HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

TYPE	PROGRAM	NUMBER
1	R01	CA 16896-01
REVIEW GROUP		FORMERLY
COUNCIL (Month, Year)		DATE RECEIVED

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR (Items 1 through 7 and 15A)

1. TITLE OF PROPOSAL (Do not exceed 53 typewriter spaces)
Genetics of Bacteria

2. PRINCIPAL INVESTIGATOR

2A. NAME (Last, First, Initial)
Lederberg, Joshua

2B. TITLE OF POSITION
Professor of Genetics

2C. MAILING ADDRESS (Street, City, State, Zip Code)
Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

2D. DEGREE
Ph.D.

2E. SOCIAL SECURITY NO.
[redacted]

2F. TELEPHONE DATA — Area Code: 415 — TELEPHONE NUMBER AND EXTENSION: 497-5801

2G. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT (See Instructions)
Department of Genetics

2H. MAJOR SUBDIVISION (See Instructions)
School of Medicine

3. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
FROM: 15 May 1974
THROUGH: 15 May 1977

4. TOTAL DIRECT COSTS REQUESTED FOR PERIOD IN ITEM 3
$195,000

5. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH PERIOD
$ 60,000

6. PERFORMANCE SITE(S) (See Instructions)
Department of Genetics
Stanford University School offMedicine
Stanford, California 94305

Congressional District No. 17

7. Research Involving Human Subjects (See Instructions)
A. [XX] NO B. [ ] YES Approved: _____ Date
C. [ ] YES – Pending Review

8. Inventions (Renewal Applicants Only - See Instructions)
A. [XX] NO B. [ ] YES – Not previously reported
C. [ ] YES – Previously reported

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY (Items 8 through 13 and 15B)

9. APPLICANT ORGANIZATION(S) (See Instructions)
Stanford University
Stanford, California 94305

IRS No. 94-1156365

Congressional District No. 17

10. NAME, TITLE, AND TELEPHONE NUMBER OF OFFICIAL(S) SIGNING FOR APPLICANT ORGANIZATION(S)
Robert D. Simmons
Contracts and Grants Manager
c/o Sponsored Projects Office
Telephone Number (s) 415) 497 2883

11. TYPE OF ORGANIZATION (Check applicable item)
[ ] FEDERAL [ ] STATE [ ] LOCAL [XX] OTHER (Specify)
Private non-profit university

12. NAME, TITLE, ADDRESS, AND TELEPHONE NUMBER OF OFFICIAL IN BUSINESS OFFICE WHO SHOULD ALSO BE NOTIFIED IF AN AWARD IS MADE
K. D. Creighton
Deputy Vice President for Business and Finance
Stanford University, Stanford, California 94305
Telephone Number 415) 497 2251

13. IDENTIFY ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES (See Instructions)
01 School of Medicine

14. PHS ACCOUNT NUMBER (Enter if known)
458210

15. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

SIGNATURES (Signatures required on original copy only. Use ink, "Per" signatures not acceptable)	A. SIGNATURE OF PERSON NAMED IN ITEM 2A	DATE
		25 April 1974
	B. SIGNATURE(S) OF PERSON(S) NAMED IN ITEM 10 Robert D. Simmons	DATE APR 2 8 197.

PHS-398
Rev. 3-70

DETAILED BUDGET FOR FIRST 12-[illegible]TH PERIOD | FROM 5/15/74 | THROUGH 5/15/[illegible]

1. PERSONNEL (List all personnel engaged on project)		TIME OR EFFORT	AMOUNT REQUESTED (Omit cents)
NAME (Last, first, initial)	TITLE OF POSITION	$/HRS.	TOTAL
Lederberg, Joshua	Principal Investigator or Program Director	15%	
Ehrlich, Stanislav	Research Associate	75%	
Elkana, Yehudit	Sr. Res. Assistant	100%	
Bursztyn, Hela	Sr. Res. Assistant	75%	
Evans, Peter	Lab. Technician	50%	
Jennings, Johnnye	Lab. Technician	75%	
Secretary	Secretary	20%	
		TOTAL →	$ 45,689
2. CONSULTANT COSTS (Include Fees and Travel)			$
3. EQUIPMENT (Itemize)			$ 4,000*
4. SUPPLIES			
Research chemicals, laboratory glassware			$ 8,000
5. STAFF TRAVEL (See Instructions)	a. DOMESTIC		$ 1,000
	b. FOREIGN		$
6. PATIENT COSTS (Separate Inpatient and Outpatient)			$
7. ALTERATIONS AND RENOVATIONS			$
8. OTHER EXPENSES (Itemize per instructions)			
Reprints and publication costs, reference materials, Equipment maintenance, communications			$ 1,311
9.	Subtotal – Items 1 thru 8 →		$ 60,000
FOR TRAINING GRANTS ONLY — 10. TRAINEE EXPENSES (See Instructions)			
a. STIPENDS	PREDOCTORAL No. Proposed ____		$
	POSTDOCTORAL No. Proposed ____		$
	OTHER (Specify) No. Proposed ____		$
	DEPENDENCY ALLOWANCE		$
	TOTAL STIPEND EXPENSES →		$
b. TUITION AND FEES			$
c. TRAINEE TRAVEL (Describe)			$
11.	Subtotal – Trainee Expenses →		$
12. TOTAL DIRECT COST (Add Subtotals, Items 9 and 11, and enter on Page 1) →			$ 60,000

