5-2 lys1-1 can1-100 trp5-48 ade2-1 met1-1 ura3-1 SUP4	YGSC
X2383-5A	α his5-2 lys1-1 can1-100 trp5-48 ade2-1 ura3-1 SUP7	YGSC
X1687-12B	α his5-2 lys1-1 trp5-48 ade2-1 arg4-17 gal2	YGSC

YGSC = Yeast Genetics Stock Center, M.C. = Michael Culbertson

TABLE 3

Strain List II

Strain	Genotype										Source
XD26	**a**	ade2-1	his5-2 • lys1-1	can1-100	SUP7 •	+	SUP4 •	trp1-1	+		This work
	α	ade2-1	his5-2 lys1-1	can1-100	+	ilv3-1	+	+	asu		
XD72	**a**	ade2-1	his5-2 • lys1-1	can1-100	+ SUP7 •	+	SUP4 •	trp1-1	+		This work
	α	ade2-1	his5-2 lys1-1	can1-100	ura2-1 +	ilv3-1	+	+	asu		
XD99	**a**	ade2-1	his5-2 • lys1-1	can1-100	+ SUP7 •	+	SUP4 •	trp1-1			This work
	α	ade2-1	his5-2 lys1-1	can1-100	ura2-1 +	ilv3-1	+	+			
XD83	**a**	his4-519 • leu2-3	lys1-1	• +	arg4-4	+	ade2-18	$[\rho^+, \varepsilon^S, c^S]$			This work
	α	his4-519 leu2-3	lys1-1	pet1	+	arg4-17	+				

asu = antisuppressor

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GENEALOGY OF MUTATION/RECOMBINATION STRAIN
(XD83)
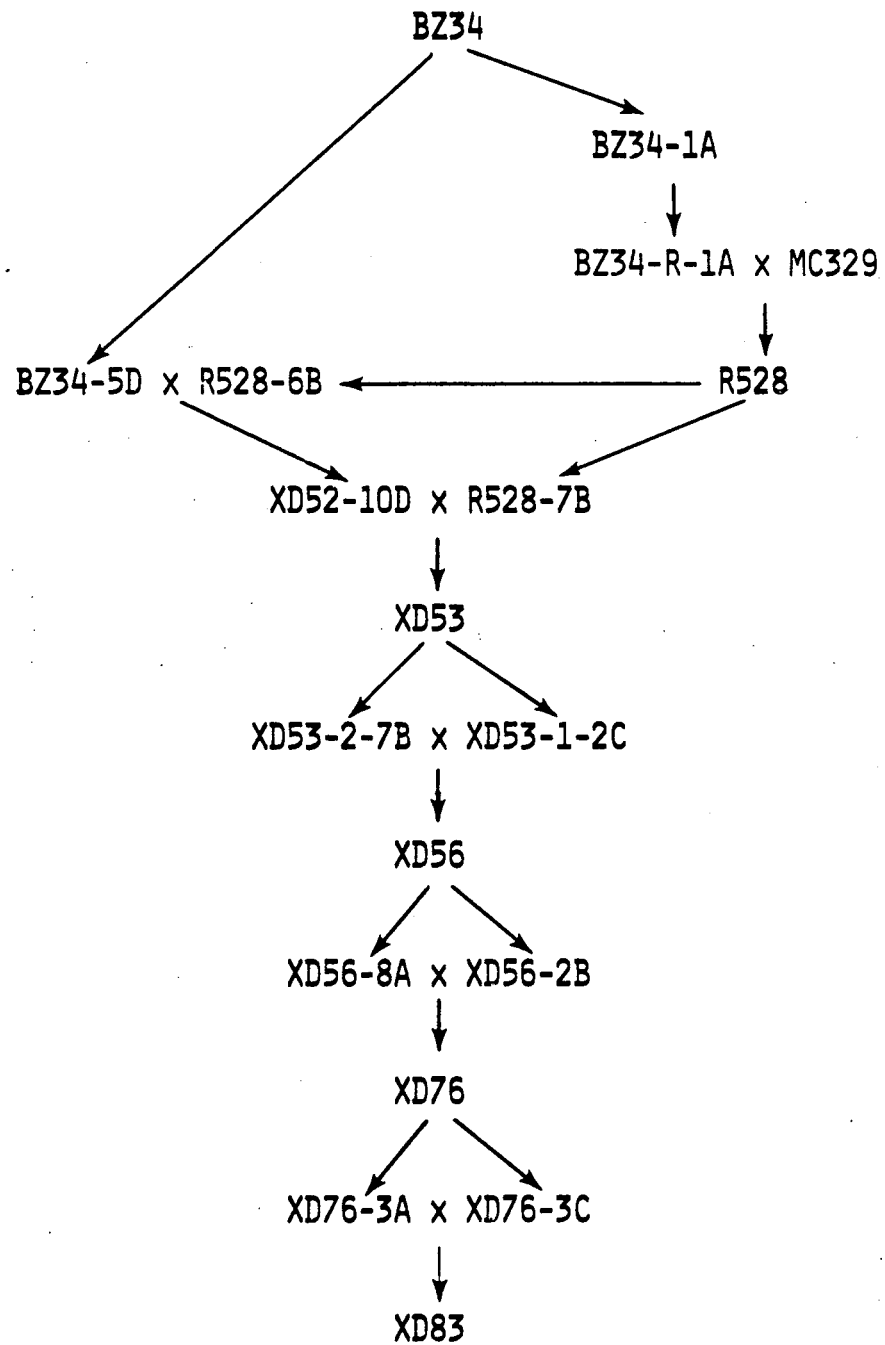
Figure 2. Genealogy of mutation/recombination strain (XD83)

GENEALOGY OF CHROMOSOME LOSS STRAINS
(XD26, XD72, XD99)

To put ilv3 into strain:

X2316-3C x X3424-5D

R512

R512-2-13A x X1687-12B

To put ura2 into strain:

XD25 ──────────────────────────────→ XD25-19D x S1780C

XD25-6C x XD57-6B ◄──────── XD57

To put SUP4 and SUP7 into strain:

X2283-5A x X2182-3D(C59)

XD1

XD64

XD25-19D x XD1-15C   XD1-15C x XD64-4D

XD26   XD72

To cross out antisuppressor

XD72-7A x XD72-9A

XD79

XD79-5-14B x XD79-5-14D   XD79-5-16A x XD79-5-16D

XD93-2   XD84

XD84-1-7A x XD84-1-7B

XD94

XD93-2-7A x XD94-3D

XD99

Figure 3.   Genealogy of chromosome loss strain (XD99)

suppressible by tyrosine-inserting ochre (UAA) suppressors.

ade2-18: adenine auxotrophy; red pigment accumulates in colony. Not ochre-suppressible.

arg4-4: arginine auxotrophy. Gene product: argininosuccinate lyase. Map position: 8R. Nonsense mutation; suppressible by tyrosine-inserting ochre suppressors. It is a non-complementing allele of arg4-17, but complements certain other arg4 alleles (see section F.2.).

arg4-17: arginine auxotrophy. A non-complementing allele of arg4-4.

can1-100: resistance to cananvanine, a toxic arginine analogue. Gene product: arginine permease. Map position: 5L. Nonsense mutation; suppressible by tyrosine-inserting ochre suppressors.

his4-519: histidine auxotrophy. Gene product: phosphoribosyl-AMP cyclohydrolase. Map position: 3L.

his5-2: histidine auxotrophy. Gene product: histidinol-phosphate aminotransferase. Map position: 9L. Nonsense mutation; suppressible by tyrosinse-inserting ochre suppressors.

ilv3-1: isoleucine plus valine auxotrophy. Gene product: dihydroaciddehydratase. Map position: 10 R. Nonsense mutation; suppressible by tyrosine-inserting ochre suppressors. Centromere linked. One study indicates it is 6.3 cM from the centromere (Mortimer and Hawthorne, 1966) and another study indicates it is 16.0 cM

from the centromere (Lawrence et al., 1975). The combined
data give an average centromere distance of 14.6 cM
(Mortimer and Schild, 1980).

leu2-3: leucine auxotrophy. Gene product:
β-isopropylmalate dehydrogenase. Map position: 3L.

lys1-1: lysine auxotrophy. Gene product: saccharopine
dehydrogenase. Map position: 9R. Nonsense mutation;
suppressible by tyrosine-inserting ochre suppressors.

trp1-1: tryptophan auxotrophy. Gene product:
phosphoribosylanthranilate isomerase. Map position: 4R.
An amber mutation, not suppressible by ochre suppressors.
Centromere linked: 0.45 cM from centromere (Mortimer and
Schild, 1980).

ura2-1: uracil auxotrophy. Gene product: aspartate
carbamoyltransferase. Map position: 10L. Nonsense
mutation; suppressible by tyrosine-inserting ochre
suppressors.

SUP4: dominant tyrosine-inserting ochre suppressor.
Gene product: tRNA^{Tyr}. Map position: 10R.

SUP7: dominant tyrosine-inserting ochre suppressor.
Gene product: tRNA^{Tyr}. Map position: 10L.

pet1: will not grow on nonfermentable substrate; lacks
cytochromes a, b, and c1. Map position: 8R. Centromere
linked: 4.5 cM from centromere.

[ρ^+]: wild-type for mitochondrial genome. Will grow
on nonfermentable substrates.

[ρ^-]: respiratory deficient. Usually lacking 20-99.9%

of the information in the mitochondrial genome from large deletions (Borst and Grivell, 1978). Will not grow on nonfermentable substrates.

$[E^S]$: wild-type mitochondrial sensitivity to erythromycin. The development of cytochromes $(a+a_3)$, b, and c_1 and some respiratory enzymes is inhibited when cells are grown on a nonfermentable substrate containing erythromycin. This results in cells which are phenocopies of a cytoplasmic ρ^- mutant since the cells cannot grow on a nonfermentable subtrate without functional mitochondria. Antibiotic resistant mutants are selected, therefore, on antibiotic-containing glycerol medium (Sherman et al., 1979). A sensitive strain is defined as one which does not grow on YPG medium containing 0.4 mg/ml erythromycin.

$[E^R]$: mitochondrial resistance to erythromycin. See above. A resistant strain is defined as one which shows growth on YPE containing 4.0 mg/ml erythromycin. This high concentration of erythromycin primarily allows mitochondrially resistant mutants to grow whereas low erythromycin concentrations allow more nuclear erythromycin resistant mutants to grow (Birky, 1973).

$[C^S]$: wild-type mitochondrial sensitivity to chloramphenicol.

$[C^R]$: mitochondrial resistance to chloramphenicol. Both erythromycin and chloramphenicol inhibit protein synthesis on mitochondrial ribosomes (Tzagoloff, 1982).

C. Chemicals

Chemicals were obtained as follows: mitomycin C, N-methyl-N'-nitro-nitrosoguanidine (Aldrich); ethylene glycol (J.T. Baker); L-canavanine sulfate, (Calbiochem); Ilotycin gluceptate (Dista); 4-nitroquinoline-N-oxide (ICN-K&K); dimethyl sulfoxide (Mallinckrodt); dimethyl sulfoxide (MC&B, spectroquality); ICR-170 (Polysciences); chloramphenicol, erythromycin, ethidium bromide, ethyl methanesulfonate, DL-para-fluorophenylalanine, mitomycin C, (Sigma); and methyl benzimidazole-2-yl-carbamate (gift of Beth Rockmill).

D. Media

YEPD: 1% yeast extract, 2% Bacto-peptone, 2% dextrose, and 2% agar. The agar was omitted for liquid YEPD.
SYNTHETIC COMPLETE: 0.67% Difco yeast nitrogen base without amino acids, 2% dextrose, 2% agar, and the following amino acids and bases: 10 mg/l of adenine sulfate; 20 mg/l each of l-histidine, l-methionine, uracil, l-isoleucine; 50 mg/l each of l-arginine, l-lysine, l-phenylalanine, l-tryptophan, l-tyrosine, l-valine; 100 mg/l each of l-leucine, l-threonine; and 1 mg/l of l-aspartic acid.
OMISSION: Synthetic complete medium minus one or more of the above amino acids or bases.

YPG: 1% yeast extract, 1% Bacto-peptone, 0.025% dextrose, 3% (v/v) glycerol, and 2% agar.

YPE: 1% yeast extract, 2% Bacto-peptone, 2% ethanol (v/v), and 2% agar.

YPDG or YPDE: 1% yeast extract, 2% Bacto-peptone, 0.1% dextrose, 3% glycerol or 2% ethanol, and 2% Bacto-agar. Media to distinguish between petite and grande colonies.

SPORULATION: Either 1) 3% potassium acetate, 0.002% raffinose, and 2% agar or 2) 1% potassium acetate, 0.1% dextrose, 0.25% yeast extract, and 2% agar.

ERYTHROMYCIN: Either 1) 0.5% erythromycin in 2% ethanol, 1% yeast extract, 2% Bacto-peptone, and 2% agar in either distilled water or 0.05M phosphate buffer (pH 6.2-6.3) or 2) 4.0 mg/ml Ilotycin gluceptate, 1% yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, and 2% agar. The erythromycin was added to the media after it had been autoclaved and while it was still hot. The plates were stored in the refrigerator after drying and used within two weeks.

CHLORAMPHENICOL: 0.4% chloramphenicol dissolved in 2% ethanol, 1% yeast extract, 2% Bacto-peptone, 2% agar in either distilled water or 0.05M phosphate buffer (pH 6.2-6.3). The chloramphenicol was added to the medium after it had been autoclaved and while it was still hot. The plates were stored in the refrigerator after drying and were used within two weeks.

CANAVANINE: arg$^-$ omission medium plus 60 mg/l

L-canavanine sulfate.

HYPERTONIC: YEPD plus one of the following: 0.5, 1.0, 2.0, or 2.5M ethylene glycol; 1.0, 1.5, or 2.0M potassium chloride; 1.0 or 2.5M sodium chloride, glycerol, sorbitol, or dextrose. Except for preliminary testing of hypertonic media, 2.5M ethylene glycol in YEPD was used for all tests.

SUPPLEMENTED MEDIA:

arg ±: arg⁻ omission medium supplemented with 0.4 mg/l arginine.

hist ±: his⁻ omission medium with 0.3mM histidinol supplemented with 0.4 mg/l histidine.

leu ±: leu⁻ omission medium supplemented with 0.4 mg/l leucine.

lys ±: lys⁻ omission medium supplemented with 0.4 mg/l lysine.

E. Ultraviolet Light Source

Three 8-watt General Electric UV germicidal lamps (G875, 90% intensity at 2537 A) were used for ultraviolet light exposures. The flux at the distance used for irradiation was approximately 27 ergs/mm^2/sec as measured by a photocell (No. 935 RCA) or a Spectroline DM-254N UV fluence meter form Spectronics Corporation. All irradiations were performed by irradiating the yeast cells

after plating on solid media.

F. Genetic Analyses

1. Detection of suppressors

The homozygous ochre suppressible markers ade2-1, his5-2, lys1-1, and can1-100 were used to monitor the presence of the suppressors. The presence of one suppressor was indicated by a 2:2 cosegregation of the suppressible markers. A combination of 2:2, 3:1, and 4:0 segregation of the suppressible markers indicated the presence of two suppressors.

2. Distinguishing between arg4-4 and arg4-17 alleles

A number of criteria may be used to distinguish the arg4-4 allele from the arg4-17 allele (Fogel et al., 1979; R.K. Mortimer, personal communication). The parental strains and the final strains were tested according to the criteria described below. During the strain constructions, however, the arg4-17 allele was followed by using the closely linked pet1 marker.

a. Properties of the arg4-4 allele: It is complemented by arg4-3, arg4-16, and arg4-27. It also has a low revertibility with ultraviolet light.

b. Properties of the arg4-17 allele: It is not

complemented by <u>arg4-3</u>, <u>arg4-16</u>, or <u>arg4-27</u>. It has a high revertibility with ultraviolet light.

3. Mitochondrial versus nuclear resistance

The high erythromycin concentration (4 mg/ml) used in the tests favors the selection of mitochondrial resistance over nuclear resistance (Birky, 1973). In addition, since the strain is diploid, expression of nuclear recessive erythromycin resistance mutations is effectively eliminated. Further confirmation of the mitochondrial or nuclear origin of the antibiotic resistance was accomplished in certain cases by the segregation pattern of the resistance in tetrad dissections: a non-Mendelian segregation pattern indicating mitochondrial resistance and a Mendelian segregation pattern indicating nuclear resistance.

G. Test Procedures

1. Suspension tests

A single clone which had been checked for its phenotype and/or genotype was inoculated into YEPD (liquid) and grown 3-4 days on a shaker at $30^{\circ}C$ to reach stationary phase. This yeast stock solution was stored at $4^{\circ}C$ until use.

P-450 induction: To induce the cytochrome P-450

system of the yeast which is necessary for the activation
of some procarcinogens to carcinogens the yeast were grown
to exponential phase. Yeast stock was inoculated into
fresh YEPD (liquid) in the proportion of 0.5 ml stock/1
liter YEPD (liquid). This was incubated on a shaker at
30°C for 10-16 hours. The culture was microscopically
checked to insure that budding cells were present.

The cells were harvested and washed 2X with distilled
water or buffer. Resuspension of the cells was made in
water, buffer, or growth media to the original
concentration or to a lesser concentration if the growth
medium was used to allow a few rounds of division.
Post-treatment growth was usually required with the
chromosome loss strain. The test substance in an
appropriate solvent was combined with the yeast suspension
and the combination was incubated on a shaker for a
specified time (usually 1/2 to 1 hour in the
mutation/recombination test and 4 hours in the chromosome
loss test). The test substance was tested over a wide
range of concentrations and a solvent control was included
in the test. With light sensitive chemicals, reduced or
gold light was used and the relevant labware was covered
with foil. Treatment was performed either at room
temperature or 30°C. The reaction was terminated by
adding an excess of cold buffer, distilled water, or 5-10%
sodium thiosulphate. The suspension was centrifuged and
washed 2X with distilled water or buffer.

To allow for the expression of the "mutant" phenotype, the cells were allowed to undergo additional growth either in supplemented or non-selective media (Murthy et al., 1976; Zimmermann et al., 1966b). The mutation/recombination media was supplemented to allow this. To allow expression in the chromosome loss strain the cells were resuspended after treatment in YEPD (liquid) at a concentration to allow a few cell divisions. The suspension was incubated with shaking for 48 hours at 30°C. The suspension was centrifuged and the cells were washed 2X with water or buffer. The cells were then resuspended with water or buffer and serial dilutions were made: 0.1 to 0.2 ml of the appropriate dilution were plated on the media. The plates were incubated at 30°C for 3 days to 2 weeks depending upon the media. Plates were counted after appropriate incubation. For the chromosome loss test, presumptive monosomics growing on hypertonic medium or canavanine medium were streaked and checked for other markers by replica plating.

2. Spot tests

A single clone which had been checked for its phenotype and/or genotype was inoculated into YEPD (liquid) and grown for 3-4 days on a shaker at 30°C to reach stationary phase. This stock solution was stored at 4°C until use. The cells were then harvested and washed 2X with

distilled water or buffer. The cells were resuspended with water or buffer and serial dilutions were made. 0.1 to 0.2 ml of the appropriate dilution was plated on the media. The suspension was allowed to soak into the media before the chemical of choice was added in microliter amounts to a paper disc in the center of the plate. The plates were then incubated at $30^{\circ}C$ for 3 days to several weeks depending upon the media. Figure 4 diagrams the idealized pattern seen in a positive result from a spot test.

In recombination and mutation experiments strain XD83 was plated directly on selective media. However, in chromosome loss experiments strain XD99 was plated first on non-selective medium (synthetic complete usually) to allow the cells to grow in the presence of the chemical before replica plating to the selective medium. Further confirmation of chromosome loss was made by replica plating the cells from the selective medium to other media indicative of chromosome loss.

3. Ultraviolet light tests

All irradiations were performed under reduced light conditions by irradiating the yeast cells after plating appropriate dilutions on solid media. Incubation was at $30^{\circ}C$ in the dark. Incubation times and counting were similar to those used in suspension tests.

SPOT TEST

revertants

disc with chemical

zone of killina

background

Figure 4. Spot test

MUTATION/RECOMBINATION STRAIN

Nuclear   Mitochondrial

Genotype:

a	his4-519	leu2-3	lys1-1	+ arg4-4 +	ade2-18	ρ^+	(ant^S)
α	his4-519	leu2-3	lys1-1	pet1 + arg4-17	+		

Phenotype:

His⁻	Leu⁻	Lys⁻	Arg⁻	white	grande	antibiotic sensitive
↓	↓	↓	↓	↓	↓	↓
frameshift mutation	frameshift mutation	base pair substitution	gene conversion	mitotic crossing-over	large deletions	point mutation or small deletion
↓	↓	↓	↓	↓	↓	↓
His⁺	Leu⁺	Lys⁺	Arg⁺	red/white sector	petite	antibiotic resistant

Figure 5. Mutation/Recombination strain (XD83).

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(Donahue et al., 1982, 1981; respectively). The his4-519 mutation, they found, is due to a +1 G addition to a glycine GGG codon which shifts the reading frame and eventually produces a premature termination signal. Donahue et al. (1981) studied five ICR-170-induced mutations at the HIS4 locus which were +1 G:C additions in a run of G:C base pairs. Some of these mutations were suppressible and some were not. Since all the externally, suppressible frameshift mutations occurred in glycine and proline codons to give the four-base codons GGGU, GGGG and CCCU, it appears likely that suppression of these four-base codons in yeast, as in bacteria, involves a four-base anticodon or its functional equivalent (Donahue et al., 1981). One of these suppressors shows an altered chromatographic profile for glycyl-tRNA (Culbertson et al., 1977). Since all the Group II mutations are suppressed by the same suppressors, Donahue et al. (1981) have proposed that all are +1 additions in glyine codons. Figures 6 and 7 show the reactions controlled by the HIS4 region and the location of the his4-519 mutation within the HIS4A region. Although the LEU2 gene has been sequenced (Andreadis et al., 1982), the leu2-3 mutation has not been sequenced yet. However, since both his4-519 and leu2-3 were induced by ICR-170 and are suppressible by some of the same external suppressors (Culbertson et al., 1977 and 1980), it is likely that leu2-3 is also a +1 G:C frameshift mutation.

The lys1-1, arg4-4, and arg4-17 alleles are ochre

site of his4-519
mutation

A   B   C

PRAMP ⟶ BBMⅡ   PRATP ⟶ PRAMP   HISTIDINOL ⟶ HISTIDINE

3   2   10
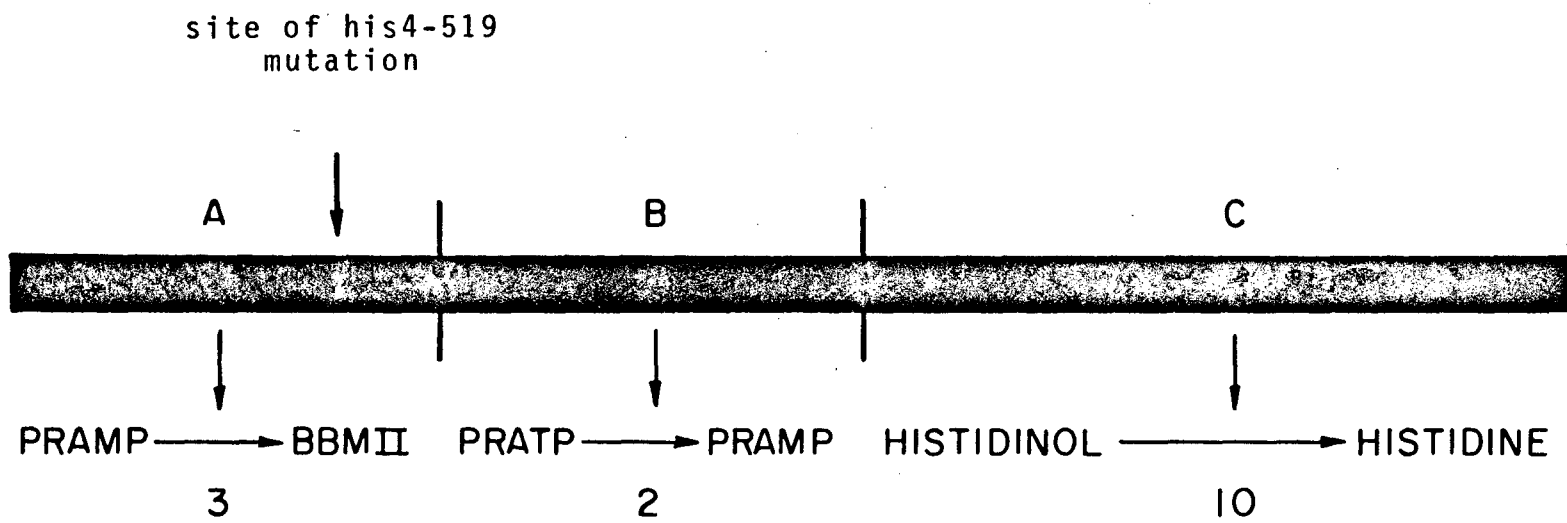
XBL837-3873

Figure 6.   Reactions controlled by <u>HIS4</u> region. A, B, and C are the segments of his4 responsible for catalyzing the 3rd, 2nd, and 10th steps (respectively) in the histidine biosynthetic pathway. PRAMP = Phosphoribosyl-AMP; BBMII = N-(5'-phospho-D-ribulosylformimino)-5'-amino-1-(5'' phosphoribosyl)-4-imidizolecarboxamide; PRATP = phosphoribosyl-ATP. (Adapted and redrawn from Fink and Styles, 1974)
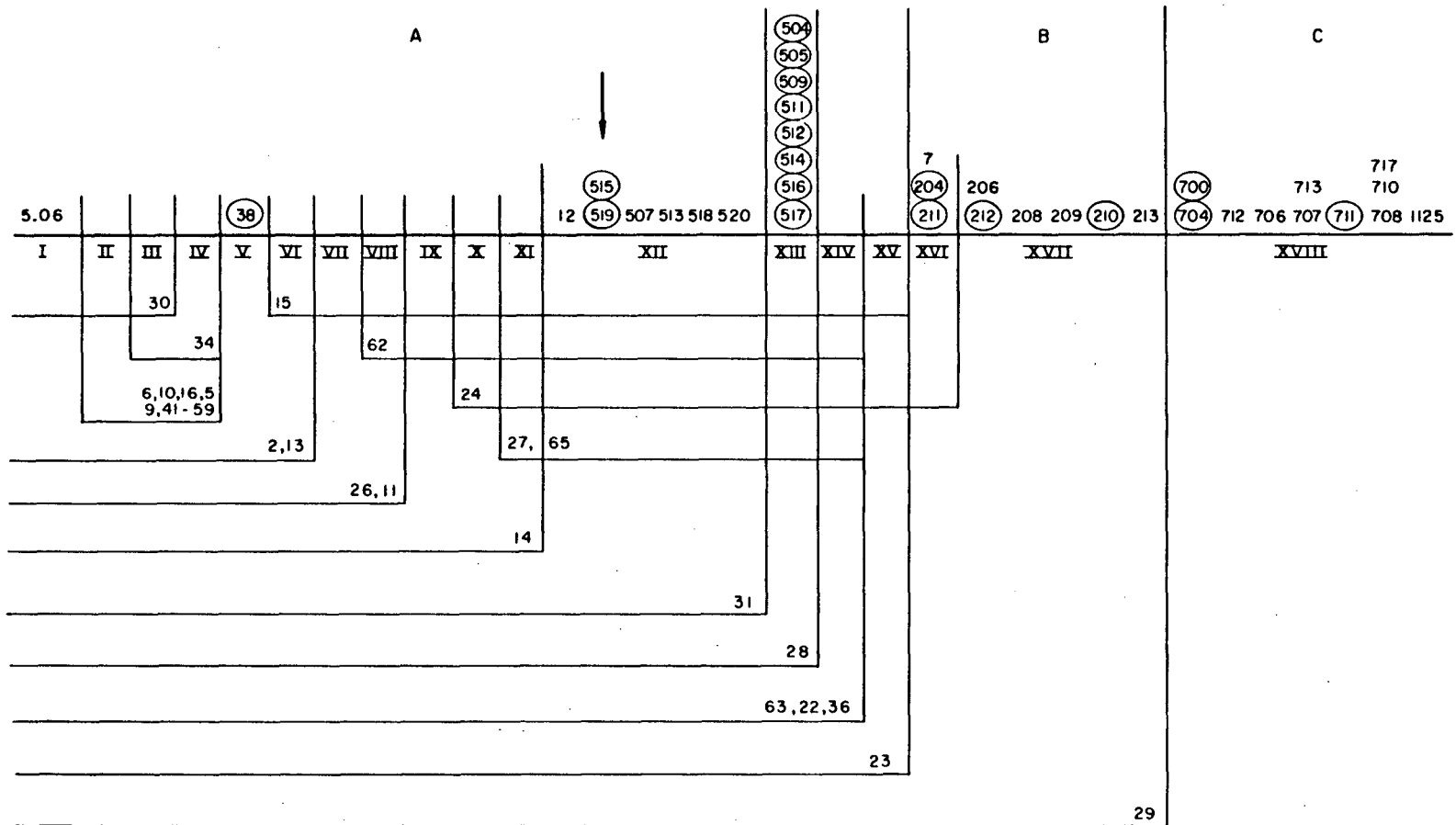
Figure 7. Map of ICR-170-induced mutations in the HIS4 region (arrow shows location of his4-519 mutation) The dark solid line represents the chromosome. The horizontal lines show the extent of the deletions. (Culbertson et al., 1977)

XBL837-3874

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mutations. Reversion of the lysine marker from a base pair substitution mutation may also be due to intragenic reversion or intergenic suppression. The latter is distinguished by the simultaneous reversion of the arginine and lysine loci. Gene conversion is the usual mechanism for the mitotic segregation of prototrophs from heteroallelic diploids (Roman and Jacobs, 1958; Hurst and Fogel, 1964; Wildenberg, 1970). The spontaneous frequency of gene conversion is several orders of magnitude higher than that for mutation. However, simultaneous reversion of the arginine and lysine loci can also be used to distinguish extragenic suppression from gene conversion. In addition, ochre suppressors may be detected by their failure to grow on hypertonic media (Singh, 1977) as discussed earlier. The arg4-4 and arg4-17 alleles are non-complementing. The mutations in these alleles are at opposite ends of the arginosuccinase locus which increases the likelihood of detecting gene conversion at this locus. The fine structure of the ARG4 locus is shown in Figure 8.

Mitotic crossing-over of the heterozygous ade2-18/+ strain may result in a red/white sectored colony. The red sector is homozygous for the ade2-18 mutation which blocks the adenine biosynthetic pathway resulting in the accumulation of a red pigment (Roman, 1956). The white sector is homozygous wild-type for adenine. A summary of some of the possible variant (red and sectored red) phenotypes and their possible explanations are shown in
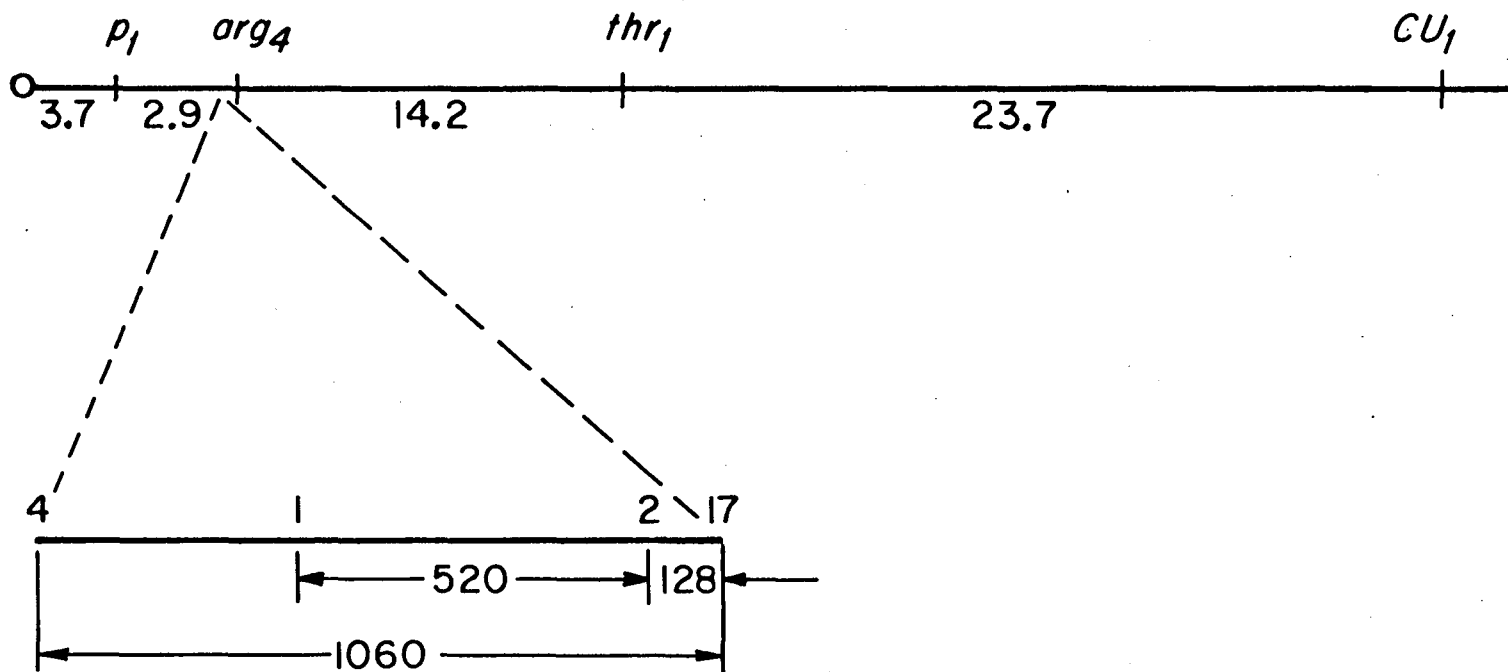
Figure 8. Fine structure of ARG4
Intergenic distances are shown in centimorgans and intragenic
distances are shown in nucleotides (redrawn from Fogel and
Mortimer, 1969).

XBL837-3871

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Figure 9. The frequency of variant colonies is a good estimate of the frequency of mitotic recombination because mitotic recombination occurs more frequently than the other phenomena listed (Johnston, 1961). Although the frequency of variants was taken in this study as an approximation of the frequency of mitotic crossing-over, it is not actually possible to distinguish between mitotic crossing-over and gene conversion without laborious genetic analyses and the relative proportion of variants from mitotic crossing-over and gene conversion varies from agent to agent (for reviews on mitotic recombination see Resnick, 1979 and Esposito and Wagstaff, 1981).

Wild-type yeast which have functioning mitochondria and are, therefore, respiratory sufficient are termed grande. The mitochondrial petite phenotype is due to a respiratory deficiency which is usually the result of a drastic change caused by large deletions (usually 20-99.9% of the genome) in the mtDNA. This deletion process is followed by a tandem duplication of the remaining DNA and an amplification which restores the original quantity, but not information content, of the mtDNA (Borst and Grivell, 1978). Small deletions as well as point mutations can be monitored in the mitochondria by the induction of antibiotic resistance (Grivell et al., 1973). Chloramphenicol and erythromycin, for example, inhibit mitochondrial protein synthesis by binding with the large ribosomal subunit of the mitochondria (Tzagaloff, 1982).

