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iii. Ordering of mutants within the close neighborhood of each other can be done by two and three factor crosses by generalized transduction and also by a new episome complementation method developed and described by Robert N. Reeves and John R. Roth, *JMB* 56, 523 (1971). Use of automatic techniques will allow the enormous labor required to make an intensive map to be done easily using transduction, mating, and other techniques that can be carried out on agar. The establishment of a large library of temperature sensitive and more completely characterized mutants covering the chromosome map thoroughly would have very many applications in the study of bacteria and especially of yeasts and higher organisms. We propose to begin the work with bacteria for which the techniques seem straightforward and extend it later to higher organisms.

Significance:

i. By periodic measurement of map distances by cotransduction or interrupted mating one can monitor increases and reductions of the chromosome by the net effect of gene doubling, recombination, deletion and other processes that may affect its size. With a large number of standard markers and standard procedures the machine can keep a steady picture of the state of integration or autonomy of various plasmids, of the chromosome number, if that is subject to change, and of the size of the chromosome. It seems more likely that the size of the chromosome is not an accurately conserved quantity but there will be variations in the population and it is hoped that methods of measurement will be sensitive enough to make some description of this distribution and how it changes when the parent strain of the population contains various mutations especially affecting DNA replication and repair.

ii. There may be regions of the map for which no temperature sensitive mutants or other conditionally lethals can be found. It is of great interest to know how much of the DNA specifies no function and is functionless except for its role in evolution of new genes to carry out new functions or for structural functions at the DNA or RNA level.

iii. When the whole map or at least regions of it are densely filled with markers it may be possible to discern overall patterns of placement and organization of the genes according to their function or evolutionary history and thus to understand better evolutionary or physiological demands that led to this particular pattern or structure. The operon concept is the most obviously important fact of this type but there may be others as yet unrecognized.

iv. It will be possible to supply large numbers of densely located temperature sensitive and other kinds of mutants in particular regions of the map for intensive further study of particular problems in this and other laboratories. We intend to use the method immediately for trying to generate large numbers of mutants in the neighborhood of known sites for DNA regulatory mutations hoping to discover other DNA regulatory mutants in the same neighborhood. As the techniques develop we will probably be able to supply other laboratories with large numbers of mutants important to their particular interests.

If automation makes it possible to follow changes in a densely-mapped bacterial chromosome subjected to a variety of mutational situations, the resulting insights into chromosome mechanics will be extremely valuable. Extension of this approach to chromosomes of higher organisms has important consequences for understanding a wide range of cellular mechanisms for cells in which good biological mechanisms of genetic recombination are available.

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- 4) Genetic characterization of the chromosome terminus and the regulation of cell division in E. coli.  
David R. Zusman, Assistant Professor of Bacteriology, University of California, Berkeley.

Methods:

Recent evidence from gene frequency measurements (1-3) autoradiography (4) and biochemical analysis (5) demonstrate bidirectional chromosome replication in E. coli. The origin of replication appears to map at about 75 map min while the terminus has been mapped at about 30 min (6). The termination of chromosome replication appears to be necessary for chromosome segregation and subsequent septum cross wall formation (7-9).

It has been suggested that chromosome termination may trigger division by the transcription of division related genes, located at the chromosome terminus, at the time of their replication. (7,10-15). This hypothesis has recently received some experimental support: (a) studies of cell division following DNA, RNA, and protein inhibition at the time of chromosome termination in synchronous cultures (16-18) indicate that the specific replication of the last 0.5% of the chromosome (0.45 map min) is required for subsequent cell division; blocking protein synthesis during this replication will block the subsequent cell division. (b) Several filament forming septation mutants have been obtained which map near 30 min, the chromosome terminus (15, 19-20).

Unfortunately, the region of the genetic map around 30 min is one of the most poorly understood areas (21). Very few markers have been identified; a stable F' has never been isolated for this region (22). We therefore propose to study this region of the E. coli map in great detail using the automated techniques now available. Hopefully the study of this region will help us understand the nature of the link between chromosome termination and cell division.

We have isolated a man<sup>-</sup> mutant (30.5 map units) that is non-reverting. We propose to use the transducing phage P<sub>1</sub> to cotransduce mutagenized markers (23) from a man<sup>+</sup> strain to our man<sup>-</sup> strain. Transductants grown on mannose minimal medium will be plated out using Dr. Glaser's automation equipment, replica plated at different temperatures, and temperature sensitive colonies obtained. These colonies will be characterized for nutritional defects or division defects. The nutritional mutants will be saved to help us map this region of the chromosome. The division-membrane mutants will be studied more carefully to determine possible relationships with chromosome structure and/or regulation of division.

Complementation studies should indicate the specific number of division related genes localized in this region of the chromosome and the possible existence of a division operon. Double mutants will be prepared so that the in vivo interaction (epistasis) of known mutants of different phenotypes can be studied (15). This approach can lead to the sequencing of related gene functions and is the first step necessary to determine the ordered pathway for septation in a manner similar to the study of T-even phage development and other self assembly systems (24).

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Significance: Detailed understanding of the relationship between DNA synthesis and cell division in E. coli may give important insights into the same relationship for proliferating animal cells, which generally do not synthesize DNA except in preparation for cell division.

- 5) Studies in biochemical evolution in E. coli and B. subtilis.  
Joshua Lederberg, Professor of Genetics and Biology and Chairman of the Genetics Department, School of Medicine, Stanford University, Stanford, California.