GPO 930-791

Supplementary Information for Personnel Costs Requested

It is the policy of the DHEW to omit specific salary rates or amounts for individuals from those copies of grant applications which are made available to reviewing consultants. Therefore, a substitute budget page is enclosed and must be used in lieu of the present budget page in all competing applications. Note that individual salaries will not be shown on the substitute budget page; however, if a grant is to be awarded as a result of this application, the awarding unit will need information concerning specific salaries. Please use the space below to list all personnel shown on the substitute budget page, and include the salary and fringe benefits requested for each, following the appropriate guidelines in the Information and Instructions.

THIS INFORMATION WILL BE USED ONLY BY PHS ADMINISTRATIVE STAFF.

DESCRIPTION *(Itemize)* PERSONNEL		TIME OR EFFORT *%HRS.	AMOUNT REQUESTED *(Omit cents)*		
NAME	TITLE OF POSITION		SALARY	FRINGE BENEFITS	TOTAL
Lederberg, Joshua	PRINCIPAL INVESTIGATOR	15%	--	--	$ --
Ehrlich, Stanislav	Research Associate	75%	9,000	1,530	10,530
Elkana, Yehudi	Sr. Res. Assistant	100%	9,000	1,530	10,530
Bursztyn, Hela	Sr. Res. Assistant	75%	8,625	1,466	10,091
Evans, Peter	Lab. Technician	50%	4,500	766	5,266
Jennings, Johnnye	Lab. Technician	75%	5,925	1,007	6,932
Secretary	Secretary	20%	2,000	340	2,340
			39,050	6,639	45,689
TOTAL - Same as total for personnel on Substitute Detailed Budget Page					45,689

INDIRECT COST *(See Instruction)*

DATE OF DHEW AGREEMENT:

____ % S&W*

47 % NTDC June 26, 1973

☐ WAIVED

☐ UNDER NEGOTIATION WITH:

*IF THIS IS A SPECIAL RATE *(e.g. off-site)*, SO INDICATE.

BUDGET ESTIMATES FOR (_ YEARS OF SUPPORT REQUESTED FR(PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)

DESCRIPTION	1ST PERIOD (SAME AS DETAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED (This application only) 2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	$45,689	$48,384	$51,233				
CONSULTANT COSTS *(Include fees, travel, etc.)*							
EQUIPMENT	4,000	4,500	4,500				
SUPPLIES	8,000	9,000	10,000				
TRAVEL – DOMESTIC	1,000	1,000	1,000				
TRAVEL – FOREIGN							
PATIENT COSTS							
ALTERATIONS AND RENOVATIONS							
OTHER EXPENSES	1,311	2,116	3,267				
TOTAL DIRECT COSTS	$60,000	$65,000	$70,000				
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD *(Enter on Page 1, Item 4)* →					$ 195,000		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

* Equipment: The budgeted amount of $4,000 is based on past experience for requirements for replacement and updating of centrifuges, spectrophotometers and other laboratory instrumentation, as well as for autoclaves and other laboratory hardware. We are now reviewing a number of items that are approaching obsolescence or may require major repairs (e.g. a $3,000 item on a leaky, large autoclave, and portions of a distilled water system), and are working out strategies for coping with these requirements – in some cases, too pressing to tolerate a 9 month delay. We will therefore offer detailed justification for specific items at a later date during the review of this application, and in any event will of course require specific approval from NIH for major purchases.

A. INTRODUCTION

1. Objective: The central research objective of this laboratory for many years has been the understanding of genetic mechanisms in bacteria. For the last decade, we have put aside studies on conjugal exchange in Escherichia coli and phage-mediated transduction in Salmonella (Lederberg, 1958 (4)) in favor of the DNA-mediated transfer of genetic information in Bacillus subtilis. Our early studies were among the first to be concerned with linkage and with the details of DNA integration in this system.