Figure 9.   ABERRANT SEGREGATIONS

$$\frac{ade2-18}{+}$$

Variant colonies	Possible mechanisms
	1. mitotic crossing-over followed by lethal sectoring 2. mutation 3. chromosome loss 4. deletion 5. gene conversion 6. other multiple events
	mitotic crossing-over or other aberrant event with segregation at first mitosis after plating (twin spots)
	mitotic crossing-over or other aberrant event with segregation after first mitosis after plating or slow growth of one sector or unequal delay of mother and bud or late lethal sectoring (examples of some possible variants)
a. b.	mitotic crossing-over, etc. and petite induction a. YEPD medium (2% dextrose) b. YEPD medium (8 or 10% dextrose) or synthetic complete medium low in adenine

Antibiotic resistant strains probably have a lower binding
affinity of the affected component for the antibiotic
(Tzagaloff, 1982). Since chloramphenicol resistance can
also be induced by a single, partially dominant nuclear
mutation which appears to alter the uptake of
chloramphenicol by the cell (Rank et al., 1975), the
induction of chloramphencol resistance was used as a
measure of both nuclear and mitochondrial forward mutation.

2. Selection media for detecting mutation and recombination

    a. Supplemented media: Supplementation was used in
the media for detecting gene conversion, frameshifts, and
base pair substitution to allow a few rounds of cell
division for expression of the revertant phenotype,.

    b. Detecting mitotic crossing-over: Variant colonies
were recorded as whole, half, quarter, and hairline
sectors. Only the sum of the different types of variant
colonies was plotted, however. Petite induction, which is
common with many carcinogens/mutagens (Egilsson et al.,
1979), can interfere with the development and, therefore,
scoring of the red color unless the medium used is high in
dextrose (Reaume and Tatum, 1949) or low in adenine.
Corrections were made for this petite interference.

    c. Mitochondrial changes: Generally, petite induction
was estimated by counting the fraction of very small, very
white colonies on the rich, fermentable YEPD medium (for

example, see Figure 13A). The petite nature of these very
small, very white colonies was confirmed by testing a
number of these putative petites for lack of growth on
non-fermentable medium. This phenotypic change from the
wild-type grande colonies which are large and off-white
colored results from the loss of the cytochromes in the
petite colonies.

The high erythromycin concentration (4 mg/ml) used in
the tests favors the selection of mitochondrial resistance
over nuclear resistance (Birky, 1973). In addition, since
the strain is diploid, expression of nuclear recessive
erythromycin resistance mutations is effectively
eliminated. Moreover, a dominant nuclear mutation for
erythromycin resistance shows a Mendelian meiotic
segregation pattern whereas a mitochondrial erythromycin
resistance mutation has a non-Mendelian meiotic segregation
pattern.

The frequency of chloramphenicol resistance was also
measured for some agents. Again the meiotic segregation
pattern can differentiate between nuclear and mitochondrial
mutations. Only limited data are available on erythromycin
and chloramphenicol resistance induction because of early
technical problems with the media.

B. Tests: Suspension and Spot

A select number of agents were tested which were

positive for one or more genetic effects in other screening systems. These were used as a test of the various endpoints in this system. A variety of means of statistically evaluating the results of microbial mutagenicity assays have been used and each have their strengths and weaknesses (see Chu et al., 1981 for a discussion of seven approaches). In this study the null hypothesis was tested and the linear correlation coefficient was calculated for the suspension test results.

The null hypothesis was tested between the control, zero dose and the highest dose of the agent only. The level of significance, P, of falsely rejecting the null hypothesis (Type I error) was estimated using a single-tailed (one-sided) statistical test, unless otherwise stated, since the usual only alternative to non-mutagenic was mutagenic. However, when a chemical could be also be anti-mutagenic, then two-sided confidence intervals and statistical tests were used. The null hypothesis of no significant difference or effect was accepted if the significance estimate gave $P > 0.05$. Statistical formulae were generally those suggested by Ehrenberg (1976) for testing for genetic toxicity. To avoid underestimating the lower and upper limits of the confidence interval, a square root transformaion was done to stabilize the variance when the number of mutants was low, that is, less than six. When no mutants were found, the confidence interval had only an upper limit and a

method described by Ehrenberg (1976) was used to calculate
this upper limit. Error bars on the dose-response curves
show the standard error. Since a zero frequency can not be
plotted on a semi-logarithmic graph, the upper limit of the
error bar was used in the dose-response curve and was
connected to the other data points by a dashed line.

Most of the induced mutagenesis data appear to have a
linear component in their dose-response curves (Ehrenberg,
1976). Therefore, the linear correlation coefficient, r,
was calculated for each dose-response curve. When the
results were not significant at the 0.05 level, generally
the linear correlation coefficient was not stated if it was
between -0.5000 and 0.5000.

The frequency data were plotted against dose and
statistically analyzed for each agent. The approximate
relative increases in the induced frequencies to the
spontaneous frequencies are given for different endpoints,
but it should be noted that these relative increases are
subject to fluctuations in the spontaneous frequencies.
Frequency data show mutants per surviving cell, whereas
yield data show mutants per treated cell. Since yield data
are a measure of the net effect of the lethal and genetic
effects of the agent, they can be used in comparative
studies of the relative mutagenic or recombinogenic
efficiencies of different agents (Haynes and Kunz, 1981;
Eckardt and Haynes, 1980). Although the yield data were
not plotted in this study, qualitative yield effects were

noted for each endpoint and agent. Spot tests also measure the net effects of the lethal and genetic effects of an agent and are, therefore, essentially a physical representation of the yield versus dose. The number of cells in a spot test which are exposed to a given concentration of a chemical, however, varies depending upon the distance of the cells from the center of the plate. Consequentially, few cells are exposed to the highest concentrations of the chemical.

The treatment conditions for the suspension tests are shown in table 4. Preliminary experiments were done for all agents except ICR-170 and 4-nitroquinoline-N-oxide. The results, however, are not given unless qualitatively different from the stated results. The concentrations, amounts, and solvents used for the spot tests are indicated on the spot test figures.

1. Ethidium bromide

In stationary cells ethidium bromide, an intercalating agent and highly selective mitochondrial mutagen, did not induce significant cell killing at doses up to 10 ug/ml on either synthetic complete medium or non-fermentable YPE medium (Figure 10A). Although a very slight induction of cell killing was seen on rich YEPD medium, the dose-response curve had a low linear correlation coefficient ($P < 0.05$, $r = 0.3309$, Figure 10A). No significant

TABLE 4

TREATMENT CONDITIONS

Agent	Test medium			Test conditions	
	Distilled water	Phosphate buffer	Citrate buffer	Treatment time	Culture stage
ethidium bromide	X			1 hour	Stationary
ethidium bromide	X			1 hour	Exponential
ethyl methanesulfonate		X (pH 7.0)		1 hour	Stationary
ICR-170		X (pH 7.4)		1 hour	Stationary
N-methyl-N'-nitro-N-nitrosoguanidine			X (pH 5.0)	½ hour	Exponential (late)
4-nitroquinoline-N-oxide		X (pH 7.4)		2 hours	Stationary
ultraviolet light		X (pH 7.0)		N/A	Stationary

General treatment conditions: non-growing, room temperature, aerobic, shaking. All buffers at 0.1 M. Treatment terminated either with sodium thiosulphate or dilution.

A.

Surviving fraction

1.0

YPE
SC
YEPD

0.5

0.2

0.1

0   2   4   6   8   10
Ethidium bromide (μg/ml)

B.

% Petite

100

10

1

0.1

0.01

0   2   4   6   8   10
Ethidium bromide (μg/ml)

C.

Ery^R per 10^8 survivors

100,000

10,000

1000

100

10

1

0   2   4   6   8   10
Ethidium bromide (μg/ml)
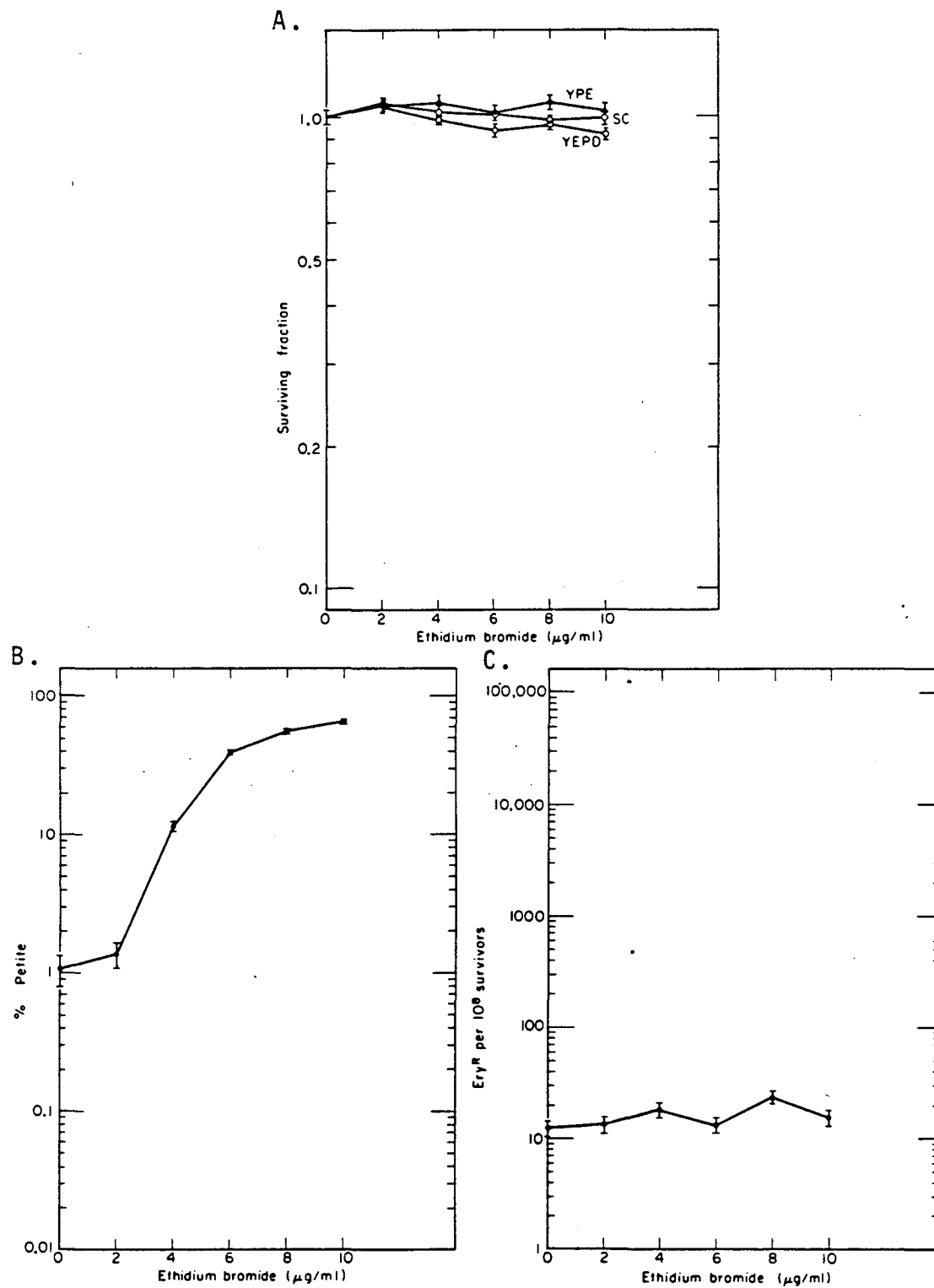
XBL836-3825

Figure 10.   Ethidium bromide suspension tests
             (stationary phase cells)

effects were seen on the frequencies of mitotic
crossing-over, frameshift and base pair substitution
mutations (data not shown). An earlier, preliminary
experiment suggested a very slight increase in the
frequency of lysine base pair substitution mutations.
Considering that the linear correlation coefficient was
high for the leucine frameshift dose-response curve,
$r=0.7843$, and moderately high for the lysine base pair
substitution dose-response curve, $r=0.5903$, more extensive
testing with higher doses might show slight, significant
effects for both. Although a very slight decrease in the
frequency of gene conversion was seen in one experiment
($P<0.05$, two-tailed; $r=-0.9613$; data not shown), this
effect was not observed in another similar experiment. The
decrease in gene conversion seen in the one experiment may
have been an artifact due to the high induction of petite
colonies whose slow growth may have led to an underestimate
of the colony counts at the higher doses.

The major effect observed was a pronounced induction of
petites ($P<0.01$, $r=0.9694$, Figure 10B). This petite
induction was not reflected in an increase of cell killing
on the YPE versus YEPD medium, however. No significant
effect was seen on the frequency of mitochondrial
erythromycin resistance although the linear correlation
coefficient was moderately high ($r=0.5296$, Figure 10C).
The high frequency of petite induction, however, so reduced
the population of cells which were capable of detecting

mitochondrial mutation and mitotic crossing-over that only
a large effect on either would have been observable.

Exponentially growing cells treated with ethidium
bromide showed a slightly different pattern of effects. No
significant effect was seen on cell killing on the rich
YEPD or synthetic complete medium (Figure 11A) at doses up
to 8 ug/ml. However, a fairly large induction of cell
killing was observed on the non-fermentable YPE medium
(P<0.01, r=-0.8850, Figure 11A). No significant effect
was seen on the induction of gene conversion nor on
histidine frameshift mutation (data not shown). A slight
decrease in frequency of leucine frameshift mutation was
seen but this was not significant at the 0.05 level.
Additionally, the dose-response curve showed a high
negative linear correlation coefficient, r=-0.7466. More
extensive testing with higher doses need to be done to test
for a possible slight anti-mutagenic effect. What appeared
to be a very slight, anti-mutagenic effect was seen for
base pair substitution (P<0.05, two-tailed; r=-0.9214).
However, this small decrease in the frequency could be an
artifact resulting from the slow growth of the petite
colonies--a problem which was discussed above.
Unfortunately, the low numbers of grande colonies
interfered with the determination of the frequencies of
mitotic crossing-over.

Most striking was the induction of petite colonies
which rapidly reached nearly 100%, a 600-fold increase over

A.

B.

C.
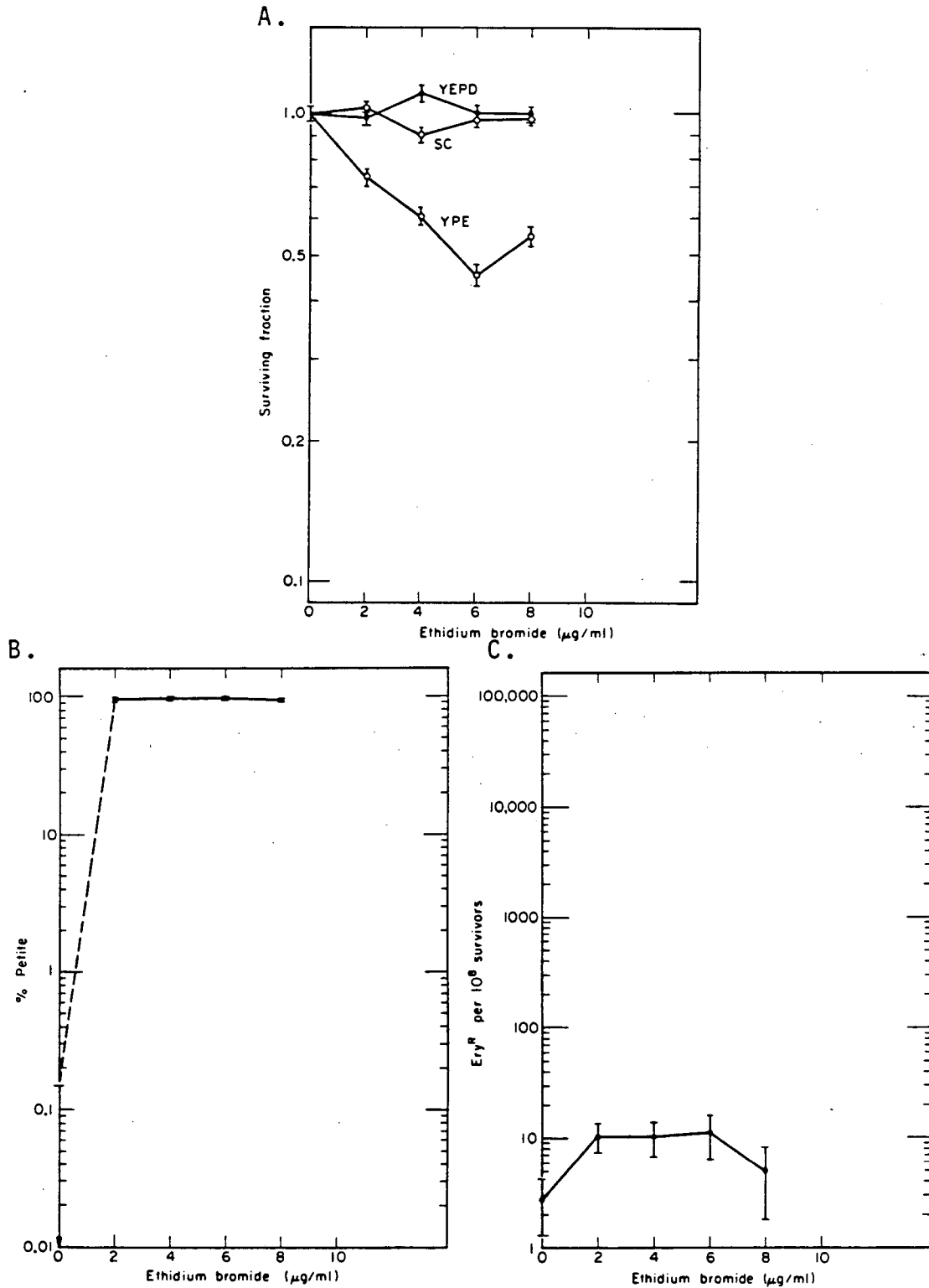
XBL836-3823

Figure 11.   Ethidium bromide suspension tests
(exponential phase cells)

the control frequency (P<0.01, r=0.7063, Figure 11B). As mentioned earlier with the stationary cells, the fraction of petites indicated by cell killing on the non-fermentable YPE versus the fermentable YEPD medium was not in agreement with the fraction of petites indicated by very small, very white colonies on the YEPD medium. Clearly, the two methods are not equivalent in indicating petite induction. This nonequivalence will be discussed in the DISCUSSION section. No significant effect was observed on the frequency of mitochondrial erythromycin resistance although the high induction of petites again interfered with this determination (r=0.2292, Figure 11C).

Ethidium bromide in exponentially growing and stationary cells had little or no effect on the mutant and recombinant yields. However, in accord with the frequency data, the yield of petites increased dramatically.

Ethidium bromide in spot tests (Figure 12) showed no effect on frameshift and base pair substitution mutations, gene conversion, forward mitochondrial erythromycin resistance, or forward chloramphenicol mutation. Higher doses of ethidium bromide may be necessary to induce nuclear mutation and crossing-over than for mitochondrial petite induction.

Differential growth patterns on three types of media indicated petite induction in spot tests. The rich YEPD medium allowed the growth of both grande and petite colonies (Figure 13B), the non-fermentable YPG medium

61

XBB 837-5902

Control   Ethidium bromide
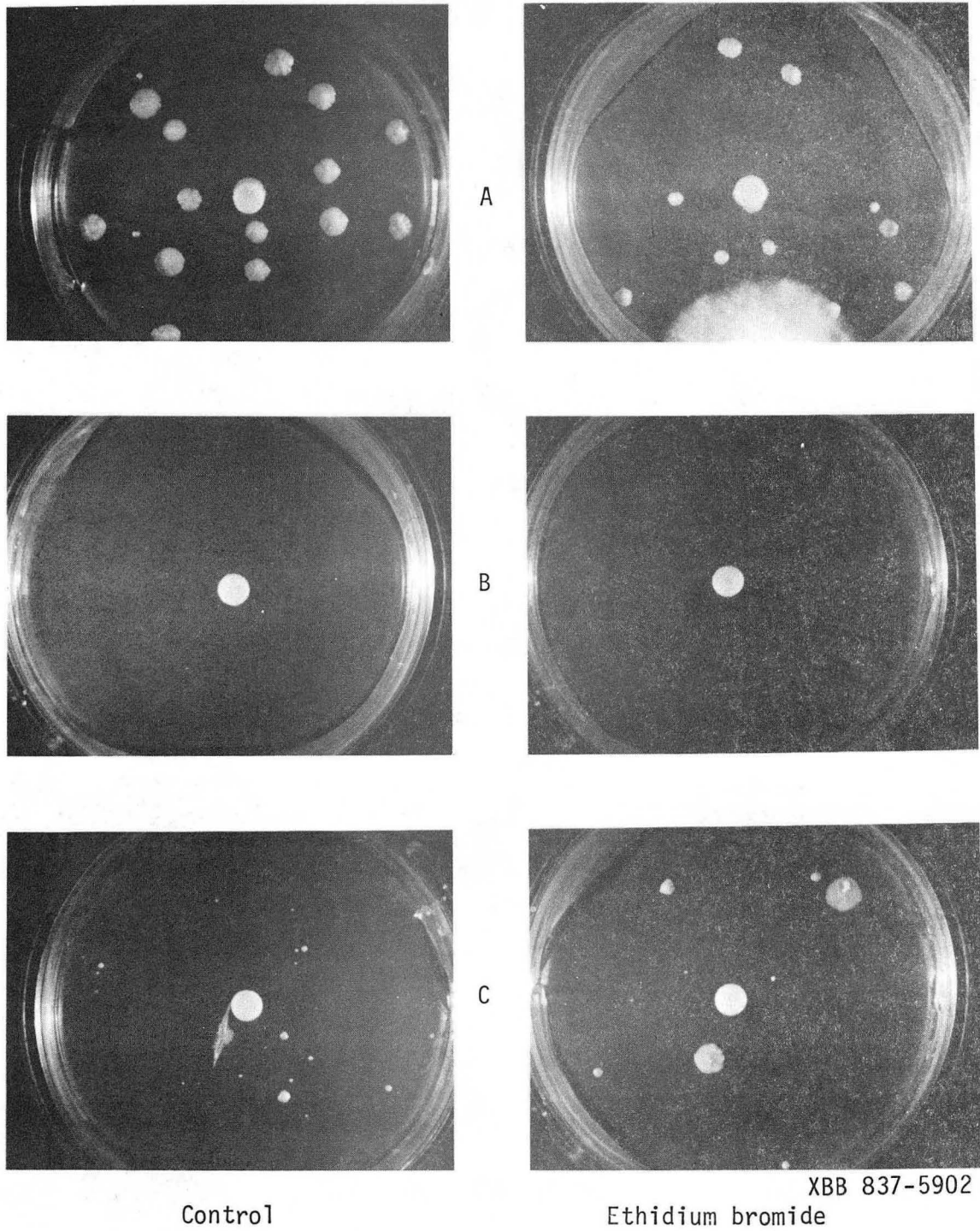
Figure 12. Ethidium bromide spot tests (20 to 25 µl of 5 mg/ml solution;
control: distilled water). A. Base pair substitution on
lys±. B. Frameshift mutation on his±. C. Frameshift muta-
tion on leu±.

XBB 837-5904

Control   Ethidium bromide

Figure 12. Ethidium bromide spot tests (continued). D. Gene conversion
on arg+. E. Forward mutation on chloramphenicol. F. Mito-
chondrial mutation on erythromycin.

Figure 13. Ethidium bromide and petite induction
    A. Suspension test showing grande and petite colonies on YEPD medium.
    B. Spot test on rich, fermentable YEPD medium allows growth of grande and petite colonies.
    C. Spot test on non-fermentable YPG allows growth of grande colonies only.
    D. Spot test on YPDG allows growth of grande colonies. Note inner circle of petite colonies on plate D which are missing from plate C.

Figure 13.

ethidium bromide

A.

B.

C.

D.

XBB 837-6113

64
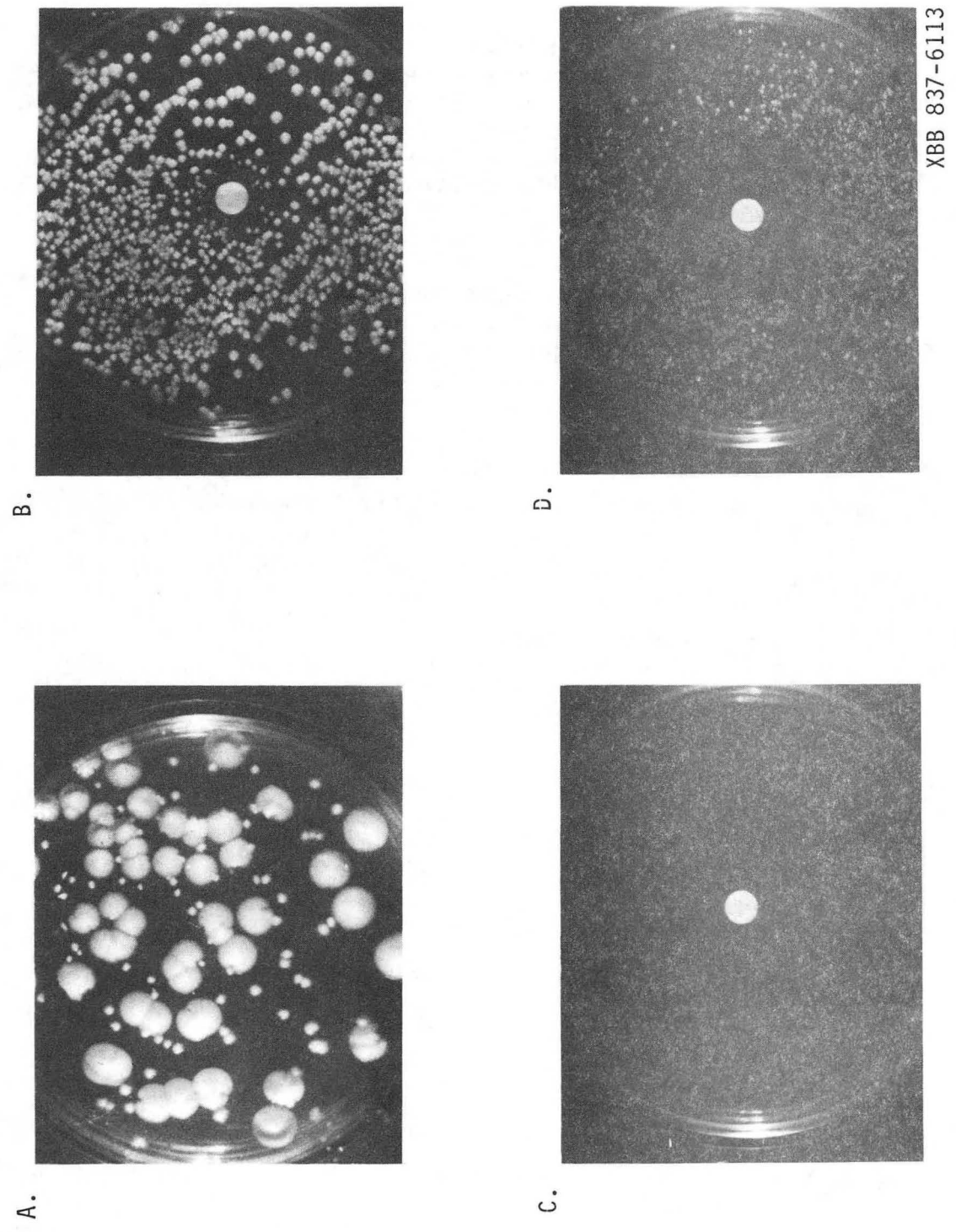
allowed only the growth of grande colonies (Figure 13C), and the YPDG (YPG with 0.1% dextrose) allowed the growth of both grande and petite colonies but petite colonies were extremely small due to the limiting amount of dextrose (Figure 13D).

2. Ethyl methanesulfonate (EMS)

The alkylating agent ethyl methanesulfonate induced a wide spectrum of effects. A significant ($P < 0.01$) induction of cell killing was seen on YEPD ($r = -0.9514$), synthetic complete ($r = -0.9024$), and YPE ($r = -0.9885$) as shown in Figure 14A. The frequencies of nuclear mutations were all significantly ($P < 0.01$) increased by 35 to 90-fold (Figure 14B). The dose-response curves for frameshift mutation on low histidine and low leucine media were quite similar ($r = 0.9764$ and $r = 0.9924$, respectively). The induction of base pair substitution mutation using low lysine medium, although with a higher spontaneous frequency, almost paralleled the slope of the frameshift dose-response curves ($r = 0.8786$). Additionally, a 20-fold increase in variant colonies ($P < 0.01$, $r = 0.9008$, Figure 14C) and 5-fold increase in gene conversion ($P < 0.01$, $r = 0.9917$, Figure 14D) were observed.

A 7-fold increase in the frequency of chloramphenicol resistance was observed ($P < 0.01$, $r = 0.7707$, Figure 14E). Although there was a 5-fold increase in the frequency of
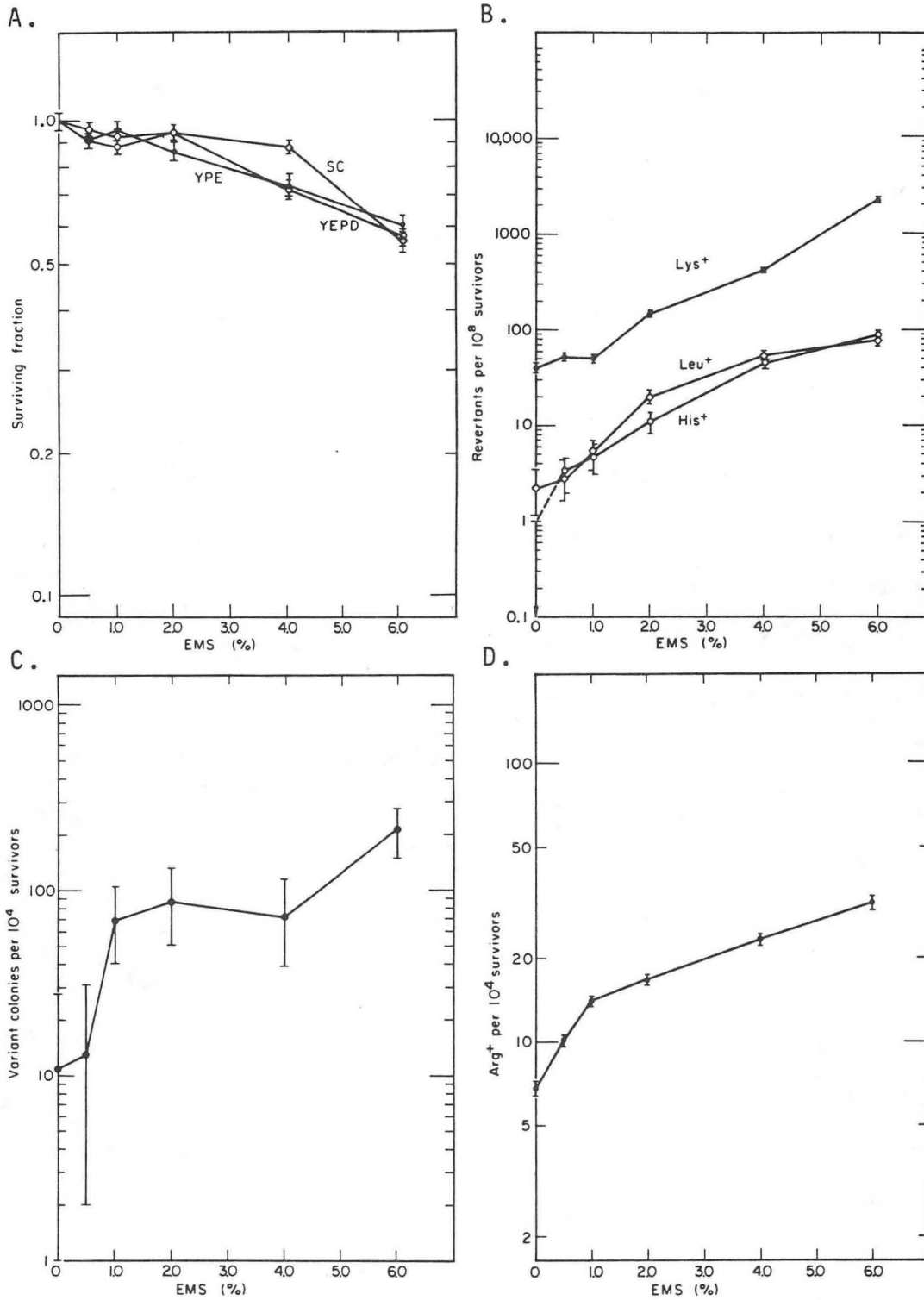
XBL 836-3822

Figure 14. Ethyl methanesulfonate suspension tests
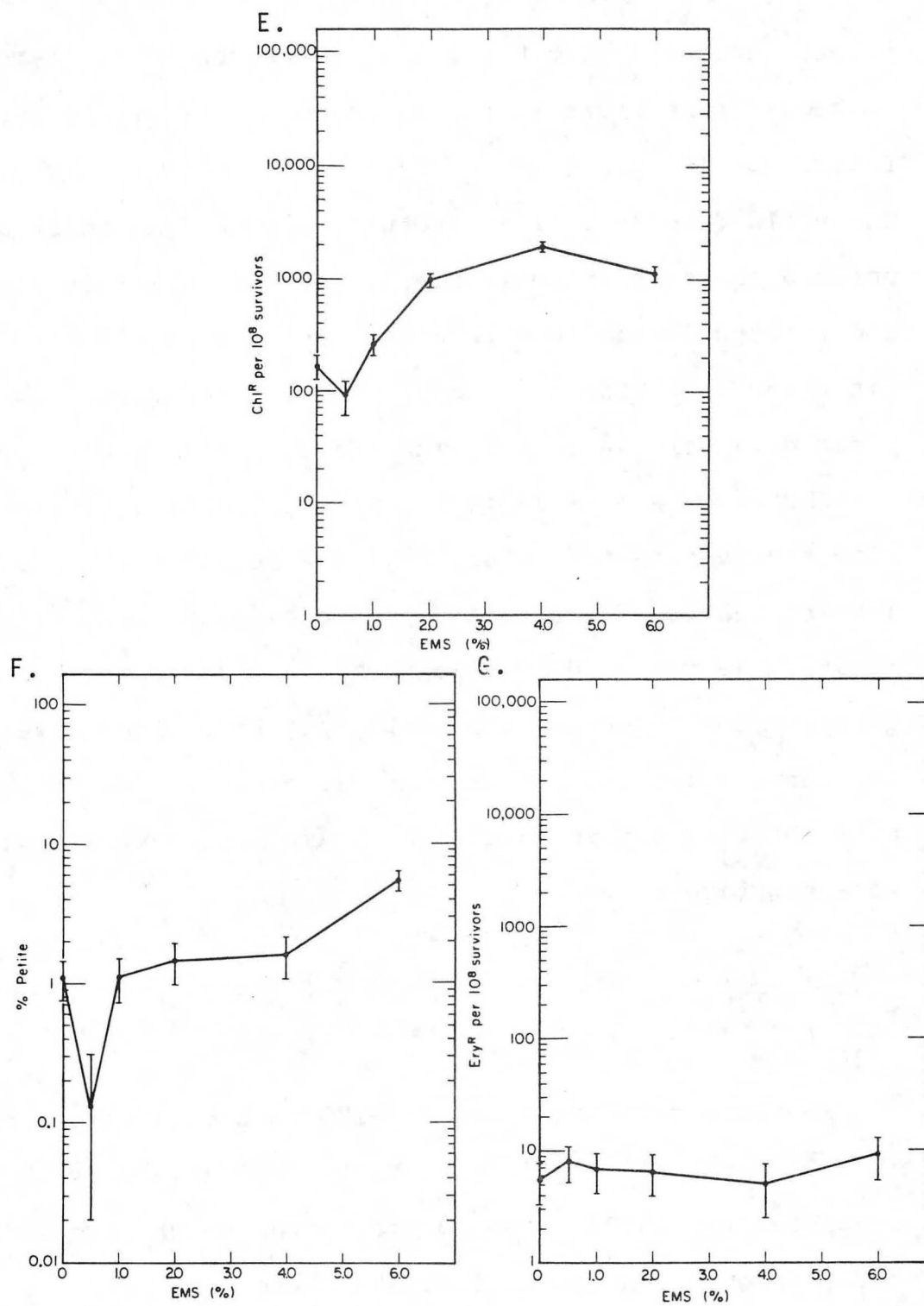
Figure 14.   Ethyl methanesulfonate suspension tests
             (continued)

XBL836-3821

petite induction (P<0.01, r=0.8725, Figure 14F), there was no significant increase in the frequency of erythromycin resistance (Figure 14G).