We wish to observe alterations in polypeptide products resulting from mutations in synthetic genes (generally synthetic homopolymer sequences) which have been inserted into the genomes of E. coli and B. subtilis bacteria. Immunochemical methods will be used for detecting these alterations by examining large numbers of small colonies for which no biological selection condition is known. By observing evolution of a polypeptide, much can be learned about the genetic code and about rates of various kinds of mutations in different nucleotide environments.

- 6) Genetic organization of the E. coli chromosome: mutation rate versus map position of the translocated lactose operon.  
Gordon Edlin, Associate Professor of Genetics, University of California, Davis, California.

The purpose of these experiments is to probe the genetic organization of the E. coli chromosome. Ultimately we would like to understand why genes are located at particular sites in the chromosome. One approach to this question is to measure frequency of mutations in a gene (or genes) which have been translocated to a number of different sites in the chromosome. A model system for these experiments is provided by the lactose operon. A set of strains exist which are genetically uniform except that the lactose genes have been translocated to a

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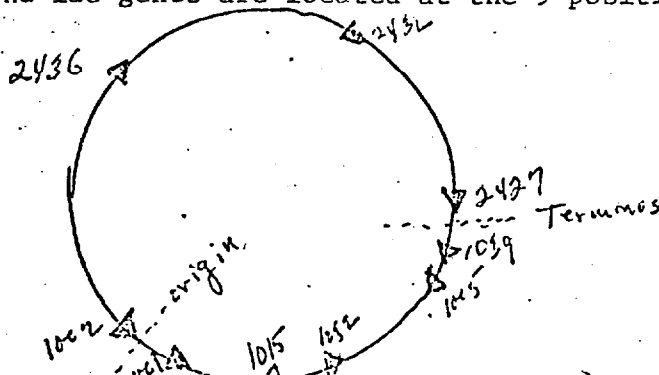


number of different sites in the chromosome.

These strains will be mutagenized with a variety of mutagens (nitrosoguanidine, ethylmethane sulfonate, U.V. light, etc.) and the frequency of lac<sup>+</sup> → lac<sup>-</sup> cells will be measured. Preliminary studies have shown that the frequency of mutations in the lactose genes are a function of chromosomal location.

After analysis of the lactose genes, the same analysis can be applied to other genetic systems such as an amino acid biosynthetic pathway, ribosomal protein, etc. Genetic techniques for constructing the appropriate bacterial strains already exist.

"Enclosed is a brief statement for your grant. We would like to go ahead on this as soon as possible since it is all worked out and is basically a matter of cranking out the data. The diagram shows the nine strains we want to test. The lac genes are located at the 9 positions



We will mutagenize with EMS and nitrosoguanidine for starters. We can measure the mutagenesis here by measuring the number of valine resistant colonies. That gives us a number to use to normalize the mutagenic effectiveness. We would then bring down the mutagenized culture to be sprayed onto trays. We want to test the number of lac<sup>-</sup> cells. I think the easiest way to do this is to place them on EMB lactose agar. Lac<sup>+</sup> are red and Lac<sup>-</sup> are white. We probably need to photograph at 2 or 3 times to reliably distinguish the 2 types and probably have to set some limits in the computer as to what it calls white and what it calls red so we probably need a dry run. Once that is determined we can run them as fast as time allows. I presume we will work with Phil on this. Let me know how and when you want to proceed."

- 7) Recombination deficient mutants of E. coli.  
Alvin J. Clark, Professor of Molecular Biology and Bacteriology and Immunology, University of California, Berkeley.

Method: "Our work in large measure stems from the discovery of recombination deficient mutants of various recombination proficient strains of E. coli. In doing the necessary mutant hunts the present bottle-neck is the picking of colonies of survivors of mutagenic treatment and patching them in geometric array. I am very interested in testing the dripper you have invented as a means of depositing cells in geometric array prior to testing their clones for recombination deficiency. It is very possible this may facilitate many experiments we have been sitting on because of the labor involved in picking and patching."

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- 8) New Salmonella typhimurium tester strains for detecting mutagens and carcinogens among environmental chemicals.

Bruce Ames, Professor of Biochemistry, University of California, Berkeley

Method: This work is an extension of work already published to special cases for which the labor of mutant isolation and characterization is limiting.

1. Ames, N. B., Lee, F. D., and Durston, W. E., Proc. Nat. Acad. Sci. U. S., 70, 782 (1973).
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- 9) Fine structure mapping in the histidine operon.

Bruce Ames.

Method: This work is an extension of work already published to special cases for which the labor of mutant isolation and characterization is limiting.

1. Ames, N. B., Lee, F. D., and Durston, W. E., Proc. Nat. Acad. Sci. U. S., 70, 782 (1973).
2. Ames, N. B., Durston, W. E., Yamasaki, E., and Lee, F. D., Proc. Nat. Acad. Sci., 70, 2281 (1973).

- 10) Metal ion mutagenesis and plasmid curing in Salmonella typhimurium.

Peter Flessel, Assistant Professor of Biology, University of San Francisco, San Francisco, California

Method: "I have been looking at the interactions of metals with bacteria using two assay systems. First, I have been studying metal ion mutagenesis and second, plasmid curing by metal salts. The decision to focus on metals was based on the near presence of a colleague in the chemistry department who had been studying metal carcinogenesis for fifteen years and was eager for some company.

