

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE  
PUBLIC HEALTH SERVICE  
NATIONAL INSTITUTES OF HEALTH

RECOMBINANT DNA MOLECULE PROGRAM ADVISORY COMMITTEE

MINUTES OF MEETING

JULY 18-19, 1975

The Recombinant DNA Molecule Program Advisory Committee was convened for its third meeting at 9:00 a.m. on July 18, 1975 at the National Academy of Sciences Summer Study Center, Woods Hole, Massachusetts. Dr. DeWitt Stetten, Jr., Deputy Director for Science, and Dr. Leon Jacobs, Associate Director for Collaborative Research, NIH, presided. In accordance with Public Law 92-463 the meeting was open to the public from 9:00 a.m. to 5:30 p.m. on July 18, and from 8:30 a.m. to 2:00 p.m. on July 19, and closed to the public from 2:00 p.m. to 2:30 p.m. on July 19 for the review and discussion of a fellowship application.

Committee members present were:

Dr. Edward A. Adelberg  
Dr. Ernest H.Y. Chu  
Dr. Roy Curtiss, III  
Dr. David S. Hogness  
Dr. John W. Littlefield  
Dr. Jane K. Setlow  
Dr. Wacław Szybalski  
Dr. Charles A. Thomas  
Dr. William J. Gartland, Jr., Executive Secretary

A Committee roster is attached. (Attachment I)

The following ad hoc consultants to the Committee were present:

Dr. Peter Day, Connecticut Agricultural Experiment Station, New Haven, Conn.  
Dr. Elizabeth Kutter, Evergreen State College, Olympia, Washington  
Dr. John Spizizen, Scripps Clinic and Research Foundation, La Jolla, Calif.

National Science Foundation representative was:

Dr. Herman Lewis

Energy Resources and Development Administration representative was:

Dr. George Shepherd

Medical Research Council of Canada representative was:

Dr. Louis Siminovitch, University of Toronto, Toronto, Ontario, Canada

Others in attendance were:

Dr. Emmett Barkley, NCI; Mrs. Betty Butler, NIGMS; Dr. Irving Delappe, NIAID; Dr. Myron Levine, University of Michigan; Dr. Malcolm Martin, NIAID; Dr. John Nutter, NIAID; Dr. Bernard Talbot, OD, NIH.

I. CALL TO ORDER

Dr. Stetten called the meeting to order and welcomed Committee members and visitors.

II. CONSIDERATION OF MINUTES

The Minutes of the May 12-13, 1975 meeting were approved and accepted with the following correction:

Page 3, III, first paragraph  
The first sentence should read:

"After considering alternative ways of reviewing grant applications involving recombinant DNA molecules, the Committee reaffirmed its recommendation that institutional biohazards committees be involved in the review of applications in the moderate and high risk categories."

III. GUIDELINES FOR RESEARCH

Dr. Hogness introduced for discussion a draft proposal for guidelines for research on recombinant DNA molecules. The draft proposal was prepared by a planning group consisting of Drs. Hogness (Chairman), Chu, Helinski and Szybalski with the assistance of Drs. Emmett Barkley and Peter Day. The planning group had met at Stanford, California on July 2 and 3, 1975.

The full Committee discussed in-depth and modified the draft proposal. The resulting document is attached (Attachment II). The Committee suggested that these recommendations be referred to as "current guidelines". During the review of this document the Committee discussed whether the term "cloning vehicle" or "vector" is more appropriate. The Committee voted to retain the use of the work "vector" in the document.

During discussion of the section on Responsibility, the Committee voted that in regard to moderate and high risk experiments involving recombinant DNA molecules, the function of the institutional biohazards committees should be limited to certification that applicants have adequate facilities to comply with NIH guidelines for the level of risk assessed by the applicant. The adequate training of laboratory personnel would be the responsibility of the principal investigator.

#### IV. CONSTRUCTION AND TESTING OF SAFER HOSTS AND VECTORS

Dr. Nutter reported that the National Institute of Allergy and Infectious Diseases is well along in the preparation of five Request for Proposals relating to the construction and testing of safer hosts and vectors. The RFPs relate to construction of safer plasmid vectors, construction of safer phage vectors, construction of a safer E. coli K12 host, survival testing in man and the environment, and independent certification of the genotype and tests for transmissibility. The subcommittee composed of Drs. Curtiss, Falkow, Helinski and Szybalski will review the final drafts of these RFPs.

Dr. Nutter reported that the NIAID manages a research reference collection consisting mostly of viral reagents. This facility might be utilized for the storage and distribution of safer hosts and vectors.

#### V. TRAINING IN MICROBIOLOGICAL TECHNIQUES

Dr. Barkley reported that the National Cancer Institute has sponsored a course focused on the safe handling of oncogenic viruses. The NCI is able to set up a two day course dealing with basic microbiological safety with emphasis on the control of biohazards of recombinant DNA molecules. The course would include laboratory exercises on the production and control of aerosols. The proposed course would be oriented towards principal investigators; it is not intended to train laboratory workers at the present time. Approximately 20 investigators could be accommodated per course, and NCI would sponsor 2 courses during the coming year. The Committee recommended that NCI proceed with plans for these courses. The question was raised as to whether it would be possible to develop a training film on microbiological techniques. It was pointed out that production of such a film would be a major undertaking, but would have the potential for wide dissemination.