The yield of base pair substitution, both frameshift and forward chloramphenicol mutants; recombinants; convertants; and petites increased with dose. Only the yield of mitochondrial mutants was approximately constant. The yield data were in accord with the frequency data.

EMS spot tests (Figure 15) were in partial agreement with the suspension tests. Positive results were seen for leucine frameshift mutation, gene conversion, and forward mutation as measured by chloramphenicol resistance. Contrary to the suspension tests, positive effects were seen in the induction of erythromycin resistance and base pair substitution and histidine frameshift mutation results were negative.

3. ICR-170

The intercalating agent ICR-170 induced significant (P<0.01) cell killing in rich YEPD (r=-0.9219), synthetic complete (r=-0.6804), and non-fermentable YPE (r=-0.6490) media (Figure 16A). Both frameshift and base pair substitution mutations were induced to a great extent. Frameshift mutations (Figure 16B) were induced on histidine medium over 4000-fold (P<0.01, r=0.9538) and on leucine medium over 300-fold (P<0.01, r=0.9701). Base pair
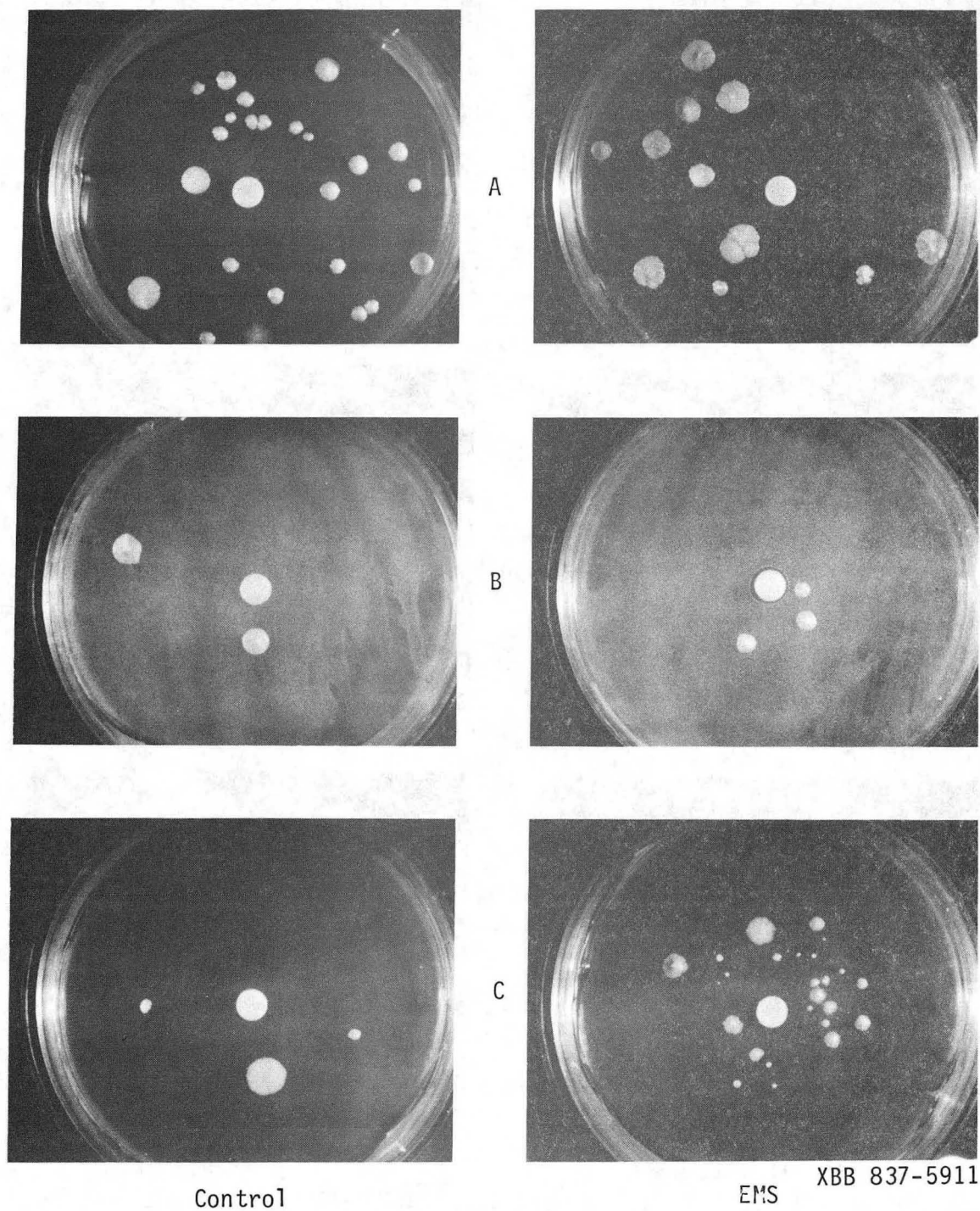
Figure 15. Ethyl methanesulfonate spot tests (25 μl undiluted; control: nothing). A. Base pair substitution on lys±. B. Frameshift mutation on his±. C. Frameshift mutation on leu±.
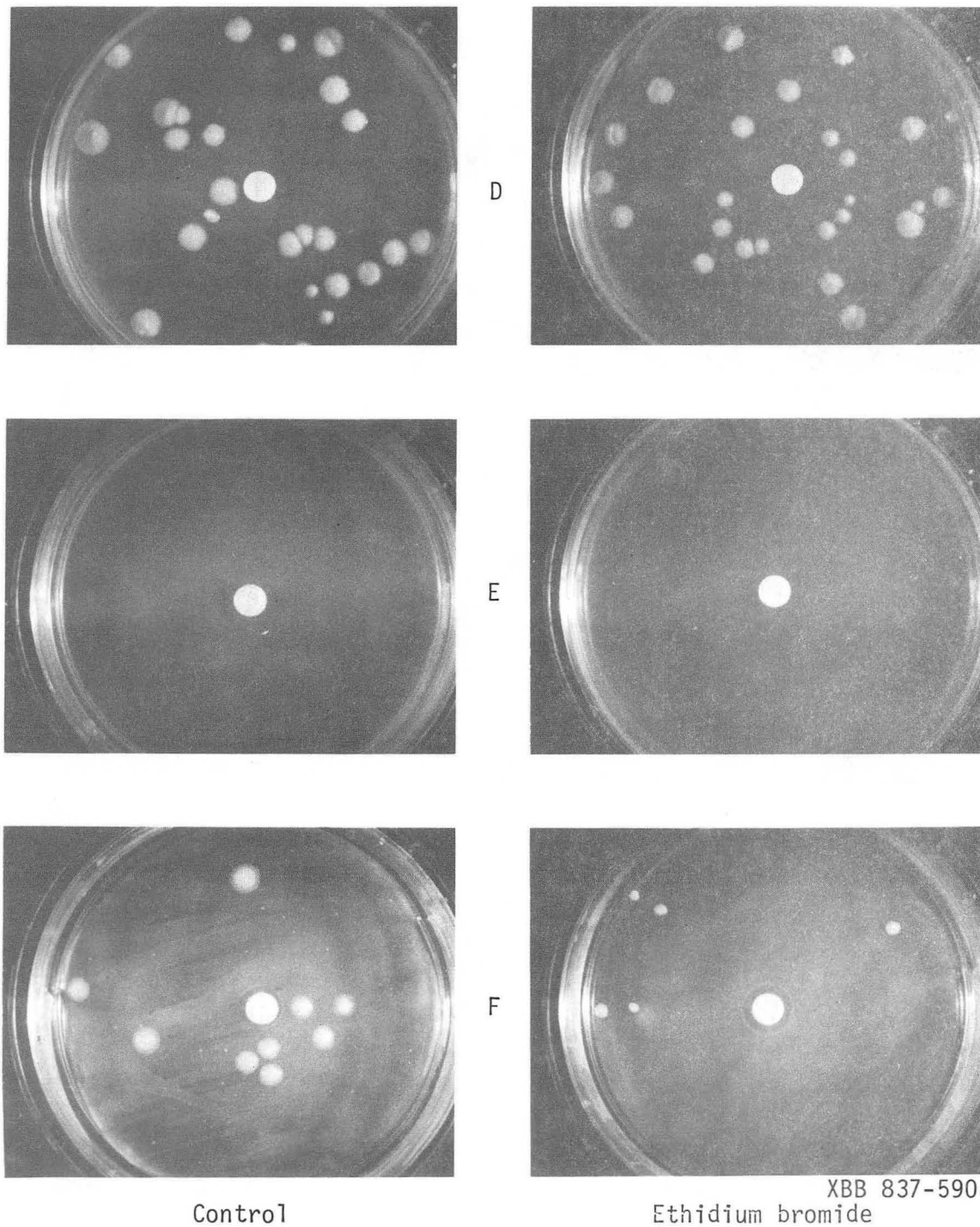
Control EMS

XBB 837-5910
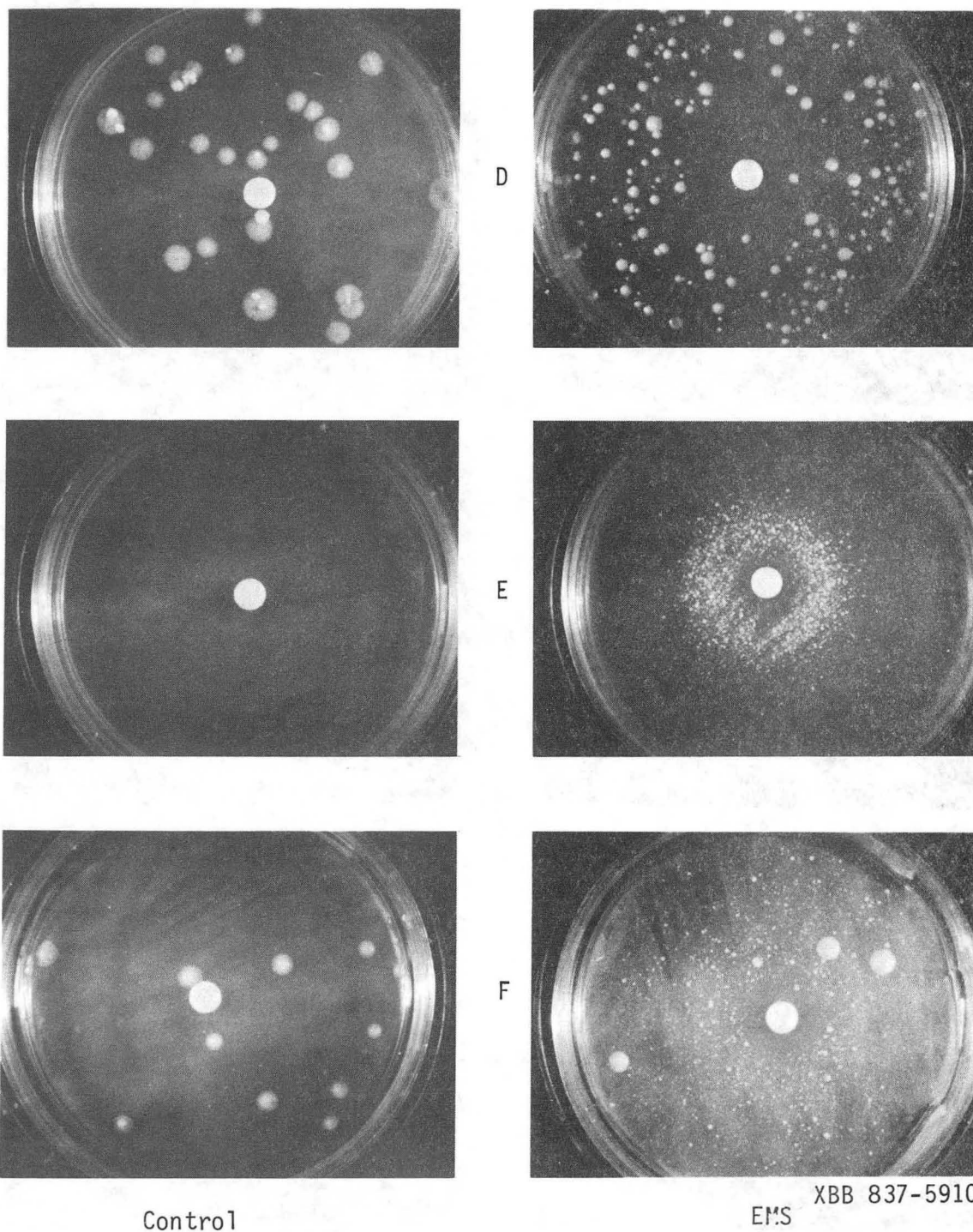
Figure 15. Ethyl methanesulfonate spot tests (continued). D. Gene
conversion on arg+. E. Forward mutation on chloramphenicol.
F. Mitochondrial mutation on erythromycin.
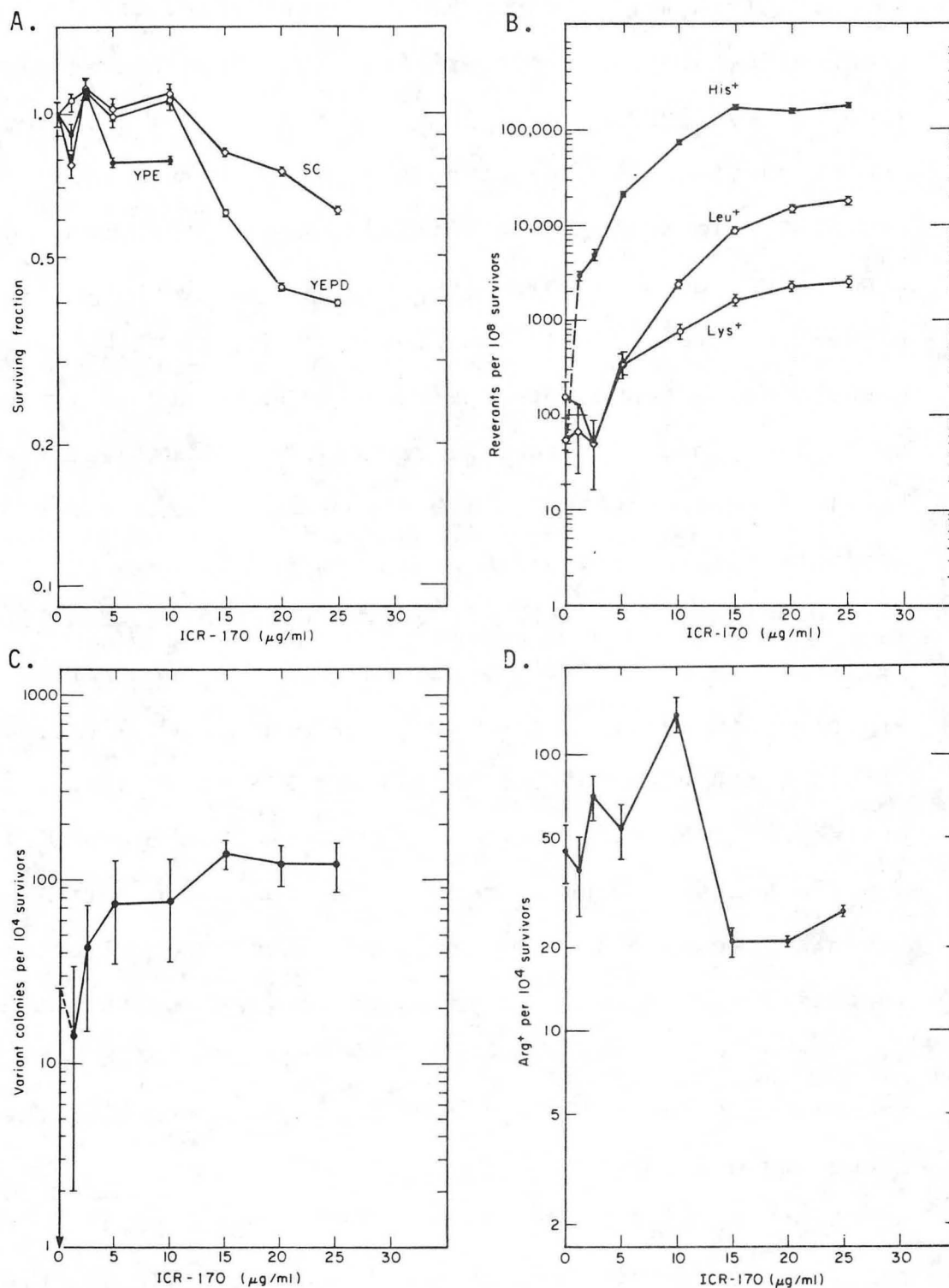
XBL836-3820

Figure 16.   ICR-170 suspension tests
             (Note on Figure 16F: dotted line is dose-response
             curve with YPE medium as control; solid line is
             dose-response curve with petites on YEPD as control)

substitutions were induced to a lesser extent than frameshift mutations, but still were induced over 15-fold (P<0.01, r=0.9857, Figure 16B). Although over a four-fold induction of mitotic crossing-over was observed (P<0.05, r=0.8910, Figure 16C), no significant effect on gene conversion was seen comparing only the spontaneous and highest dose Figures (Figure 16D). However, the dose-response curve for gene conversion seemed to increase up to 10 ug/ml and then fell rapidly below the initial level of gene conversion. This possible narrow range of activity will be discussed in the next section.

Additionally, ICR-170 greatly increased the fraction of petites (P<0.01, r=0.9617, Figure 16E). Mitochondrial erythromycin mutation also showed a significant increase (P<0.01, r=0.8846 and r=0.8945 using YPE as a control or petites on YEPD as a control, respectively; Figure 16F).

The yield of base pair substitution and both frameshift mutants, recombinants, petites, and mitochondrial mutants increased with dose. The yield of convertants increased with dose up to a maximum and then decreased with increasing doses. The yield data is in accord with the frequency data.

Spot tests with ICR-170 (Figure 17) were positive for histidine frameshift and base pair substitution mutations. Both the forward mitochondrial mutation on erythromycin and forward mutation on chloramphenicol results appeared slightly positive. Gene conversion and leucine frameshift
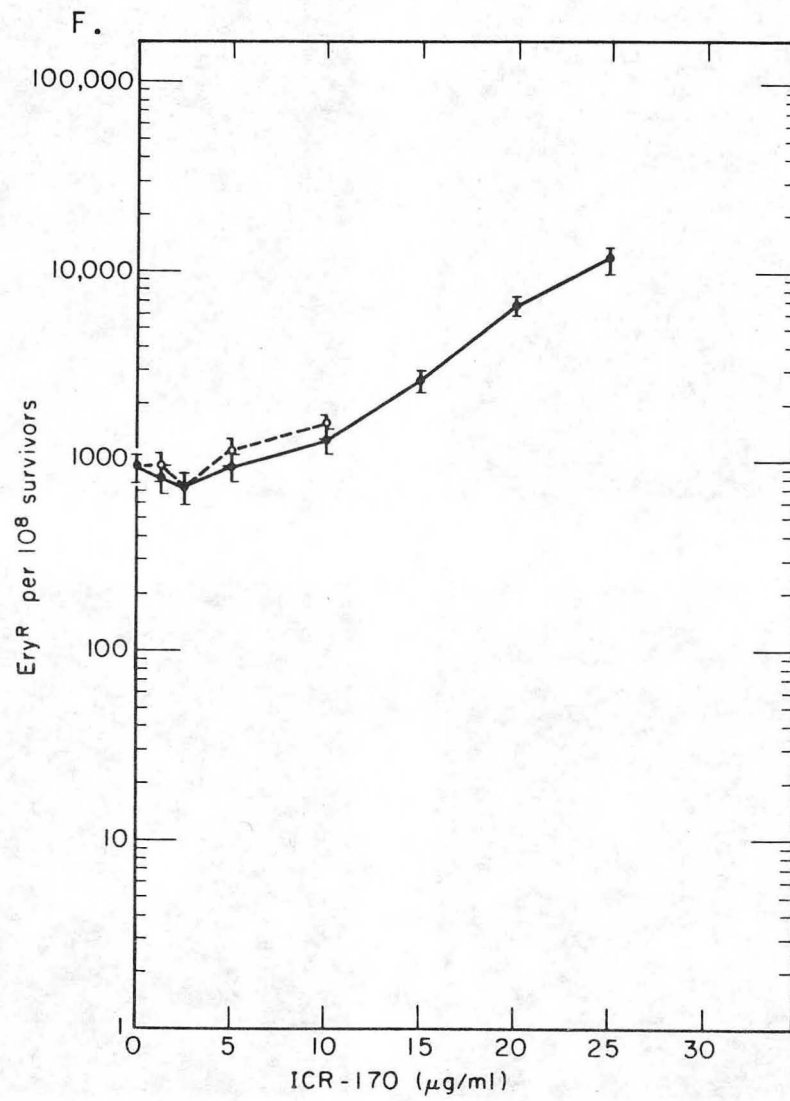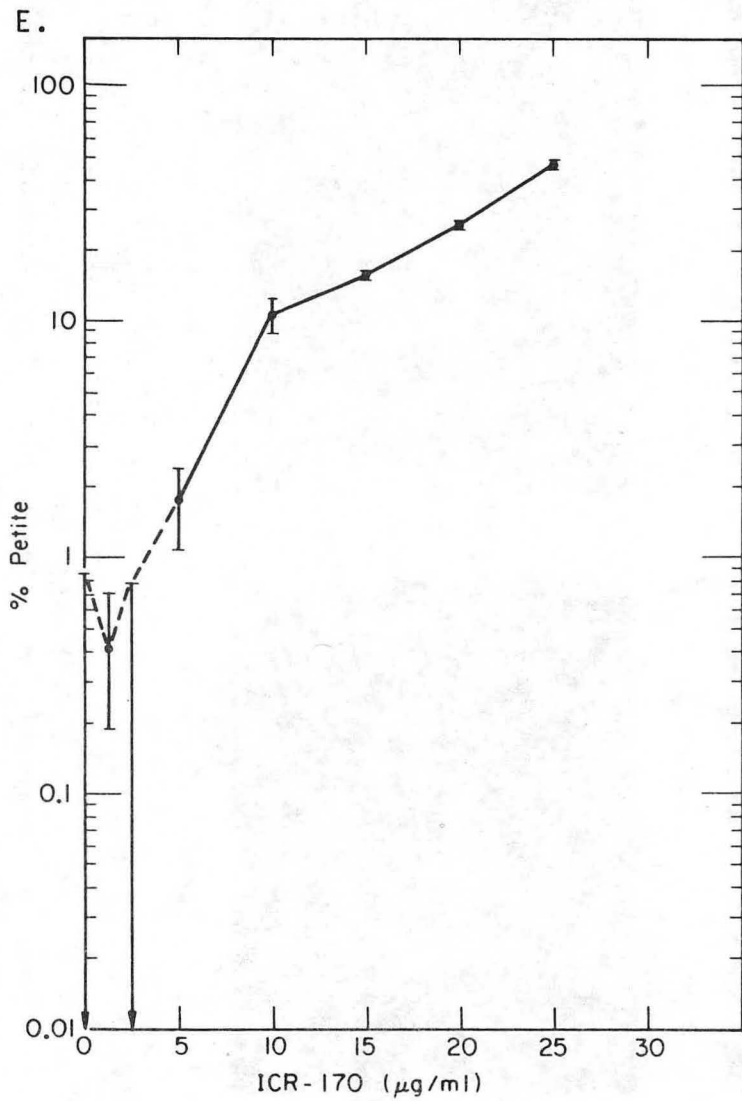
Figure 16.   ICR-170 suspension tests (continued)

XBL836-3824

73
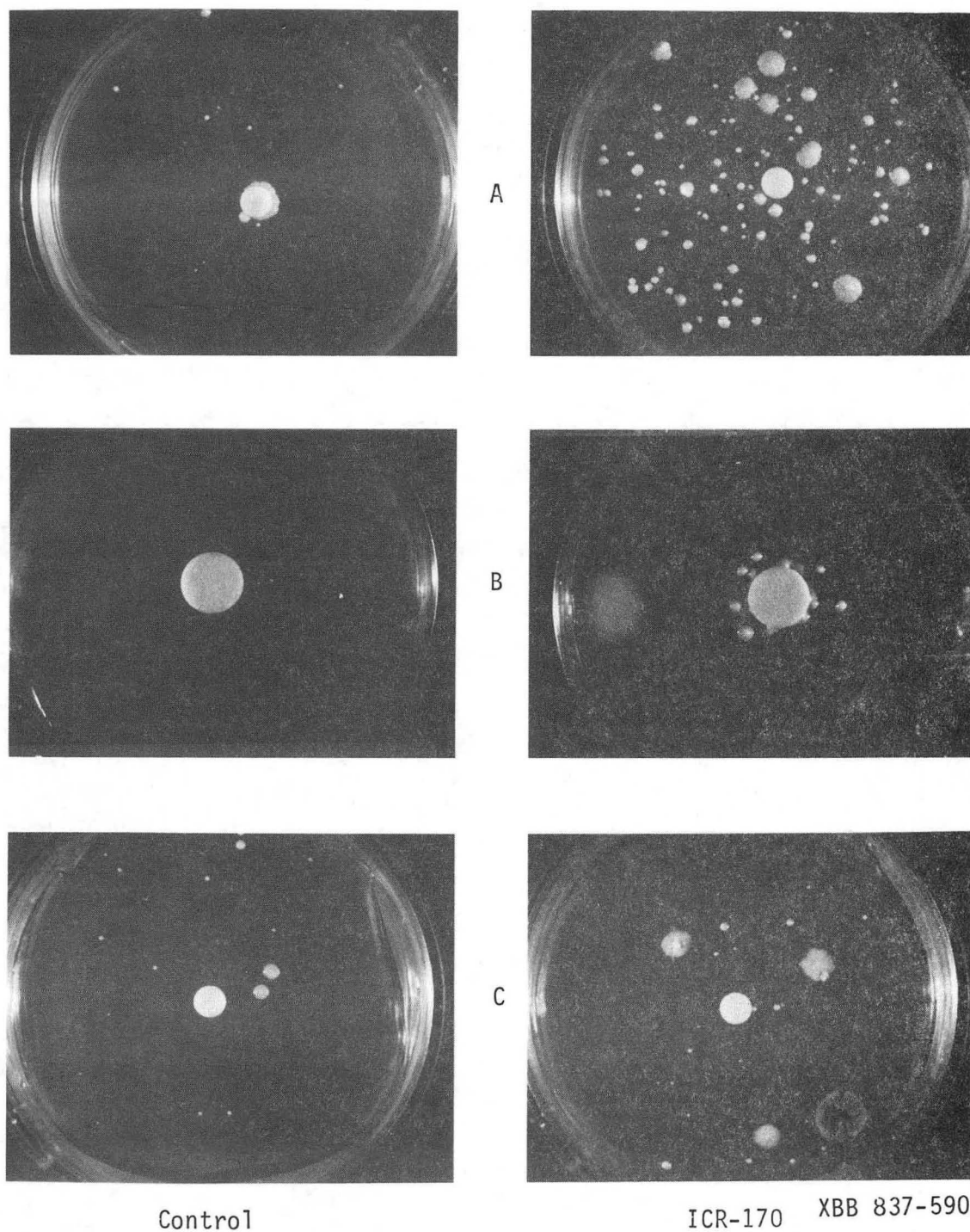
Figure 17.   ICR-170 spot tests (25 or 40 μl of 10 mg/ml solution except Figure 31B which is 50 μl of 5 mg/ml solution; control: distilled water). A. Base pair substitution on lys±. B. Frameshift mutation on his±. C. Frameshift mutation on leu±.

75

Control   ICR-170   XBB 837-5903
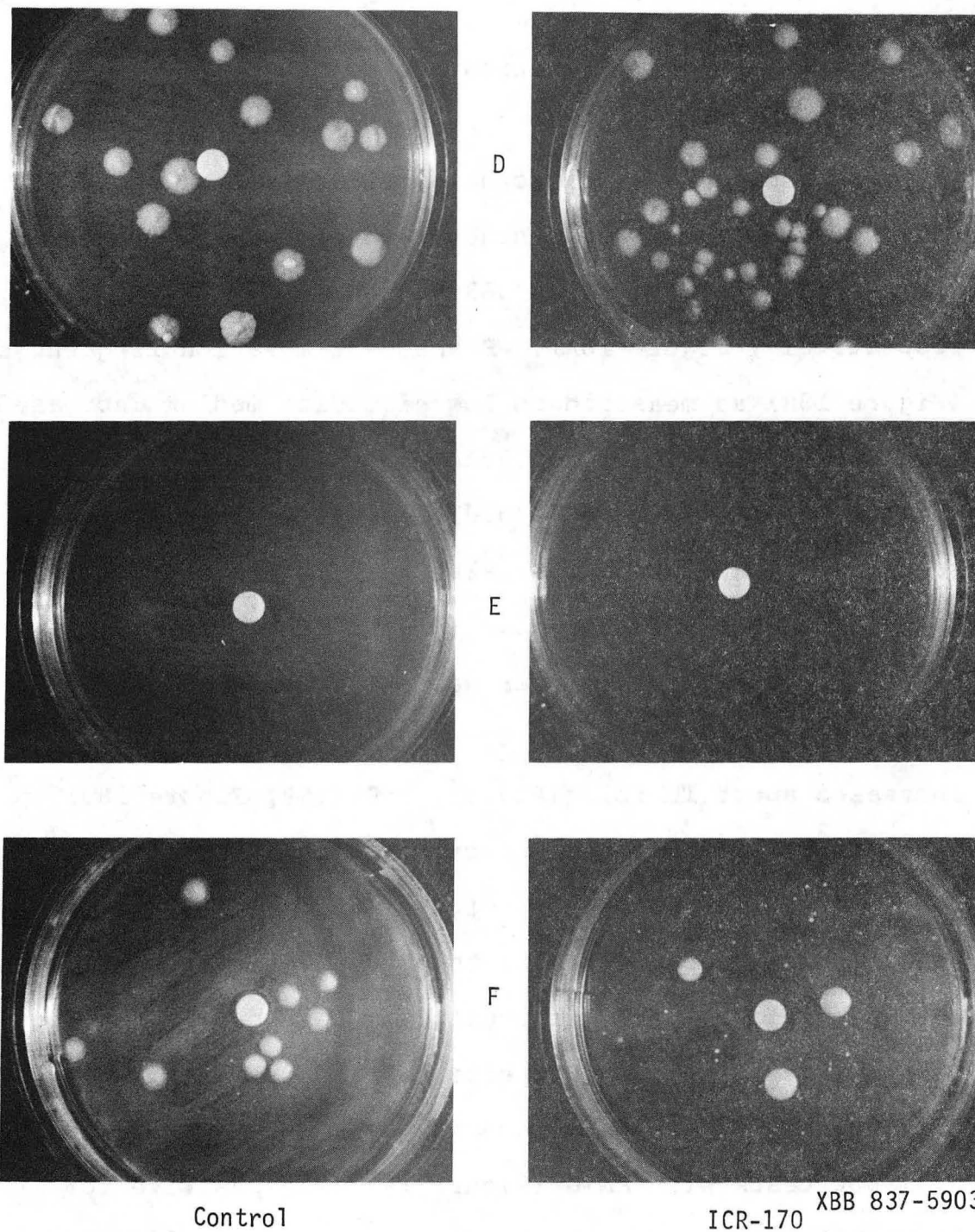
Figure 17.   ICR-170 spot tests (continued).   D.   Gene conversion on arg+.
             E.   Forward mutation on chloramphenicol. F. Mitochondrial
             mutation on erythromycin.

76

mutation were not noticeably affected.

4. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

MNNG induced a significant amount of cell killing on all three media: YEPD, synthetic complete, and YPE (P<0.01 for all and r=-0.8583, r=-0.8380, and r=-0.7768, respectively; Figure 18A). Frameshift mutation frequencies (Figure 18B) as measured on low histidine medium increased over 100-fold (P<0.01, r=0.9934) and on low leucine medium increased over 300-fold (P<0.01, r=0.9343). Base pair substitution mutations were also heavily induced, almost 10-fold, on low lysine medium (P<0.01, r=0.9842, Figure 18B). Mitotic crossing-over was increased about 20-fold (P<0.01, r=0.9223, Figure 18C) and gene conversion was increased about 10-fold (P<0.01, r=0.8699, Figure 18D).

A large, significant induction of petites was also observed (P<0.01, r=0.9251, Figure 18E). No tests were done for the induction of erythromycin resistance.

The yield of both frameshift and base pair substitution mutants, recombinants, convertants, and petites increased with dose. The yield and the frequency data are in accord.