"The work to date has been basically an extension of Bruce Ames' scheme applied to metals. So far I have shown that  $MnCl_2$  and  $NiCl_2$  are mutagens in S. typhimurium. Our search for new metal mutagens is continuing and I suspect we will find others in the next few months. The mechanism of metal mutagenesis has not been thoroughly explored. It is not known, for example, whether metal ion penetration of the cell membrane is a prerequisite to mutagenesis. To find out, I would like to select for mutants which are temperature sensitive for resistance to metals. The assumption is that resistance would be a reflection of the failure to take up the metal. I would select for growth at 42 (permease denatured) and no growth at 37 (permease functional) in the presence of the metal. Having obtained such mutants I would test them for susceptibility to metal mutagenesis at both temperatures. I realize my proposal is perfectly straightforward. If I carry it out with the time and resources at my disposal, it is at least a year's work. With the "dumbwaiter" I think I could have the first mutants in several weeks."

- 11) Genes affecting virulence in Salmonella and isolation of mutants suitable for a live vaccine.

Bruce Stocker, M.D., Professor and Acting Chairman, Department of Medical

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Microbiology, School of Medicine, Stanford University, Stanford, California.

For experiments on the mapping of genes affecting the virulence of Salmonella species, it is expedient to obtain genetically marked sublines in particular strains. For example, in lines of S. typhimurium which differ from the available genetically marked stocks of strain LT2 by their high virulence for the mouse, on intraperitoneal inoculation. It has been the experience of several workers that auxotrophic mutants obtained by mutagen treatment of virulent strains of Salmonella commonly have unwanted additional mutations causing reduced virulence, by unknown mechanisms. Therefore, in theory, the best method of procedure would be to introduce chosen negative alleles, determining nutritional requirements or inability to ferment particular substrates, by transduction. To do this by ordinarily available methods, even with the aid of penicillin enrichment, is hardly practicable, because of the amount of labor required to detect the rare transductants, which cannot be selected for. Dr. Glaser's apparatus should make possible the detection and isolation of the desired transductants by an automated procedure. A second problem, in the same general field, is the isolation of particular classes of auxotrophic, etc. mutant in mouse-virulent strains, for possible use as live vaccines, stably non-virulent because of, for instance, growth factor requirement, but otherwise unaltered. Mutants blocked in the synthesis of the diaminopimelic acid component of the bacterial cell wall should be unable to multiply in the tissues of a mammalian host because of absence of this substance, a component of bacterial but not of eukaryotic organisms. Heavy mutagen treatment of the bacterial strain to be used would be likely to cause additional, unwanted mutations: furthermore, it is unlikely that such mutants can be selected for by the penicillin enrichment technique. Probably the only way to isolate such mutants is by direct examination of a bacterial population for cells able to produce small colonies on defined medium supplemented with a small amount of diaminopimelic acid and able to resume growth on provision of additional diaminopimelic acid. Dr. Glaser's methods and apparatus should make this feasible, whereas it is hardly so by other methods.

- 12) Proline non-utilizing mutants of Salmonella typhimurium.  
John R. Roth, Associate Professor of Molecular Biology, University of California, Berkeley

We've been analysing the proline degradative pathway. It involves an operon containing three genes, two genes for degradative enzymes and one permease. Permease mutants can be obtained by positive selection. The other two classes are more difficult to obtain. Because even wild type cells grow rather slowly on proline, the standard penicillin enrichment works very poorly. Screening of mutagenized cells with your apparatus should permit mutant isolation. These will be strains which fail to grow on proline as sole N. source but can use either  $\text{NH}_3$  or glutamate as a nitrogen source."

- 13) Isolate mutants of Bacillus subtilis deficient in DNA synthesis at high or at low temperatures.
- 14) Isolate mutants of Bacillus subtilis resistant to certain phage and to drugs like p. hydroxyphenylazouracil for studies on DNA synthesis.  
A. T. Ganesan, Professor of Genetics, Stanford University, School of Medicine, Stanford, California.

Our research project involves the study of the mechanism of DNA replication and

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its genetic control in Bacillus subtilis, a transformable bacteria. We have isolated several temperature sensitive mutants that are defective in DNA synthesis. The thermosensitive protein has been studied in a few cases. There are about 9 groups of genes that control DNA synthesis. There may be even more. We isolate these mutants routinely by conventional, slow and laborious procedures. The automated petri dish machine would be ideal for the above project. We are specifically interested in both low and high temperature sensitive mutants, and mutants that are resistant to drugs like p. hydroxyphenylazouracil. This drug specifically inhibits DNA polymerase III in Bacillus subtilis. Polymerase III is directly involved in DNA synthesis. Resistant mutants would help to locate the position of the gene for the enzyme. The system is also adaptable to test phage mutants which are currently studied. The instrument is a very valuable and unique tool for our projects. We would very much like to collaborate with Dr. Glaser in obtaining several important mutants and adapting the machine for other related projects in cell biology.

- 15) Screening for possible mutagens among environmental chemicals by mutations affecting sporulation in Bacillus subtilis.  
Lawrence E. Sacks, Research Microbiologist and  
James T. MacGregor, Research Pharmacologist  
United States Department of Agriculture, Agricultural Research Service,  
Western Regional Laboratory, Berkeley, California.

Thousands of chemicals, whose biological effects are little understood, have been disseminated into our environment and the food we eat by modern technological society. Most frightening of these chemicals are the mutagens, with their potential for teratogenic effects, cancer, and unknown long-term effects of alteration of germ-cells.