#### VI. NIH EXTRAMURAL PROCEDURES

Dr. Jacobs asked the Committee to comment on a draft NIH Manual issuance dealing with the control of hazardous microbiological agents in extramural research. The Committee felt that it is not appropriate to group recombinant DNA molecules, which are potentially dangerous, with agents which are known to be extremely hazardous. The Committee recommended that a separate document be prepared for recombinant DNA molecules, following the Committee's recommendations and guidelines.

#### VII. NEXT MEETING

A tentative meeting was scheduled for Friday, October 10, 1975 in Bethesda, Maryland. If it is felt that there is no need for this meeting, it will be cancelled. The following meeting will be held in early December, 1975 in conjunction with the Workshop on the construction of safer hosts and vectors.

RECOMBINANT DNA MOLECULE PROGRAM ADVISORY COMMITTEE

CHAIRMAN

STETTEN, DEWITT, JR. 1979  
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ADELBERG, EDWARD A. 1978  
PROFESSOR & CHAIRMAN  
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HOGNESS, DAVID S. 1978  
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CHU, ERNEST H.Y. 1976  
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DARNELL, JAMES E., JR. 1977  
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SZYBALSKI, WACLAW 1977  
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HELINSKI, DONALD R. 1976  
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THOMAS, CHARLES A., JR. 1976  
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EXECUTIVE SECRETARY

GARTLAND, WILLIAM J., JR.  
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In designing these guidelines we have adopted the following principles, which are consistent with the general conclusions that were formulated at the International Conference on Recombinant DNA Molecules held at Asilomar Conference Center, Pacific Grove, California in February 1975(3): (i) There are certain experiments for which the assessed potential hazard is of such a serious nature that they should not be attempted at the present time. (ii) The remainder can be undertaken at the present time provided that appropriate safeguards are incorporated into the design and execution of the experiment. In addition to an insistence on the practice of good microbiological techniques, these safeguards consist in providing both physical and biological barriers to the dissemination of the potentially hazardous agents. (iii) The level of containment provided by these barriers should match the estimated potential hazard for each of the different classes of recombinants. For projects in a given class, this level should be highest at initiation and modified subsequently only if there is a substantial change in the assessed risk or in the applied methodology. (iv) The guidelines should be subjected to periodic review (at least annually) and modified to reflect improvements in our knowledge of the potential biohazards and of the available safeguards.

## II. Containment

Effective biological safety programs have been operative in a variety of laboratories for many years. Considerable information therefore already exists for the design of physical containment facilities

and laboratory procedures applicable to organisms carrying recombinant DNAs(4-13). The existing programs rely upon mechanisms that, for convenience, can be divided into two categories: (1) a set of standard practices that are generally used in microbiological laboratories, and (2) special procedures, equipment and laboratory installations that provide physical barriers which are applied in varying degrees according to the estimated biohazard.

Experiments on recombinant DNAs by their very nature lend themselves to a third containment mechanism - namely, the application of highly specific biological barriers. In fact, natural barriers do exist which either limit the infectivity of a vector or vehicle (plasmid, bacteriophage or virus) to specific hosts, or its dissemination and survival in the environment. The vectors that provide the means for replication of the recombinant DNAs and/or the host cells in which they replicate can be genetically designed to decrease by many orders of magnitude the probability of dissemination of recombinant DNAs outside the laboratory.

As these three means of containment are complementary, different levels of containment appropriate for experiments with different recombinants can be established by applying different combinations of the physical and biological barriers to a constant use of the standard practices. We consider these categories of containment separately here in order that such combinations can be conveniently expressed in the guidelines for research on the different kinds of recombinant DNAs (Section III).

A. Standard practices and training - The first principle of containment is a strict adherence to good microbiological practices. Consequently, personnel involved in experiments on recombinant DNAs should receive adequate instruction. This should include training in aseptic techniques and instruction in the biology of the organisms used in the experiments so that the potential biohazards can be understood and appreciated.

In addition to aseptic experimental techniques, standard practices generally include the following procedures when the experiments involve organisms that may be pathogenic or may undesirably alter the basic ecology. (1) Materials and equipment that contain or have come in contact with these organisms should be disinfected or sterilized by autoclaving prior to disposal, and work surfaces should be decontaminated. (2) Cotton plugged pipettes may be used where the hazard is minimal, but mechanical pipetting devices are preferable and should be required for more hazardous material. (3) Sharp, pointed syringe needles should be avoided wherever possible. (4) Eating, drinking and smoking in the work area should not occur while experiments with potentially hazardous material are in progress and at least until the decontamination indicated above is completed. (5) Laboratory personnel should wash hands after experiments involving these materials. (6) Laboratory doors should be closed while such experiments are in progress. (7) Appropriate clothing such as laboratory coats, or similar apparel, and closed shoes should be worn when handling potentially hazardous organisms. Laboratory coats should not be worn outside the work area.