More recently, and up to the present time, our efforts are directed at the gamble of finding ways to achieve molecular translocation, that is to introduce genetic information, according to the free choice of the experimenter, into the genome of say B. subtilis. Despite the epochal advances in technical facility and theoretical understanding that many workers have achieved with respect to the replication of DNA, and to its integration in the process of genetic transformation in a variety of bacteria, genetic exchange has still been restricted to pairs of rather closely related species. It would be of great theoretical and practical importance to be able to introduce, for example, sequences of synthetic polynucleotides or of mammalian DNA into a bacterium or a virus genome. However, the transforming systems that have been described to date all show a high degree of specificity; evidently a critical step in the integration of an entering fragment of DNA is the probative formation of a heteroduplex between the chromosome and the fragment, and the rejection of unmatchable, or poorly matched, pairs.

Our previous efforts to surmount this obstacle will be summarized below; they have been superseded by Sgaramella's discovery (2), elaborated here, that the polynucleotide ligase coded by phage T4 (T4-ligase) grown in E. coli is capable of mediating the terminal joining of two DNA duplexes in addition to the already known function of sealing nicks within a well formed duplex. With certain tricks the sealing function can also be exploited for the purpose of achieving molecular translocations, as has been done in parallel studies by Paul Berg and his associates (3).

Our specific objective is the further exploration of the chemical mechanism and biological effect of terminal joining of biologically specific and active DNA molecules, the correlation of chemical and biological linkage, the facilitation of preparing DNA from various sources to allow such joining - in short, THE DEVELOPMENT OF MOLECULAR TRANSLOCATION AS A ROUTINE METHOD OF STUDY OF CELLULAR GENETICS.

There is hardly a problem in genetics, pure or applied, that might not be influenced by the technical ability to study the function of a DNA segment in a well standardized context of a bacterial or a viral genome. However, we do not underestimate the difficulties of reaching that goal, and specific applied problems that we hope to attack in this way are under the heading "D. Significance" rather than as realistic expectations for the immediate period of this prospective grant. It is to be expected, and certainly not to be discouraged, that many other investigators will capitalize on these applications, as has been the case, of course, for such discoveries as conjugation, transduction and lambda-lysogeny.

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2. Background: The general features of transformation are well known, and discussed in much detail in numerous reviews and texts (e.g., Stent (445), Watson (543), Hayes (826). A review by Hotchkiss and Gabor (337) cites 241 references through 1969, almost all of them relevant to the present discussion. We will then not undertake a monographic survey or complete bibliography here.

The specificity of transformation, e.g. the rejection of E. coli DNA by B. subtilis, pervades the literature. However, few published reports are directed at pushing the empirical upper limits, perhaps < 10^{-9}, on the relative efficiency of gene transfer between these species, or similar situations. Undoubtedly, we have followed a common procedure in not bothering to publish such negative results in detail.

Gene transfer between B. subtilis and other B. spp. is greatly hindered, but nevertheless does occur (cf. Wilson and Young, 1972 (848), whose results concur with our own, R. Harris, unpubl.). The behavior of "hybrids" is consistent with the model of a requirement for regular duplex formation in local segments mentioned above (in contrast, for example, with a restriction (and host-modification?) system as is reported for Hemophilus (Smith and Wilcox, 1970 944). Hotchkiss (733) has discussed the relative exclusion of some markers arising by mutation possibly involving heterogeneity at a single base pair. These findings help to delineate, rather than to solve, the problem of achieving molecular translocation involving arbitrary, alien sequences.

An overview of mutation in bacteria compared to eukaryotes also suggests a basic difference in the role of chromosome inversion and translocation. These processes dominate variety and species formation in eukaryotes; they occur only exceptionally in bacteria, and then perhaps mainly in relation to the integration and de-integration of episomes.

For this reason, in previous grant applications, I had postulated that translocation-mediating enzymes were a later evolution, connected with mechanisms of gene-regulation more complex than the sequential expression of markers in a linear (or circular, or simple multi-segmented) pattern - the concept associated with the operon model of gene regulation in prokaryotes. (One could also argue that eukaryote chromosomes have evolved multiple recognition sites, i.e., that this is one role of "redundant-sequence DNA" across which translocations could be sealed without invoking new enzymes. The report of translocations between mouse and human chromosomes in somatic cell hybrids (Ruddle, 1972 (41) 10 tends to argue against such a role). For this reason, some of our previous efforts were directed at the examination of DNA-repair mechanisms of a eukaryote in vivo, that is in eggs of Xenopus injected with bacterial DNA. These studies, reported further in "4. Progress Report", have, however, been overtaken by Sgaramella's findings with T4-ligase.