In screening for possible mutagenic chemicals, microorganism systems offer the advantages of speed, simplicity, and economy over animal systems. A disadvantage of microbial systems now in use is that they test only for mutations occurring in one or a few genes. A bacterial system sensitive to mutations on many genes, scattered throughout the chromosome, would seem to offer important advantages over currently used systems (1). We believe such a system is that governing sporulation in the genus Bacillus. Sporulation is a very complex process requiring the participation of a minimum of 28 operons for the sporulation process alone (2). Other systems (e.g. TCA cycle) are required for successful sporulation. Eight hundred genes have been estimated to be required for successful sporulation (3).

Selection of asporogenic mutants is simplified by their characteristic white color easily distinguished from the wild-type brown colonies, colored by formation of a pigment late in the sporulation of B. subtilis, Marburg strain. Using a highly transformable strain of this organism, and a wide variety of mutagenic agents, many sporulation genes have been mapped (4) in programs designed to unravel the genetic control of sporulation. We propose only to invert this procedure, and to use sporulation mutants to identify new mutagenic agents.

Dr. Glaser's instrument, capable of identifying single mutants in huge populations, will be of great value in identifying mutagenic activity at very low concentration levels. This combined use of a bacterial system involving over a hundred genes with scanning by an instrument capable of identifying mutation rates below  $10^{-7}$  should result in a very rapid, extremely sensitive method for identifying mutagenic chemicals.



We summarize below some advantages of the proposed system:

1. It is based on forward mutation, the most general type of detection system. Any type of mutation which inactivates or substantially alters a gene essential for sporulation will be detected.
2. A large number of genes are involved in sporulation (2, 3). Some mutagens are specific for particular regions of the DNA. The more genes surveyed, the less chance of excluding mutagenic "hot-spots".
3. Sporulation mutants are often characterized by a block at a particular stage in their morphological development. The frequency of occurrence of particular stages of arrest will permit an assessment of the randomness (or specificity) of each mutagen.
4. The B. subtilis system is well-suited to genetic studies. Highly transformable strains exist, and many genes have already been mapped (4). Dr. Glaser's scanning system, however, is not limited to the pigmented B. subtilis colonies. Other well-studied species (e.g. B. megaterium, B. cereus) may also be employed.

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16) Proline degradation mutants in yeast.  
 John R. Roth, Associate Professor of Molecular Biology, University of California, Berkeley.

We've started looking at proline degradation in yeast. Here the available mutant enrichment techniques generally work poorly. Most people seek yeast mutants in a fairly "brute-force" sort of way. A large set of proline-non-utilizing mutants would be very useful to us. This hunt would need to follow the Salmonella hunt and probably should follow preliminary work (in progress) on the few available mutants. In this way the most advantageous conditions can be determined.

17) Saturation mapping of one yeast chromosome.  
 John R. Roth

Yeast has roughly 4-5 times as much DNA as bacteria. Roughly one hundred genes have been located. The spacing of these genes are wide enough to make it difficult to map new markers. A saturation map (or even a very sub-saturation map) would give new markers for mapping and a minimum estimate of the gene density.

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Strains will be obtained through Robert Mortimer which are monosomic for a small chromosome. Other strains will be obtained which are monosomic for a large but poorly marked chromosome. These strains are diploid for all but one chromosome.

A hunt for temperature-sensitive mutants should yield mutants carrying lesions in the chromosome for which these strains are haploid. These can then be analysed. Determining the number of genes involved is fairly easy because of the simplicity of yeast complementation tests. I'd like to try this in a year or so after I've gotten back from sabbatical leave. (I'll be doing yeast genetics during that time.)

18) Genetic mapping in Saccharomyces cerevisiae

Robert K. Mortimer, Professor and Chairman of Medical Physics Department, University of California, Berkeley.

The availability of detailed genetic maps is an important component in determining the suitability of an organism for genetic and molecular studies. For a number of years, we have carried on a program of genetic mapping in the yeast Saccharomyces cerevisiae as an adjunct to our other studies. These mapping studies have resulted in a genetic map which establishes the location of more than 150 genes on 17 chromosomes. However, because of the large number of chromosomes and the high frequency of genetic recombination in yeast, very few heavily mapped regions are available. Such regions are important for studies we wish to carry out on gene conversion and its relation to mechanisms of genetic recombination. We believe the instrument developed by Professor Glaser could help to speed up the further development of genetic maps in this organism. The approach we propose to use is based on the random spore technique described in our recent mapping paper (Mortimer and Hawthorne, Genetics 74: 53-54 (1973)). A series of strains that each carry one of a set of ochre suppressors in combination with a suppressible canavanine resistance gene and an additional selection of nutritional genes will be crossed to a large series of temperature sensitive lethals. The resultant crosses will be sporulated, and the asci will then be treated with glucylase followed by sonication. The sonicated suspension will be inoculated onto complete medium containing canavanine. Only spores lacking the suppressor and carrying the resistance gene will grow. These can then be transferred by replica plating to a series of "drop-out" plates to score the nutritional genes and to a "36°" environment to score the conditional genes. The patterns of growth: nongrowth on these various media can then be recorded automatically by the scanner and the resultant data analyzed for linkage by a suitable computer program. In this procedure it will be necessary to inoculate at a concentration that reduces to a low level the probability of clones developing from more than a single spore. The instrument should greatly facilitate random spore analysis both by permitting larger samples to be analyzed and by automatically recording and analyzing the results.

## 19) Gene conversion and recombination in unselected mitotic yeast cells.

Seymour Fogel, Professor and Chairman of Genetics Department, University of California, Berkeley.

## 20) Post-meiotic segregation and heteroduplex DNA.

Seymour Fogel.