B. Physical containment levels - A variety of combinations (levels) of special procedures, equipment and laboratory installations that provide additional physical barriers can be formed. For example, 31 combinations are listed in "Laboratory Safety at the Center for Disease Control" (4); four levels are associated with the "Classification of Etiologic Agents on the Basis of Hazard" (5), and with the "Summary Statement of the Asilomar Conference on Recombinant DNA Molecules" (3); and the National Cancer Institute uses three for research on oncogenic viruses (6). We define only four levels of physical containment here, both because the accuracy with which one can presently assess the biohazards that may result from recombinant DNAs does not warrant a more detailed classification, and because additional flexibility can be obtained by combination of the physical with the biological barriers. Though different in detail, these four levels (P1 < P2 < P3 < P4) approximate those given for the classification of etiologic agents (i.e., classes 1 through 4; ref. 5), in the Asilomar summary statement (i.e., minimal, low, moderate and high; ref. 3), and by the NCI (low, moderate and high; ref. 6), as is indicated by the P-number or adjective in the following headings:

P1 level (minimal) - Requires standard microbiological practices (See A above).

P2 Level (low) - (1) Access to the laboratory is controlled when handling potentially hazardous organisms requiring P2 containment, but not otherwise. During the controlled period appropriate biohazard signs should be posted at access points to the laboratory, and only authorized persons who have been advised of the potential biohazard should enter when such signs are posted. The signs should

be removed upon completion of the hazardous procedures. Experiments of no or less biohazard can be carried out concurrently in the same laboratory. (ii) Mouth pipetting is prohibited; mechanical pipetting devices are required. (iii) Specific precautions are required for those procedures that have a high potential for release of aerosols containing potentially hazardous material - e.g., centrifugation should be carried out in airtight cups or rotors; sonication and blending should be similarly contained or carried out in biological safety cabinets.

P3 Level (moderate) - In addition to the P2 procedures, this level requires the following: (i) Operations with potentially hazardous organisms that require P3 containment should be carried out in a laboratory that is separated from areas where less than P3 containment is practiced. This laboratory should be operated under negative pressure(34). The exhaust air from these laboratories should be discharged to the atmosphere in an appropriate manner. If recirculated, the air must be decontaminated. Appropriate biohazard signs should be posted at access points to the laboratory, and only persons specifically authorized by the principal investigator should enter when these signs are posted. Normally, authorized persons should be limited to those who work in the laboratory. (ii) Biological safety cabinets, meeting appropriate NIH performance standards, should be used for all transfer operations and for all procedures likely to produce aerosols. (iii) Gloves should be worn during the handling of biohazardous materials. (iv) Vacuum lines should be protected by filters.

P3-level research may be conducted in restricted laboratories where negative pressure cannot be provided if the following additional safeguards are maintained. (i) Sealed negative pressure cabinets should be used for all transfer operations and all procedures likely to produce aerosols. (ii) Negative pressure biological safety cabinets should be used for all other operations involving the hazardous organisms (e.g., centrifugation, growing cells on shakers, etc.). (iii) Transfer from one negative pressure area to another should be carried out in sealed, unbreakable containers.

P4 Level (high) - Work areas are in a special facility of the type designed to contain highly infectious and hazardous microbiological agents. These areas are isolated by airlocks, a negative pressure environment, personnel clothing change and shower rooms, and treatment systems to inactivate or remove biohazardous agents contaminating exhaust air, liquid and solid wastes. All persons occupying these areas should wear protective laboratory clothing and shower at each exit from the facility. The handling of the biohazardous agents should be confined to biological safety cabinets in which the exhaust air is incinerated or passed through Hepa filters.

C. Biological Containment Levels - Biological barriers are specific to each host-vector system. Hence the criteria for this mechanism of containment cannot be generalized to the same extent as for physical containment. This is particularly true at the present time when our experience with existing host-vector systems and our predictive knowledge about projected systems is sparse. Furthermore the classification of experiments with recombinant DNAs that is necessary for the construction of the experimental guidelines (Section III) can be accomplished with least confusion if we use the host-vector system as the primary element and the source of the inserted DNA as the secondary element in the classification. It is therefore convenient to specify the nature of the biological containment under the host-vector headings given in the next section.

### III. Experimental Guidelines

A general rule that, though obvious, deserves statement is that the level of containment required for any experiment on DNA recombinants shall never be less than that required for the most hazardous component used to construct and clone the recombinant DNA (i.e., vector, host and inserted DNA). In most cases the level of containment will be greater, particularly when the recombinant DNA is formed from species that ordinarily do not exchange genetic information.

This rule by itself effectively precludes certain experiments - namely those in which one of the components is in Class 5 of the "Classification of Etiologic Agents on the Basis of Hazard" (5),

as these are excluded from the United States by law and USDA administrative policy. There are additional experiments which may engender such serious biohazards that they should not be performed at this time. These are considered prior to presentation of the containment guidelines for permissible experiments.

A. Experiments that should not be performed - We recognize that it can be argued that certain of the recombinants placed in this category could be adequately contained at this time. Nonetheless, our estimate of the possible dangers that may ensue if that containment fails are of such a magnitude that we consider it the wisest policy to at least defer experiments on these recombinant DNAs until there is more information to accurately assess that danger and to allow the construction of more effective biological barriers. In this respect, these guidelines are more stringent than those initially recommended (1).