This recent paper, which is appended, is the main foundation of our intended further work. Previous work had indicated terminal joining of two synthetic polynucleotides (Sgaramella et al., 1970 1143, each of which had based-paired ends (a deoxynucleoside 5' phosphate paired with a complementary 3'hydroxyl), a condition of the ends that may be termed "flush". The present paper takes advantage of the known condition of phage P22 DNA as a flush clipped ensemble of segments which are terminally redundant, but produced as if by a random cut in a circular molecule. Therefore, almost every end is different, and the ease

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of terminal joining then argues against sequence-specificities for the ligase.

Furthermore, P22 did not join terminally to linearized SV-40 DNA, the latter having thus been shown to have not a clipped but a cohesive end, i.e., an overlapping simplex that would seek its complement on another strand. This SV-40 DNA (obtained by the action of a sequence-specific nuclease restriction enzyme on circular SV-40) could, however, be homo-oligomerized either with T4 or E. coli ligase, both enzymes being able to seal a contrived, doubly nicked duplex. The paper itself gives a more complete, and possibly clearer, account.

The problem of molecular translocation can then be reduced to that of securing flush ends on biologically interesting DNA. Alternatively, various restriction enzymes might have just the appropriate specificity to generate useful cohesive ends, or terminal polymerases may be used to add synthetic homo-polymer cohesive ends to existing DNA molecules, an approach also contemplated in the previously submitted applications in this series. Berg's group will be emphasizing the second and third of these approaches, and have, of course, already achieved an outstanding result with SV-40 and lambda; we will be concentrating on terminal joining.

In order to pursue the biological activity of the oligomers, we have developed a transfection system (see Progress Report) for P22 DNA. (In the light of unforeseeable hazards with derivatives of SV-40, we prefer not to pursue work with this as an animal virus in tissue culture). Although most promising, the transfection system needs further improvements (which we foresee should be possible) before we can efficiently test the oligomers for biological activity.

3. Rationale: This is difficult to state differently from the operational and situational (historical) aspects of our proposal. The ultimate rationale is the DNA theory of heredity, namely that the information encoded in base-sequences of DNA molecules is the material basis of heredity. Hence, manipulations by chemical, physical and enzymatic means of DNA molecules afford a way of understanding and controlling the hereditary processes of cells.

The particular cells we are concerned with are bacteria (and their viruses); and the specific manipulation that now gives us new opportunities for genetic experimentation is the end-to-end joining of DNA segments, and eventually the insertion of new genes into established genomes.

4. Comprehensive Progress Report:

a. Period: September 1968 to December 1972.

b. Summary: (see also A.2.Background) We tried various approaches to the chemical cross-linking between DNA molecules, the addition of synthetic cohesive ends, and laying the basis for identifying a terminal-joining enzyme in frog eggs. These false starts were superseded by the identification of terminal-joining activity in T4-ligase and its use in the formation of oligomers of P22 DNA (24).

We have also searched for deletion mutations in B. subtilis and were surprised to find that they are very rare, if they occur at all in our strain. Heterospecific transformations (B. subtilis x B. globigii) were studied and

the model that sequence hemology is required for efficient transformation was supported.

We have made a detailed analysis of the effects of chlorine on DNA, but eventually concluded that its effects on DNA breakage were incidental to effects on proteins, and that the latter was the principal site of cell damage. However, chlorine is a (feeble) mutagen, and has an enigmatic effect on the burst size as well as viability of treated phage (lambda). It therefore deserves further study as a possible environmental pollutant of biological consequence.

A variety of other incidental findings are indicated in the list of published titles.

c. Detailed Report: The most salient findings have already been outlined under "Background" and in the Sgaramella paper (1972, 23, 24), which should be regarded as part of this application.

A range of other studies avowedly of lesser importance is detailed in the remaining list of publications (see d.).