Spot tests with MNNG (Figure 19) were positive for frameshift mutation on leucine, gene conversion, forward mitochondrial mutation on erythromycin medium, and forward mutation on chloramphenicol medium. No noticable effects were seen on base pair substitution and frameshift mutation
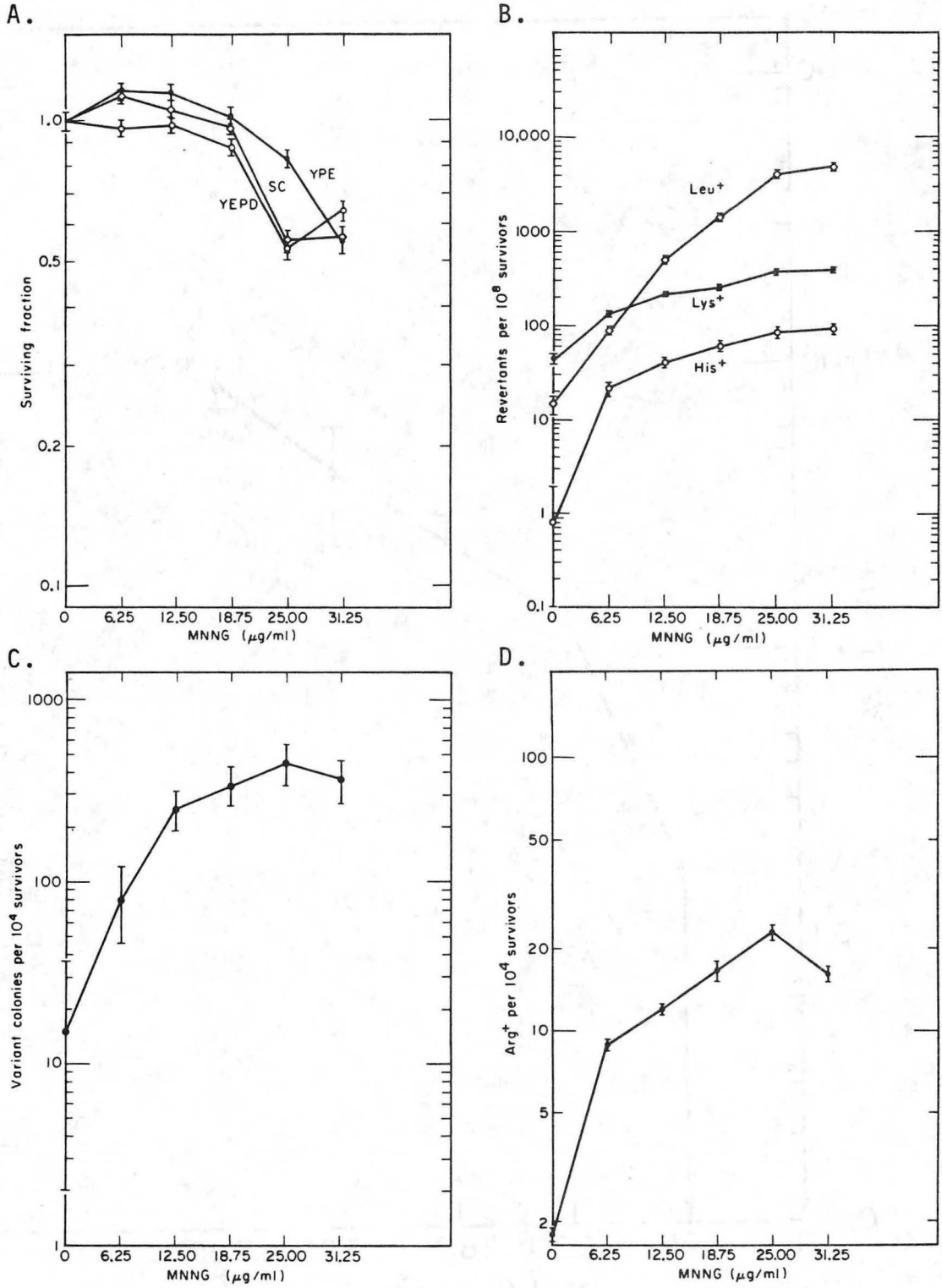
XBL836-3819

Figure 18. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) suspension
            tests

Figure 18.   N-methyl-N'-nitro'-N-nitrosoguanidine   XBL831-3532
              (MNNG) suspension tests (continued)
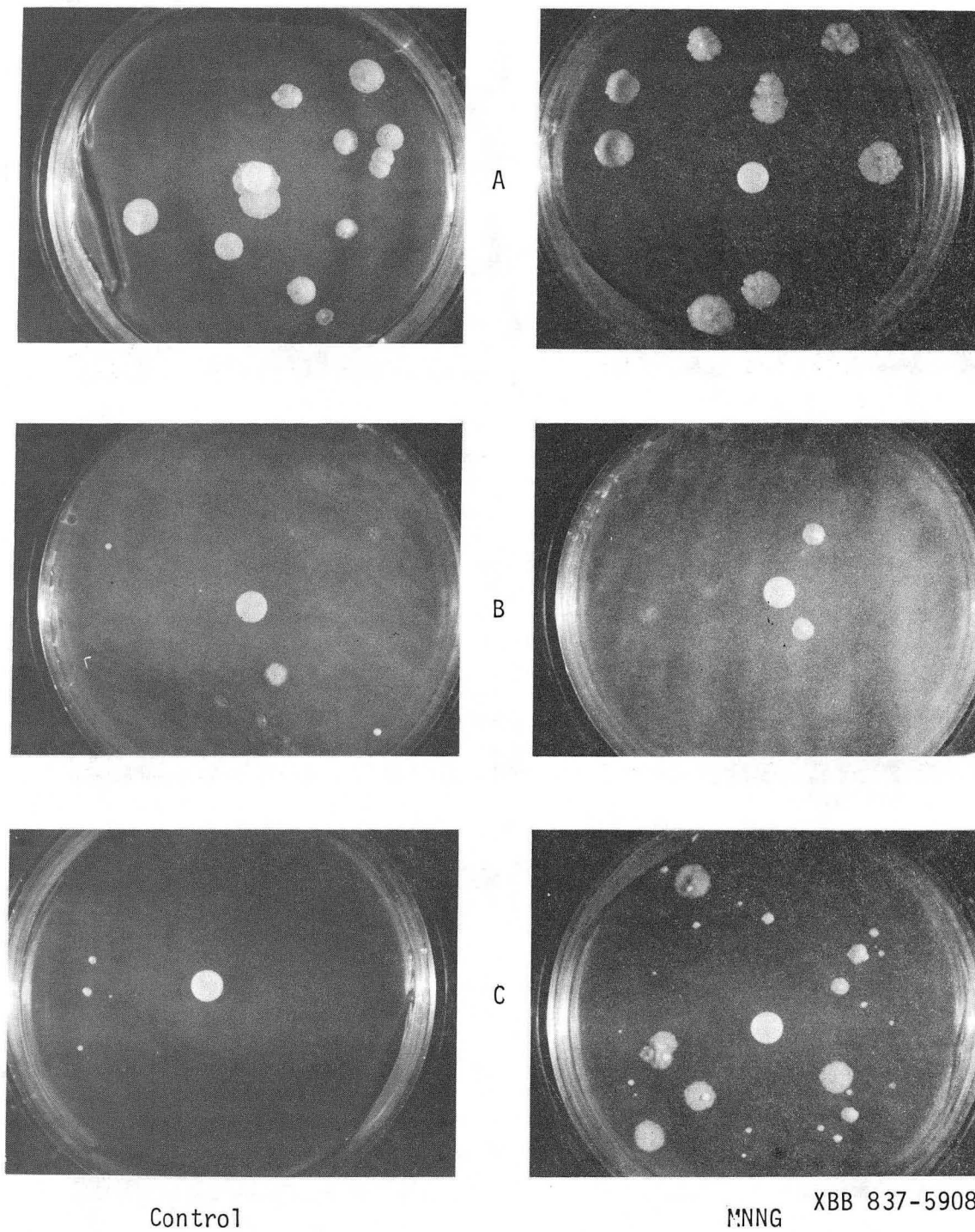
Figure 19. N-methyl-N'-nitro-N-nitrosoguanidine spot tests (5 or 10 μl
of 4 mg/ml solution; control: 0.1 M citrate buffer, pH 5.0).
A. Base pair substitution on lys±. B. Frameshift mutation
on his±. C. Frameshift mutation on leu±.

on histidine.

5. 4-Nitroquinoline-N-oxide (4-NQO)

4-NQO induced cell killing almost identically on YEPD
and synthetic complete media (P<0.01 for both, r=-0.9670
and r=-0.9748, respectively; Figure 20A). Cell killing was
even more apparent on YPE medium (P<0.01, r=-0.9147, Figure
20A), although the slope of killing paralleled that on the
two fermentable media after the lower doses, <2 ug/ml. The
slopes of the dose-response curves were similar for both
frameshift mutations and base pair substitution mutation
(Figure 20B) although the spontaneous frequencies were
considerably different. The increases in frameshift
mutation frequencies as measured on low histidine medium
and on low leucine medium were very large, 800 and 80-fold,
respectively (P<0.01 for both; r=0.9942 and r=0.9953,
respectively). Base pair substitution mutation was also
increased 300-fold over control values (P<0.01, r=0.9886).
Mitotic crossing-over and gene conversion were both
induced, 25 and 10-fold respectively (P<0.01 for both,
r=0.7898 and r=0.9663, respectively; Figures 20C and 20D).

Petite induction reached a high plateau in the early
dose range (P<0.01, r=0.5088, Figure 20E). No data are
available on mitochondrial erythromycin reistance
frequencies.

The yield of base pair substitution and both frameshift
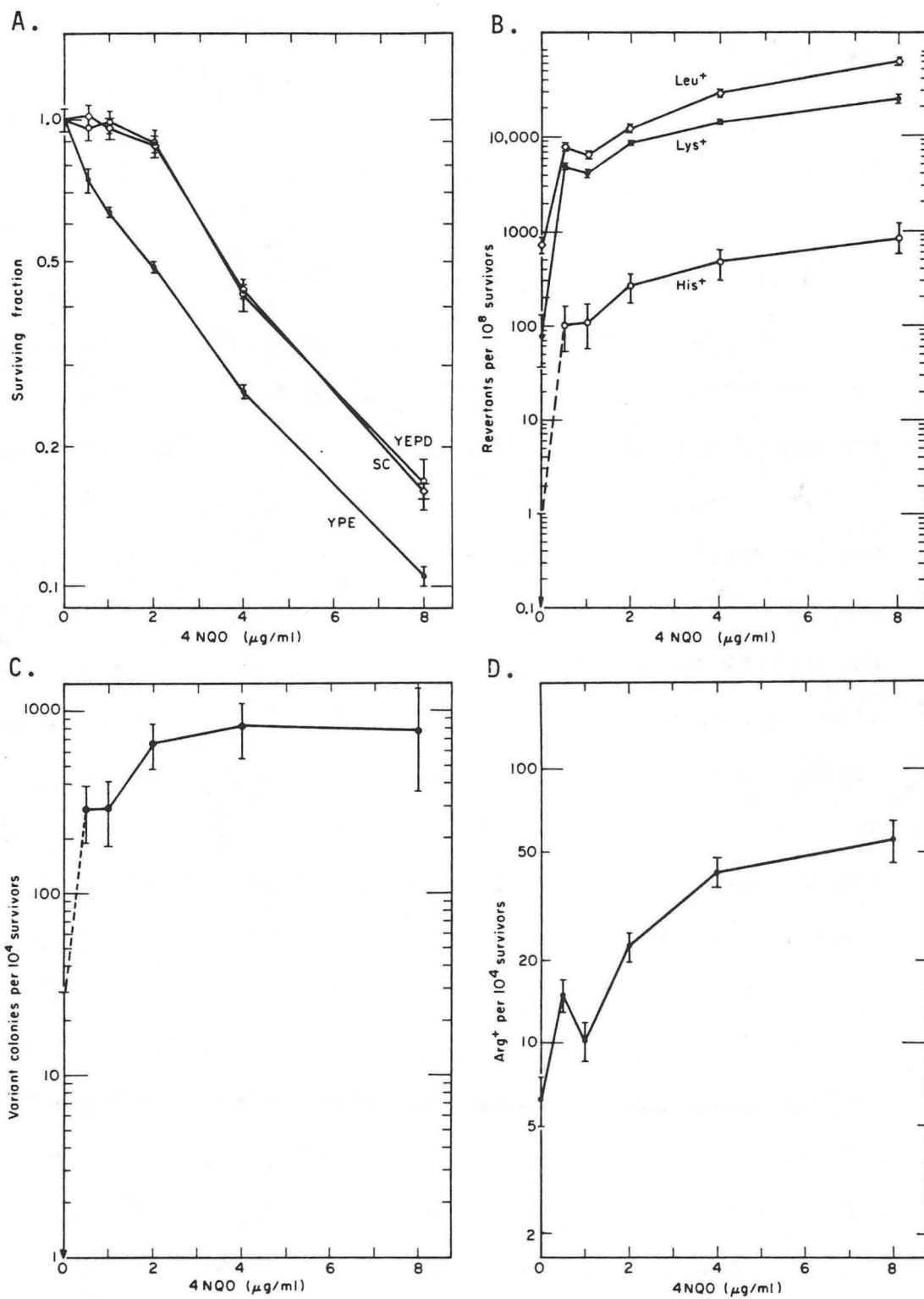
Figure 20. 4-Nitroquinoline-N-oxide suspension tests

XBL836-3818
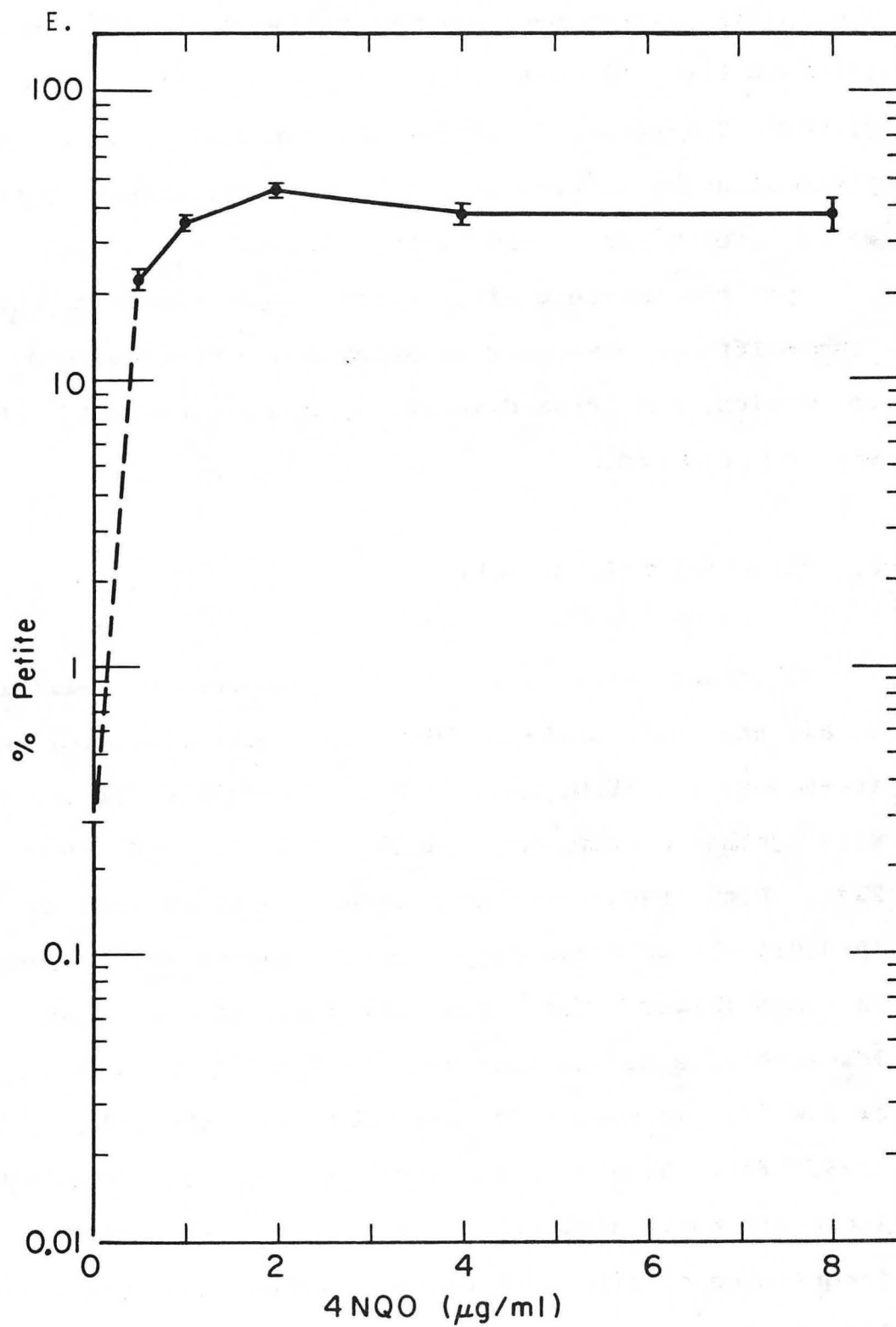
XBL831-3533

Figure 20. 4-Nitroquinoline-N-oxide suspension tests (continued)

mutants, recombinants, and convertants increased with dose
generally and then decreased slightly at the highest dose
although the yield was still greater than on the control
plates. The yield of petites increased with dose. The
yield data was in general accord with the frequency data
which leveled off at the highest doses.

Spot tests were positive with 4-NQO (Figure 21) for
frameshift and base pair substitution mutations, gene
conversion, and forward mutation on chloramphenicol and
erythromycin media.

6. Ultraviolet light (UV)

UV induced significant ($P<0.01$) amounts of cell killing
on all the media tested: YEPD, synthetic complete, and YPE
($r=-0.9894$ for YEPD, $r=-0.9799$ and $r=-0.9696$ for two trials
with synthetic complete, and $r=-0.9446$ for YPE, Figure
22A). High frequencies of nuclear mutations were induced
($P<0.01$) although the dose-response curves were dissimilar
in shape (Figure 22B). Frameshift mutation frequency on
low histidine medium increased 200-fold ($r=0.7989$) whereas
on low leucine medium it increased over 5000-fold
($r=0.9242$). Base pair substitution mutation frequency
increased about 1000-fold ($P<0.01$, $r=0.9748$). High
frequencies of mitotic crossing-over and gene conversion,
100 and 15-fold respectively, were also induced ($P<0.01$ for
both, $r=0.9295$ and $r=0.9941$, respectively, Figures 22C

XBB 837-5907

Control   4-NQO
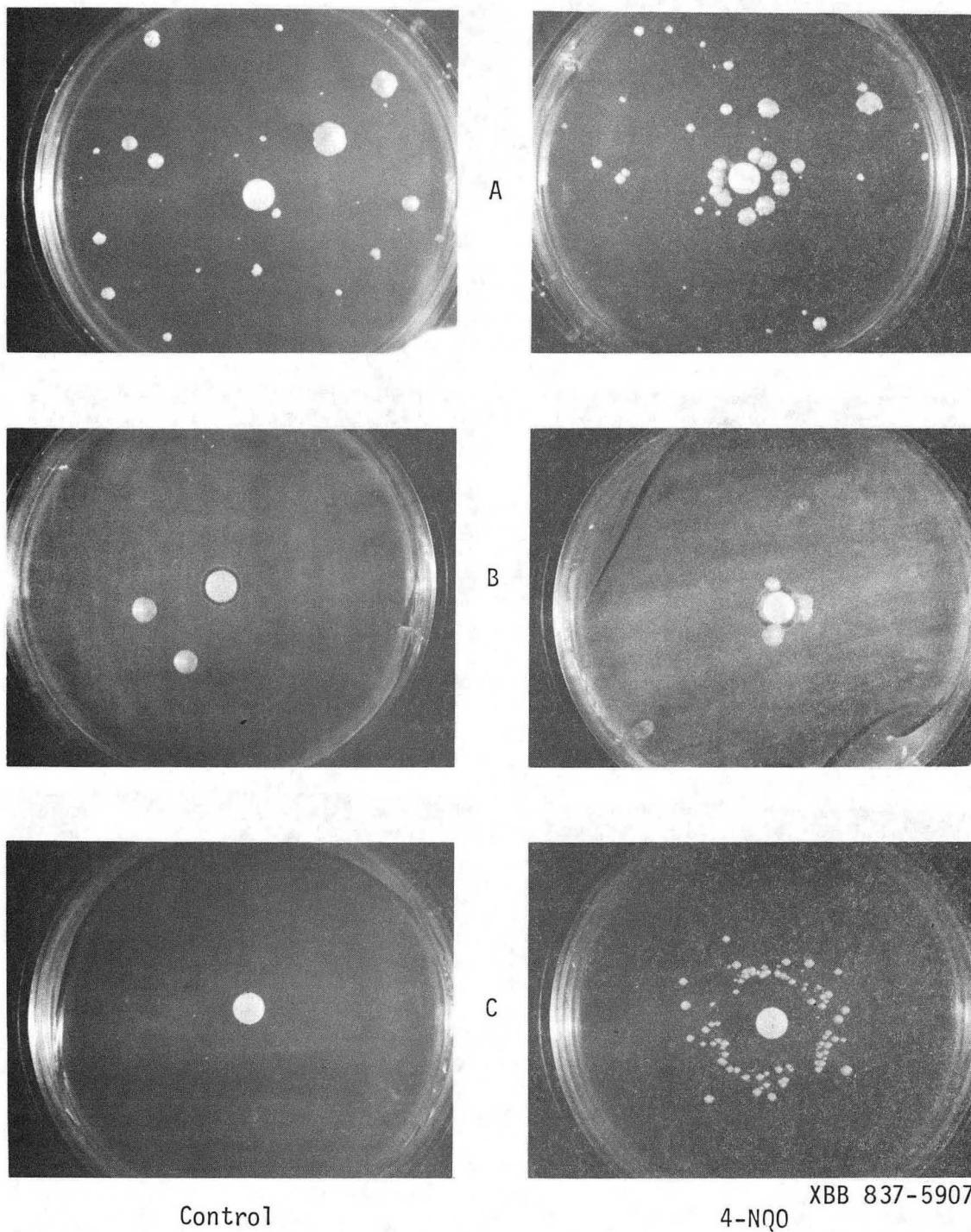
Figure 21. 4-nitroquinoline-N-oxide spot tests (15 or 25 µl of 0.5 mg/ml
            solution; control: DMSO). A. Base pair substitution on lys+.
            B. Frameshift mutation on his±. C. Frameshift mutation on
            leu±.

XBB 837-5905

Control   4-NQO
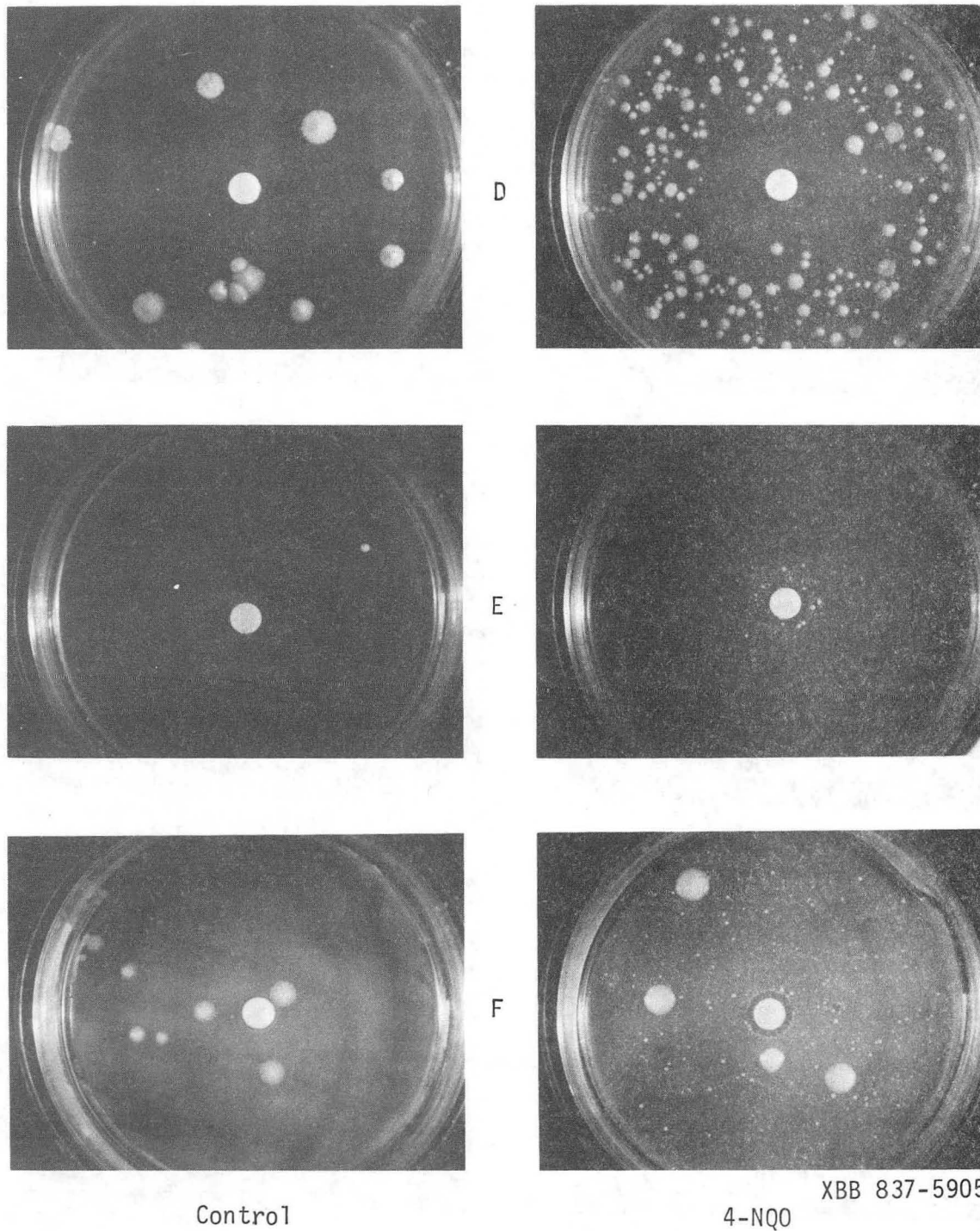
Figure 21. 4-nitroquinoline-N-oxide spot tests (continued). D. Gene conversion on arg±. E. Forward mutation on chloramphenicol. F. Mitochondrial mutation on erythromycin.
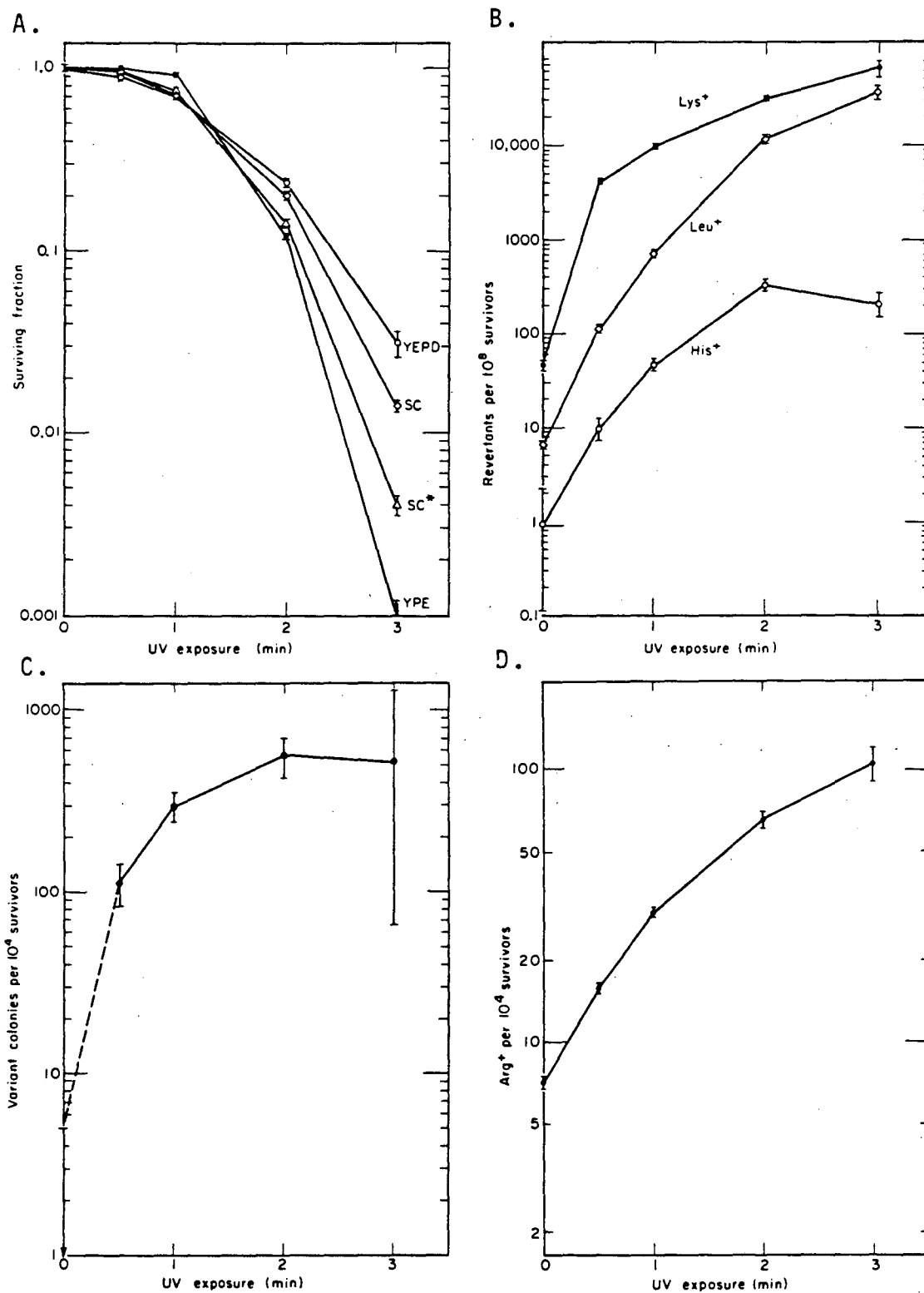
Figure 22. Ultraviolet light plate tests (flux = 27 ergs/mm^2/sec)

XBL836-3817

and 22D).

Forward mutation as measured by the induction of chloramphenicol resistance increased 2000-fold ($P<0.01$, $r=0.7932$, Figure 22E). In addition, UV induced a large fraction of petites ($P<0.01$, $r=0.9100$, Figure 22F). Similar t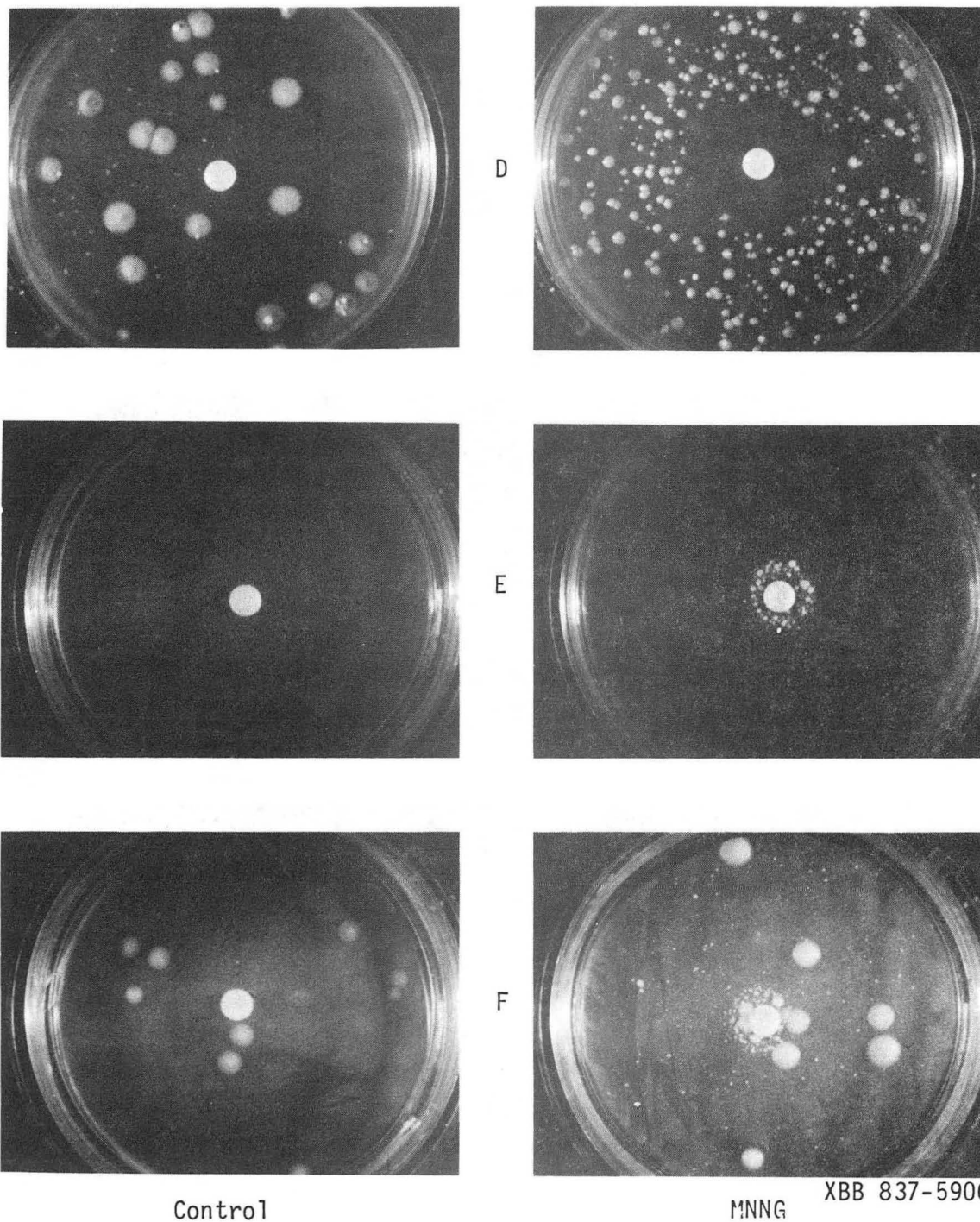o the chlorampehnicol results, mitochondrial mutation on erythromycin medium also increased 2000-fold ($P<0.01$, $r=0.7995$, Figure 22G). In summary, UV induced significant ($P<0.01$), large effects on the frequency of every event measured. The yield of base pair substitution, both frameshift and forward chloramphenicol mutants; recombinants; convertants; petites; and mitochondrial mutants increased with dose.