We therefore strongly advise that the following experiments not be initiated at the present time. (i) Cloning of recombinant DNAs derived from the highly pathogenic organisms in Classes 3, 4 and 5 of "Classification of Etiologic Agents on the Basis of Hazard" (5), regardless of the vector-host system used. (ii) Deliberate formation of recombinant DNAs containing genes for the biosynthesis of toxins of very high toxicity (e.g., botulinum or diphtheria toxins). (iii) Deliberate creation from plant pathogens of recombinant DNAs that are likely to increase virulence and host range.

In addition, we recommend that large scale experiments (e.g., more than 10 liters of culture) with recombinant DNAs known to make harmful products not be carried out at this time. We differentiate between small and large scale experiments with such DNAs because the probability of escape from containment barriers normally increases with increasing scale. However, specific experiments in this category that are of direct social benefit may be excepted from this rule if special containment precautions and equipment designed for large scale operations are used, and provided that these experiments are expressly approved by the Recombinant DNA Molecule Program Advisory Committee of the NIH.

B. Containment guidelines for permissible experiments -- It is anticipated that most recombinant DNA experiments initiated before these guidelines are next reviewed (i.e., within the year) will employ E. coli K12 host-vector systems. These are also the systems for which we have the most experience and knowledge regarding the effectiveness of the containment provided by existing hosts and vectors, and necessary for the construction of more effective biological barriers. We therefore consider DNA recombinants cloned in E. coli K12 before proceeding to other host-vector systems that we suppose will be used less frequently and for which we have less information.

1. Experiments using E. coli K12 host-vectors - We consider the following three levels of containment with these host-vectors.

EK 1 host-vectors - These are host-vector systems that can be estimated to provide a moderate level of containment, and include most of the presently available systems. The host is always E. coli K12,

and the vectors include nonconjugative plasmids (i.e., pSC101, Col E1 or derivatives thereof, such as mini-ColE1; refs. 14-21) and variants of bacteriophage  $\lambda$  (22-24).

The *E. coli* K12-nonconjugative plasmid system is taken as an example to illustrate the approximate level of containment referred to here. The available data from feeding experiments with humans and calves [25-27] indicate that *E. coli* K12 do not colonize the normal bowel, and exhibit little if any multiplication while passing through the alimentary tract even after feeding high doses (e.g.,  $10^9$  - to -  $10^{10}$  bacteria per human). However, as they can survive this passage,† transfer of the plasmid vectors from *E. coli* K12 to resident bacteria in the gut must also be considered.

The nonconjugative plasmid vectors cannot promote their own transfer, but require the presence of a transfer plasmid for mobilization. Thus ingested *E. coli* K12 containing a nonconjugative plasmid must first conjugate with resident bacteria containing a transfer plasmid before the nonconjugative vectors can in turn be transferred. Estimates for the frequency of this series of events are in the range of  $10^{-12}$  to  $10^{-14}$  per 24 hr/g of feces [27].

These observations indicate the remoteness of the possibility of dissemination of such vectors by accidental ingestion, which would probably involve only a few hundred or thousand bacteria provided that at least the standard practices (II-A) are maintained, particularly the avoidance of mouth pipetting. The probabilities of colonization and hence of transfer are increased, however, if the normal flora in the bowel is disrupted by, for example, antibiotic therapy. For this reason, persons receiving such therapy should not work with DNA recombinants formed with *E. coli* K12 host-vector systems during the therapy period and for seven days thereafter; similarly, persons who have functional gastrointestinal disorders or who have had surgical removal of part of the stomach or bowel should avoid such work.

The observations on the fate of *E. coli* K12 in the human alimentary tract are also relevant to the containment of recombinant DNAs formed with bacteriophage  $\lambda$  variants. Accidental dissemination in this case depends either (i) on survival of the mature phage, followed by the finding and productive infection of some suitable *E. coli* in nature, or (ii) on the establishment of  $\lambda$  prophage or plasmids. Although the probability of survival and infection of resident *E. coli* in the human gut by ingested mature  $\lambda$  has not been directly determined, it is estimated to be very small, given the high sensitivity of  $\lambda$  to the low pH of the stomach, the insusceptibility to  $\lambda$  infection of encapsulated

† For example, viable *E. coli* K12 can be found in the feces after feeding humans  $\geq 10^7$  bacteria in broth [25] - the most likely mode of accidental ingestion, or after feeding  $\geq 3 \times 10^4$  bacteria protected by suspension in milk [26].

E. coli cells (the type that normally reside in the gut), and the failure to detect  $\lambda$  in human feces after ingestion of  $10^9$   $\lambda$  particles (28). Establishment of stable lysogeny in host cells should also be very infrequent for most of the described  $\lambda$  vectors (22-24) since they lack the att and int functions (estimated lysogenization frequency  $< 10^{-5}$  to  $10^{-6}$ ; 29-31). The estimated frequency of plasmid formation by these vectors is also low (ca.  $10^{-6}$ ; 32).

While not exact, the containment estimates for these host-vectors are at least as accurate as those for physical containment, and are sufficient to indicate that both vector systems provide a moderate level of biological containment. Other nonconjugative plasmids and bacteriophage that, in association with E. coli K12, can be estimated to provide the same approximate level of moderate containment are included in the EK1 class.