Two rather specific proposals for use of the automated microbiological equipment are presented. These focus on fundamental yet health related issues. Thus, the proposals concerning mitotic recombination in unselected cells and post-meiotic segregation in yeast could provide, as a model system, a framework essential to understanding higher eucaryotic systems. With only minor changes the procedures

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strategies and overall rationale may be carried over to address such seemingly unrelated though central problems as chromosome nondisjunction, or screening mutagens, carcinogens, fungicides and antibiotics for their genetic effects.

### I. Gene conversion and recombination in unselected mitotic yeast cells

Our current understanding of intragenic recombination in cells committed to a mitotic cycle emerges from data generated by selective methods. In effect, these depend on appropriate signal devices that lead to the detection and recovery of only wild type or prototrophic recombinants. However, we have recently demonstrated the occurrence of mitotic co-conversion in hybrids marked by three or four heterozygous sites in a single structural gene, and it must be emphasized that multisite conversions do not typically generate wild type recombinants. Thus, though co-conversions might represent the most frequent event class, they remain undetected and unscored in conventional selective procedures. By analogy to our studies on unselected complete meiotic tetrads, we propose to analyze (in the same hybrids) an unselected population of mitotic cells for all conversional events falling within a defined genetic region.

Mitotic gene conversion in yeast occurs with an average frequency of the order  $10^{-4}$  to  $10^{-5}$ . Accordingly, collecting a sample of  $10^2$  or  $10^3$  unselected conversional events involves screening a total population of  $10^6$  -  $10^8$  cells, or a sample beyond the capability of routine microbiological methods. Automated microbiology equipment, however, augurs well for the successful completion of this and similar studies.

Our analytical strategy requires a) automated single cell inocula; b) replicating the derivative clones; c) irradiating the replica prints (UV or X-ray); d) detecting, locating, and retrieving sectored clones; e) finally, complete genetic diagnosis of each sectored clone by random spore or tetrad analysis of each segment.

### II. Post-meiotic segregation and heteroduplex DNA.

Common to all molecular models seeking to account for genetic recombination are enzymatically mediated steps that eventuate in heteroduplex or hybrid DNA production. At the in vivo genetic level, the presence of unresolved heteroduplex DNA is detected by post-meiotic segregation (PMS). PMS is comparatively frequent among the total aberrant octads of *Ascombolus* or *Sordaria*. However, technical difficulties with these forms, including a paucity of genetic markers, preclude total and critical analysis. With automated microbiological procedures adapted to random spore or tetrad analysis based on diploid yeasts suitably marked with 7-10 heterozygous sites (i.e., loci and alleles of known meiotic conversion frequencies), we could readily assess the frequency, extent and distribution of heteroduplex DNA in the yeast genome on a statistically reliable base. Sectored ascospore clones, otherwise concordant for all segregating markers will be considered as PMS events.

Also, from the distribution of PMS events among spores produced by heteroallelic diploids of the type  $+/12$  or  $1+/2$  (notation as before) where the mutant allele pairs may be chosen from extensive fine structure maps to represent a range of genetic distances, the regularities and basic attributes of heteroduplex DNA relative to gene conversion and recombination could be inferred.

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20a) Mammalian somatic cell genetics.

Frank Ruddle, Professor of Biology and Human Genetics, Department of Biology, Yale University, New Haven, Connecticut.

I believe that your machine has particular possibilities with regard to the recovery of conditional temperature sensitive mutants in tissue culture populations. As we have previously discussed, it would seem possible to establish colonies in the machine and then to shift to higher temperature and examine the colonies for retardation in the rate of increase of colony size. It would be possible to maintain the cultures at 34°C as a permissive condition and then to increase the temperature to 38.5° for 3 hr. periods out of a total period of 24 hrs. and carry this regimen forward for a period of one or two weeks. It would seem to me that this would not kill the temperature sensitive mutants but would result in a decided difference in their colony size which could be easily monitored by your photographic equipment. The isolation and characterization of temperature sensitive mutants will, I believe, be one of the most important aspects of somatic cell genetic work in the next decade. It should be possible by this means to obtain mutants which affect the biosynthesis of cell membranes, nucleic acid, and protein. It is also possible to pick up mutants which specifically affect the ability of mammalian cells to progress orderly through the cell cycle. All of these mutants can be analyzed by genetic complementation tests involving cell hybridization and chromosome segregation. For this purpose it would be best to make use of Chinese hamster cells or mouse cells as the population in which the mutants are recovered.

It seemed to me that your machine could be adapted also for recovery of mutants in differentiated cells. Quite a number of tissue culture cell lines which express specific differentiated traits are now available. For example, we are growing hepatoma cell lines which produce albumin. The albumin is secreted into the medium at high levels. It would seem to be possible to maintain colonies and then test the individual colonies for albumin production perhaps using a fluorescent reagent. One can then examine a large number of colonies for cells which fail to produce albumin. This would represent an excellent method for picking up non-producers. These cannot at the present time be enriched by selection techniques. One could also test for reversion to capacity to produce the differentiated product using the non-producing mutant as the base population. This kind of procedure could be adapted to cell lines which produce hemoglobin, myocin, nerve specific protein, etc.

When your machine is sufficiently developed to make use of mammalian cell populations, I would very much like to be in touch with you with regard to these possibilities. If you are interested in pursuing these possibilities I'd be more than happy to come out to Berkeley and spend a month or so in this connection.

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- 21) Isolate and characterize a large number of steroid- and cyclic AMP-resistant clones of mouse lymphoma cells.