Work not yet published includes:

i. (R. Harris). A study of the specificity of a nuclease produced by B. globigii and not by B. subtilis. This nuclease attacks B. subtilis DNA more rapidly than that from B. globigii and may therefore resemble "restriction enzymes" in specificity. The DNA from different hybrids is being examined to look into the role and nature of a host-modification system. However, there is no evidence of such an enzyme with differential specificity in the competent strain of B. subtilis as might be hypothesized to account for the specificity of DNA in transformation.

ii. (I. Majerfeld). The cross-linking of DNA with various agents, of which glyoxal and glutaraldehyde appear to be the most promising. (The rationale was that chemically linked DNA might be copied with DNA polymerase with a mere skip across the link in the template. However, material isolated so far has not been well enough defined for a cogent test of the concept). The work was interrupted by Mrs. Majerfeld's emigration to England with her husband prior to the completion of her Ph.D. research. She may, however, resume it at Sussex.

iii. (B. Brandt and J. Wachtel, in press). On the hypothesis that eukaryotes possessed an enzyme for molecular translocation, we tried to develop a system for studying the effect of frog egg enzymes, in vivo, on injected bacterial DNA. Following Gurdon,[12] DNA synthesis was demonstrable on the injected templates. However, it proved to be too difficult to recover workable amounts of "repaired" DNA from injected eggs, and attemtion was then directed to the enzymes in the extracts. These have been shown to contain a DNA-polymerase with variations in template specificity similar to those reported for the intact eggs by Gurdon. That is, Xenopus laevis DNA, undenatured (but treated with DNAse I) was a preferred primer, compared to E. coli DNA or to poly d(A,T). E. coli polymerase I prefers denatured DNA from various sources. Larvae and immature ovaries yielded enzymatic activities with still different patterns of preference. However, on 50-fold purification, the egg extract enzyme was less discriminating, perhaps owing to the removal of a DNAse-inhibitor.

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A ligase has also been demonstrated in these extracts, but has not yet been characterized, i.e. for temrinal joining activity.

iv. (V. Sgaramella and H. Bursztyn, manuscript in preparation). Transfection in P22. The more or less fortuitous amenability of P22 DNA to terminal joining by T4 ligase naturally led to an inquiry on the biological activity of the oligomers. Published work in transfection with P22 DNA has been remarkably discouraging, efficiencies about 10^{-9} having been reported with spheroplasted hosts (13).[28] In order to attempt correlative transduction, we would prefer intact bacteria. Attempts to condition Salmonella by cold shock and $CaCl_2$ which has given spectacular results with E. coli (14)[29] were unrewarding. However, supernates of shocked E. coli cells were found to condition an R strain of S. typhimurium LT2 to a low rate of transfective competence. (The supernate factor is, of course, under close study). Subsequently it was found that limited treatment of P22 DNA with exonuclease would further augment the efficiency of transfection to a level now in the range of 10^{-7} to 10^{-8}. We have not evidently exhausted the possibility of further improvement which would furnish another valuable tool. These rates are still too low to expect the transduction of bacterial markers to be observed - nor have we done so as yet.

d. Publications. See page —

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B. SPECIFIC AIMS

We have now passed the probing stage, and with the T4 ligase intend a systematic study of ways to achieve molecular translocation.

Perhaps this is also the place to remark about my personal involvement in this research. During the past five years, I have undertaken heavy commitments in pursuing the social and ethical aspects of science as a matter of self-education and in relation to national policy (e.g. as a consultant to the ACDA on the Biological Warfare Treaty negotiations, and as a member of the National Advisory Mental Health Council); and in public education and understanding of science as a weekly columnist for the Washington Post, and as an occasional witness before Congressional committees.

Such activities, however one evaluates them, inevitably compete with laboratory research and I could hardly point to this period as one of notable productivity in molecular genetics. For almost a year, however, I have concluded that I had now made my main contribution to public service and could concentrate again on my laboratory responsibilities - and this has been greatly reinforced by the work on the T4 ligase summarized herein. While I still must own to heavy administrative and some public responsibilities, and am still involved in other research projects unrelated to this (involving computer intelligence and participation in the Mars 1975 Viking mission) I am fortunate that, for various reasons, these also promise to be less demanding. And, of course, the graceful termination of my regular column writing is a particular bonus from this standpoint.

I should stress that the present grant is the only substantial support to which I can look for laboratory work in molecular genetics - the rest being limited to sharing in the department's training grant for stipends for graduate students and fellows. My name is associated with these and other funds in an administrative more than a personal capacity, and I do not have recourse to them for this line of research.

C. METHODS OF PROCEDURE

The detailed methodology is outlined in attached and referenced papers, and this section will address a strategic outline.

RESEARCH PLAN - OUTLINE

Work in progress sets a substantial momentum. Our plans are a combination of opportunistic forays from recent discoveries and systematic exploration of plausible alternatives. In relation to the overall goal of molecular translocation we perceive the following set of interrelated issues:

(i) How generally to achieve proper flush clipping of DNA;

(ii) The choice of donor DNA;

(iii) The choice of co-donor and of recipient genomes and the associated biological assay systems. We have also to consider

(iv) The functions of terminal joining in phage and by hypothesis in eukaryotes and further study of ligase specificities;

(v) Other problems that may attend the integration and functioning of inserted sequences, and

Squeeze (vi) here?