EK 2 host-vectors - These are host-vectors that have been genetically designed to provide a high level of biological containment as determined from data obtained in cultures or in other environments created in the laboratory. The genetic modification of the E. coli K12 host and/or the EK 1-type vectors should increase the containment determined in this manner by at least  $10^6$ -fold over that for the parent EK1 system. Whereas useful phage vectors can be obtained that, in effect, do not form stable combinations with the host so that the vector is the only element that must be contained<sup>†</sup>, this is not the

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For example, a  $\lambda$  vector that has already been constructed has the following features (33):

1. Formation of the prophage state is blocked by deletion of the att site and the genes int, xis and ci.
2. Plasmid formation is blocked by the nin5 deletion and the c17 mutation.
3. Phage recombination functions red and int are deleted.
4. The phage is highly lethal to its hosts, including even  $\lambda$ -immune lysogens encountered in nature, because of its virulence (resulting from a combination of the ci and nin5 deletions with the c17 mutation).
5. The phage yields are high ( $10^{10}$  -  $10^{11}$  per ml.)

Such a virulent phage vector and its host form a two-component system, each component alone being unable to disseminate the recombinant DNA unless the phage vector and a suitable host in nature are brought together. Moreover, once such an encounter occurs, the probability that such  $\lambda$  vectors will transfer the recombinant DNA and establish a combination capable of disseminating the DNA is extremely small. The phenotypes and genetic stabilities of the mutations and chromosome alterations included in this vector indicate that it should provide an increase in containment well in excess of the required  $10^6$ -fold.

case for plasmid vectors. For systems involving nonconjugative plasmids we recommend that the increase in containment should be at least  $10^6$ -fold in survival of the host and/or plasmid and at least  $10^6$ -fold in plasmid transmissibility<sup>†</sup>.

It should be emphasized that even higher levels of containment than specified here are desirable, and, while not required, should be used when applicable.

EK 3 host-vectors - These are EK 2 systems for which the increased containment has been confirmed by tests in animals and, if possible, in other relevant natural environments. If tests do not exist which are sufficiently sensitive to quantitate the increased degree of containment, a negative finding at the maximum sensitivity of the test will be considered as confirmation.

In the following classification of containment criteria for different kinds of recombinant DNAs, the stated levels of physical and biological containment are minimums. It is recommended that higher levels of biological containment (~~EK 1~~ EK2 > EK1) be used if they are available and are equally appropriate for the purposes of the experiment. In this case consideration may be given to a corresponding decrease in the required level of physical containment.

#### <a> Shotgun Experiments

These experiments involve the production of recombinant DNAs between the vector and the total DNA from the specified cellular source or any fraction thereof that has not been rigorously purified and defined. Recombinants formed from rigorously purified DNAs will be considered separately.

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<sup>†</sup> Examples of mutations in the host/plasmid that would increase containment are: (1) Temperature-sensitive mutations in the plasmid or host resulting in failure of the plasmid to replicate at mammalian body temperature; (2) Suppressible mutations in the plasmid restricting replication of the plasmid to a host bacterium possessing specific suppressor genes; (3) Mutations (e.g. *dap*<sup>-</sup>, *Sm*<sup>D</sup>) in the host bacterium to reduce survival of the organism in nature; (4) Mutations in the host bacterium that reduce the organism's ability to accept a conjugative plasmid (e.g. conjugation-deficient mutants); (5) Mutations or deletions of plasmid DNA that result in a reduction in mobilizeability (e.g. mini-ColE1 is mobilizeable at a considerably lower frequency than ColE1). Combinations of these stable mutations should increase containment of the plasmid at least  $10^6$ -fold with respect both to the survival of the host and/or plasmid and the transmissibility of the plasmid.

(1) Eukaryotic DNA recombinants

Mammals - P3 physical containment + an EK 2 host-vector.

Warm-blooded animals other than mammals - P3 physical containment + an EK 1 host-vector or P2 physical containment + EK 2 host-vector.

Cold-blooded animals and all other lower eukaryotes - P2 physical containment + an EK 1 host-vector. If the eukaryote in this class is a known pathogen (i.e., an agent listed in Class 2 of ref. 5 or a plant pathogen) or carries such an agent, the containment should be increased to P3 + EK2.

Higher plants - P2 physical containment + an EK 1 host-vector. If the plant carries a known pathogenic agent or makes a product known to be dangerous, the containment should be increased to P2 + EK 2.

(ii) Prokaryotic DNA recombinants

Prokaryotes that naturally exchange genetic information with E. coli -

The level of physical containment is directly determined by the rule of the most dangerous component (see introduction to Section III).

Thus P1 conditions can be used for DNAs from those bacteria in Class 1 of ref. 5 ("Agents of no or minimal hazard...") which naturally exchange genes with E. coli ; and P2 conditions should be used for such bacteria if they fall in Class 2 of ref. 5 ("Agents of ordinary potential hazard..."), or are plant pathogens. EK 1 host-vectors can be used for all experiments requiring only P1 physical containment; in fact, experiments in this category can be performed with E. coli K12 vectors exhibiting a lesser containment than EK1 vectors (e.g., conjugative

plasmids). Experiments with DNA from species requiring P2 physical containment which are of low pathogenicity can use EK 1 host-vectors, but those of moderate pathogenicity should use EK 2 host-vectors.

