CORRELATION BETWEEN INTRAMOLECULAR BASE COMPOSITION HETEROGENEITY OF DNA AND CONTROL OF TRANSCRIPTIONAL EXPRESSION IN *E. coli* TEMPERATE PHAGE P2

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ABSTRACT

Intramolecular base compositional heterogeneity has been demonstrated in the DNA of the phage P2 by following the optical density of solutions at 260 nm as a function of increasing temperature. First derivative curves of the functions thus generated have been obtained for P2 DNA and the DNA of two closely related phages. Comparison of these curves reveals similarities at the high temperature (high GC) end and differences at the low temperature (low GC) end. All three of these phages can, by supplying the genes concerned with phage particle maturation, serve as helper for the defective phage P4.

The strands of P2 DNA have been separated on the basis of their buoyant densities in CsCl solutions when complexed with poly UG. In vivo transcription patterns from these strands in cells infected with various P2 mutants have demonstrated that the "heavy" strand is the one predominantly transcribed, that some "light" strand transcription originates from an operon coding for proteins involved in phage head assembly (thus fuling out a "read-through" mechanism for late gene activation), and that early "light" strand transcription does not originate from DNA deleted in two mutants which is in the right half of the physical map. There is, however, some "light" strand transcription early in infection.

P2 DNA has been sheared in half and the halves separated on the basis of Hg^{++} binding in Cs_2SO_4 density gradients. Electron microscopic analysis of partially denatured molecules in these preparations have fixed them with respect to the physical map. In vivo transcription originates primarily from the right, or low GC half early in infection and then shifts

to the left, or high GC half. Mutants in genes A and B, which are located in the right half of the genetic map, are defective in both DNA replication and in effecting this shift. Infection with one polar amber mutant under non-permissive conditions has demonstrated that this operon lies in the right half of the DNA and thus helps to fix the physical map with respect to the genetic map.

Two of those regions of the phage DNA which are known to be transcribed from a repressed genome, namely the prophage, appear to be of quite low GC content.

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INTRODUCTION

The study of temperate bacteriophages has long been fruitful for molecular biologists interested in mechanisms controlling the expression of genetic information. By definition, temperate phages can either multiply lytically at the expense of their host or establish a symbiotic relationship with their host (lysogeny). During this latter tenure the host-destructive functions of the phage are repressed, and the repression system confers upon the cell immunity to further infection by the same phage. Only those functions which benefit the host and/or are compatible with the continued existence of the pair are allowed expression. This symbiotic coexistence continues until some event circumvents or disables the phage's repression system. When this happens, phage multiplication and destruction of the host may occur, leaving inert but infectious phage particles to carry on the cycle under (perhaps) more favorable circumstances. These two modes of propagation require that the phage possess two at least partially separate sets of functions and a switching system sensitive to varying circumstances. The lytic cycle itself requires that the phage be able to subvert the bacterial cellular machinery to serve its purposes. Considerable effort has been expended in the study of how these switches are effected, and how the phage programs its lytic multiplication (for reviews see Calendar, 1970; Hershey, 1971).

A. Two classes of temperate E. coli bacteriophages

The phage P2 was first isolated by Bertani in 1951 from the Lisbonne and Carrere strain of Escherichia coli. This bacterial strain was isolated in 1923, and forms the basis for the discovery of lysogeny. In addition to P2, this strain carries P1, a favorite tool of the bacterial

geneticist, as well as another P2-related phage called P3 (Bertani and Bertani, 1971). λ was discovered by Ester Lederberg in E. coli K12 in 1951. In order to compare P2 with other lysogenic phages, the Bertanis isolated 42 different temperate phages able to attack E. coli from specimens obtained at the Los Angeles County Hospital. Of these, 16 were serologically related to P2 and 12 were related to the far more widely studied λ (Bertani and Bertani, 1971). There thus seem to be at least two large classes of temperate E. coli phages.

A physical distinction, at least among several representatives of each group, has been established (Mandel and Berg, 1968; Baldwin et al., 1966; Kaiser and Wu, 1968): The DNA molecules of the phages of both groups possess "cohesive ends" i.e. 5' terminated short stretches of single-stranded DNA at either end of the molecule which are complementary, allowing the formation of hydrogen-bonded "circles", dimers, trimers, etc. The cohesive ends of phages within a group appear to be similar since they form mixed dimers. They are not similar to the cohesive ends of members of the other group since they will not form mixed dimers.