(vi) Specific problem-oriented applications of these new methods.

Research Plan – detailed

(i) FLUSH CLIPPING DNA. The availability of monomer P22 DNA of standard size facilitates the assay for flush clipping of other DNA's of different length or composition. If these are differentially labeled, terminal joining will give stable covalent complexes that can be separated in the centrifuge.

We plan then to explore the flush clipping of various phage and bacterial DNA's by various enzymes and physical treatments, and also followed by exonuclease or repair-polymerase intended to rectify the ends. We do know that simple sonication does not work. Some evidence that ravelled ends bind the enzyme needs to be elaborated: if ravelled ends do compete we need to find ways to purify the flush clipped component of mixtures.

Some endonucleases that reputedly flush clip may leave short simplex ends that could, however, be rectified by exonuclease or polymerase.

(ii) DONOR DNA. P22 and FRAGMENTS. The P22-P22 (homo) oligomers may give the first opportunity to examine a biological correlate of terminal joining - if they are biologically active. Obviously, testing these for infectivity or for rescuable markers is high on our agenda. If this succeeds much interesting work remains on the further dissection of the fragments. It also opens the opportunity to look at the addition of torn fragments of P22 (with one flush end) to intact P22 with respect to the genetic information that may then be introduced from marked phage genotypes.

P.11 K.D.

E. coli episomes offer a wide range of DNA sequences with predictable biological activity - for example, the tryptophan sythetase of ϕ80 (E. coli). If inserted into a continuous subtilis sequence the complex might transform B. subtilis auxotrophs to give an easily recognizable alien gene product. As always, the proper clipping of these inputs is crucial. Defective bacterial phages of B. subtilis, reported ~~to have intact and perhaps~~ with nearly if not perfect flush ends (15,34), might afford ready-made fragments for temrinal joining to phage or to bacterial DNA. However, a preliminary examination of such a phage DNA did not give evidence of terminal joining and this may leave open some question as to its fine molecular structure.

Even more specific sequences of DNA are now available by the dissection of DNA-DNA and DNA-RNA-hybrids homologous over a limited range, and by the reverse transcription of m-RNA's. A technically easier compromise is the partial fractionation of total B. subtilis DNA by differential melting (16). Reverse transcription of purified m-RNA from differentiated eukaryotic cells may be the most practical general method of concentrating natural genetic specificity to a useful degree.

We foresee the further possibility of inserting synthetic homopolymer sequences which should sometimes result in the production of the corresponding homopeptides. Pfuderer and Rogers have claimed (17) such an effect with TMV RNA by poly-A, but without detailed substantiation - the issue being the persistent replication of such modified sequences; it is entirely reasonable that ehy will be translated into polylysine as predicted by the genetic code). Dr. Elkana's experience in the identification of synthetic homopeptides prompts

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her interest in joining this study. Along the same lines, we can, of course, foresee the eventual possibility of inserting artificial genes, e.g. one emulating the code for a transfer RNA as has been synthesized by Khorana's group (and with which Dr. Sgaramella was associated before coming to Stanford).

(iii) CO-DONOR AND RECIPIENT DNA. P22. The role of the cohesive ends in the infectivity of P22 may be illuminated by further studies of the transfection system. They presumably are sufficiently important that terminal additions to P22 will be rejected but this must be empirically substantiated. If not, we would be in the fortunate position of being able to extend the genome by a single addition rather than requiring an insertion and a double terminal joining. Dimers, if they were functional, could have the advantage of allowing the disruption of some genetic information by insertions at random positions in one sequence while the other remained intact. With careful handling the cohesive ends might allow non-covalent circle formation, clipping and rejoining of an inserted segment to the two ends of the single molecule. It would be asking a great deal to expect such processes to occur with very high efficiency at an early stage of our investigation. However, it should be possible to contrive genetic selective systems whereby even rare successful insertion, according to this protocol, could be detected. For example, a P22 genome, augmented with bacterial DNA coding for tryptophane synthetase, might form viral clones capable of growing in tryptophane-dependent host-bacteria. (Transducing phage particles have been observed to exhibit substantial "escape synthesis" of the corresponding enzymes even in the absence of specific conditions for induction (~~18~~).47