Spot tests were not applicable.
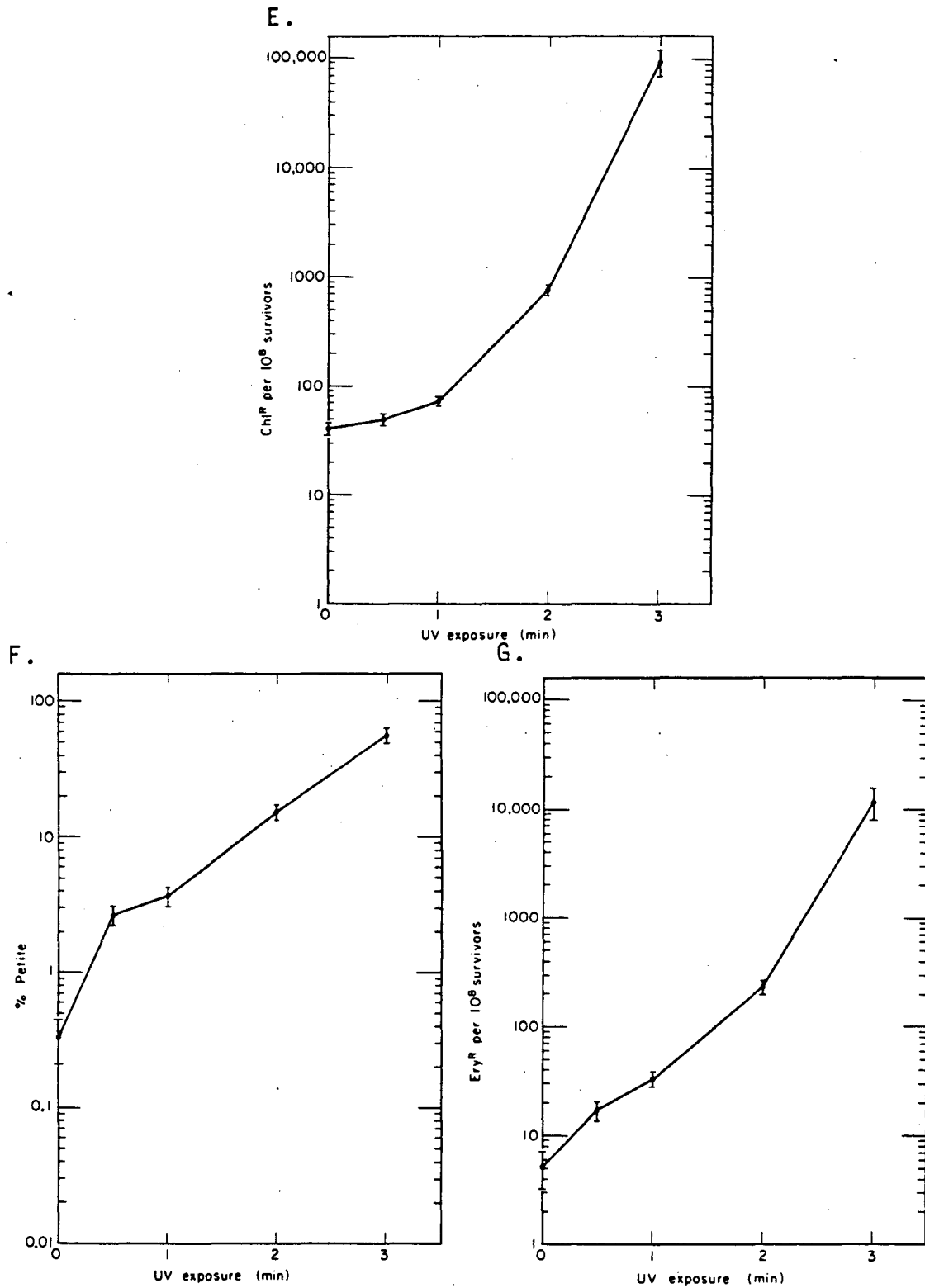
Figure 22. Ultraviolet light plate tests (flux = 27 ergs/mm^2/sec)
(continued)

XBL836-3816

IV. DISCUSSION OF MUTATION AND RECOMBINATION STUDIES

Mutational assay systems have been highly sensitive and accurate in detecting carcinogens as mutagens. Certain carcinogens, however, are not readily detected by reverse mutational systems, but can be detected in recombinational assay systems or in forward mutational systems. Strain XD83 represents a combination of a number of assay systems in one strain: forward mutation as measured by the induction of chloramphenicol resistance, back nuclear frameshift and base pair mutation, nuclear intragenic and intergenic recombination, mitochondrial forward mutation, and large mitochondrial deletions. All of these genetic changes have been suggested as possible steps in the carcinogenic process. A small, select number of widely used genotoxic agents were tested both in suspension tests and spot tests with this system. A good agreement was obtained between the two methods. Those agents which induced a significant change in a given endpoint usually demonstrated a strong linear component of induction as well in the dose range used.

A. Test Results

1. Ethidium bromide

General: Ethidium bromide may be carcinogenic and is mutagenic in the _Salmonella_/microsome assay (Ames' test) with S-9 activation (McCann et al., 1975a). It is an aromatic amine and an intercalating agent. In yeast ethidium bromide binds preferentially to mitochondrial DNA (Mahler and Bastos, 1974) and is used routinely to induce mitochondrial petites, both ρ^O and ρ^- (Slonimski et al., 1968; Dujon et al., 1981; Sherman et al., 1979). In other organisms it has mutated mitochondria, chloroplasts and other plasmids and in some organisms it has also mutated nuclear DNA (Levy and Ashri, 1975).

Nuclear mutation: Hixon and Burnham (1979) did not observe ethidium bromide-induced reverse mutation in yeast strains D7 or N123 in growing cells with or without light and without exogenous activation. No significant effects on reverse frameshift or base pair substitution mutations were observed in either stationary or exponentially growing cells of XD83 without light in suspension or spot tests. However, no cell killing was apparent at these doses and possibly higher doses might show effects.

Nuclear recombination: No recombination was seen in yeast with growing C3116 or D7 cells with or without light (Hixon and Burnham, 1979; Takahashi, 1972).   Hixon and Burnham (1979) also did not observe gene conversion in D7 growing cells with or without light. However, Ito and Kobayashi (1977) did observe gene conversion in stationary cells of D4 under light conditions. In XD83 there was no

increase in gene conversion. Instead there was a decrease in gene conversion which may have been an artifact due to the high induction of petites with high doses which leads to slow growth. It was difficult to observe mitotic crossing-over due to the high frequency of induced petites. Either high glucose or low adenine medium might give more conclusive results.

Mitochondrial mutation: Ethidium bromide as expected from the work of other investigators (Slonimski et al., 1968) induced a high frequency of petites both in stationary and exponentially growing XD83 cells. The higher rate of induction seen with the exponentially growing cells may have been due to the greater permeability of the cells during exponential growth. Several carcinogens have been shown to have greater effects on exponentially growing cells (Parry et al., 1976). Cottrell (1982) observed that the peak uptake of ethidium bromide and petite mutagenesis coincided with bud formation and the beginning of nuclear DNA synthesis although there was a considerable uptake and mutagenesis at all times in the cell cycle. Another possibility is the differences in metabolism of the cell during exponential growth. For example, the cytochrome P450 system of yeast is induced in growing yeast cells, but not in non-growing cells (Callen and Philpot, 1977; Wiseman and Lim, 1975b).

A greater induction of petites was observed on the YEPD plates than was reflected by killing on the YPE plates.

However, it appears that petites may actually grow to some
extent on yeast extract, peptone and agar without dextrose
(or ethanol) and, therefore, the YPE medium may not fully
select against petites (J.B. Bassel, personal
communication).

Ethidium bromide is highly specific for mitochondrial
mutation and it may be that much higher doses are needed
for induction of nuclear mutations. Possibly ethidium
bromide does not reach very high concentrations in the
nucleus. It appears that in those cases in which ethidium
bromide did induce mutations, mutagenesis appeared to be
specific to certain types of damage and only occurred under
specific conditions.

In XD83 the high induction of petites interfered with
the determination of mitochondrial erythromycin resistance
induction. Petite induction was marked in both stationary
and exponentially growing cells although it was highest and
most rapid in exponentially growing cells. The high
induction of petites by ethidium bromide was even visible
in a spot test.

Ethidium bromide showed very little cytotoxicity at the
doses used. Possibly higher doses might result in
cytotoxicity, nuclear mutation and recombination. However,
petite induction was observed at even very low doses of
ethidium bromide. Even the relatively high doses used in
spot tests resulted in no observable effects other than
petite induction.

2. Ethyl methanesulfonate

General: Ethyl methanesulfonate is a powerful alkylating agent and a known carcinogen. It reacts by a borderline S_N1/S_N2 mechanism and shows a high reactivity towards O-atom sites in DNA besides reacting strongly with the N-7 of guanine and less strongly with the N-3 of adenine, N-1 of adenine, N-3 of cytosine and other sites (Lawley, 1974a and b; Singer, 1975). A S_N1 mechanism is a two-step reaction in which a bond between the substrate and leaving group is broken before the new bond is established whereas a S_N2 reaction is a bimolecular nucleophilic substitution reaction. S_N1 reagents react relatively more extensively at O-atom sites in DNA or RNA than do S_N2 reagents which tend to react with O-atom sites to form phosphotriesters (Lawley, 1974a and b). Therefore, since EMS reacts by an intermediate mechanism, besides alkylation of O-atoms of guanine and pyrimidines, phosphotriesters are formed (Lawley, 1974a and b; Singer, 1975). Depurination or hydrolysis of the phosphotriesters can lead to strand breakage (Lawley, 1974a and b; Singer, 1975). Singer (1982) has found that ethylating agents react more with oxygen than do analogous methylating agents and are frequently also more mutagenic at lower treatment doses. Whether the mutagenicity of EMS is due to faulty base-pairing or to faulty repair of the

alklated bases is not known. EMS appears to induce and
revert primarily base-pair substitution mutations,
primarily transitions from guanine:cytosine to
adenine:thymine (Krieg, 1963; Prakash et al., 1973). EMS
fairly specifically induced base pair substitution mutation
in the Ames' test (McCann et al., 1975a). It does not
require an exogenous activation system.

Nuclear mutation: Lindegren et al. (1965) induced
forward mutations in yeast at a number of sites. Since a
greater proportion of these were multiple site than would
be expected from independent events, these investigators
postulated that chromosomal rearrangements might be
occurring in addition to gene mutations. Fahrig (1979) and
Nesvera (1973) observed forward mutation in stationary
yeast cells. Kern and Zimmermann (1978) observed reverse
mutation in stationary cells of D7 and D7 derivative
strains with either rad3 or rad6 mutations. Reverse
mutation in stationary yeast cells was also observed by
Shanin and von Borstel (1977) and Parry (1969).

Van Zeeland et al. (1983) found relatively small
differences in the DNA alkylation levels from EMS in
Neurospora and yeast compared to those previously
determined for E. coli and mammalian cells in culture. The
ethylations per nucleotide increased at a rate slightly
less than proportional to the concentration of EMS.
However, the mutation kinetics were considerably different
for the different species. They reported that Neurospora

and yeast, like E. coli, showed an exponential increase
of genetic effects. Forward mutations at the adenine loci
were scored in Neurospora and yeast. The slope of the
log-log dose-response curve was 1.5 for yeast and 2.6 for
Neurospora. The slopes are linear on a log-log plot and
indicate a power log curve (although they describe the
curve as exponential). The Neurospora test can pick up
multi-locus deletions. The haploid yeast test does not.
They postulate, therefore, that if EMS induces multi-locus
deletions as well as other types of genetic damage, then
this might explain the different slopes. Unfortunately, no
comments were made concerning the log-log plot of the
induced reversion frequency per survivor versus the EMS
exposure concentration and error bars were absent.
However, a replotting of the lys1 reversion data on a
linear plot indicated that the data would also fit a linear
curve quite well. Actually, the forward mutation
dose-response curve for yeast is quite linear except for
the last dose point. Possibly the mammalian and Drosophila
dose-response curves are linear only because relatively low
doses were plotted. It is unfortunate that error bars were
not included in the graphs since it is often possible to
draw many curves through the same points.

Prakash and Sherman (1973) found that EMS induced
selective guanine:cytosine to adenine:thymine transitions
in an iso-1-cytochrome c mutant of yeast in a base pair
substitution test. Although most of the radiation repair

(rad) mutants tested did not affect EMS-induced mutation frequencies, four of sixteen of these rad mutants did affect mutation frequencies (Prakash, 1976). Prakash (1976) concluded that a functional repair system and misrepair is required for EMS mutagenesis (Prakash, 1974).

As expected, EMS induced a high frequency of base pair substitution in strain XD83. However, it also induced very high frequencies of frameshift mutations. A high linear component was observed for mutation induction especially for frameshift mutations at the doses used.

Shukla and Auerbach (1981) concluded that at least 60% of EMS-induced point mutations in a Drosophila test system were dose-independent small deletions although an alternative possibility which has some support is that EMS tends to produce clusters of linked mutations. The high induction of frameshift mutation in the EMS treated XD83 strain agrees with the findings of Shukla and Auerbach.

Recombination: EMS-induced recombination in yeast has been observed by a number of experimenters (Brusick and Andrews, 1974; Fahrig, 1979; Parry, 1969). Gene conversion has also been observed in strain D4 (Brusick and Andrews, 1974; Fahrig, 1973). Kern and Zimmermann (1978) observed mitotic crossing-over and gene conversion in stationary cells of D7 with EMS. D7 with rad3 or rad6 mutations were sensitive to much lower doses of EMS than the wild type D7. Zimmermann et al. (1975) found that EMS induced mitotic crossing-over, gene conversion, and reversion mutation in

stationary phase cells. The dose-response curves for mitotic gene conversion and reversion were exponential whereas the curve for induction of mitotic crossing-over as indicated by twin sectors reached a plateau at doses giving about 30% killing. Gene conversion of several other strains has been observed also (Parry, 1969; Parry et al., 1979a; Fahrig, 1979). Fahrig (1973) found that EMS induced gene conversion in an intraperitoneal host-mediated assay using stationary D4. Yost et al. (1967) have also observed EMS induced mitotic gene conversion.

Murthy and Sankaranarayanan (1978a) determined the radiation equivalence of EMS for induction of gene conversion using the diploid strain BZ34, a parent strain to XD83. They found that the induction of gene conversion appeared to be non-linear (sigmoidal) in the concentration range that they studied (six doses from 0.025% to 1.0%, 2 hour treatment). In this study, however, there appeared to be a linear induction of gene conversion in the dose range used (six doses from 1 to 6%, 1 hour treatment).,

Mitochondrial mutation: Polakowska and Putrament (1981) found the lethal action of EMS to be greater on the mitochondrial genome than on the nuclear genome of yeast. EMS induced high frequencies of petites, but had little or no effect on the induction of mitochondrial erythromycin resistance. However, forward mutations to mit$^-$ were induced by EMS. Therefore, EMS is capable of inducing at least some mitochondrial mutation. They point out that

since there are many mitochondrial genomes in a single
yeast cell, mutation in a few of the genomes may go
unobserved and the true frequencies of induction of EMS may
be underestimated. The suspension tests with EMS in XD83
showed no significant increase in the induction of
erythromycin resistance whereas the spot tests indicated at
least a slight induction of erythromycin resistance. Both
spot and suspension tests showed an increase in the
induction of chloramphenicol resistance. In this study a
greater induction of petites or mitochondrial lethality was
found than nuclear lethality.

EMS induced a very wide range of genetic changes: base
pair substitution mutations, frameshift mutation,
recombination, mitochondrial point mutations, and
mitochondrial deletions. Suspension and spot tests were in
general agreement.

3. ICR-170

General: ICR-170 has an acridine ring with a nitrogen
mustard sidechain. It induces and reverts primarily
frameshift mutations (Ames and Whitfield, 1966 and Malling,
1967). It is an intercalating agent and a known
carcinogen. It was positive in the Ames' test (McCann et
al., 1975a) and a host of other assay systems (Hollstein et
al., 1979). It is routinely used to induce frameshift
mutations and is often used as a diagnostic mutagen to

distinguish frameshift mutations from base pair substitution mutations. It is highly mutagenic in Drosophila and Neurospora, but is not as mutagenic in Salmonella (Carlson and Oster, 1962; Brockman and Goben, 1965; Ames and Whitfield, 1966). It was developed by Dr. H. J. Creech and his associates as one of several potential antitumor agents (Creech et al., 1960) and has been used in chemotherapy. Malling (1967) found that ICR-170 induced primarily base-pair insertions and deletions in Neurospora crassa and to a lesser extent base-pair substitutions. Comparative studies using structurally related compounds led to the conclusion that the nitrogen mustard group is essential for ICR-170's mutagenicity, that the acridine ring is necessary for frameshift reversions, and that the base-pair substitutions are due to the monofunctional nitrogen mustard group (Malling, 1967).

Nuclear mutation: Brusick (1970) found that ICR-170 induced mutations in four haploid strains of yeast. The sensitivity to killing and to mutagenesis varied greatly depending upon the growth-phase of the cells. Starved, log-phase cells were strikingly most sensitive probably due to an increased uptake of the ICR-170 in the log-phase cells. Brusick (1970) indicates that the lower uptake of ICR-170 in stationary cells may explain the low mutagenicity seen by Parker and Sherman (1969) of ICR-170 for yeast. Brusick (1970) found that 14 of 18 ICR-170-induced auxotrophs were reverted with ICR-170 and

only slightly if at all with MNNG. One mutant reverted only with MNNG and 3 reverted strongly with both ICR-170 and MNNG. ICR-170 induced primarily single-site mutations that were revertible by ICR-170 but relatively stable to the action of MNNG. Brusick (1970) found his results were consistent with the evidence that ICR-170 induces predominantly frameshift mutations in eukaryotes. Pittman and Brusick (1971) have also observed a presumptive specific induction of frameshift mutations in a spot test with ICR-170 which showed an enhanced reversion in log-phase cells as compared to stationary-phase cells. Parker and Sherman (1969) reported an apparent lack of specificity of ICR-170 for the induction of frameshift mutations.

Sequence analysis of ICR-170-induced mutations at the HIS4 locus indicate that ICR-170 causes frameshift mutations in G-C regions of yeast DNA and, furthermore, these frameshifts are +1 G-C additions (Donahue et al., 1981). External suppressors of these mutations appear to involve either a four-base anticodon or its functional equivalent (Donahue et al., 1981).

ICR-170 in XD83 induced a high frequency of reversion of the known frameshift mutation his4-519 and the presumptive frameshift mutation leu2-3. The base pair substitution mutation induction which was also observed is not surprising considering the alkylating side chain on the acridine ring (Malling, 1967).

Recombination: Brusick and Andrews (1974) report that ICR-170 induces gene conversion and mitotic crossing-over in stationary phase cells in Zimmermann's D3, D4, and D5 strains. However, ICR-170 did not show a graded dose-response. They state this narrow range of activity is often characteristic of acridine type compounds. Strain D4 (for gene conversion) was more resistant to the effects of ICR-170 than were the two mitotic crossing-over strains, D3 and D5. Although ICR-170 induced mitotic crossing-over in strain XD83, it did not induce gene conversion in a graded dose-response in suspension tests. Actually the dose response curve for gene conversion in XD83 seemed to increase up to 10 ug/ml ICR-170 and then fell rapidly below the initial level of gene conversion. This may be comparable to the narrow range of activity discussed by Brusick and Andrews. No effect on gene conversion was observable in the spot test.

Mitochondrial mutation: ICR-170 most effectively induces petites in exponentially growing yeast although it does induce petites in stationary cells as well (Brusick, 1970). The results in this study show a strong dose-related increase in mitochondrial mutation and petite induction in suspension tests. Spot tests for mitochondrial point mutations showed a slight positive response.

Suspension and spot tests were in general agreement. Although the high induction of frameshift mutations is the

most striking effect of ICR-170, in this study it proved to be a potent mutagen and recombinogen with a wide range of genotoxicity. ICR-170 did not show a high degree of specificity in its spectrum of genetic damage. The treatment conditions (stationary, non-starved cells in the dark) may have related to this lack of specificity.

4. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

General: MNNG is a known carcinogen and a potent mutagen (for reviews see Neale, 1976 and Gichner and Veleminsky, 1982). MNNG, a monofunctional alkylating agent, is known to attack the DNA at the replication point and frequently induces closely linked secondary mutations in bacteria (Cerda-Olmeda et al., 1968). This propensity of MNNG to attack replication points has been exploited in yeast in efforts to map the temporal order of chromosome replication and to locate sites of simultaneous DNA replication (Carter and Dawes, 1978; Burke and Fangman, 1975; Dawes et al., 1977). MNNG is a direct mutagen and does not require metabolic activation. It is primarily considered to induce base pair substitution mutations and induced base pair substitution mutations fairly specifically in the Ames' test although some frameshift mutation was also observed (McCann et al., 1975a; McCann and Ames, 1976). Yourno and Heath (1969) and Yourno et al. (1969) found direct evidence for MNNG-induced frameshift

mutations. Oeschger and Hartman (1970) have found that
MNNG will revert about half of the ICR-induced frameshift
mutations in the his operon of Salmonella typhimurium and
have proposed that MNNG can cause single base deletions,
but not additions, with low efficiency in addition to
inducing base pair substitution mutations. In addition,
there is evidence that MNNG can also induce duplications,
strand breaks and large deletions (see reviews by Neale,
1976; Singer, 1975; and Gichner and Veleminsky, 1982).
MNNG is known to react by the $S_N 1$ mechanism and shows a
high reactivity towards O-atoms of guanine and pyrimidines
and also forms phosphotriesters (for reviews see Neale,
1976; Singer, 1975; and Gichner and Veleminsky, 1982).

Nuclear mutation: Forward mutation has been observed
in stationary yeast cells (Fahrig, 1979). Reverse mutation
at six loci was observed by Brusick (1970). Prakash and
Sherman (1973) found that MNNG induced specifically
guanine:cytosine to adenine:thymine transitions in a mutant
of iso-1-cytochrome c in a base pair substitution test. A
functional repair system is required in yeast for mutation
induction by MNNG (Prakash, 1974).

Calderon and Cerda-Olmeda (1983) found that contrary to
the results in bacteria, yeast cells were more sensitive
to MNNG killing and mutagenesis during stationary phase
than during exponential phase. The mutant frequency
depended upon the survival level and not so much on the
treatment conditions used. The mutation frequency

differences between exponentially growing and stationary phase cells were due to expression and not induction of the mutations. The frequency of mutants reached a maximum at 20% survival and then decreased. A number of differences between yeast and bacteria were observed regarding the optimal mutagenesis conditions and the relationship between mutagenesis and survival.

Calderon and Cerda-Olmeda (1982) also report that MNNG mutagenesis did not result in closely linked mutations using the HIS4 gene cluster. Similar results were obtained by Dawes et al. (1977). Calderon and Cerda-Olmeda (1982) suggest that possibly differences in the organization of eukaryotic DNA into chromatin and its lower replication velocity may result in smaller mutable regions. Multiple replcation forks would also obscure comutagenesis. MNNG mutagenesis is dependent upon the presence of the wildtype alleles of RAD6, RAD9 and possibly RAD15 according to Prakash (1976). Calderon and Cerda-Olmeda (1982) speculate that the lack of closely linked mutations observed in their system might be due to the repair of premutagenic lesions. Dawes et al. (1977) found MNNG induces a high frequency of multiple mutations in yeast. Since this comutation of loci is fairly specific but not clustered, they interpret comutation as MNNG acting to enhance mutagenesis at sites of simultaneous DNA replication within the cell. Carter and Dawes (1978) reported that mutation to nuclear erythromycin resistance is enhanced during nuclear DNA

replication and this enhancement is dependent upon DNA replication at the time of treatment.

In XD83 MNNG induced base pair substitution as expected, but primarily it induced extremely high frequencies of frameshift mutation. The relative increase in frameshift reversion was quite different for the two endpoints, histidine and leucine reversion. The dose-response curve for the reversion of the histidine requirement was roughly similar to that of the lysine requirement after the zero dose. Unfortunately, much of the data on MNNG mutagenesis in yeast is on base pair substitution or forward mutation so it is difficult to compare this study's results with those of other investigators. It seems that one cannot simply compare frameshift mutagenesis and base pair substitution mutagenesis at only one site to determine the relative amounts of each.

Nuclear recombination: In yeast MNNG induced recombination in the D3 system of Zimmermann (Simmon, 1979) in stationary cells with and without exogenous activation. Recombination was also observed in a number of other strains in stationary cells (Zimmermann et al., 1966a; Koeda and Hirano, 1979; Fahrig, 1979). Gene conversion was also observed by several investigators in stationary strain D4 (Fahrig, 1979; Zimmermann and Schwaier, 1967). Gene conversion in stationary D4 was also observed in intraperitoneal host-mediated assays (Fahrig, 1979).

MNNG induced both mitotic crossing-over and gene conversion in XD83. The relative increase in mitotic crossing-over was most striking. In fact, the relative increase in mitotic crossing-over was higher than that of base pair subsitution mutation. The relative increases in gene conversion and base pair subsitution were similar. Brusick and Mayer (1973) observed gene conversion in a qualitative plate test. This was also observed in this study in a spot test.

Mitochondrial mutation: Dawes and Carter (1974) observed nuclear and mitochondrial mutation to erythromycin resistance in growing yeast. The frequency of erythromycin resistance mutation, they found, was a function of cell age. Two peaks of susceptibility to MNNG were found: the first of these peaks coincided with nuclear DNA synthesis and the second occurred late in the cell cycle. Petite induction, on the other hand, was induced at all stages of the cell cycle. Nordstrom (1967) also observed the induction of petites with MNNG. Calderon and Cerda-Olmedo (1983) observed that MNNG-induced petites are cytoplasmic and their induction increases with dose until they compose essentially the entire population. However, using different conditions, Mayer and Legator (1970) found that approximately half of the low dosage MNNG-induced petites were nuclear. In XD83 a high induction of petites were produced by MNNG. Although no attempt was made to classify their origin, it is unlikely than these petites were of

nuclear origin since strain XD83 is diploid. Both forward
mutation to chloramphenicol resistance and erythromycin
resistance were seen in spot tests of XD83. The high
erythromycin concentration which was used makes it most
likely that the resistance was of mitochondrial origin
(Birky, 1973).

In the system described in this study MNNG primarily
induced frameshift mutations, but this agent also induced a
broad spectrum of genetic damage including base pair
substitution mutation, gene conversion, mitotic
crossing-over, mitochondrial point mutations and petite
induction. It was also highly cytotoxic. The spot tests
were in good agreement with the suspension tests.

5. 4-Nitroquinoline-N-oxide (4-NQO)

General: 4-NQO is a known carcinogen and a mutagen.
It induces primarily base pair substitution mutations, both
transitions and transversions, although it also induces
some frameshift mutations in bacteria (McCoy et al., 1981).
4-NQO induced base pair substitutions fairly specifically
in the Ames' test (McCann et al., 1975a). The enzymes
needed for the activation of 4-NQO appear to be present in
most microbial and mammalian cells (for a review of the
metabolism of 4-NQO see Tada, 1981). 4-NQO can interact
with DNA in three distinct ways: the quinoline group can
intercalate between the base pairs, covalently bound purine

adducts can be formed, and DNA-strand breakage can occur
(Nagao and Sugimura, 1976). The reduction of the nitro
group seems to be necessary for mutagenic activity (McCoy
et al., 1981). Although it is similar in many respects to
ultraviolet light in its damage spectrum, the lesions
introduced by 4-NQO are repaired by both long-patch and
short-patch repair whereas the repair of UV damage may
involve only long-patch repair (Dollery et al., 1983).
Fibroblast cell lines from classical and variant Xeroderma
pigmentosum subjects were more sensitive to the lethal
effects of 4-NQO than were those of a normal cell line
(Dollery et al., 1983). In human leukocytes 4-NQO induced
chromatid breaks (Epler et al., 1977).

Nuclear mutation: Fahrig (1976) found that 4-NQO
induced reverse mutation in strain D4.

Prakash et al. (1974) observed that 4-NQO induced both
transitions and transversions of guanine:cytosine base
pairs in iso-1-cytochrome c mutants of yeast and that this
specificity was not strongly influenced by neighboring base
pairs in those strains studied (Prakash and Sherman, 1974).
Prakash (1976) found that a functional repair system is
required for the induction of mutation by 4NQO. Damage
induced by 4-NQO is repaired by some of the same systems
which repair UV damage but some of the pathways seem to
operate on one type of damage and not on the other since
the mutagenic responses to UV and 4-NQO in
radiation-sensitive mutants are somewhat different

(Prakash, 1976; Lawrence and Christensen, 1976; Prakash et
al., 1974). 4-NQO induced an insignificant amount of
frameshift mutations (Prakash et al., 1974).

In XD83 4-NQO induced predominantly frameshift
reversion at the histidine site, followed by base pair
substitution, and then frameshift reversion at the leucine
site. The relative increase in frameshift mutagenesis at
the histidine and leucine sites were different by an order
of magnitude. However, the dose-response curves were quite
similar for frameshift and base pair substitution
mutagenesis for all doses beyond the control zero dose.

Nuclear recombination: In yeast, Simmon (1979) found
that 4-NQO was the most recombinogenic agent that was
tested of 101 chemicals in the D3 system of Zimmermann
(1973a). In fact, it was 10 times more active than MNNG on
an equimolar basis. Koeda and Hirano (1979) found induced
recombination in stationary strain D5. Fahrig (1976) found
that 4-NQO induced gene conversion in strain D4. The
dose-response curves for gene conversion and reverse
mutation were parallel and linear on a double logarithmic
scale. Fahrig also found recombination and gene conversion
in strain MP1. Fahrig (1973) found that 4-NQO induced gene
conversion also in an intraperitoneal host-mediated assay
in strain D4. Fahrig (1973) found that 4-NQO was more
active than MNNG in inducing gene conversion in vitro and
less active in a host-mediated assay. However, in this
study the results are quite similar for the induction of

gene conversion _in vitro_ by both MNNG and 4-NQO. Mitotic crossing-over was especially effectively induced.

Mitochondrial mutation: Nagai (1969) observed the effective induction of petites in yeast with 4-NQO both in glucose and glycerol media. The petites were of cytoplasmic origin primarily.

In XD83, 4-NQO induced both frameshift and base pair substitution and frameshift mutations. It also induced gene conversion, mitotic crossing-over, and petites. The dose-response curves were linear for mutation and gene conversion at the doses tested. Its damage spectrum was similar in the spot tests. The spot tests also indicated forward mutation to chloramphenicol resistance although this was not tested in the suspension tests.

6. Ultraviolet light (UV)

General: Ultraviolet light is a known carcinogen and mutagen. Its effects have been studied extensively. In humans it is known to be correlated with skin cancer. A genetic disease in humans, _Xeroderma pigmentosum_, is thought to be due to a defect in the repair of ultraviolet light damage and patients with this disease are extremely sensitive to light and are plagued with high incidences of skin tumors where their skin is exposed. UV has induced a broad spectrum of genetic damage in a wide variety of organisms. It induces pyrimidine dimers as well as strand

breaks, cross-links, and base damage. In yeast there are

three known pathways for the repair of radiation-induced

damage. These pathways have been extensively studied (for

a recent review see Haynes and Kunz, 1981). The repair of

UV damage involves an error-free and an error-prone

pathway. The strain used for these studies was wild-type

for the repair genes. Blocking a repair pathway for UV

damage can result in increased lethality and/or mutagenesis

or decreased mutagenesis (rad6).

Nuclear mutation: UV induced base pair substitution,

but not a specific type, in iso-1-cytochrome c mutants of

yeast (Prakash and Sherman, 1973). The previous

specificity for adenine:thymine to guanine:cytosine

transitions seen in revertants of iso-1-cytochrome c

mutants (Stewart et al, 1972; Sherman and Stewart, 1974)

was not seen in mutants at the RAD6 locus instead a variety

of base pair substitutions were seen (Lawrence et al.,

1974). The functioning of the RAD6 and REV3 genes appears

to be necessary for UV mutagenesis at all sites and of all

types (Lawrence and Christensen, 1976). Resnick (1969)

compared mutation induction in yeast in a UV-sensitive

strain (uvs9-3) and a wild-type strain. He found both

base-pair substitution and frameshift mutations. The

wild-type strain had a significant number of lesions due to

pyrimidine dimers while in the sensitive strain pyrimidine

dimer damage was seen only when reversion was due to

frameshift mutations. The intact repair system allowed the

removal of 80-90% of the UV-induced damage that would
normally cause mutation in the sensitive strain. Reversion
of nonsense mutations occurred primarily by reversion at
the loci rather than by suppressors which is contrary to
what is found in bacteria. The forward mutations in the
wild-type strain appeared to be due mainly to lesions which
were not pyrimidine dimers. Lesions due to pyrimidine
dimers and those not due to pyrimidine dimers both tended
to produce base pair substitution mutations (greater than
75%). Moustacchi (1969) found an increase in sensitivity
for the lethal and mutagenic effects of UV light in a
UV-sensitive mutant compared to wild-type. About 90% of
the mutations in the sensitive strain are prevented by
repair in the wild-type strain. Eckardt and Haynes (1977b)
observed a linear increase in forward mutation with dose in
a non-selective system up to a maximum frequency of about
3×10^{-3} mutants per survivor which then declined in
both wild-type and rad2 strains. They concluded that for
both strains the major type of premutational lesions are
unexcised pyrimidine dimers since the mutation frequencies
plotted against survival are almost identical over the
whole survival range.

In this study with XD83 UV induced high frequencies of
base-pair substitution and frameshift mutation. The
greatest increase was seen for frameshift reversion of the
leucine marker. The frequency increase of base pair
substitution of the lysine marker was increased more than

the increase in frameshift reversion at the histidine marker.

Nuclear recombination: Nakai and Mortimer (1969) using a multiply marked strain found that approximately 80% of sectored colonies induced by UV could be explained by single mitotic crossing-over events. Mitotic gene conversion was also observed. Similar results were obtained by Esposito (1968). James and Lee-Whiting (1955) found that the most likely explanation for homozygosis following UV at one or more loci in a heterozygous vegetative cell was mitotic crossing-over. Recombination within a gene has been found to be primarily due to gene conversion events (Roman and Jacob, 1958; Hurst and Fogel, 1964; and Wildenberg, 1970). Moustachhi (1969) found that UV-induced recombination was higher in a UV-sensititive strain than in a wild-type strain. Since high levels of UV-induced recombination can be reversed by photoreactivation (Parry and Cox, 1965), it is concluded that much of the recombination is due to lesions, primarily pyrimidine dimers, in DNA. These lesions could also lead to the synthesis of enzymes which might be required for mitotic recombination (Holliday, 1975).

In this study recombination increased significantly following UV exposure. Although the frequency of gene conversion was increased to a great extent, the increase in mitotic crossing-over was even more striking.

Mitochondrial mutation: The frequency of UV-induced

petites in a UV-resistant strain is actually higher than in
a UV-sensitive strain (Moustachhi, 1969). In fact, the
frequency of UV-induced cytoplasmic petites stays almost
constant with increasing UV doses in the UV-sensitive
strain. Mayer and Legator (1970) found that UV induced
petites were primarily of cytoplasmic origin in contrast to
MNNG-induced petites which were approximately half of
nuclear origin.

UV induced a high frequency of petites in this study.
In addition a dramatic increase in the frequency of
mitochondrial point mutations was also observed. The
increase in forward mutation was comparable to that seen in
mitochondrial point mutation.

In the present system UV induced a high frequency of
every type of genetic damage that was assayed.

B. General Discussion of Results

1. Lack of specificity

With the exception of ethidium bromide, a questionable
carcinogen, all the carcinogenic agents induced a wide
spectrum of genetic change. Actually, almost all of the
agents induced every genetic change monitored. The
relative amounts varied, however. Ethidium bromide
specifically induced mitochondrial petites although certain
changes were difficult to detect due to the high induction

of petites interfering with the determination of

mitochondrial point mutations and nuclear mitotic

crossing-over.

2. Dose-response curves for frameshift mutations

The dose-response curves for the induction of

frameshift mutations at the his4-519 and leu2-3 frequently

had different slopes. Therefore, it appears that the locus

involved may partially determine the kinetics of induction.

In comparisons of the relative amounts of frameshift versus

frameshift or frameshift versus base pair substitution

mutations, the relative frequencies can vary at different

dose points (for example, see Figure 18B). Caution is

indicated, therefore, in declaring a mutagen to be either a

frameshift or a base pair substitution mutagen since the

mutagen's effect appears to vary with the dose. Further

experiments are necessary, however, to confirm the kinetics

of induction seen in this study.

3. Spot tests versus suspension tests

The suspension and spot tests were in good general

agreement. The lack of agreement was usually seen in the

nuclear mutation tests. This is probably due to the

frequency of the mutations being too low to detect in the

limited number of cells which are used in the spot test.

Addition of the HOL1 mutation to the strain in the future

may remedy part of this problem. This mutation allows

growth on histidinol if the strain has a functional his4C

region. This should increase the length of DNA within

which a frameshift mutation can occur to revert the strain.