Prokaryotes that do not ordinarily exchange genetic information with E. coli - The minimum containment conditions for this class consist of P2 physical containment + an EK 1 host-vector, and apply when the risk that the recombinant DNAs will increase the pathogenicity or ecological potential of the host is judged to be minimal. Experiments with DNAs from pathogenic species (Class 2 of ref. 5 plus plant pathogens) should use P3 + EK 2 conditions if of low pathogenicity and P3 + EK 3, or P4 + EK 2 if of moderate pathogenicity.

(iii) Characterized clones of DNA recombinants derived from shotgun experiments - When a cloned DNA recombinant has been highly characterized and there is sufficient evidence that it does not contain harmful genes, then experiments involving this recombinant DNA can be carried out under P1 + EK 1 conditions if the inserted DNA is from a species that naturally exchanges genes with E. coli, and under P2 + EK 1 conditions if not.

<b> Purified cellular DNAs other than plasmids, bacteriophages and other viruses.

The formation of DNA recombinants from cellular DNAs that have been highly purified by physical and chemical techniques (i.e., not by cloning) and for which there is sufficient evidence that they do not contain harmful genes, can be carried out under lower containment conditions

than used for the corresponding shotgun experiment. In general, the containment can be decreased one step in physical containment (P4 + P3 + P2 + P1) while maintaining the biological containment specified for the shotgun experiment, or ~~one~~ step in biological containment (EK 3 + EK 2 + EK 1) while maintaining the specified physical containment - provided that the new condition is not less than that specified above for characterized clones from shotgun experiments (section <a> - iii).

<c> Plasmids, bacteriophages and other viruses.

Recombinants formed between EK-type vectors and other plasmid or virus DNAs have in common the potential for acting as double vectors because of the replication functions in these DNAs. The containment conditions given below apply only to propagation of the DNA recombinants in E. coli K12 hosts. They do not apply to other hosts where they may be able to replicate as a result of functions provided by the DNA inserted into the EK vectors. These are considered under other host-vector systems.

(i) Animal viruses - P3 + EK2 conditions should be used to form DNA recombinants that include all or part of the genome of an animal virus. P3 + EK 1 or P2 + EK2 conditions can be used when forming recombinants from highly purified and characterized segments of viral genomes for which there is sufficient evidence that they do not contain harmful genes and, in the case of oncogenic viruses, derive from the non-transforming regions of the genome.

(ii) Plant viruses - P3 + EK 1 or P2 + EK 2 conditions should be used to form DNA recombinants that include all or part of the genome of a plant virus. P2 + EK 1 conditions can be used when forming recombinants from highly purified DNA segments and for which there is sufficient evidence that they do not contain harmful genes.

(iii) Eukaryotic plasmid DNAs - The containment conditions given below apply only when the plasmid DNA has been highly purified; otherwise the conditions given under shotgun experiments apply. Mitochondrial DNA from mammals: P3 + EK 1 or P2 + EK2. Mitochondrial or chloroplast DNA from other eukaryotes: P2 + EK 1.

(iv) Prokaryotic plasmid and phage DNAs -

Plasmids and phage from hosts that naturally exchange genes with E. coli - Experiments with DNA recombinants formed from plasmids or phage genomes that have not been characterized with regard to pathogenic components or are known to significantly contribute to the pathogenicity of their normal hosts should use the containment conditions specified for shotgun experiments with DNAs from the respective host. If the DNA recombinants are formed from plasmids or phage that are known not to contain pathogenic components, or from highly purified and characterized plasmid or phage DNA segments for which there is sufficient evidence that they do not contain such components, the experiments can be performed with P1 physical containment + an EK 1 host-vector.

Plasmids and phage from hosts that do not naturally exchange genes with E. coli - The rules for shotgun experiments with DNA from the host apply to their plasmids or phages, with the following qualifications. Experiments with DNA recombinants formed with plasmids or phage containing resistance genes to clinically significant antibiotics should use P3 physical containment + an EK 2 host-vector. The minimum containment conditions for this category (P2 + EK 1) can be used for plasmid

and phage, or for highly purified and characterized segments of plasmid and phage DNAs, when the risk that the recombinant DNAs will increase the pathogenicity or ecological potential of the host is judged to be minimal.

Note: Where applicable, cDNAs (i.e., complementary DNAs) synthesized in vitro from cellular or viral RNAs are included within each of the above classifications. For example, cDNAs formed from cellular RNAs that are not highly purified and characterized are included under <a>, shotgun experiments; cDNAs formed from highly purified and characterized RNAs are included under <b>; cDNAs formed from viral RNAs are included under <c>; etc.

## 2. Experiments with other prokaryotic host-vectors

Other prokaryotic host-vector systems are at the speculative, planning or developmental stage, and consequently do not warrant detailed treatment at this time. However, the containment criteria for different types of DNA recombinants formed with E. coli K12 host-vectors can, with the aid of some general principles given here, serve as a guide for containment conditions with other host-vectors when appropriate adjustment is made for their different habitats and characteristics.