Other differences are known which differentiate between the two groups: λ and its relatives are inducible, i.e. exposure of a lysogen of these phages to UV irradiation, mitomycin C, or thymine starvation induces excision of the phage DNA and lytic multiplication. Exposure of lysogens of the P2 class of phages to the same conditions does not usually induce (Bertani and Bertani, 1971). Members of the λ group are able to replicate their DNA in bacteria lacking the rep function (Denhart et al., 1967), those of the P2 family are not (Calendar et al., 1970). Members of the λ group exhibit a recombination frequency more than 100-fold

greater than P2 (Lindahl, 1969a; Campbell, 1971). Members of the P2 group, although possessing less DNA (approximately 20 million daltons as opposed to approximately 30 million daltons for λ Geisselsoder and Mandel, 1970) put together a somewhat more elaborate package: P2 and its relatives have rigid contractile tails with base-plates, spikes, tail fibers, collars, etc. (Inman et al., 1971; Geisselsoder and Mandel, 1970; Baldwin et al., 1966), while λ has a flexible non-contractile tail with only a single short fiber (Kellenberger and Edwards, 1971). Finally, although both insert their DNA into the bacterial chromosome, lysogens of members of the λ group have their DNA integrated at only one of two unique sites on the bacterial chromosome, one near the genes for galactose utilization, the other near those for tryptophan biosynthesis. On the other hand, individual lysogens of P2 can carry phage DNA in more than one site, and lysogens carrying a single P2 copy vary with respect to its location (Six, 1963; Bertani and Six, 1958). The most common P2 attachment sites are cotransducible with his and ilv respectively. The P2 related phage 186 attaches to the chromosome near recA (Abe and Tomizawa, 1971). Another P2-related phage, 299, shares at least one attachment site with P2 (Sunshine, 1972).

The emergent picture is that of two groups of bacteriophages which perform quite similar functions in somewhat dissimilar fashions. A closer look at the similarities and differences of the physical properties of their DNA and its transcriptional expression should yield information on the control mechanisms they utilize.

B. Positioning on the genetic map

Figures 1 and 2 demonstrate that the genetic maps of both phages

Fig. 1

The Lambda Map

Distances between genes are taken from Campbell (1971), transcription patterns and % GC distributions are taken from Szybalski, (1970), and the expanded "regulatory region" is taken from Reichardt and Kaiser (1971).