III. RESULTS OF MITOTIC CHROMOSOME LOSS STUDIES

A. <u>Detection</u> <u>of</u> <u>Mitotic</u> <u>Chromosome</u> <u>Loss</u>

1. The strain

Mitotic chromosome loss is a relatively rare event in yeast (Esposito et al., 1982; Wood, 1982; Parry and Zimmerman, 1976; Campbell et al., 1974). Therefore, to screen for such events a positive system using selective media is highly desirable. To achieve this end, advantage was taken of two dominant, tyrosine inserting ochre (UAA) suppressors, <u>SUP4</u> and <u>SUP7</u> (Hawthorne and Mortimer, 1963), located on opposite arms of chromosome X (Hawthorne and Mortimer, 1968). They suppress a large number of known ochre mutations. A diploid strain was constructed which has these two suppressors on one homologue of chromosome X (Figure 23). The other homologue of chromosome X bears the <u>ilv3-1</u> and the <u>ura2-1</u> ochre-suppressible markers. Additionally, the strain is homozygous for the ochre-suppressible markers <u>ade2-1</u>, <u>his5-2</u>, <u>lys1-1</u>, and <u>can1-100</u> located on various other chromosomes. The loss of the homologue of chromosome X bearing the two suppressors allows the expression of the ochre mutations including the <u>can1-100</u> mutation (Figure 23). The <u>CAN1</u> gene codes for arginine permease (Whelan et al., 1979). Wild type cells,

CHROMOSOME LOSS STRAIN

Genotype:

$$\frac{a}{\alpha} \quad \frac{ade2-1}{ade2-1} \quad \frac{his5-2}{his5-2} \quad \frac{lys1-1}{lys1-1} \quad \frac{can1-100}{can1-100} \quad \frac{+ \quad SUP7 \quad \bullet \quad + \quad SUP4}{ura2-1 \quad + \quad \bullet ilv3-1 \quad +} \quad \frac{\bullet trp1-1}{\bullet \quad +}$$

Phenotype:

| Ade$^+$ (white) | His$^+$ | Lys$^+$ | canavanine sensitive | Ura$^+$ | osmotic sensitive | Ilv$^+$ | Trp$^+$ |

LOSS OF CHROMOSOME X HOMOLOGUE BEARING THE TWO OCHRE SUPPRESSORS

| Ade$^-$ (red) | His$^-$ | Lys$^-$ | canavanine resistant | Ura$^-$ | osmotic resistant | Ilv$^-$ | Trp$^-$ |

Figure 23. Chromosome loss strain

which can transport arginine, are sensitive to the toxic arginine analogue, canavanine (Grenson et al., 1966).

Mutant canl-100 cells lack permease function and are, consequently, resistant to canavanine. In the presence of the two ochre suppressors, however, this canl-100/ canl-100 strain is sensitive to canavanine. A chromosome loss event involving the loss of the two suppressors allows the strain to grow on canavanine containing medium. Previous chromosome loss systems in yeast (Parry and Zimmerman, 1976; Esposito et al., 1982) have used positive selection procedures for the loss of one arm of a chromosome. Further identification of the loss of the other arm relied upon a color change. Growth on canavanine medium selects for the simultaneous loss of both arms of chromosome X which increases the selectivity of the system. The strain also undergoes a color change from the wild-type cream color to the mutant red color associated with the loss of the suppression of the adenine mutation.

2. Selective media for detecting chromosome loss

a. Canavanine medium: Initial tests using canavanine for positive selection were unsuccessful in detecting chromosome loss because canavanine resistance was frequently found without loss of both of the suppressors. This was indicated by the continued suppression of the other ochre suppressible markers. A number of possible

explanations were investigated including faulty medium,
additional arginine mutations (argRI, II, and III were
later found to be wild-type by F. Hilger, personal
communication), and improper strain construction. Testing
discounted these possibilities, however. It now appears
that a heterozygous antisuppressor gene, affecting the
efficiency of suppression, was involved in this unexpected
canavanine resistance. Technical difficulties in detecting
the antisuppressor and in distinguishing between the
presence of one or two suppressors made initial crosses to
eliminate this gene unsuccessful. An alternative to
canavanine in the selective medium was found, however.

b. Hypertonic media: Singh (1977) found that
hypertonic media inhibit the growth of many nonsense
suppressors. Although the mechanism for this inhibition is
not understood, the inhibition appears to be correlated
with the efficiency of suppression. A variety of
hypertonic media described by Singh were tested with
chromosome loss strain XD26. The results are shown in
Table 5. The 2.5M and 2M ethylene glycol media
demonstrated the greatest fractional accuracy (.97) in
detecting the presence or absence of the ochre suppressors.
Data from ten tetrad dissections of strain XD26 showed that
both media had a sensitivity (true positive fraction) of
1.00 and a specificity (true negative fraction) of 0.96 for
detecting suppressor presence or absence. Two cases of
canavanine resistance without suppressor loss were seen in

Table 5

ACCURACY OF DETECTING SUPPRESSORS WITH HYPERTONIC MEDIA

	Fraction correct	
Media Used	with suppressor	without suppressor
2.5M ethylene glycol	24/25	11/11
2.0M " "	24/25	11/11
1.0M " "	19/25	11/11
0.5M " "	1/25	11/11
2.0M potassium chloride	24/25	8/11
1.5M " "	18/25	9/11
1.0M " "	9/25	10/11
2.5M sodium chloride	25/25	0/11
1.0M " "	7/25	10/11
2.5M glycerol	17/25	9/11
1.0M "	0/25	11/11
2.5M sorbitol	25/25	0/11
1.0M "	0/25	11/11
2.5M dextrose (modified procedure)*	5/25 25/25	8/11 8/11
1.0M dextrose (modified procedure)*	0/25 25/25	11/11 8/11
YEPD (control)	0/25	10/11

Strain XD26: 10 tetrads dissected; 90% spore viability
25 spores with 1 or 2 suppressors; 11 without suppressors

* In modified procedure colony color was used in determination
of suppressor presence in addition to growth or lack of growth
on the medium.

the segregants. These cases were probably due to the
presence of an antisuppressor since the adenine mutation
was only partially suppressed also. The 2.5 and 2M
ethylene glycol and the 2M potassium chloride media
correctly detected the suppressor's presence in one case
and not in the other. Eight other segregants were
partially suppressed for adenine and either partially or
fully suppressed for canavanine. All of the 1M hypertonic
media allowed all of these partially suppressed segregants
to grow whereas the 2.5 and 2M ethylene glycol and the 2M
potassium chloride media did not allow any to grow. The
advantage of the ethylene glycol media compared to the
potassium chloride medium was that on the former growth
occurred for all of the nonsuppressor bearing segregants.
For the potassium chloride medium this did not occur. The
2.5M sodium chloride and the 2.5M sorbitol did not support
the growth of any segregants. This, however, may have been
due to aging of the media. Singh (1977) has reported that
certain batches of hypertonic media do not support the
growth of any yeast strain if the media is allowed to dry
for more than a few days. This was also observed in this
study with certain batches of hypertonic media including
ethylene glycol media.

The ethylene glycol hypertonic media supported the
growth of the nonsuppressor bearing segregants slightly
better than the control YEPD medium. This result may be due
to the hypertonic media affecting one or more osmotic

remedial alleles (Hawthorne and Friis, 1964) which are expressed by the loss of the suppressors. Agents which can penetrate into the cell like ethylene glycol are especially efficient osmotic remedial agents (Bassel and Douglas, 1970).

In suspension tests the ethylene glycol media were much more selective for chromosome loss events than canavanine medium. Tests with canavanine in the selective medium resulted in only a few colonies with the chromosome loss phenotype, whereas, on control plates 30-75% of the colonies selected on ethylene glycol medium had lost the two suppressors (Figures 28C, 29C, and 30C) and about 30-60% of those had the chromosome loss phenotype (Figures 28D, 29D, and 30E). However, because the hypertonic medium was not completely selective and some false growth was encountered, it was necessary to test putative chromosome loss colonies for the total chromosome loss phenotype: Ade⁻, His⁻, Ilv⁻, Lys⁻, Ura⁻, and canR.

During the testing, it was found that petites did not grow on the hypertonic media. This agrees with Singh's (1977) finding that strains which grow poorly on glycerol medium also grow poorly on hypertonic media. An adjustment in the calculations was made to compensate for this. In addition, loss of the suppressors did not allow the strain to develop the full red color when the strain was grown on hypertonic media. When clones were picked off the media and then grown on YEPD, however, the full red color did

develop in those clones which had lost the suppressors.

c. Elimination of the antisuppressor: As previously stated, the unexpected high frequency of canavanine resistance without the concommittant loss of the suppressors indicated the possible presence of an antisuppressor. The pattern of canavanine resistance seen upon dissection of the relevant clones indicated that the antisuppressor was nuclear, heterozygous, recessive and probably affected only one of the suppressors. The spectrum of suppression with and without the antisuppressor is shown in Table 6. Presumably, the antisuppressor became homozygosed during the testing and made its presence known following the loss of one of the suppressors. Further preliminary genetic evidence suggested that the antisuppressor modified the efficiency of SUP7 only when alone and not that of SUP4 and SUP7 in combination. Furthermore, this antisuppressor did not abolish, it only reduced, suppressor activity. Suppression of canavanine resistance was most reduced, adenine suppression was moderately reduced, histidine suppression was sometimes slightly reduced, and lysine suppression was not noticeably affected. Similar results were seen with antisuppressors to SUP7 by Gorman et al. (1977) and to the ochre suppressor, ocSUPO2 (McCready and Cox, 1973).

Since hypertonic media are difficult to use because they age quickly and are subject to concentration and strain effects (Singh, 1977; this study), efforts were

TABLE 6

SPECTRUM OF SUPPRESSION WITH AND WITHOUT THE ANTISUPPRESSOR

Genotype			can	ade-	his-	ilv-	lys-	ura-	S.C.	EG
ASU	sup4+	sup7+	R	-	-	-	-	-	+	+
asu	sup4+	sup7+	R	-	-	-	-	-	+	+
asu	SUP4	SUP7	S	+	+	+	+	+	+	-
ASU	SUP4	sup7+	S	+	+	+	+	+	+	-
asu	SUP4	sup7+	S	+	+	+	+	+	+	-
ASU	sup4+	SUP7	S	+	+	+	+	+	+	-
asu	sup4+	SUP7	R	±	+/±	+	+	+	+	-
asu	SUP4	SUP7								
+	+	+	S	+	+	+	+	+	+	-
±	SUP4	SUP7								
+	+	+	S	+	+	+	+	+	+	-

asu = antisuppressor; ASU = wild-type allele of antisuppressor; SUP4 and SUP7 = mutant alleles of wild type sup4 and sup7, respectively; and + = wild-type gene (in genotype column). R = canavanine resistant, S = canavanine sensitive, + = growth on media, ± = slight growth on media, and - = no growth on media.

made to cross out the troublesome antisuppressor gene so
that canavanine medium could be used for selection.
Additionally, elimination of the antisuppressor was
expected to increase the selectivity of the hypertonic
media for true chromosome loss events. To improve the
selectivity for chromosome loss, strain XD72 was
constructed. This strain is similar to strain XD26 except
that it is also heterozygous for a uracil marker on the
left arm of chromosome X (Table 3).   Strain XD72 through a
series of dissections and crosses was used to finally make
strain XD99 which lacks the antisuppressor (Figure 3).
The antisuppressor probably entered the strain through one
of the haploid parents. Each haploid was phenotypically
canavanine sensitive, however, so it was not obvious which
parent brought the antisuppressor into the strain.
Possibly the antisuppressor had a rather negligible effect
on the suppressor strain which initially carried it and a
more noticable effect on the other suppressor which was
introduced later through the crosses. A number of
antisuppressors to SUP7 (Gorman et al., 1977) have been
observed with a similar pattern to that observed here. In
the diploid strain there should be no selection for a
recessive antisuppressor.

Gilmore and Mortimer (1966) and Gilmore (1967) reported
that two ochre suppressors from class I, set 1 when
combined in one haploid cell resulted in morphologically
aberrant cells and retarded growth. They postulated that

this could be due to excessive readthrough at normal chain-terminating codons. During construction of the chromosome loss strains, this two-suppressor effect was not observed although the suppressor strains used (Table 2) were descendants of the original suppressor strains used by Gilmore and Mortimer. Even after the antisuppressor was crossed out of the strain, the two-suppressor effect was still not observed. In fact, the colony size was frequently smaller without the two suppressors than with them. However, the chromosome loss strains were all heterozygous at the ILV3 locus and since small colony size was seen in conjunction with expression of the ilv3-1 marker, the expression of the ilv3 marker and the other markers were probably responsible for obscuring the two-suppressor effect.

3. Events Which Can Mimic the Chromosome Loss Phenotype

a. Coincident crossing-over: The simultaneous crossing-over between SUP7 and the centromere and the centromere and SUP4 may result in the loss of both suppressors. The resultant phenotype mimics that of chromosome loss. However, the frequency of this coincident crossing-over should be quite low since two independent, relatively rare, events are required because the genes involved are located on opposite arms of chromosome X (Hawthorne and Mortimer, 1968). Additionally, the ilv3-1

mutation is between the centromere and SUP4 so that if a double crossing-over event has occurred, then many of the resulting Sup$^-$ clones should have an Ilv$^+$ phenotype and these clones can be discounted. When chromosome loss is the dominant reason for the loss of the two suppressors, then the fraction of Ilv$^-$ clones should be high, close to one. Unfortunately, mitotic map distances for this region of the chromosome are not available so that it is not possible to estimate the expected fraction of Ilv$^-$/Ilv$^+$ clones from recombination. When coincident crossing-over is the dominant reason for the simultaneous loss of the suppressors, then the fraction of Ilv$^-$ clones should be much lower. If both events are occurring, then the fraction of Ilv$^-$ clones should have an intermediate value between the two extremes. Chromosome loss should result in the loss of the URA2 and ILV3 markers leaving the strain hemizygous for ura2-1 and ilv3-1 or homozygous for these markers if the remaining homologue replicates itself to restore euploidy. The possible restoration of euploidy will be discussed later (see DISCUSSION).

b. Mutation: In order to mimic the chromosome loss phenotype, four independent mutations are required. Mutations are unlikely, therefore, to contribute a significant fraction of mimicing events and can be ignored.

c. Gene conversion: Two independent gene conversion events spanning the SUP7-URA2 and ILV3-SUP4 regions or two

independent gene conversion events at <u>SUP7</u> and <u>ILV3</u> with recombination of the flanking markers (or a combination of both) can mimic the chromosome loss phenotype. Two such independent and rather specific gene conversion events appear unlikely to constitute a significant fraction of the chromosome loss phenotype. Loss of the two suppressors with retention of the heterozygosity of the <u>URA2</u> locus also can indicate possible gene conversion events. Colonies arising from such events can be discounted since such colonies do not have the complete chromosome loss phenotype.

    d. Deletion: Deletions involving both arms of chromosome X could mimic chromosome loss. However, deletions seem to be rare events in yeast (Fink and Styles, 1974). McCusker et al. (1981) found that deletions in chromosome III often led to mitotic instability. Therefore, those deletions which do occur could result in a chromosome loss event. Deletions at the <u>SUP4</u> locus, however, occur at an unusually high frequency. Rothstein and Helms (1981) found the frequency to be 3×10^{-7}. They believe this high deletion frequency might be promoted by recombination between the two direct repeats of the delta sequences which flank the <u>SUP4</u> gene. Even this "unusually high" frequency combined with the deletion frequency of the <u>SUP7</u> locus would result in an extremely low frequency for the simultaneous loss of the two suppressors. Therefore, this source of error is probably

insignificant.

e. Antisuppressor: An antisuppressor only partially mimics the chromosome loss phenotype. Although a clone containing an antisuppressor might behave much like a clone which has lost its suppressors, usually ochre antisuppressors do not totally abolish suppression (Gorman et al., 1977; McCready and Cox, 1973). In addition, the clone would still be Ilv^+ and Ura^+.

f. Combinations of events: Although a combination of the above events could mimic chromosome loss, the frequency of such events is likely to be too low to be of significance.

4. Distinguishing Chromosome Loss from Coincident Crossing-over Events

In those cases where the colonies growing on the selective medium are sectored, it is possible, although laborious, to distinguish probable chromosome loss events from probable coincident crossing-over events.

a. Chromosome loss: Figure 24 illustrates two mechanisms by which loss of the chromosome X homologue bearing the two ochre suppressors can result. Nonreciprocal loss of the chromosome can occur through failure of a chromosome to replicate or segregate or through chromosome fragmentation. The resulting colony is sectored red and
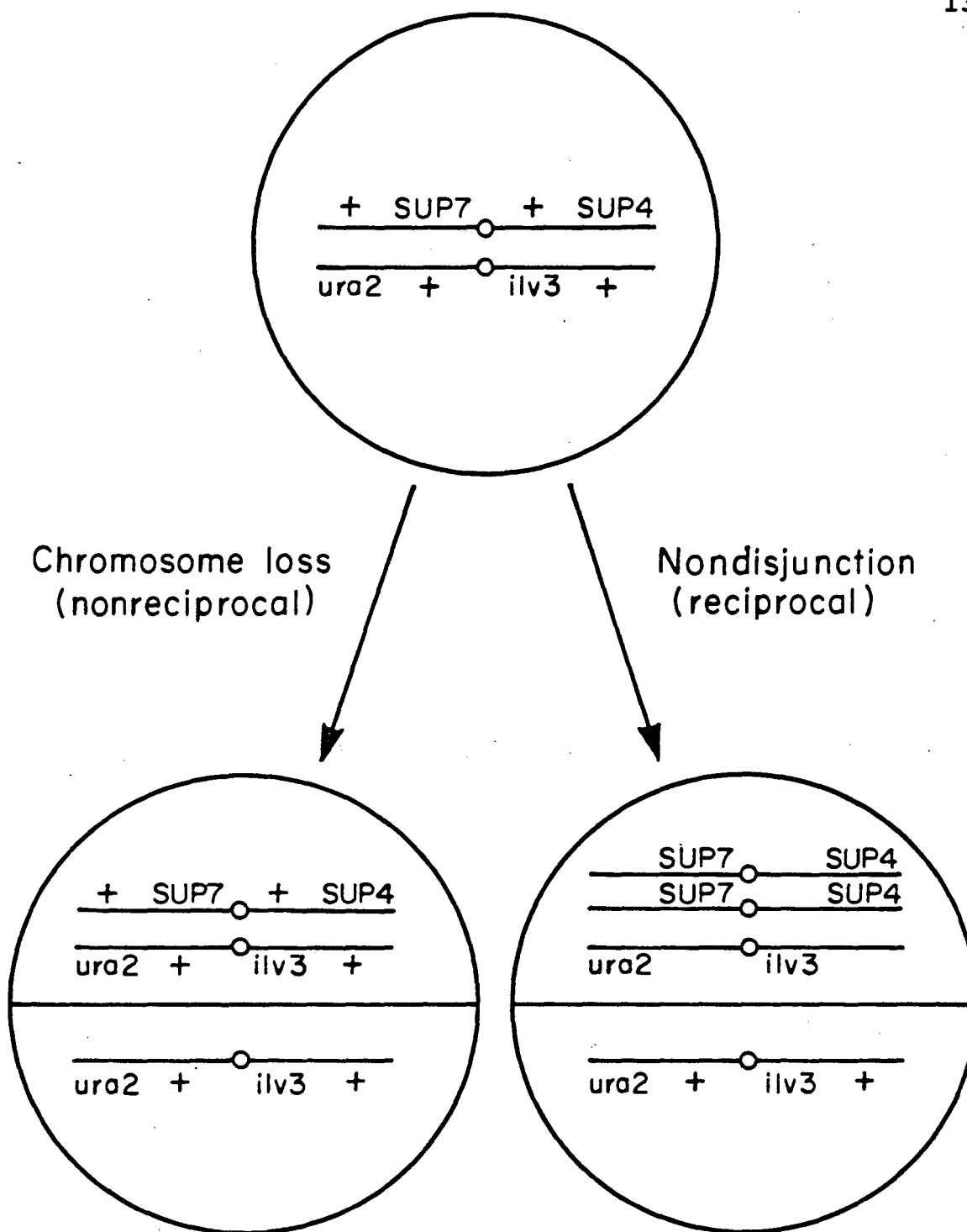
Figure 24. Chromosome loss and nondisjunction may result in sectored colonies.

white. The red sector is hemizygous for _ilv3-1_ and _ura2-1_ and the white sector is heterozygous for the two suppressors and the isoleucine-valine and uracil markers. Reciprocal loss of the chromosome can occur through nondisjunction. In this case the red sector is hemizygous as in nonreciprocal loss, but the white sector is heterozygous and trisomic for chromosome X.

b. Mitotic crossing-over: Figure 25 illustrates how mitotic crossing-over can mimic the chromosome loss phenotype. Case 1A, crossing-over in regions 1 and 3, results in a red and white sectored colony. The red sector is without the two suppressors, heterozygous for the isoleucine-valine marker, and homozygous for the _ura2-1_ marker. The white sector is homozygous for the two suppressors, heterozygous for the isoleucine-valine marker, and homozygous wild-type for uracil. Case 2A, crossing-over in regions 1 and 2, results in a red and white sectored colony. The red sector is without the two suppressors and is homozygous for _ilv3-1_ and _ura2-1_. It is this sector which is likely to be mistaken for a chromosome loss event. However, the white sector which may accompany this sector is homozygous for the two suppressors and homozygous _ILV3_$^+$ and _URA2_$^+$ whereas the white sector accompanying a chromosome loss event is heterozygous for all the relevant markers. Case 1B and 2B result in non-sectored white colonies which are heterozygous for the relevant markers and which do not have
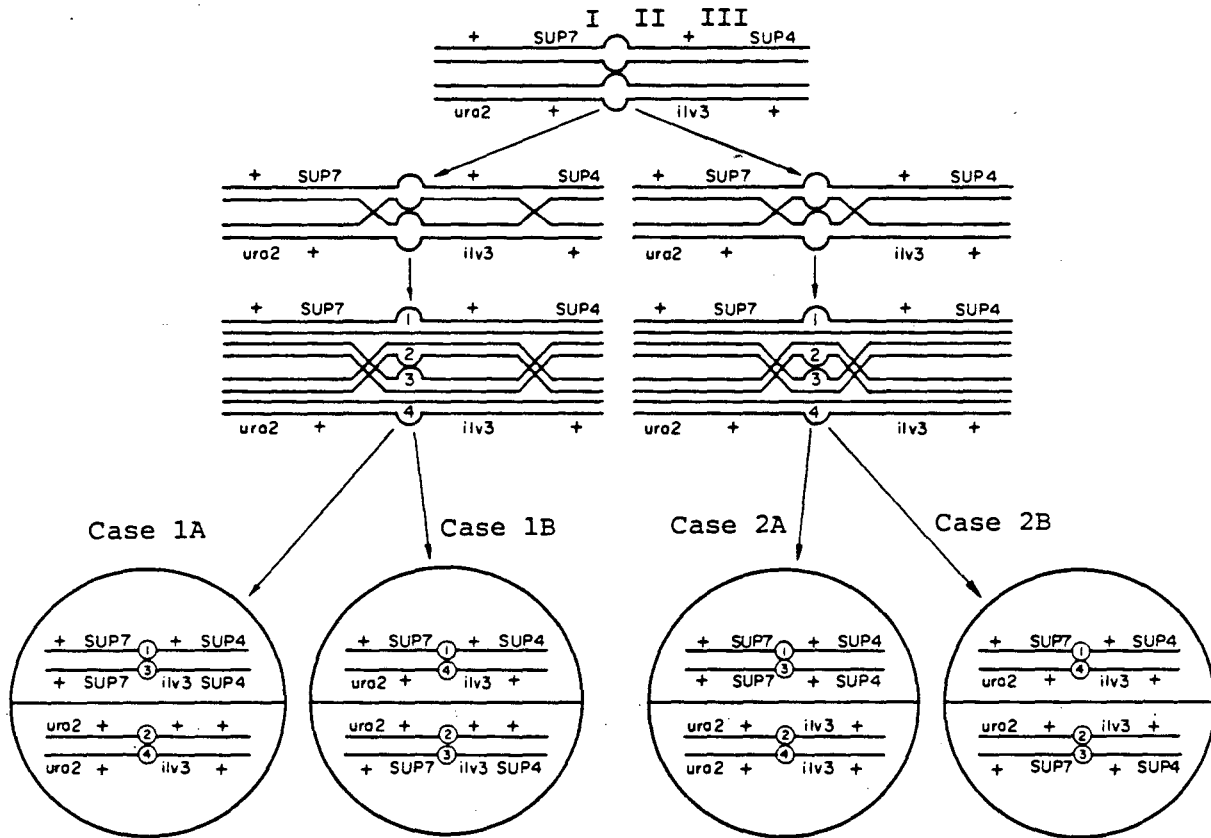
XBL834-3689

Figure 25. Coincident crossing-over on both sides of cenX may result in sectored or nonsectored colonies.

the chromosome loss phenotype because the suppressors are
still present.

To summarize, a sectored red and white colony is
probably the result of coincident crossing-over if both
sectors are homozygous. If the white sector is
heterozygous, then probably chromosome loss has occurred.

5. Tests for Monosomy and Trisomy

As shown in Figure 23, putative chromosome loss can be
partially verified after selection on ethylene glycol (or
canavanine) medium by testing for the ochre suppressible
markers. In addition, the strain bears a centromere linked
amber mutation, trp1-1. Dissections of the sporulated
putative monosomic clones were expected to show a 2:2
segregation for a centromere linked lethal confirmable by
the segregation of the trp1-1 marker (Figure 26). This
lethal pattern was never observed, however, in any of the
dissections. Some of the putative monosomics did not
sporulate, but of those which did, most contained four
viable spores per ascus. Those clones which had only two
viable spores per ascus had both TRP1$^+$ and trp1-1
spores segregating. Either coincident crossing-over had
occurred in these putative monosomic clones or chromosome
loss was followed by restoration of the euploid (2n) state
had occurred (see DISCUSSION).

Since monosomy for chromosome X may not be stable,
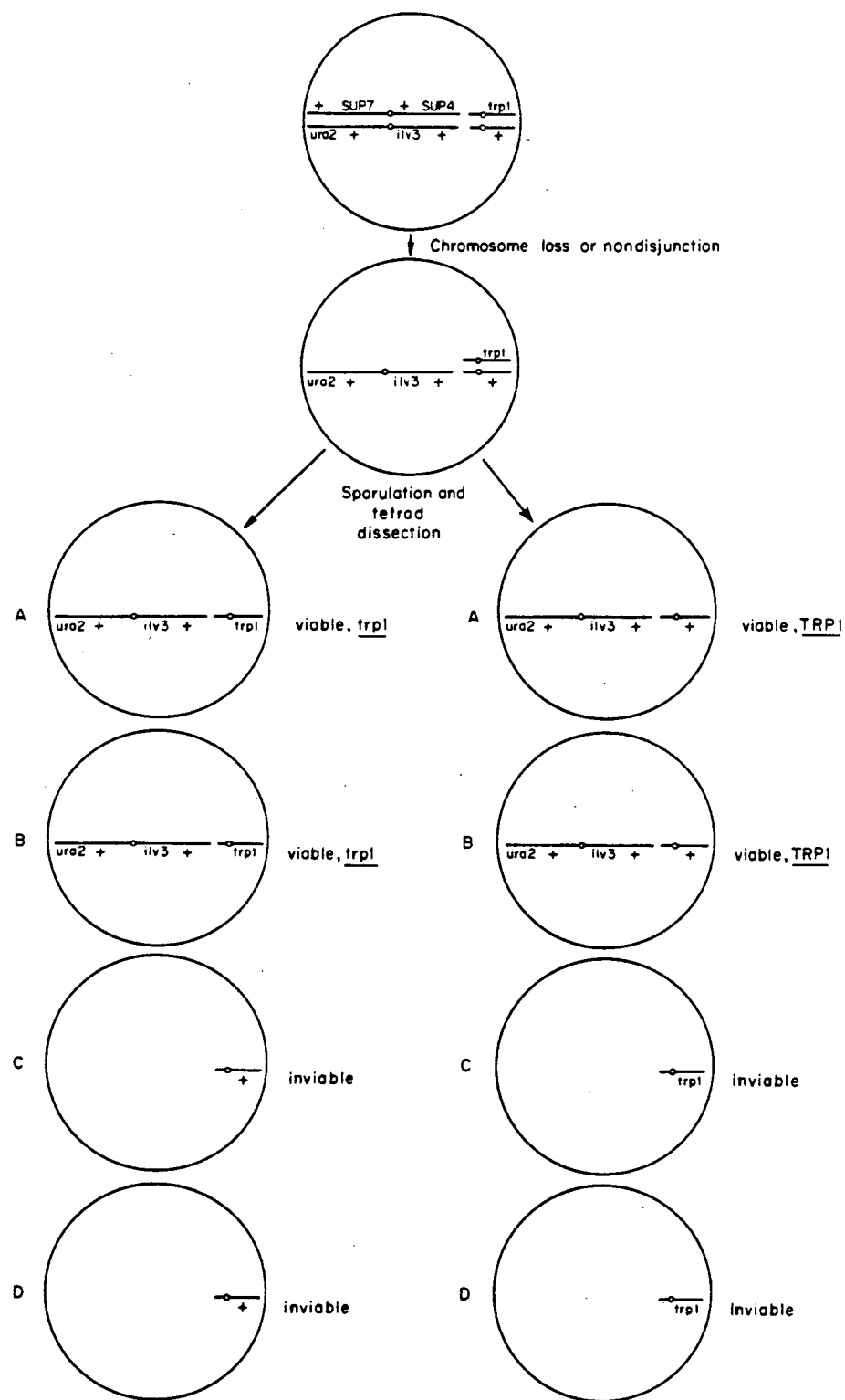
Figure 26. Segregation of a centromere-linked 2:2 lethal following chromosome loss (without restoration of euploidy).
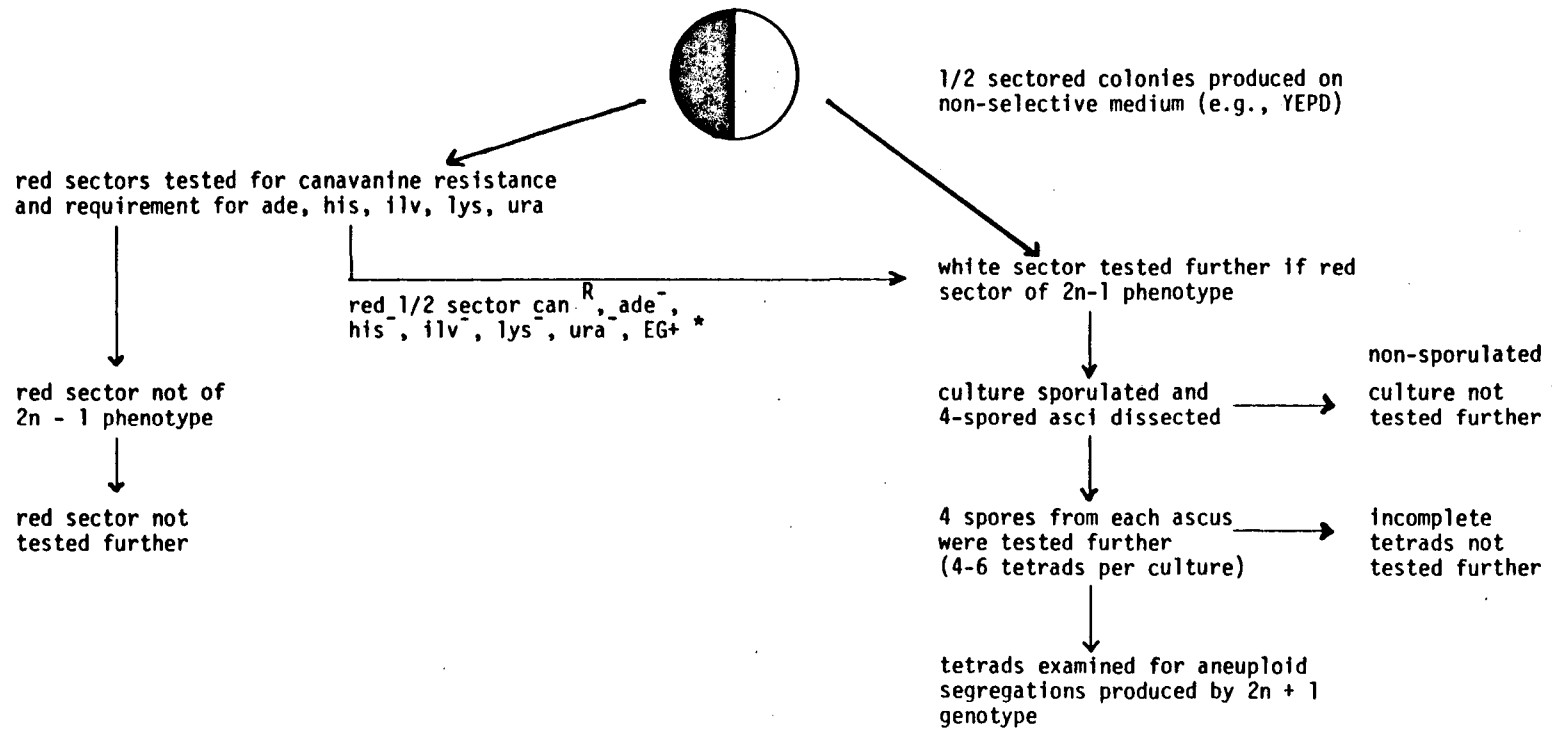
further verification of possible chromosome loss events had
to be studied by using another technique. A certain
proportion of clones growing on the hypertonic selective
media are sectored. Presumably, these sectors could be
due to a chromosome loss event. The red sector resulting
from the loss of the suppressor bearing homologue and the
white sector possibly containing the lost homologue plus
the other two usual homologues. In other words, the white
sector could be trisomic. If the monosome is not stable,
possibly the trisome is and might be dissected to show an
aneuploid segregation indirectly confirming the loss of the
homologue from the red sector. This analytical scheme
(modified from Parry et al., 1979a and b) is shown in
Figure 27.

B. Tests

1. Suspension Tests

   a. Para-fluorophenylalanine (PFA): Para-
fluorophenylalanine, a toxic analogue of phenylalanine, was
selected as a test chemical for the chromosome loss system
because it is a known inducer of chromosome loss in a
number of organisms including yeast (Stromnaes, 1968;
Emeis, 1966; Parry et al., 1979a). Strain XD26 was treated
under growing conditions for one day and for three days in
two experiments. In this study PFA was found to be

1/2 sectored colonies produced on
non-selective medium (e.g., YEPD)

red sectors tested for canavanine resistance
and requirement for ade, his, ilv, lys, ura

red_1/2 sector can ^{R}, ade$^-$,
his$^-$, ilv$^-$, lys$^-$, ura$^-$, EG+ *

white sector tested further if red
sector of 2n-1 phenotype

red sector not of
2n - 1 phenotype

non-sporulated

culture sporulated and
4-spored asci dissected

culture not
tested further

red sector not
tested further

4 spores from each ascus
were tested further
(4-6 tetrads per culture)

incomplete
tetrads not
tested further

tetrads examined for aneuploid
segregations produced by 2n + 1
genotype

After Parry, J. M., D. Sharp, R. S. Tippins and E. M. Parry. Radiation-induced mitotic and meiotic aneuploidy in the yeast
Saccharomyces cerevisiae, Mut. Res. 61 (1979) 37-55.