Gordon M. Tomkins, Professor of Biochemistry, University of California, San Francisco.

General Objectives: For some years our laboratory has been studying biological regulatory mechanisms in cultured mammalian cells. We have concentrated primarily on the action of the steroid hormones but more recently have become interested in the cyclic nucleotides as well. The bulk of our work heretofore has been a biochemical analysis of the molecular mechanisms of cell-hormone interaction. Quite recently, however, we have begun to explore genetic techniques to pursue our objectives. For this purpose we have been using cultured mouse lymphoma cells which are killed on prolonged exposure to either the adrenal glucocorticoids or to cyclic AMP. This response occurs at physiological levels of the effector molecules and presumably reflects the well known immunosuppressive action of the glucocorticoids and of agents which elicit cyclic nucleotide synthesis. In any event, we have been able to select variant lymphoma cells resistant to the killing actions of the steroids, cyclic AMP or both agents. Our results to date indicate that the transitions from effector-sensitive to effector-resistant occur at random at a rate, in the case of the steroids, of  $3 \times 10^{-6}$  per cell per generation and for cyclic AMP, of approximately  $1 \times 10^{-7}$  per cell per generation. Various mutagens increase the frequency of steroid resistant cells. Biochemical analysis of the phenotypes of steroid- and cyclic AMP-resistance had indicated that in the former case, three types of variants can be isolated: those lacking the normal cytoplasmic steroid binding activity; those where binding takes place, but in which the receptor-steroid complex is not translocated to the nucleus; and finally those in which binding and translocation occur but cell death does not result.

Preliminary investigations suggest that various phenotypes also give rise to cyclic AMP resistance. To date we have studied only cells in which the cyclic nucleotide binding protein and its associated kinase are deficient.

Specific Aims:

1. To isolate a large number of steroid- and cyclic AMP-resistant clones of lymphoma cells.
2. To determine the frequency of their occurrence and the effects of a variety of natural and artificial mutagens on the transition from sensitivity to resistance.
3. To determine the biochemical bases of cell killing.
4. To characterize the phenotypes in terms of various known steps in hormone action.
5. To carry out complementation analyses using cell hybridization techniques to determine the number of biochemical steps involved in cell-hormone interaction.
6. To determine whether the transitions result from genetic or other types of stochastic, heritable variations such, for example, as might occur during the differentiation process.
7. To investigate possible relationships between resistance to the steroids and to the cyclic nucleotides.
8. To apply similar methods to circulating malignant cells in patients with lymphoma or leukemia in an attempt to design more rational therapies for these diseases.

Significance: The projected studies bear on many aspects of cell biology and

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clinical medicine. The glucocorticoids are major therapeutic agents in leukemia and in other malignancies. Their effectiveness is limited only by the emergence of hormone-resistant cell populations. Our observations with cultured cells can therefore serve as a useful model for studying how it might be averted. The finding that certain mutagens, in particular alkylating agents, enhance the conversion from steroid-sensitivity to steroid-resistance already indicates that therapeutic regimes which employ alkylating agents together with steroids might be redesigned to avoid the possibility that steroid-resistant cells are produced in the course of therapy.

These studies also suggest that new classes of agents, such as the cyclic nucleotides or compounds which elicit their production, might be used in tumor chemotherapy. The apparently lower frequency of resistance to cyclic nucleotides holds out the hope that these agents could be more effective therapeutically than the steroids.

From a theoretical point of view, these experiments could provide novel approaches to investigations of drug and hormone action by combining genetics, with cell biology and biochemistry. It should, for example, be possible to isolate cyclic AMP-resistant variants in which adenyl cyclase or various specific membrane receptors are deleted making it possible to study the interrelation between the elements in this important regulatory circuit. The same considerations hold true for the steroid hormones and studies on their mechanism of action.

Steroid and cyclic AMP-resistance are the result of changes in structure of the receptors. Since these molecules have been identified, and to some extent, purified, the generation of resistant mutants can be correlated with altered molecules. Therefore a more complete genetic analysis can be carried out than if the selective marker (e.g. drug resistance) were not correlated with a known protein.

- 22) Linkage analysis in mammals by somatic cell genetics.  
Theodore T. Puck, Director, Institute for Cancer Research; Professor of Biophysics and Genetics, Eleanor Roosevelt Institute for Cancer Research, University of Colorado Medical Center, Denver, Colorado.

Preliminary discussion of this project has indicated the great labor of isolating mutants and establishing linkage. Feasibility studies need to be carried out before real research plans can be made. Because the genetic exchange system is so inconvenient compared with E. coli, the automation may be even more valuable for animal cells than for bacteria.

- 23) Sensitive detection of mutagenesis by changes in colony morphology-extension to additional bacterial and eukaryotic cells.  
D. A. Glaser.

Method: Since colony morphology is a highly polygenic characteristic, it should be a very sensitive detector of mutagenesis. Extremely uniform reproducible culture conditions are required to guarantee reproducible colony morphology even in the absence of mutations. For measuring gross mutagenic effects down to very low "exposures", we plan to explore the limits of colony reproducibility for a variety of organisms.

Significance: If successful, colony morphology changes provide a method of assessing mutagenic effects on a wide variety of clonable cells, even if little

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or no genetic information is available. Screening of chemical and physical mutagens is an obvious application.