In general, the strain of any prokaryotic species used as the host should conform to the definition of Class I etiologic agents given in ref. 5 (i.e., "Agents of no or minimal hazard..."), and the plasmid or viral vector should not make the host more hazardous. In addition, it is recommended that the newly developed host-vector systems offer

some distinct advantage over the E. coli K12 host-vectors - for instance, host-vectors, such as thermophilic organisms, whose major habitats do not include humans and/or economically important animals and plants.

At the initial stage, the host-vector should exhibit at least a moderate level of biological containment comparable to EK 1 systems, and be capable of modification to obtain high levels of containment comparable to EK 2 and EK 3. The type of confirmation test(s) required to move a host-vector from an EK 2-type classification to an EK 3-type will clearly depend upon the preponderant habitat of the host-vector. For example, if the unmodified host-vector propagates mostly in or on higher plants, but not appreciably in warm-blooded animals, modification should be designed to reduce the probability that the host-vector can escape to and propagate in or on such plants, and it is that lower probability which should be confirmed.

The following principles should be followed in using the containment criteria given for experiments with E. coli K12 host-vectors as a guide for other prokaryotic systems. Experiments with DNA from prokaryotes (and their plasmids or viruses) should be classified according to whether the prokaryote in question naturally exchanges genes with the host-vector or not, and the containment conditions given for these two classes with E. coli K12 host-vectors applied.

Experiments with DNAs from eukaryotes (and their plasmids or viruses) can also follow the criteria for the corresponding experiments with E. coli K12 vectors if the major habitats of the given host-vector

overlap those of E. coli. If the host-vector has a major habitat that does not overlap those of E. coli (e.g., root nodules in plants), then the containment conditions for some eukaryotic recombinant DNAs should be increased (for instance, higher plants and their viruses in the preceding example), while others may be reduced.

### 3. Experiments with eukaryotic host-vectors

#### <a> Animal host-vectors.

Host cells should derive from cultures expected to be of minimal hazard. Cells within the animal should not be used as hosts until the recombinant DNA has been well tested in cell cultures. DNAs from mitochondria or from minimal or low risk viruses may be used as vectors. When viral DNAs are used, preference should be given to viruses that are not known to be pathogenic or oncogenic in mammals or economically important animals or plants.

The host-vector system should exhibit at least a moderate level of biological containment at the initial stage, and be capable of modification to a high level of containment. Since the host cell lines generally will have little if any capacity for propagation outside the laboratory, the primary focus for containment is the vector. Very little is known about the ability of mitochondrial DNAs to serve as vectors, but they offer the potential of high containment as they are not packaged to form infective agents. Consequently, we urge that the vector capabilities of mitochondrial DNAs be investigated.

At present, however, viral DNAs form the most obvious group of potential vectors. They can be modified according to many of the same principles applied to phage vectors to increase the biological containment. An important criterion for the selection of a viral vector therefore is that it has already been sufficiently characterized to facilitate genetic modifications that will yield a highly contained vector.

Experiments on recombinant DNAs formed between the initial, moderately contained vectors and DNA from a source that is not itself pathogenic or does not contain pathogenic agents or genes may use P3 physical containment. Experiments involving the latter types of DNA should not be done unless there is strong justification and then only under P4 conditions. It is suggested that when the possibility of encapsidation of recombinant DNA exists, suitable assays for infectivity should be carried out.

The development and use of host-vector systems that exhibit a high level of biological containment permit a decrease of one step in the physical containment (P4 → P3 → P2) specified above.

<b> Plant host-vectors

Cells in tissue cultures, seedlings or plant parts, (e.g., tubers, stems, fruits and detached leaves) or whole mature plants of small species (e.g., arabidopsis) can be handled under the P1 - P4 containment conditions that we have specified previously. However, cells in most whole plants pose additional problems. P2 physical containment conditions can be provided by: (1) the best insect-proof

greenhouses, (2) sterilization of contaminated plants, pots, and soil by autoclaving, and (3) adoption of the other standard practices for microbiological work. P3 physical containment can be sufficiently approximated by confining the operations with whole plants to growth chambers like those used for work with radioactive isotopes, provided that (1) such chambers are modified to produce a negative pressure environment with the exhaust air appropriately filtered, and (2) that other operations with infectious materials are carried out under the specified P3 conditions. The P2 and P3 conditions specified earlier are therefore extended to include these cases for work on higher plants.

The host cells for experiments on recombinant DNAs may be cells in culture, in seedlings or plant parts, or in whole plants. However, we recommend that cells in whole plants that can not be adequately contained not be used as hosts for shotgun experiments at this time, and that attempts to infect whole plants with DNA recombinants cloned elsewhere not be initiated until their effects on host cells in culture, seedlings or plant parts have been studied.

DNAs from mitochondria, chloroplasts or viruses of minimal or low pathogenicity to plants may be used as vectors. In general, the same preference criteria for selecting host-vectors given in the preceding section on animal systems apply to plant systems, where chloroplast and mitochondrial DNAs can be grouped together as offering the potential of highly contained vectors that should be investigated.