Figure 27. Technique used to detect trisomic (2n + 1) cells produced during mitotic cell
division

cytotoxic (p<0.01, r=-0.8945) and induced petites (Figures 28A and 29A). Both one and three day treatments showed a significant induction of putative chromosome loss events (p<0.01; r=0.7204 and r=0.7765, respectively) although the three day treatment was most effective (Figures 28B and 29B). As mentioned earlier, petites do not grow on hypertonic media and a correction must be made for this. This petite interference was observed after the experiment with the one day treatment, therefore, corrected data are shown for the three day treatment only (Figure 29B).

Figures 28C and 29C show that the fraction of clones growing on the hypertonic medium which were due to suppressor loss increased with dose indicating that coincident loss of the two suppressors increased with increasing dose. In addition, the high proportion of suppressor minus clones growing on the hypertonic medium indicates that this medium selects well for suppressor loss. Coincident suppressor loss is most likely due to either chromosome loss or coincident crossing-over (see section A.3.). However, with coincident crossing-over, many of the suppressor minus clones should be Ilv⁻. On the other hand, chromosome loss should result in suppressor minus clones which are also Ilv⁻. As Figures 28D and 29D show, the fraction of Ilv⁻ clones among the the suppressor minus clones also increased with dose and approached one indicating that chromosome loss was the most probable explanation for the observed phenotype.
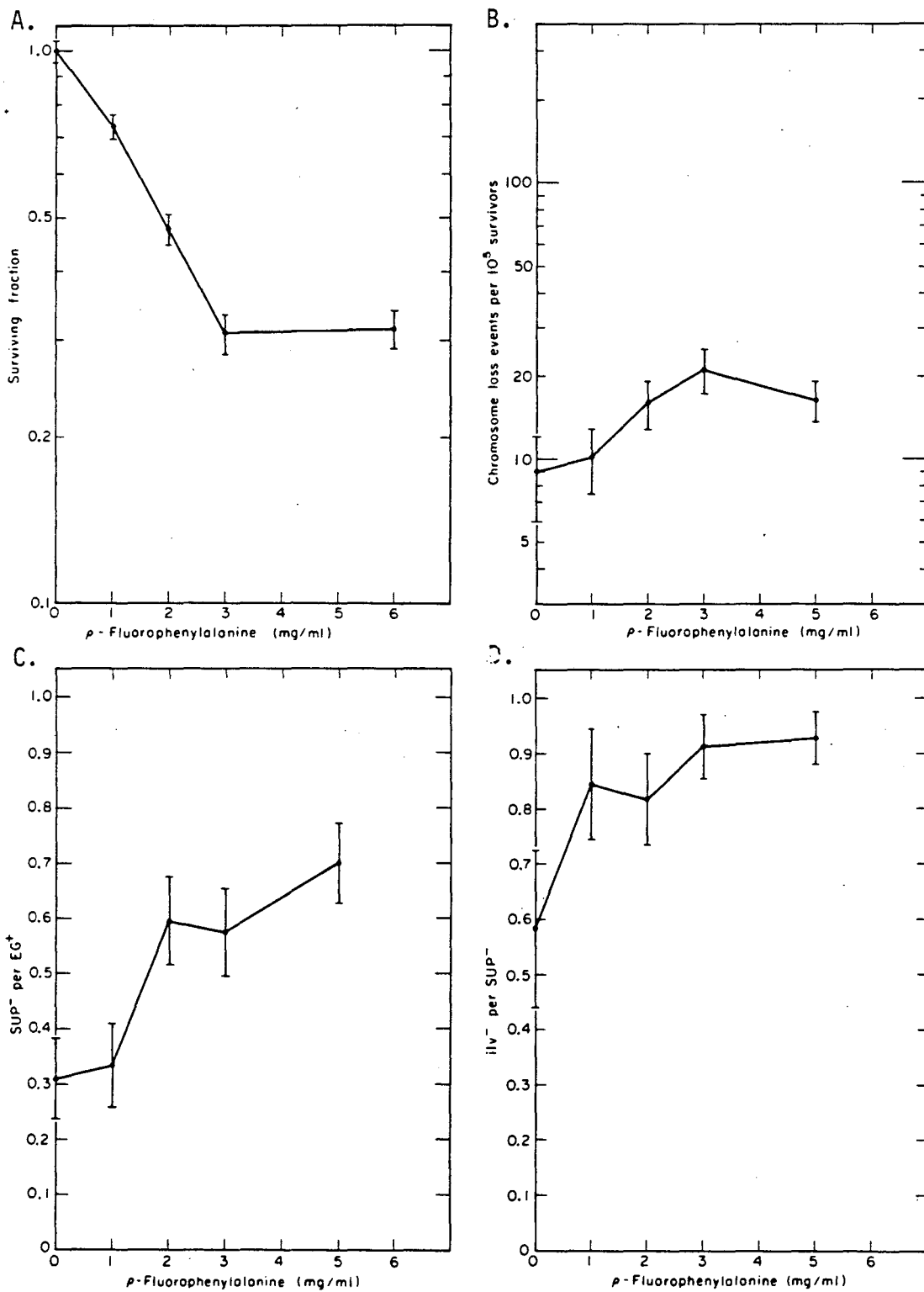
XBL836-3814

Figure 28. Para-fluorophenylalanine suspension test
(one day treatment)
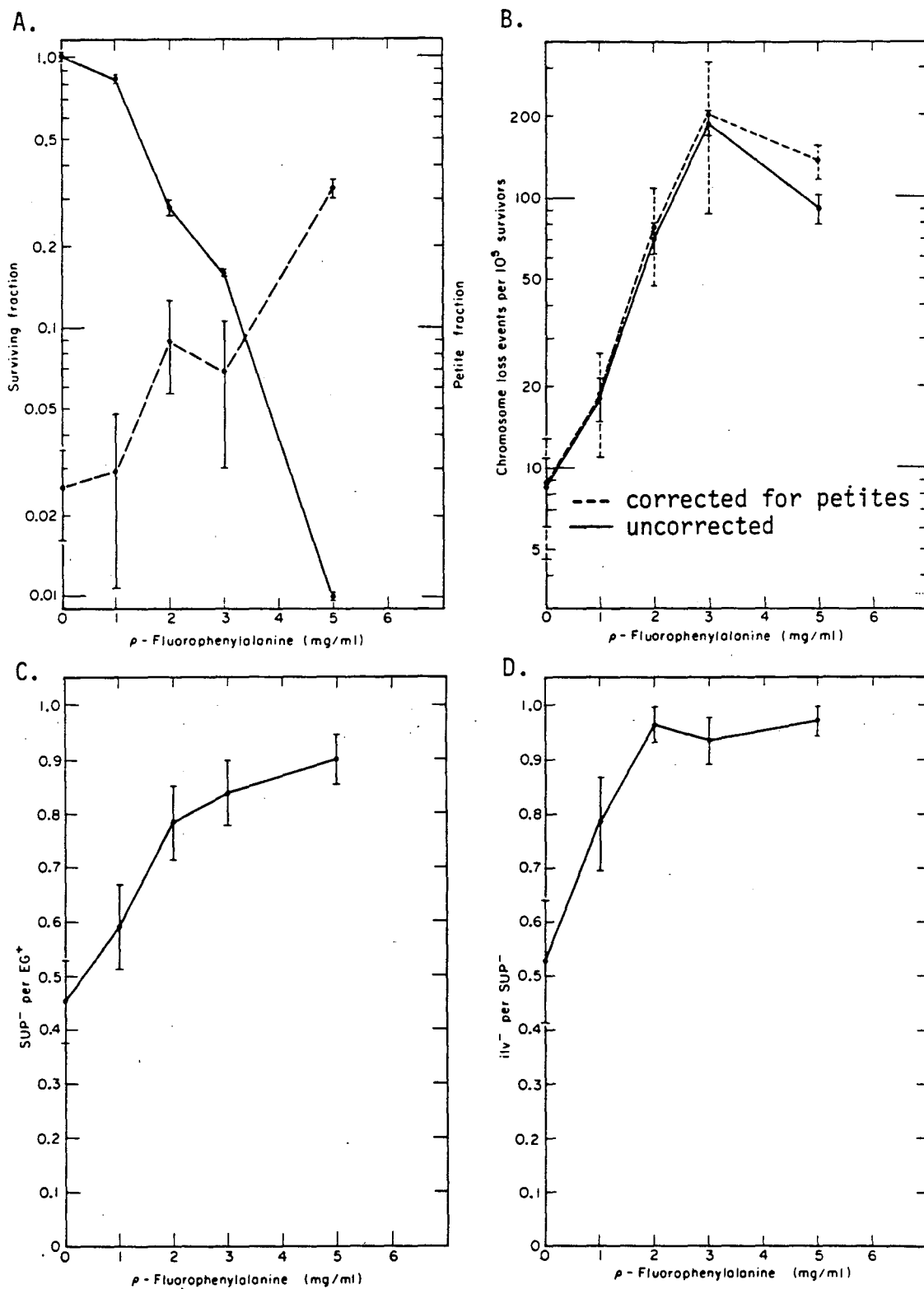
Figure 29. Para-fluorophenylalanine suspension test
(three day treatment)

XBL836-3813

In fact, at the highest doses of para-fluorophenylalanine, almost all of the clones growing on the hypertonic medium appeared to be have undergone a chromosome loss event. Therefore, para-fluorophenylalanine induces chromosome loss.

b. Mitomycin C: The bifunctional cross-linking antibiotic, mitomycin C, is a known recombinogen in yeast (Holliday, 1964; Takahashi, 1974) as well as an inducer of chromosome loss (Parry et al., 1979a). Strain XD72, which is closely related to strain XD26, was treated under growing conditions for two days with mitomycin C. Mitomycin C was cytotoxic (Figure 30A, $p<0.01$, $r=-0.9889$) but did not induce petites (data not shown). The frequency of putative chromosome loss events (Figure 30B) increased significantly with dose ($p<0.01$, $r=0.9188$) and so did the fraction of suppressor minus clones growing on the hypertonic medium (Figure 30C). Once more the hypertonic medium showed a good selection for suppressor loss. In fact, almost all of the clones growing on the hypertonic medium were suppressor minus. The fraction of Ilv^- clones, however, did not increase (Figure 30D). On the contrary, the fraction of Ilv^- clones decreased significantly with dose ($p<0.01$, $r=-0.55539$). Furthermore, some of the Ilv^- clones were Ura^+ (compare Figures 30D and 30E) which is not expected with chromosome loss events. A number of clones were seen also which had lost the two suppressors and were Ilv^+ and Ura^-. This phenotype
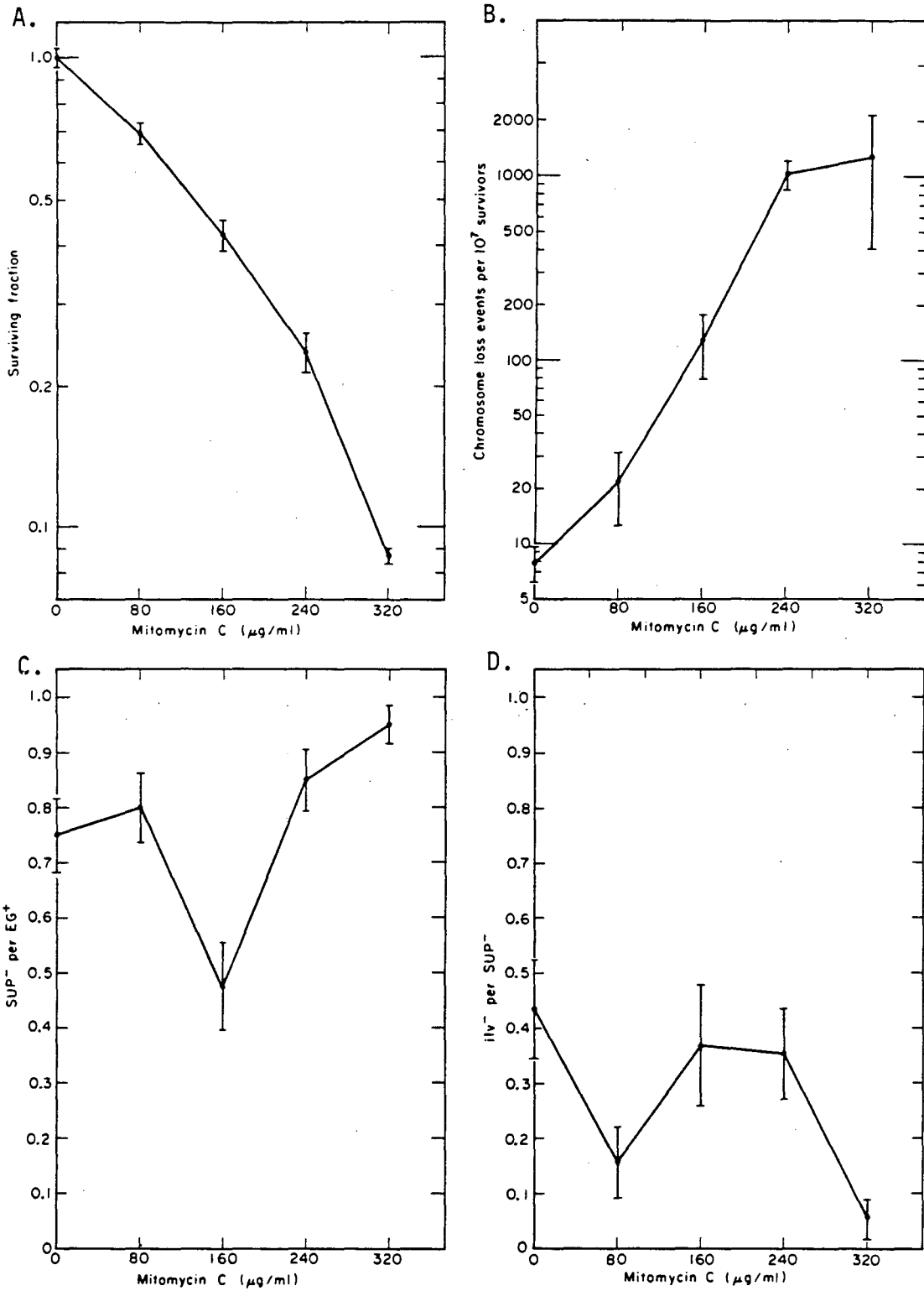
Figure 30.   Mitomycin C suspension test
(two day treatment)
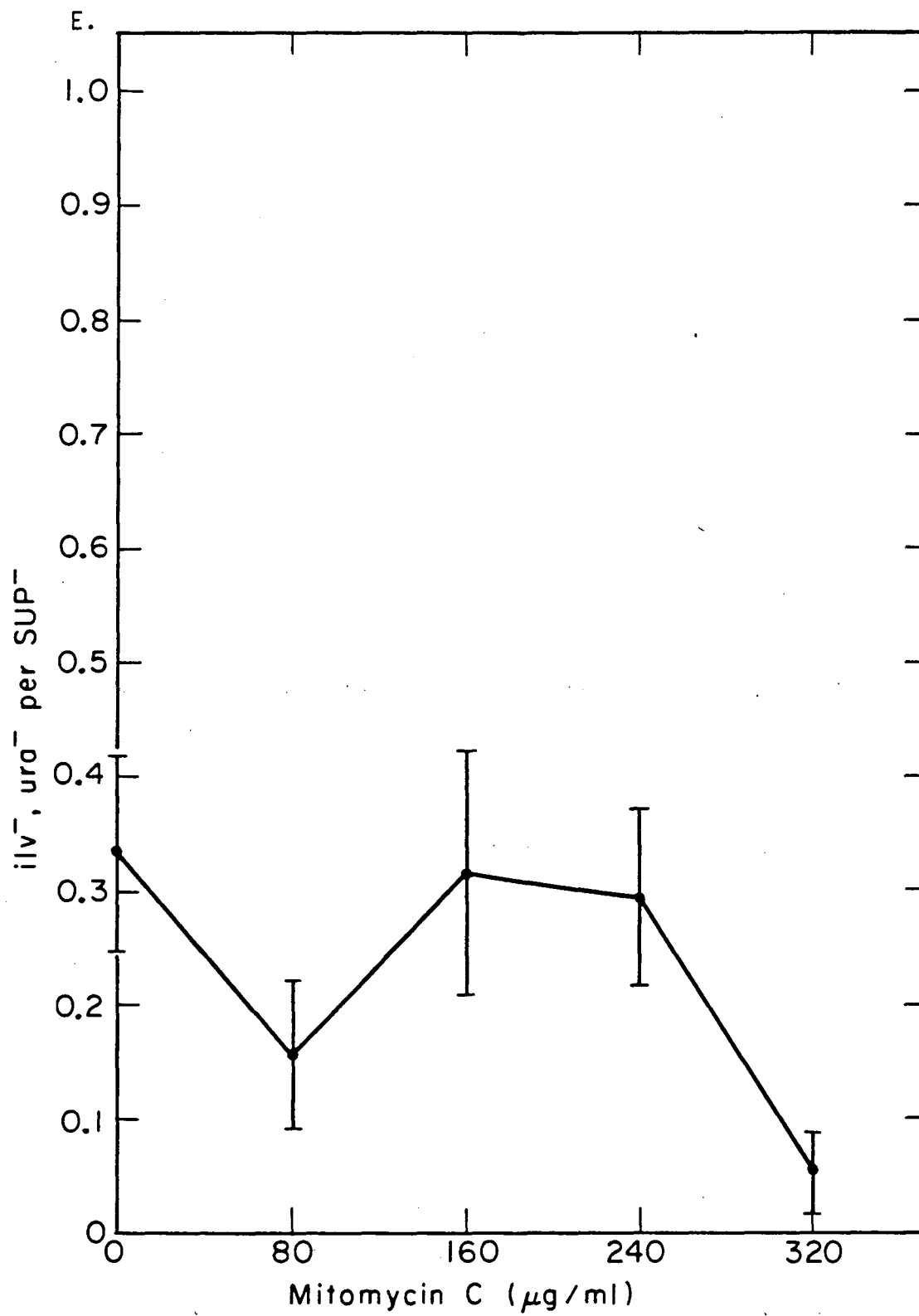
XBL 836-3815

144

Figure 30. Mitomycin C suspension test
            (two day treatment) (continued)

XBL832-3597

also is not indicative of chromosome loss. These results contrast sharply with the results obtained for para-fluorophenylalanine. A significant fraction of the increase in the chromosome loss phenotype with mitomycin C is most likely due to an increase in recombination. Of course, chromosome loss may be occurring as well as coincident crossing-over. Further testing is necessary to distinguish chromosome loss from mitotic crossing-over for this chemical and to ascertain whether or not chromosome loss is occurring at all. A method for distinguishing between the two events was discussed earlier (see section A.4.).

c. Spontaneous tests with canavanine: Early tests using canavanine medium for selection of chromosome loss events in strains XD26 and XD72, both of which contained an antisuppressor, were unsuccessful. Extremely few of the canavanine resistant colonies had the chromosome loss phenotype and most still contained at least one suppressor. Therefore, hypertonic ethylene glycol medium was used as a first step in the selection for chromosome loss events. Later, strain XD99 was constructed to avoid the antisuppressor problem encountered with the earlier strains (see section A.2.c.). Strain XD99 is isogenic to strain XD72 except that it lacks the antisuppressor. When this strain was plated to canavanine medium, 96 of 128 canavanine resistant colonies which were tested had the chromosome loss phenotype. The antisuppressor, therefore,

appears to have been the reason for the initial failure of the canavanine medium to select for chromosome loss events. Strain XD99 should be able to be used with both hypertonic and canavanine media to select for chromosome loss events. This new strain XD99 was used to develop the spot tests discussed below.

2. Growth Curves

The treatment times involved for the chromosome loss experiments were relatively long. Therefore, growth curves were performed to see if induction of chromosome loss was occurring or selective growth of monosomic cells. Figures 31A, 31B, and 31C show the growth curves for strains XD26, XD72, and XD99. Strains isogenic to these strains, but without the two suppressors were isolated. These strains were designated XD26-CL, XD72-CL, and XD99-CL respectively. These latter strains have the chromosome loss phenotype and presumably have undergone a chromosome loss event. Growth curves for these strains have also been plotted (Figures 31A, 31B, and 31C). In each case, the strain with the two suppressors grew faster than the isogenic strain without the two suppressors. These data indicate that induction of chromosome loss was occurring and not selective growth following chromosome loss.

A.

B.

C.
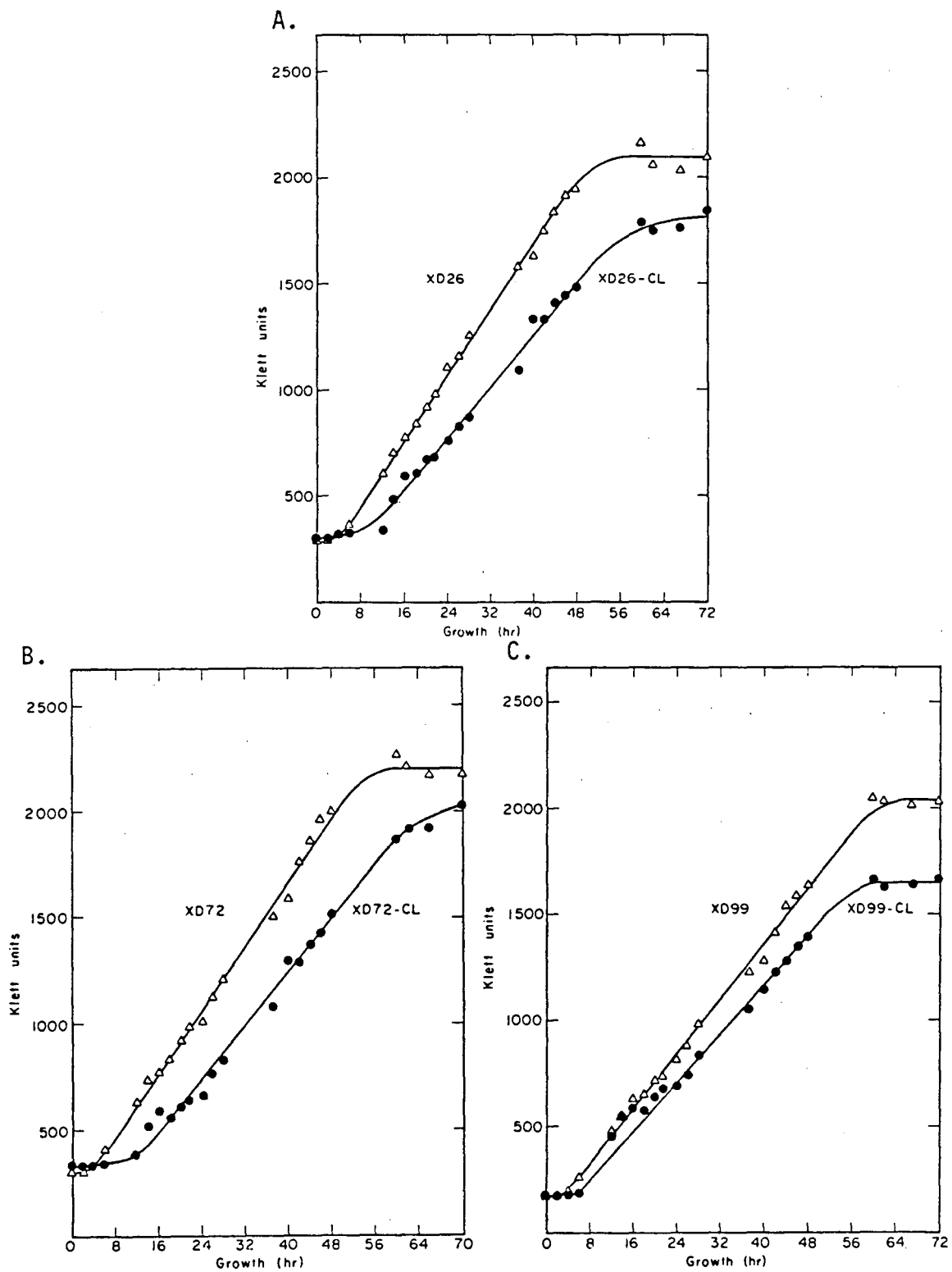
XBL837-3875

Figure 31. Growth curves

3. Spot Tests

Spot tests, although they have limitations (Ames et al., 1975), can provide fast, easy, qualitative tests for many genetic changes (Fink and Lowenstein, 1969; Pittman and Brusick, 1971; Ames et al, 1975; Kunz et al.,1980) and may be used to screen a large number of chemicals to decide on a priority for suspension testing. The selectivity of strain XD99 for the simultaneous loss of both arms of chromosome X has allowed the development of a spot test for chromosome loss.

Direct plating on canavanine medium was usually unsuccessful in detecting chromosome loss events. This may have been due to a requirement for a few cell divisions for the expression of the canavanine resistance (Lemontt, 1977). Direct plating on hypertonic medium was also unsuccessful probably again for the same reason. In addition, growth on the medium was not uniform. However, treatment of the cells on non-selective medium (such as synthetic complete) prior to replica plating to selective medium (canavanine or ethylene glycol) met with good success. Further confirmation of chromosome loss was made by replica plating to other selective media to test for the rest of chromosome loss phenotype.

A small number of chemicals were tested to develop the spot test. The cells were grown on synthetic complete with the chemical and then replica plated to canavanine medium

(Figures 32A, 32B, 32C, and 32D). The cells growing on the canavanine plates were then replica plated to YEPD (Figures 33A, 33B, 33C, and 33D) and various selective media (data not shown). Those colonies which grew on hypertonic medium and were canavanine resistant, red on YEPD, and isoleucine-valine, histidine, lysine, and uracil deficient had presumably undergone a chromosome loss event for chromosome X.

4-Nitroquinoline-N-oxide (4-NQO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl benzimidazole-2-yl-carbamate (MBC) were tested. Figures 32 and 33 show the spot tests with these chemicals. A representative sample of colonies were picked off the canavanine plates and tested for the chromosome loss phenotype. Many of the colonies from the MNNG and 4-NQO-treated plates (12 out of 31 and 11 out of 19, respectively) had the chromosome loss phenotype. A lesser fraction from the EMS-treated plates (3 out of 62) had the chromosome loss phenotype. All of the canavanine resistant colonies except one (117 out of 118) from the MBC-treated plate had the chromosome loss phenotype. However, the ring pattern usually associated with spot tests was not present in the MBC tests although it was evident with the other chemicals.

Tetrad dissections were performed on a representative sample of colonies with the chromosome loss phenotype from the MBC and 4-NQO plates. Although these colonies all had

150
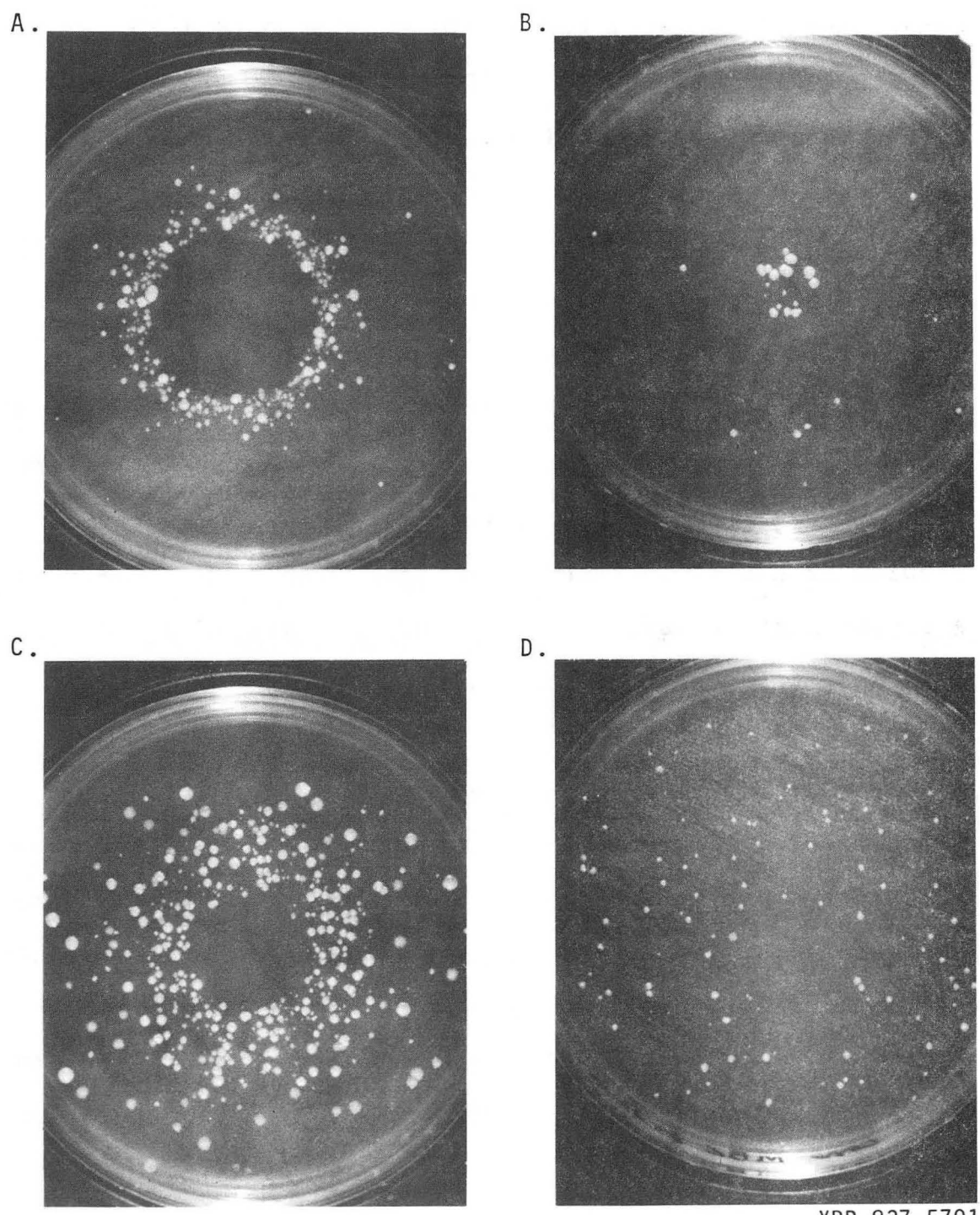
XBB 837-5701

Figure 32. Spot tests for chromosome loss I. Cells were grown on synthetic complete medium in the presence of the chemical and then replica plated to selective canavanine medium. A. 4-NQO B. MNNG C. EMS D. MBC
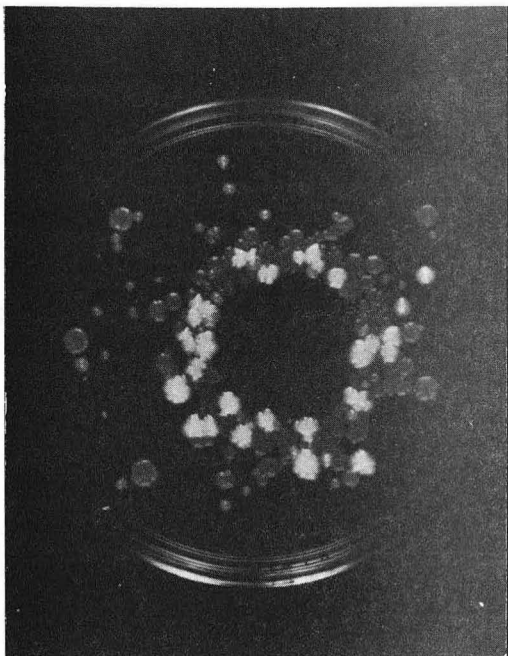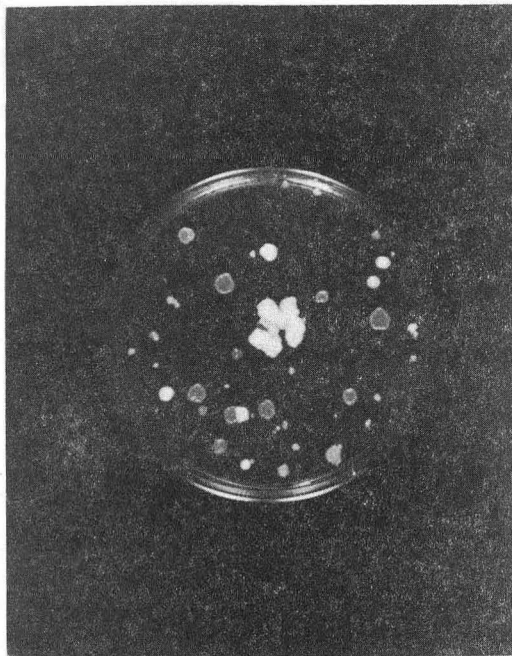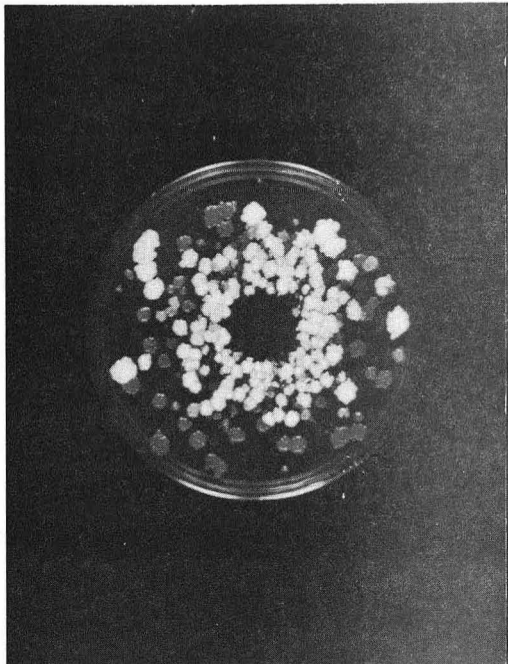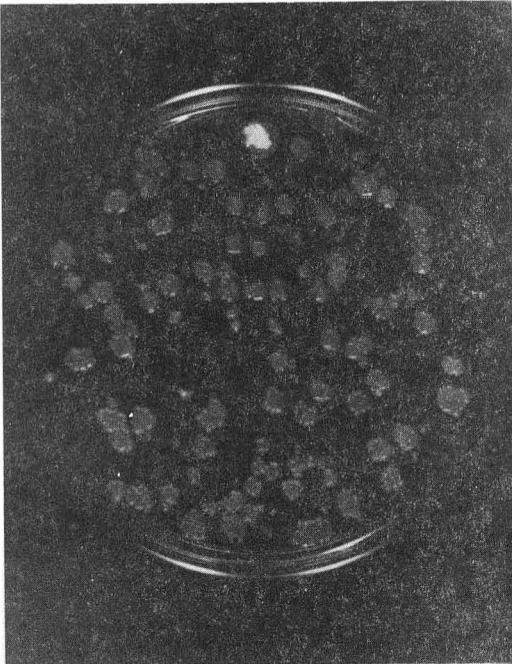
CBB 837-5994

Figure 33.   Spot tests for chromosome loss II. Cells in previous
figure were replica plated to YEPD. Cells which have
undergone chromosome loss should be dark red.
A. 4-NQO   B. MNNG   C. EMS   D. MBC

the chromosome loss phenotype, none of the cells showed a
2:2 centromere-linked lethal segregation pattern (Table 7).
4-NQO treatment did result in a high proportion of 2:2
linked lethal segregations. However, these lethal
segregations were not linked to the segregation of the
centromere-linked marker. This lack of a 2:2 lethal
segregation pattern agrees with that from the suspension
tests (see section A.5.). Although coincident
crossing-over can not be ruled out, the high proportion of
isoleucine-valine minus colonies among the suppressor minus
colonies is indicative of chromosome loss and, therefore,
the test appears to have a good selectivity for chromosome
loss.