- 24) Transformation and mutation of Mammalian Cells in vitro by low doses of mutagens and ionizing radiation.  
D. A. Glaser

"Transformation of Mammalian Cells in vitro by Low Doses of X-rays", C. Borek and E. J. Hall, Nature 243, 450-453 (1973). Embryos of golden hamsters were minced and separated into individual cells growing on agar. The cells were irradiated with 1 to 600 rads of X-rays, incubated, stained, and the colonies formed (probably about 2 mm in size) examined for forms made by transformed cells.

Table of Effect of Transformed Cells

Dose	Clones Examined	Cells Transformed
0	36,000	0
1	17,900	3
10	10,200	4
25	5,500	8

Clearly large numbers of clones were examined for the infrequent event. The ability to use larger numbers of cells and examine the clones formed from them would make the numbers found more precise and allow better description of the dose response curve at low doses.

- 25) Behavioral Mutants of Motile Organisms  
D. A. Glaser

In the original proposal for construction of the DW and scanner system, we described possible behavioral studies of motile organisms of standard or "instinctive" behavior as well as adaptive or "learned" behavior. The following is quoted as an example of the type of study we would like to pursue sometime during the next few years.

"Chemotaxis by the Nematode Caenorhabditis elegans: Identification of Attractants and Analysis of the Response by Use of Mutants", S. Ward, PNAS 70, 817-821 (1973). Known behavior mutants of this nematode were put onto gradients of an attractant on agar plates covered with agarose beads or sephadex beads. The patterns resulting differed between the wild type and the mutants. Some studies were done to understand the chemotaxis. The hunt for more mutants was proposed. Clearly, in hunting for mutants, the more worms to be examined the better. The worms are small enough to be inoculated in 0.05 ml of liquid from an Eppendorf pipette. The patterns are formed quickly and photograph well. Analysis of the path can be done by computer in the same way Berg follows the three-dimensional path of E. coli.

- 26) Further Automation Instrumentation Development.

Although the Dumbwaiter and all of its ancillary equipment is expected to be in full operation when this program-project would begin in June 1975, a number of specialized accessory instruments will probably be needed as the biological program develops. A sample of such instrumentation projects that we envision at this time follows:

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(a) Optical Cell Sorter--At the present time the vibrating nozzle inoculator is used for laying down regular rays of droplets containing bacterial suspension. In the future it will be used to deposit yeast cells and animal cells as well. If the concentration of cells is adjusted so that each droplet contains on the average 1 cell, then 1/e of the droplets will be empty, 1/e of the droplets will contain 1 cell, and the rest of the droplets will contain more than 1 cell. For most measurements the only really useful results come from colonies descended from a single cell and the empty droplets are of obviously no use for most measurements. By illuminating the droplet at the time of its formation laser light, it is possible to make dark field measurements of light scattering, color, and fluorescence, which signal the presence of a cell and give some information about it. Such instruments work well with animal cells, but require further development to detect bacteria, which are much smaller.

1. W. A. Bonner, H. R. Hulett, R. G. Sweet, and L. A. Herzenberg, "Fluorescence activated cell sorting", Rev. Sci. Instruments 43, 404 (1972).
2. M. J. Fulwyler, R. B. Glascock, R. D. Hiebert, and N. M. Johnson, "Device which separates minute particles according to electronically sensed volume", Rev. Sci. Instruments 40, 42 (1969).

None of the existing systems seems capable of detecting bacteria and we hope to build such a system sensitive to bacteria as well as to larger cells.

- (b) Increase Film-Scanning and Computing Speed--Since the Dumbwaiter can easily take one photograph per second and since the film-scanning time ranges from 10 to 20 seconds per picture depending on the experiment, the film-scanning and computing operations will be rate-limiting steps in the output of the entire system. We are, therefore, very anxious to cut the analysis time by installation of the PDP-11, PDP-10 scanner computer system as well as by some software improvements.
- (c) Install Television System--For some future experiments it will undoubtedly be useful to analyze biological systems in real time and to intervene in the experiments without having to wait for the several-hour delay of taking pictures, developing, and analyzing them. For this purpose we plan to install a television system connected directly to the computer which will eliminate photography. In addition to allowing real-time intervention, it will be a considerable saving in the cost of photographic materials. On the other hand, the television system does not have the reliability of experiments recorded on film, nor do television cameras have as high resolution as our present flying-spot scanner. We imagine, therefore, that we will use both systems depending on the needs of the experiment.
- (d) Irradiation Facility--We plan to provide a facility in the Dumbwaiter for irradiating cells with ultraviolet or infrared light and also with ionizing radiation on some schedule as required by the experiments.
- (e) Semi-micro Photography--For study of very small colonies we will need to provide a semi-micro photographic system which will photograph a 1-cm or even 3-mm square on the agar instead of the present 100-mm square. There is a trade-off between the time and cost of photography and the size of agar area covered. Optimizing the trade-off will require different magnifications for different experiments.
- (f) New Cell Manipulation Devices--Our present plans are to use colony pickers, restreakers, and replicating devices suitable for colonies of E. coli and other cells that have similar physical properties. We can well imagine that other clonable cells will require somewhat different techniques for manipulation and special cell manipulation devices will be required from time to time.

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#### D. Significance

In the discussions above of the particular biological research projects, the significance of each one was pointed out. In general, these applications of modern automation technology coupled with computer-directed pattern recognition and analysis of data offer a powerful new tool for accelerating research in a wide variety of fields of molecular biology and cell biology. They reduce enormously the labor, time, and materials required to isolate rare mutants critical at a number of stages in research as well as to measure with high accuracy frequencies of genetic and mutational events which must be known for the genetic dissection of important biological processes.