For a number of reasons, especially the versatility of genetic functions and analytical methods we would prefer to deal with a bacterial system. The primary bottleneck is the need to discover ways of flush clipping bacterial DNA in a transforming system like B. subtilis. Presumably it will still be necessary to insert new sequences in an ordered series to allow for homologous duplex formation with the recipient bacterial genome on both sides of the insertion. (This is a surmise, though a plausible one, rather than an empirical finding as we have yet to produce terminal additions of sequences to bacterial DNA). This then exhibits substantially the same difficult as was mentioned for P22. It might be surmounted by the use of circularized sequences or through other methods of immobilizing very high molecular weight DNA. For example, we envisage the examination of high molecular weight, folded chromosome complexes (~~19~~)49 of bacteria. If occasional loops of such complexes were clipped, the flush ends might still remain near enough to permit an occasional orderly insertion by terminal joinings of added fragments. Bacterial cells, coagulated with alsohol but broken to expose DNA surfaces would be an easy preparation for large scale trials of such acceptors. Density labelling of the added DNA would facilitate the preferential recovery of pieces of DNA in which insertions had occurred, for these would be expected to have an intermediate density; these concentrated fractions would then be tested for biological activity.

Fortunately, these and many other problems that can now be anticipated are amenable to being identified and solved step-by-step. We do not require an improbable global solution to find our path.

Episomal DNA, already circularized, can now be transferred with high efficiency in E. coli (~~14~~)24 and this may be regarded affording an essentially similar opportunity for the receipt of new sequences that can then be taken up by bacteria

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so as to test their genetic specificity. (I will have no hesitation in returning to E. coli if newly found methods for dealing with its DNA as a chemically isolatable entity repair the disadvantages that I had perceived it to have by comparison with B. subtilis during recent years. We have retained a very extensive library of strains of E. coli and of Salmonella that could be resuscitated for such investigations.

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(iv) FUNCTION OF TERMINAL JOINING IN PHAGE

The role of a ligase with new terminal joining activity in T4 infected cells of E. coli is obscure. Ligase-deficient mutants of T4 show increased recombination and a higher incidence of nicks but these phenomena give no hint of a terminal joining function as a natural process in phage replication.[27] On the other hand, one might plausibly speculate that terminal joining plays some role in the integration of double stranded DNA in the course of P22 transduction [redacted] but this would imply that either the genetic information or the enzyme itself is associated with transducing particles which may be free of viral genome content (20[39]). We intend to study a range of viruses including different mutants of T4 and of P22 to help ascertain whether any of the indicated functions are prominently associated with the terminal joining as distinguished with other capabilities of phage induced ligases. Mutants of P22 capable of differing frequencies of transduction would be particularly interesting for this purpose (21).[42]

According to the hypothesis adumbrated previously, terminal joining may be expected to be a feature of some or all of the ligases of eukaryotic cells and our survey will include these sources as well. Existing methods of assay for the ligase are tedious and difficult and more work for their improvement would be a fruitful investment; furthermore, our survey may well reveal more convenient and richer sources of this enzyme, which is crucial for our further studies. Similarly, existing work bearing on the specificity of the sealing versus terminal joining functions of the ligase will be continued in order to get a clearer understanding of the way in which these two functions relate to one another and of the chemical mechanism of the catalytic process. Much has still to be learned about the relationships of RNA sequences to DNA ligation, an arena expected to be included in this study. This investigation obviously fits neatly into complementary ones of looking for ways of shaping the ends of DNA molecules of biological interest.

A.15
R.Q

(v) OBSTACLES TO INTEGRATION AND FUNCTION OF INSERTED SEQUENCES?

The motive for our search for deletions in B. subtilis was to provide, by studies of transformation using DNA from the intact wild type into deletion mutants, a model of what might be expected to occur when an insertion-modified DNA confronts a bacterial genome. As indicated, we have had some difficulty in obtaining deletion mutants, but one or two putative candidates have shown a definite although modest rate of acceptance of wild type DNA. This is consistent with a long tradition of study of transduction involving deletion mutants in Salmonella. Very recently Adams has reported (22) in some detail on exactly this problem involving a large deletion recognized by arsenate-sensitivity. She concludes that transduction occurs with nearly perfect efficiency but that transformation is impaired about a thousand fold. She concluded "that the physiological state of competence is at least partially responsible for the exclusion of non-homologous DNA sequences regardless if they are of transforming or transducing origin". The reduced efficiency in transformation may reflect the rather extensive size of the deleted segment; in any case, this finding, although dampening, is not fatally discouraging and more doubtless remains to be learned about the conditions that will permit deletion mutants to be efficiently transformed. Precisely the same craftmanship will then be useful in setting up the most appropriate experimental conditions for achieving new insertions.