TABLE 7

SEGREGATION PATTERNS OF PUTATIVE MONOSOMICS FROM SPOT TESTS

| Agent | Colony forming:Non-colony forming | | | | | Total dissected spore colonies | Percent viability |
	4:0	3:1	2:2	1:3	0:4		
MBC-9	3	0	1	1	1	20	75
MBC-18	0	3	1	1	0	20	60
MBC-20	2	1	1	1	0	20	70
MBC-29	2	1	0	1	1	20	60
MBC Total	7	5	3	4	2	80	66
4-NQO-4	0	1	8	2	0	44	48
4-NQO-5	0	2	4	1	0	28	53
4-NQO-6	0	0	5	3	1	36	36
4-NQO-16	4	1	2	0	1	32	72
4-NQO Total	4	4	19	6	2	140	51
UNTREATED CONTROLS-1	2	2	1	0	1	24	67
UNTREATED CONTROLS-2	7	0	1	0	0	30	93
UNTREATED CONTROLS-3	5	5	2	2	0	56	73
UNTREATED CONTROLS-4	6	4	0	0	0	40	90
UNTREATED CONTROLS Total	20	11	4	2	1	150	82

VI. DISCUSSION OF CHROMOSOME LOSS

A. Detection of Mitotic Chromosome Loss

Strain XD99 and its related strains, XD26 and XD72,
can detect chromosome loss and nondisjunction. Two
positive selection techniques were developed which allow
for selection of putative monosomics. An advantage of this
system is that simultaneous loss of both arms of chromosome
X is selected for thereby greatly increasing the
selectivity of the system. Further confirmation of
chromosome loss is done using ochre suppressible markers in
the strain.

1. Comparison of selective media

Two types of selective media were used to detect
putative chromosome loss events. Each had advantages and
disadvantages.

a. Hypertonic media: A variety of hypertonic media
were tested. 2.5M and 2 M ethylene glycol media were the
most accurate in detecting the presence or absence of the
two ochre suppressors in the chromosome loss strains. Even
in the presence of an antisuppressor, the ethylene glycol
media were still highly selective for chromosome loss and
much more selective than canavanine medium in strains XD26

and XD72. One advantage of ethylene glycol is that sectoring can be seen for some colonies and, therefore, these sectors can be dissected to distinguish chromosome loss from coincident crossing-over. Such coincident crossing-over events are probably the major event that mimics the chromosome loss phenotype. Hypertonic media also directly selects for the loss of the two suppressors.

A number of actual and potential technical problems with hypertonic media will be discussed below, however. 1) Some hypertonic media age quickly so that nothing will grow on them, suppressor containing or not. This aging may be due to an increase in molarity from the plates drying out. In this study plates were used usually on the third day after pouring to minimize this problem. 2) Petites do not grow on these media so that a correction must be made for this. If an agent induced a very high frequency of petites, then a large source of error could develop. 3) Other mutations such as osmotic resistance can lead to growth on hypertonic media. However, corrections can be made for this since this is distinguishable from suppressor loss. 4) Certain strains do not grow at all on hypertonic media (Singh, 1977). All the suppressor minus strains used in this study grew on the hypertonic media. 5) Concentration effects can occur. In other words, a doubling of the number of cells plated did not result in a doubling of the number of cells which grew. 6) The red color due to the expression of the adenine mutation did not fully develop in

the strain after chromosome loss on ethylene glycol media.
Possibly the adenine mutation is an osmotic remedial
mutation. Hypertonic media can reverse an osmotic remedial
phenotype (Hawthorne and Friis, 1964). 7) Antisuppressor
bearing strains may not grow on these media (Singh, 1977).
However, antisuppressors to ochre suppressors seem to be
recessive (Gorman et al., 1977) and, therefore, are not
likely to be expressed in a diploid strain. In fact, in
this study two of the strains carried recessive
antisuppressors which did not seem to interfere greatly
with the execution of the tests. Agents that induce
mitotic crossing-over may, however, homozygose such
antisuppressors. 8) Ostergren (1944) has reported that 1
M ethylene glycol induces sticky chromosomes in the root
tips of Allium cepa. This raises the possibility that
the media itself might induce chromosome loss. Since the
same media were used for all doses including the zero dose,
this possibility should be under control. 9) Growth was
uneven over the plate with the greatest density of growth
in the center of the plate. 10) The mechanism of
hypertonic media growth inhibition in suppressor bearing
strains is not understood (Singh, 1977). The two most
serious problems encountered with hypertonic media in this
study were the concentration effect and the short time
period within which the media had to be used.

b. Canavanine medium: Canavanine medium detects
chromosome loss indirectly by the loss of the two

suppressors flanking cen10 that suppress canavanine resistance thereby allowing the expression of the canavanine resistance mutation. Canavanine medium was highly selective in suspension and spot tests for suppressor loss in strain XD99 but was poorly selective in the antisuppressor bearing strains, XD26 and XD72. Petite colonies were able to grow on this medium and the red color from the expression of the adenine mutation following chromosome loss also was apparent when the canavanine medium was also made low in adenine. Concentration effects were not as significant as with the ethylene glycol medium and growth on the canavanine medium did not seem to be strain dependent. The mechanism of the cytotoxic action of canavanine is also understood (Grenson et al., 1966; Whelan et al., 1979). Canavanine medium is more convenient to use than ethylene glycol medium because it does not age as quickly.

A major disadvantage of canavanine medium is that antisuppressor bearing strains will grow on it, thereby, minimizing its selectivity for chromosome loss. Any mutation in the canavanine gene which might be picked up and which is not suppressible by SUP4 or SUP7 should not pose a problem since canavanine resistance is recessive unless a recombination event results in homozygosis of the mutation.

In summary, either hypertonic media or canavanine medium may be used to select for chromosome loss in strain

XD99. For most purposes, canavanine medium has probably
the greatest technical advantages in ease of handling.
However, in strains bearing an antisuppressor such as XD26
and XD72, or when a high frequency of non-ochre
suppressible canavanine resistance is induced, hypertonic
media, such as 2 or 2.5M ethylene glycol, is more
selective.

2. Antisuppressor

The presence of an antisuppressor in strains XD26 and
XD72 was responsible for the initial lack of selectivity of
the canavanine medium for chromosome loss events. This
antisuppressor was recessive, heterozygous and decreased
the ability of either SUP4 or SUP7 to suppress the ochre
mutations ade2 and canl-100 but did not noticably affect
the ability to suppress the other ochre mutations his5-2
and lysl-1. Similar antisuppressors to ochre suppressors
have been reported by McCready and Cox (1973, 1976) and by
Laten et al. (1978). Repeated genetic crosses eliminated
the antisuppressor and resulted in strain XD99 which can be
used with either canavanine or ethylene glycol media. In
addition, spot tests are now possible using the canavanine
medium.

3. Chromosome loss versus other events

Probably the major event which mimics the chromosome loss phenotype is coincident crossing-over. A scheme was devised to differentiate the two events on ethylene glycol medium using sectored colonies. This relied primarily on the genotype of the white sector: homozygosity of the sector indicating coincident crossing-over and heterozygosity of the sector indicating chromosome loss.

4. Restoration of euploidy

Certain stable aneuploids have been found in yeast. Takahashi (1972) found monosomics were induced for chromosomes I and VIII by certain petite-inducing mutagens: Cd^{++}, Co^{++}, and acriflavine. These putative monosomics segregated a 2:2 lethal. Stromnaes (1968) also recovered probable monosomics for chromosomes I and VI. Shaffer et al. (1971) found that chromosome III disomes are relatively stable and trisomes are very stable.

Likewise, Parry and Cox (1971) noted a high tolerance of aneuploidy in yeast. This stability, however, was for disomy of one or more chromosomes. Multiple disomy for more than five out of the ten chromosomes studied was not observed (Parry and Cox, 1971). In addition, certain chromosomes were disomic more than others and certain multiple combinations of disomy occurred. Bruenn and Mortimer (1970) also noted aneuploid stability in some monosomics. However, many of their 29 sporulated

presumptive monosomics appeared to be mixtures of diploid and monosomic cells. The diploid revertants were all homozygous for the marker used to isolate the presumptive monosomy. Strains monosomic for the larger chromosomes generally grew and sporulated poorly and reverted to diploidy more rapidly than strains monosomic for the smaller chromosomes. The only stable monosomics found were those for chromosomes I and VI which appear to be short chromosomes from genetic mapping data (Mortimer and Schild, 1980). Campbell et al. (1981) observed that the multiple disomes from triploid segregations had a low number of disomic chromosomes which suggests that yeast do not tolerate high multiple disomy. Campbell et al. (1981) further suggest that five may be an upper limit of disomic chromosomes in a multiple disome from the data of Bruenn and Mortimer (1970). Although all chromosomes were found as disomes and appeared to have an equal probability of formation during triploid meiosis, Campbell et al. (1981) found a differential mitotic stability for different disomes.

A number of mutants have elevated levels of chromosome loss or nondisjunction. Liras et al. (1978) found a chromosome loss mutation in which nonrandom chromosome loss occurred in diploids during mitosis. Monosomics for chromosomes I and III were found at particularly high frequencies, for VIII and XVI at lesser frequencies, and for II, V, VI, VII, XI and XVII at low or background

frequencies. This chl mutation also induced mitotic
crossing-over and gene conversion. Mortimer et al. (1981)
have also found that the rad52 mutation loses chromosomes
at such high frequencies that near haploidy may result.
Since rad52 mutants are also sensitive to x-rays and are
defective in mitotic and meiotic recombination and
sporulation, this mutation may directly affect DNA.
Additionally, Kawasaki (1979) found that certain cell
division cycle mutants, cdc6 and cdc14 can induce
chromosome loss.

On the other hand, chromosome VII does not appear to
form stable monosomes. Parry and Zimmerman (1976) found in
D6 that there may be a restoration of euploidy following
chromosome loss in chromosome VII. They found that
putative monosomics upon sporulation did not show a 2:2
lethal segregation as might be expected. Instead, those
"monosomics" which sporulated had four viable spores. This
may occur through endoreduplication or nondisjunction.
Recently, Esposito et al. (1982) also failed to observe a
2:2 lethal segregation following putative chromosome loss
for chromosome VII in a diploid. Aspergillus nidulans is
another fungal organism in which aneuploids are unstable
(De Bertoldi and Griselli, 1980).

Although certain monosomics, especially monosomics for
chromosome I, appear to be stable, stability for monosomy
does not seem to exist for chromosome X, a relatively long
chromosome (Mortimer and Schild, 1980). Dissection of the

putative monosomic colonies gave results similar to the findings of several other studies in yeast (Parry and Zimmerman, 1976; Esposito et al., 1982; Malone et al., 1980; Wood, 1982). Putative monosomics from para-fluorophenylalanine treatment did not show a 2:2 lethal segregation although most of the chromosome loss events appeared to be due to actual loss or nondisjunction events judging from the preponderance of Ilv$^-$ clones. Rare coincident crossing-over events for both arms of chromosome X which would mimic chromosome loss and also not show 2:2 lethal segregation following dissection do not appear to explain the results. Dissections of putative monosomics from the spot tests with MBC also did not show a 2:2 lethal segregation for a centromere-linked lethal. Since MBC does not induce gene conversion and induces mitotic crossing-over at a much lower frequency than chromosome loss (Wood, 1982), this lack of lethal segregation in what is most likely chromosome loss is further evidence that restoration of the euploid state is occurring after chromosome loss. It appears, therefore, that monosomy for a number of chromosomes in yeast is not stable and is followed, possibly through further aberrant segregations, by a restoration of the euploid state.

This probable restoration of euploidy following chromosome loss has important implications. Chromosome loss has been proposed as a possible mechanism of promotion in carcinogenesis (Ohno, 1974). A recessive mutation may
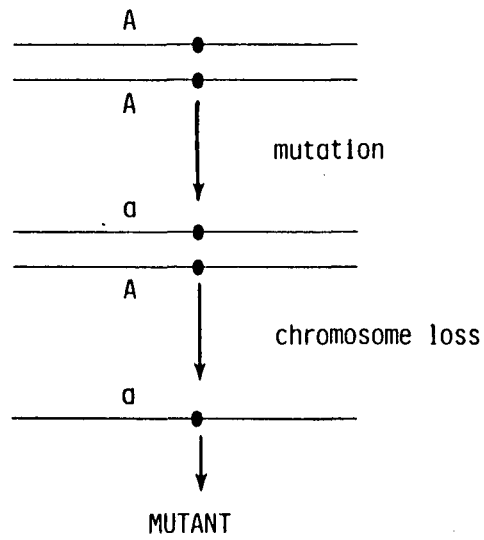
be expressed by a chromosome loss event which can lead to hemizygosity. In fact, Parry et al. (1981) found that six promoters induced mitotic chromosome loss in yeast whereas six non-promoters, four of which were phorbol analogues, did not. Kinsella and Radman (1978) objected that chromosome loss could not be a mechanism for promotion since promotion occurs only after initiation and presumably chromosome loss should be effective either before or after initiation (Figure 34). Restoration of the euploid state following chromosome loss resolves the seeming contradiction posed by Parry's results and Kinsella and Radman's objection: chromosome loss before mutation does not lead to a mutant phenotype whereas chromosome loss after mutation does (Figure 35). Chromosome loss, therefore, could be a mechanism of promotion if restoration of euploidy occurs in vertebrates as well as in yeast.

Nondisjunction may also lead to a chromosomal imbalance from the production of monosomy and trisomy.

How might tumor promoters induce chromosome loss? In fungi, mitosis is intranuclear and connections exist between the mitotic apparatus and the nuclear membrane (Carter, 1978). Tumor promoters have induced a variety of effects including membrane changes (for reviews see Berenblum and Armuth, 1981; Sivak, 1978 and 1979). Bellincampi et al. (1980) have evidence in _Aspergillus nidulans_ that membrane-damaging drugs cause mitotic nondisjunction. Therefore, it seems possible that a

Mutation ⟶ Chromosome loss:

Chromosome loss ⟶ Mutation:

A

A

mutation

a

A

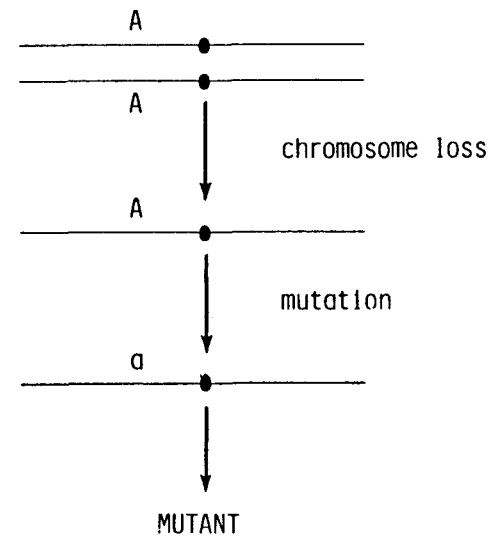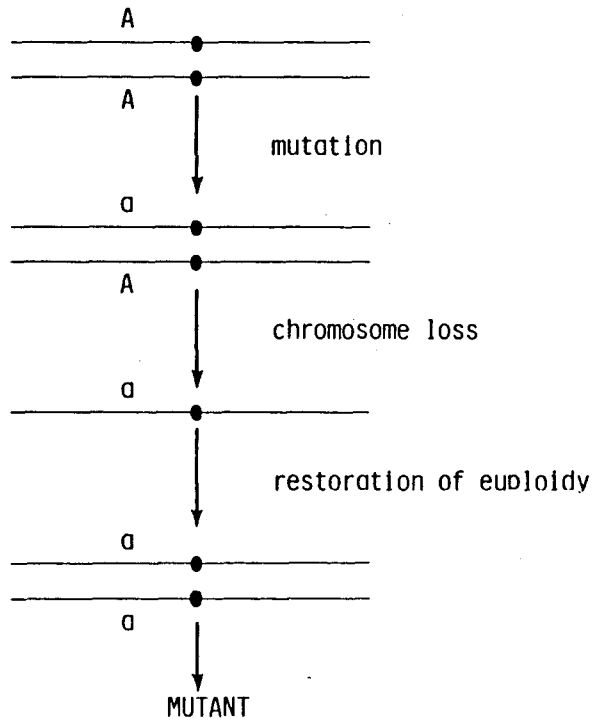chromosome loss

a

MUTANT

A

A

chromosome loss

A

mutation

a

MUTANT

Figure 34. Chromosome loss and promotion I

Mutation ⟶ Chromosome loss ⟶ Restoration of euploidy: Chromosome loss ⟶ Restoration of euploidy ⟶ Mutation:

A

A

mutation

a

A

chromosome loss

a

restoration of euploidy

a

a

MUTANT

A

A

chromosome loss

A

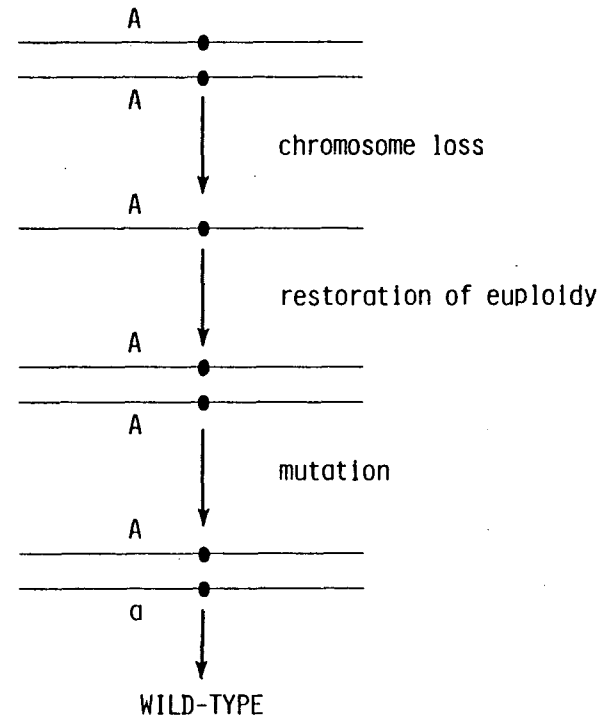restoration of euploidy

A

A

mutation

A

a

WILD-TYPE

Figure 35.   Chromosome loss and promotion II

membrane change caused by a promoter could result in chromosome loss by interfering with the attached mitotic apparatus.

In higher organisms, both plants and animals, there is also evidence that there are connections between the mitotic apparatus and the nuclear membrane (Kay and Johnston, 1973). Promoter-mediated membrane damage in these higher organisms might be from free radicals which appear to be generated from cells stimulated by tumor promoters (see Marx, 1983 for a brief review). Emerit and Cerutti (1982) suggest that a clastogenic factor may relate the initial interaction of the promoter with the cell membrane to the genome. Tumor promoters have induced strand breakage in human leukocytes by activated oxygen species liberated by phagocytes (see Birnboim, 1982 concerning strand breakage in human leukocytes).

Since malignant cells are frequently aneuploid, it is tempting to consider chromosome losses or gains as possible causative factors in carcinogenesis. However, it is uncertain at this time whether or not aneuploidy is a result or a cause of carcinogenesis.

5. Selection for or induction of chromosome loss?

Suppressor bearing strains usually grow more slowly than strains without suppressors and strains with two ochre suppressors show a markedly depressed growth (Gilmore,

1967; Gilmore and Mortimer, 1966). Therefore, a growing culture may undergo an enrichment for those cells which have lost the suppressors due to selective growth. However, growth curves of the chromosome loss strains and strains isogenic, but without the two ochre suppressors, indicate that the strains without the two suppressors actually grow more slowly. Therefore, it appears that there is an induction of suppressor loss occurring, not just a selection of prior existing suppressor minus mutants. In fact, the frequency of chromosome loss may be underestimated because, if anything, there appears to be a selection against the colonies which have lost the two suppressors. The poor growth of the strain after loss of the two suppressors is likely due to the expression of the _ilv3_, _ade2_, and other mutations since mutations for auxotrophic requirements generally tend to limit growth.

B. Chromosome Loss Suspension Tests

Two chemicals positive for the induction of aneuploidy in other systems were used to test the chromosome loss strains developed in this study.

1. Para-fluorophenylalanine

Para-fluorophenylalanine (PFA), a toxic analog of phenylalanine, appears upon incorporation to make faulty G2

division-related proteins (Wheatley and Henderson, 1974).

Morpurgo et al. (1979) suggest that it may interfere with

the spindle fibers. It was selected as a test chemical for

the chromosome loss system because it is a known inducer

of chromosome loss or nondisjunction in a number of

organisms including yeast (Stromnaes, 1968; Emeis, 1966;

Griffiths, 1979; Parry et al., 1979a) and other fungi

(Lhoas, 1961; Gutz, 1966; Morpurgo et al., 1979; De

Bertoldi and Griselli, 1980). Holliday (1964) found that

at toxic concentrations PFA did not induce mitotic

crossing-over in yeast, although Stromnaes (1968) found a

broad spectrum of genetic changes with PFA including

mitotic crossing-over, translocations, inversions, and

chromosome loss. Davies and Parry (1978) found that PFA

induced mitotic gene conversion and forward mutation in

yeast. Talmud and Lewis (1974) also observed mutation

induction in yeast with PFA. De Bertoldi and Griselli

(1980) found that PFA significantly induced mitotic

nondisjunction but not mitotic crossing-over in Aspergillus

nidulans. Morpurgo et al. (1979) found no effect of PFA

on point mutation and crossing-over in Aspergillus nidulans.

They did observe nondisjunction of germinating but not

quiescent conidia which they suggest means that PFA acts on

the spindle fiber level and not on the DNA level.

In chromosome loss strain XD26 para-fluorophenylalanine

(one and 3 day treatments) induced a dose-related increase

in chromosome loss as measured by growth on hypertonic

medium and expression of the ochre-suppressible markers. Initial problems using canavanine medium for selection were overcome by using hypertonic medium which selected for the simultaneous loss of two ochre suppressors on opposite arms of chromosome X. Loss of the two suppressors was usually associated with expression of the ilv3-1 and ura2-1 markers which provides strong evidence for chromosome loss or nondisjunction as opposed to other possible events such as coincident crossing-over. Therefore, although some coincident crossing-over may have occurred, the major source for the chromosome loss event phenotype appeared to be chromosome loss or nondisjunction. In addition, para-fluorophenylalanine was cytotoxic and also induced many petites.

2. Mitomycin C

The bifunctional cross-linking antibiotic, mitomycin C, is a carcinogen and an anti-tumor agent. It has given positive results in a number of screening systems (Hollstein et al., 1979). Results with the early standard tester strains of the Salmonella/microsome assay were inconclusive (McCann et al., 1975a), but later testing indicated that mitomycin C reverts only strains with the R-factor (the ampicillin resistance plasmid pKM101) and a functional UV-repair system (McCann et al., 1975b and Levin et al., 1982a and b). It has been found to induce

structural aberrations of chromosomes including gaps, chromatid exchanges and deletions in a wide range of organisms (for a review see Vig, 1977). The ability of mitomycin C to induce chromosome breaks has been attributed to its cross-linking capabilities. These breaks produce chromatid-type aberrations only and appear to be primarily in G-C rich regions of the genome (Vig, 1977). In vivo and in vitro studies indicate that mitomycin C usually affects the late replicating, constitutive heterochromatic regions of the chromosomes (Vig, 1977).

Mitomycin C is a potent inducer of recombination in a number of organisms (Vig, 1977) including yeast (Holliday, 1964; Takahashi, 1974; Davies and Parry, 1976; Koeda and Hirano, 1979; Parry et al., 1979a). Schewe et al. (1971) have suggested that mitomycin C may create recombinational sites by producing single strand nicks in DNA. Mitomycin C also has induced sister chromatid exchanges in a number of systems (Vig, 1977).

Sora et al. (1983) have found that mitomycin C induces diploid meiotic products in yeast but not disomic meiotic products. Griffiths (1979) similarly found that mitomycin C also did not induce meiotic disomy in a Neurospora crassa system. Takahashi (1974) found that mitomycin C primarily induced mitotic recombination events on chromosome V-some of which were double crossing-over events. Where a 2:2 lethal segregation pattern was observed, it was deduced from the segregation pattern that monosomy had not been

induced, but rather what appeared to be chromosome breakage followed by deletion of part of one arm of the chromosome. Takahashi also reported that mitomycin C decreased the frequency of petite induction. However, in contrast to the results in mitotic cells, Sora et al. (1983) did not find a significant induction of meiotic recombination. Parry et al. (1979a) have reported mitotic chromosome loss in yeast with mitomycin C using the D6 diploid strain. This agent also induced endoreduplication in PHA-stimulated human tonsillar lymphocyte cultures (Takanari and Izutsu, 1983).

In this study using chromosome loss strain XD72 mitomycin C induced putative chromosome loss events in a suspension test. However, contrary to the results with para-fluorophenylalanine, mitomycin C appeared to induce a high amount of recombination, but few if any petites.

The increase in probable coincident crossing-over on both sides of the centromere was much greater than the increase in possible chromosome loss events. In fact, the increase was so great that it is possible that what appeared to be chromosome loss was actually due to coincident crossing-over. Unfortunately, Parry et al. (1979a) did not confirm the putative chromosome loss events which they observed with mitomycin C by checking for the centromere-linked leu1 marker which is in their strain (see strain D6 in Table 1). The cyh2 marker and the ade3 marker which were used to diagnose chromosome loss are both quite distant from their associated centromere, cen7, and

coincident crossing-over may have occurred rather than chromosome loss (Figure 36). With such a potent recombinogen as mitomycin C, it is necessary to rule out possible coincident crossing-over events especially since mitomycin C is known to preferentially act on pericentric DNA in a number of organisms (Vig, 1977). Moreover, since mitomycin C has been found to induce deletions, a deletion combined with some other event might also be mistaken for chromosome loss. Of course, recombination and chromosome loss could both be occurring with mitomycin C. In fact, the two events may even be related since recombination near the centromere in yeast has been observed to be associated with an increase in chromosome loss in a disome by Campbell and Fogel (1977). A crossing-over event near the centromere preceding chromosome loss is not detectable in either the XD99 or D6 strain.

Although it appears that mitomycin C can induce diploid meiotic progeny (Sora et al., 1983), it is not clear at this time whether mitomycin C actually induces mitotic chromosome loss and/or nondisjunction in yeast. Further testing is necessary to distinguish chromosome loss from mitotic crossing-over with this chemical and other agents which induce such high frequencies of recombination.

The fraction of Ilv^-/Ilv^+ clones among putative monosomics is an important indication of chromosome loss versus mitotic crossing-over and a high frequency of Ilv^+ clones must be carefully evaluated. Confirmation
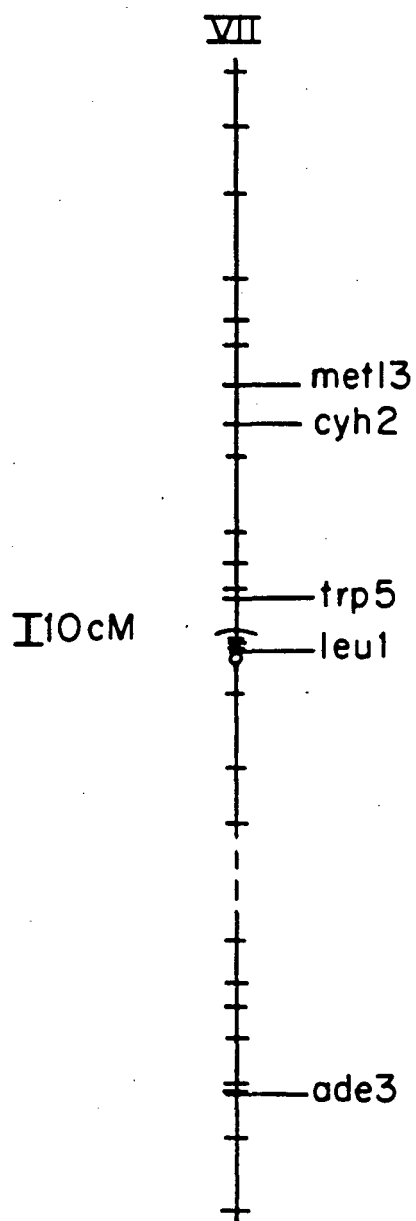
XBL837-3872

Figure 36. Chromosome VII markers used in strain D6

of chromosome loss in mitotic systems using a
centromere-linked marker is essential for agents that
induce high frequencies of recombination.

C. Chromosome Loss Spot Tests

Previous mitotic chromosome loss strains in yeast
(Parry and Zimmermann, 1976; Esposito et al., 1982) have
relied upon a positive selection (cycloheximide resistance)
for the loss of one chromosome arm and a color selection
(red to white) for the loss of the other chromosome arm.
Since only an extremely small fraction of the cycloheximide
resistant colonies were also white in spontaneous tests
with strain D6 (49 white out of 6,135 cycloheximide
resistant; see Table 1 in Parry and Zimmermann, 1976), spot
tests with this and similar strains are not feasible.
Strain XD99, however, uses a positive selection procedure
for the loss of both arms of chromosome X. This high
selectivity for chromosome loss made it possible to develop
a spot test for chromosome loss. Growth in the presence of
the chemical agent appeared to be a requirement generally
for the induction or expression of chromosome loss.

Putative chromosome loss was observed for methyl
benzimidazole-2-yl-carbamate, ethyl methanesulfonate (very
slight), N-methyl-N'-nitro-N-nitrosoguanidine, and
4-nitroquinoline-N-oxide. The typical ring pattern usually
seen in spot tests (Figure 4) was not observed with MBC.

In fact, the yield of chromosome loss colonies was constant over the entire concentration gradient for MBC. However, the other agents did induce the typical ring pattern. These differences in the chromosome loss induction patterns are not understood but may relate to the mode of chromosome loss induction for the different agents or relate in some way to the diffusion characteristics of the chemical. MBC is a spindle poison whereas EMS, MNNG, and 4-NQO are chromosome breakers or clastogens.

MBC was especially effective in inducing chromosome loss. Wood (1982) found that MBC in yeast induced little or no forward mutation, no mitotic gene conversion, and an increase in mitotic crossing-over. The increase in mitotic crossing-over was highly variable and did not correlate with any experimental parameter tested. However, the primary effect of MBC in yeast appeared to be the induction of mitotic chromosome loss, at least 20-fold more than the increase in mitotic crossing-over. This loss occurred randomly and at approximately equal frequencies for all 13 out of 16 chromosomes tested including chromosome X which was used in this study. That MBC affects the mitotic spindle and causes chromosome nondisjunction is consistent with Wood's finding of chomosome loss and gain with MBC.

Ethyl methanesulfonate has also induced mitotic chromosome loss or nondisjunction in yeast (Parry, 1977; Parry et al., 1979a). Campbell (1980) has found disomic

chromosome loss is associated with EMS-induced centromere-adjacent gene conversion. This corresponds with the earlier findings of Campbell et al. (1975) that spontaneous disomic chromosome loss and mitotic exchange in centromeric regions are associated. Although most of the chromosome loss events they observed were not related to mitotic exchanges, more than a 100-fold greater fraction than expected was correlated with exchanges. Further studies by Campbell and Fogel (1977) found that disomic chromosome loss is increased about 40-fold over basal levels among centromere adjacent mitotic gene convertants and not in convertants far from the centromere. Campbell and Fogel suggest that centromere involvement during recombination may result in faulty segregation and lead to chromosome loss. EMS has also induced effects in Chinese hamster ovary cells that could result in aneuploidy (Zimmermann et al., 1979).

In the XD99 spot test, however, only a marginal induction of chromosome loss was observed although other chromosome loss mimicking events were occurring frequently as evidenced by the pronounced ring pattern of growth on the selective plates (Figures 32 and 33). Most of the putative chromosome loss events occurred in the outermost area of the ring pattern where the concentration of chemical was relatively low.

MNNG and 4-NQO have not been tested in yeast for the induction of chromosome loss or nondisjunction. MNNG has

induced aneuploidy in somatic cells from a number of organisms including the Chinese hamster, Syrian hamster, and guinea pig and it has also induced teratogenesis in mice (see Gichner and Veleminsky, 1982 for a review). Epler et al. (1977) found that 4-NQO induces chromatid breaks in human leukocytes. It has also induced endoreduplication (Sutou and Tokuyanma, 1974). The XD99 spot test has, therefore, revealed two aneuploidy inducing agents for yeast. Follow-up suspension tests need to be done to confirm the results of the spot tests.

VII. SUMMARY AND CONCLUSIONS

Two diploid strains of the yeast Saccharomyces cerevisiae were constructed to detect a wide spectrum of genetic damage. Strain XD99 and its related strains can detect chromosome loss which is important in the induction of teratogenesis, aneuploidy, and possibly carcinogenesis. Two positive selection techniques were developed which select for the simultaneous loss of both arms of chromosome X. Para-fluorophenylalanine was found to induce chromosome loss in strain XD26 whereas mitomycin C primarily induced coincident crossing-over in the related strain XD72. Positive selection on hypertonic medium was highly selective for loss of the suppressors on the opposing arms of chromosome X. However, confirmation of chromosome loss by use of a centromere-linked marker was found to be essential to distinguish chromosome loss from coincident crossing-over.

The high selectivity of strain XD99 allowed the development of a spot test for chromosome loss. Methyl benzimidazole-2-yl-carbamate and ethyl methanesulfonate which have induced chromosome loss in other yeast systems induced chromosome loss in the newly developed spot test. Two carcinogens which have not been tested for chromosome loss in yeast before, 4-nitroquinoline-N-oxide and N-methyl-N'-nitro-N-nitrosoguanidine, also induced

chromosome loss in the spot test. Further quantitative suspension tests need be done to confirm these qualitative results. Chromosome X monosomics were found to be unstable and were restored to the euploid state. This restoration of euploidy may have important implications in regard to chromosome loss as a mechanism of promotion.

Strain XD83 can detect multiple genetic changes: nuclear frameshift and base pair substitution mutations, nuclear mitotic crossing-over and gene conversion and mitochondrial large deletions and forward point mutations. The known carcinogens ethyl methanesulfonate, ICR-170, N-methyl-N'-nitro-N-nitrosoguanidine, 4-nitroquinoline-N-oxide, and ultraviolet light and the questionable carcinogen, ethidium bromide, were tested in XD83. All of the agents induced dose-related increases in one or more of the genetic endpoints. None of the carcinogens was specific in its induced spectrum of damage. On the contrary, all five of the carcinogens induced multiple genetic effects. Only ethidium bromide, the questionable carcinogen, induced a highly specific spectrum of damage: petite induction. The generality of this lack of specificity remains to be discovered. Generally, suspension tests appeared to be more sensitive in detecting genetic change than spot tests. Although spot tests were quite sensitive to gene conversion, forward nuclear mutation as measured on chloramphenicol medium, and forward mitochondrial mutation, some improvements are needed to

make the spot tests more sensitive to nuclear frameshift and base pair substitution mutations.

This two strain system may be used to screen agents for their genetic toxicity. The variety of endpoints monitored may allow the detection of certain carcinogens and other genetically toxic agents which have escaped detection in other systems. Furthermore, this system may be useful in the study of possible mechanisms of carcinogenesis and aneuploidy.

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