In addition to the great gains expected in the speed of research in fundamental biology, the same large-scale automation techniques offer great promise for a variety of bio-assay applications, including the screening of environmental chemicals for their potential mutational and carcinogenic effects; the testing of proposed antibiotics, antineoplastic agents, and cell regulatory substances. In addition to the possibility of large scale testing of chemical agents, it seems possible to make highly accurate measurements of the effects of ionizing, as well as non-ionizing, radiation on a variety of clonable cells. The information resulting is important to studies in fundamental biology as well as the difficult problem of setting safe standards for allowable exposure to ionizing radiation among the general population and among workers in industries involving the presence of radioactive substances. With these large scale methods, it may be possible to extend the dose-effect relationship down to very low exposures and so to discover in an over-all sense whether there is a threshold or minimum dose below which repair mechanisms prevent any detectable genetic damage at the single-cell level.

Finally, the success of these applications of the cutting-edge of modern technology serves as a demonstration which may stimulate similar applications in industrial as well as medical and research sectors. We already know of several projects for strain improvement of antibiotic producing organisms that have been directly stimulated by this work. Representatives of a very large number of pharmaceutical manufacturing firms, instrumentation manufacturers, and chemical companies have visited our facilities. Suppliers of agar for medical and research purposes have also visited our facilities and have discussed with us their problems in maintaining uniform reproducible quality in their product. Variability is a source of considerable difficulty in both medical and research applications and we have agreed in a general way to measure batch to batch variations by its effect on colony morphology and growth rates in an effort to help them improve the quality of their product.

The five-year period of this proposed program-project should be ample time to carry through successfully a number of the projects we are proposing as well as to test the feasibility of a number of others and evaluate the usefulness of this kind of technology to biomedical science and industry.

#### E. Facilities Available

Virus Laboratory - Molecular Biology Department.

Many of the biological experiments described here will be developed, at least to the pilot stage, in the Molecular Biology Department and Virus Laboratory as has been done in the past. All of the usual common research facilities of these laboratories will be available as necessary. In addition, a small, well-equipped machine shop is at our disposal.



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From time to time we may call upon special shops and consult with experts from the Lawrence Berkeley Laboratory to help us with problems which they may have already encountered in their High Energy Physics and other programs. In addition we can often obtain electronic and other specialized supplies at very attractive prices and with immediate availability from the excellent stockroom facilities of the laboratory. We are very fortunate to be able to take advantage of the superb technical facilities and talent available at the Lawrence Berkeley Laboratory.

**Electrical Engineering Department**

The large-scale automatic equipment including the computer and flying-spot scanner are located in specially remodelled space provided for that purpose in the basement of the Electrical Engineering Building, Cory Hall. Members of the faculty and graduate students in Electrical Engineering have been taking an effective role in the development and use of this system. Thus collaboration with the Electrical Engineering Department and the Engineering Research Laboratories offers excellent special facilities for work of the type we are undertaking, as well as a unique opportunity for those members of the faculty and graduate students in Electrical Engineering interested in applying their special skills and knowledge to biomedical engineering.

**Campus Computer Center**

Only modest funds have been budgeted for use of the Campus Computer Center since, until now, we have been able to carry out all the computations associated with our work on our own computing system. When our own system is saturated, we may be able to reorganize our programs so that some of the pure computation can be put on magnetic tape and carried out at the Campus Computer Center which offers general facilities for large-scale computational work.

**Physics Department**

Laboratory space in the Physics Department is available for this work if needed and the excellent resources of the Machine Shop and Glassblowing Shop can be used from time to time as necessary.

**Extra-fabrication Space**

For fabrication of much of the sheet metal and welding work required for the construction and maintenance of the large-scale automated equipment, we have been granted the use of a corrugated metal building located in the parking lot of Cory Hall convenient to all of our other operations. Commonly called the "Ore House", this building was previously used for storing ores obtained from a nearby practice mining shaft.

**F. Collaborative Arrangements**

We have had extensive conversation and in some cases correspondence with all of the scientific investigators who have proposed projects using our equipment and who have visited our facilities. Since the Cyclops has been running only a short time, we have not encouraged active work in our laboratory until very recently and the precise relationship that will develop between these scientific investigators and members of our own laboratory remain to be defined. In every case the scientific

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investigators proposing projects listed here have independent support for carrying out these projects in their own laboratories and we intend to provide use of our facilities and necessary supplies without any formal arrangements or exchange of funds. If scheduling of experiments and assigning of priorities becomes difficult, we will probably invite some of the scientific investigators to join us and constitute an Advisory Committee to help plan the work schedule. It is too early to foresee accurately how all of these relationships will develop so no formal administrative structure for collaboration is being planned at this time.

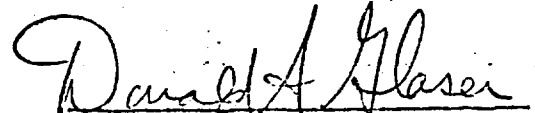
In addition to the scientific investigators named above, Professor Herbert B. Baskin and Professor Martin Graham of the Department of Electrical Engineering and Computer Science, University of California, Berkeley have been very helpful in giving advice concerning computer hardware and software. They generously agreed to continue this relationship and perhaps play a more active role in this program in coming years.

G. Principal Investigator Assurance.

The undersigned agrees to accept responsibility for the scientific and technical conduct of the research project and for provision of required progress reports if a grant is awarded as the result of this application

9 November 1973

Date

  
Donald A. Glaser  
Principal Investigator