Experiments on recombinant DNAs formed between the initial, moderately contained vectors and DNA from cells of species in which the vector DNA can replicate, either autonomously or as an integrated segment of the cell's genome, should use P2 physical containment - provided that the source of the DNA is itself not pathogenic or known to carry pathogenic agents, or to produce products dangerous to plants. In such cases, the experiments should be carried out under P3 conditions.

Experiments on recombinant DNAs formed between the above vectors and DNAs from other species can also be carried out under P2 if that DNA has been highly purified and determined not to contain harmful genes. Otherwise, the experiments should be carried out under P3 conditions if the source of the inserted DNA is not itself a pathogen, or known to carry such pathogenic agents, or to produce harmful products - and under P4 conditions if these conditions are not met.

The development and use of host-vector systems that exhibit a high level of biological containment permit a decrease of one step in the physical containment specified above (P4 → P3 → P2 → P1).

<c> Fungal, or similar lower eukaryotic host-vectors.

The containment criteria for experiments on recombinant DNAs using these host-vectors most closely resemble those for prokaryotes, rather than the preceding eukaryotes, in that the host cells usually exhibit a capacity for dissemination outside the laboratory that is

similar to that for bacteria. We therefore consider that the containment guidelines given for experiments with E. coli K12 and other prokaryotic host-vectors (Sections IIIB-1 and -2, respectively) provide adequate direction for experiments with these lower eukaryotic host-vectors. This is particularly true at this time since the development of these host-vectors is presently in the speculative stage.

#### IV. Responsibility

The principal investigator has the responsibility for estimating the potential biohazards associated with the experiments on recombinant DNAs performed in the laboratories under her or his direction, for instituting the appropriate safeguards within these laboratories, for developing procedures for minimizing the effects of accidents, for training and ensuring the proficiency of relevant personnel in the application of these safeguards and procedures, for informing them of both the potential hazards and the basis on which these hazards have been estimated, and for maintaining these practices on a continuing basis.

The experimental guidelines given here are to help the principal investigator determine the nature of the safeguards that should be implemented for experiments with different types of recombinant DNAs. Because the complexity of types is great, these guidelines are bound to be incomplete in some regards. Hence they are not meant to substitute for the investigator's own evaluation of the containment conditions

required for each experiment. Whenever this evaluation calls for an increase in containment over that indicated in the guidelines, the investigator has the responsibility for instituting such an increase. By contrast, the containment conditions should not be decreased over those called for in the guidelines without peer review (see Section V).

The institution of appropriate safeguards includes a continuing control on containment procedures, on the effective operation of the physical containment facilities, and on those aspects of the genotype of the host-vectors that are relevant to the biological containment they provide. Although the data on the phenotypic characteristics of a given host-vector that determine its level of biological containment usually will have been obtained in other laboratories, the principal investigator has the responsibility of being able to justify the overall containment determination on the basis of such data. That is, it is not sufficient that he or she simply be assured by some other person of the determination of the containment level; rather, investigators should be or become sufficiently knowledgeable to make their own determination. In addition, investigators have the responsibility of ascertaining that the hosts and/or vectors exhibit the required genotype prior to their use in experiments with DNA recombinants in their laboratories. Such ascertainment generally involves a simple phenotypic test for each relevant mutation.

V. Implementation

Implementation starts with the principal investigator's evaluation of the potential biohazards associated with a given project and of the appropriate safeguards to be applied. To help in this evaluation and

application we recommend that each institution or group of institutions where research on DNA recombinants takes place form a biohazard committee which would have the following two functions. The first and most useful function would be to serve as a source of advice and reference regarding: (1) the availability and quality of the safety equipment and laboratory installation modes required for P3 and P4 physical containment, (2) the availability and level of biological containment of different kinds of host-vector systems, (3) advice and reference regarding suitable training of personnel, and (4) more general data on the potential biohazards associated with different types of recombinant DNAs. To this end, each local biohazard committee should create a central reference file and library of catalogues, books, articles, newsletters and other communications relevant to the above subjects.

The second, more formal function of this committee would be to examine the equipment and installations in laboratories requiring P3 or P4 physical containment, and, if they meet the requirements for such containment, to so certify. It is not the responsibility of the local committee to determine either the scientific quality or the containment conditions required for a given project.

When investigators apply to an agency for funds to support research projects on recombinant DNAs, or whenever they decide to initiate or significantly change research on recombinant DNAs under existing grants, they should provide the agency with the above certification (if the research requires P3 or P4 physical containment) along with a statement

containing their evaluation of the potential biohazards and the containment conditions they will use for the proposed projects.

The peer group reviewing the scientific merit of a research proposal for the agency will then make an independent evaluation of the potential biohazards and determine whether the proposed containment conditions provide the appropriate safeguards, using the guidelines given here as their basic reference. If the review group concludes that the safeguards are appropriate, the grant would be processed on the basis of scientific merit in the usual fashion. If there is some question concerning the adequacy of the containment capability that can not be resolved at this level, then the matter may be referred to an appropriate committee.

In those cases where the investigator wishes to initiate or significantly change research on recombinant DNAs under existing grants, the agency can, without peer review, make the decision to endorse this initiation or change. However, if there is some question regarding the adequacy of the containment capability that cannot be resolved at this level, the matter should be referred to the initial review group.

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