Again, we are fortunate to have powerful selective techniques to be able to discover even rare "successful" events out of a large population of failures. Furthermore, a single insertion-transformed clone, when the genetic information comes from a different species, should be susceptible of unambiguous diagnosis by immunological or eventually amino acid sequence analysis of the protein products

(vi) PROBLEMS. Although most of this discussion is oriented toward the perfection of an invaluable technique, this can hardly be achieved without a deeper understanding of the subtleties of DNA specificity and of the cellular mechanisms which are involved in the rejection of invading DNA molecules which may often have pathogenic portent. These investigations, therefore, must lean heavily on what is already known of cellular discrimination against viruses and of the circumstances where this may be expected to break down. By the same token it also bears on the mechanisms of speciation in bacteria and on the most general evolutionary problems.

Besides the questions that are inherently incidental to the achievement of molecular translocation and which concern the panoply of enzymes concerned with DNA replication and recombination, there are a number of specific questions whose solution would be greatly facilitated by the development of these techniques. They would fall generally in two categories: (1) where the ability to conduct any DNA transformations with respect to a given genetic trait is already the crucial advance and (2) where the relocation of genetic information within the genome is of immediate importance. Under the first heading, we would have a large variety of problems that arise in the genetics of bacterial species for which recombination systems have not yet been described, but from which DNA can be extracted and which DNA is undoubtedly at the basis of their hereditary specificity. Both headings comprise a considerable range of questions that concern regulatory mechanisms in bacteria. The one of most proximate interest to us, in an extension of a long history of previous work, concerns the mechanism of phase variation in Salmonella (23 34). p. 17 R.D. Although it was established long ago that the alternation of flagellar antigenic specificity is based on an "alternation of state" at a specific locus in Salmonella, we have never been able to ascertain for sure whether this involves a substitution within the DNA sequence or some kind of epinucleic modification, possibly more akin to regulatory phenomena in higher organisms. Any system that would allow a high efficiency of transformation with purified DNA would serve to help solve this problem; for one could determine whether the alternation of state was inherent in purified DNA or not. It is problematical, however, whether this will be achieved more readily as a restricted problem in its own right or as an example of gene transfer, say, from Salmonella to Bacillus subtilis.

The same remarks apply to the application of these techniques to the study of genetic specificity of DNA from eukaryotes by transplanting segments to a bacterium or a virus. Artificial microbial complexes with genetic information from eukaryotic nuclei could presumably help answer many deep questions about differentiation, diversification in antibody formation, the genetic foundations of differences between normal and neoplastic cells, indeed represent a large scale extension of existing approaches in the genetics of somatic eukaryotic cells.

Some more directly applied implications of these techniques are addressed in section D.

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References for Research Plan are incorporated under the publication list on

D. SIGNIFICANCE

In the previous paragraphs, I have stressed some of the more theoretical outcomes that might be expected from the technique of molecular translocation. At a more applied level, one should simply say that this should give us a whole new set of tools for the artificial breeding of new kinds of hybrid cells and species, allowing for a general disregard of existing barriers to recombination. It may be a long way before such techniques can be extrapolated to crop plants with obvious implications for agriculture; and, longer still to animals; and, there are of course obvious ethical barriers to experimentation in man in this regard. As I have outlined in a recent reflective article (25), I believe that it is a great mistake to discuss genetic engineering as if man were the implied primary target: this is no more likely to be true than it has been for the application of Mendelism for genetic engineering. On the other hand, the genetic engineering of bacteria and viruses can lead to the ready availability for therapy and for prophylaxis of biological products which are now vanishingly scarce, to experimentation with new evolutions of amino acid sequences, and to a new repertoire of diagnostic procedures, particularly for situations involving genetic deviation. These "genetic engineering" applications should pose no significant ethical problems since they involve no manipulation of human or prospectively human subjects, but merely the extraction of very short sequences of DNA from a mammal or man and its implantation in a microbe. The theoretically feasible capacity to produce isolated antigenic proteins characteristic of pathogenic viruses in innocuous organisms that can be grown cheaply on a large scale would already be sufficient to repay the investment in this kind of work many times over.

E. FACILITIES AVAILABLE

The Department of Genetics and the Kennedy Laboratories for Molecular Medicine have been operating for many years as a well equipped research laboratory in this field with all the principal facilities that are pertinent to molecular biology research. Equipment requirements will have to do mainly with replacement of specific items as they are worn out or occasional updatings of specialized items. In addition, we have been fortunate in being able to assemble some quite sophisticated instrumentation capabilities, initially with help from the N.A.S.A. and subsequently from the biotechnology resources branch of NIH. While these computer and other advanced instrumentation facilities are mainly directed to other purposes they have often proven useful in solving specific analytical problems, for example the application of mass spectrometry to the identification of N-chlorocytosine (26).

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