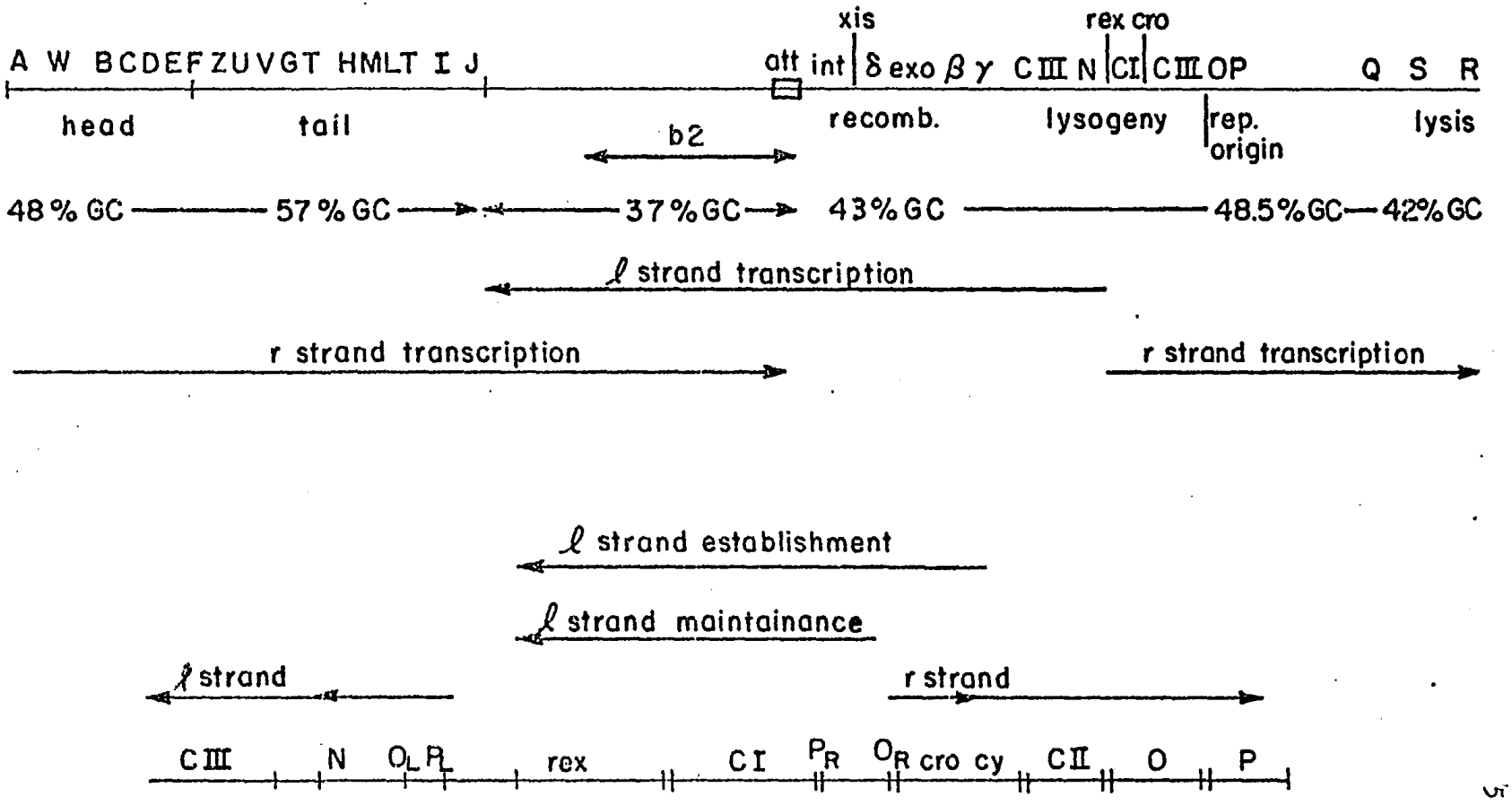
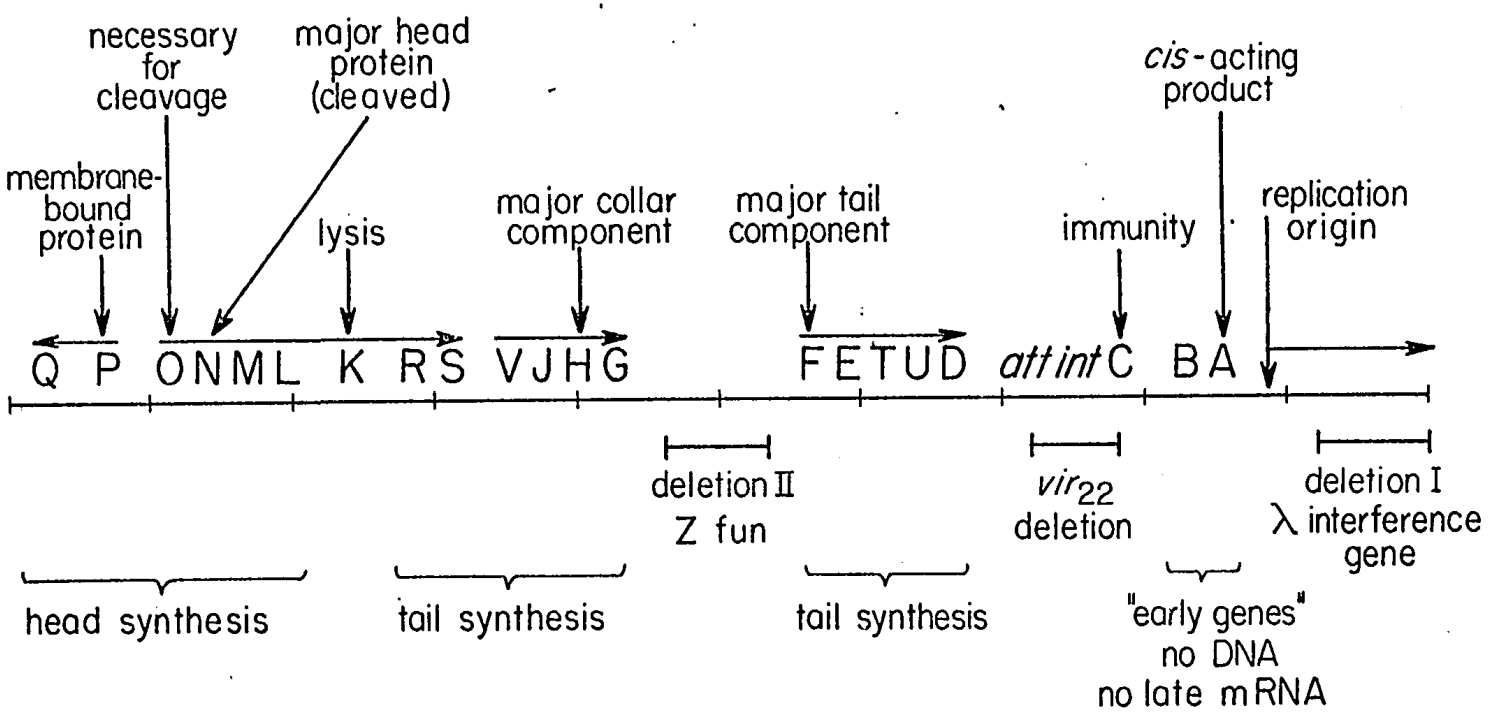

xis
rex cro
A W BCDEFZUVGT HMLT I J
att int δ exo β γ CIII N CI CIII OP Q S R
head
tail
b2
recomb.
lysogeny
rep. origin
lysis
48% GC
57% GC
37% GC
43% GC
48.5% GC
42% GC
ℓ strand transcription
r strand transcription
r strand transcription
ℓ strand establishment
ℓ strand maintainance
ℓ strand
r strand
CIII
N
OL PL
rex
CI
PR
OR cro cy
CII
O
P

Fig. 2

The P2 Genetic Map

The gene order is according to Lindahl (1969a, 1970, 1971) and Sunshine et al., (1971). The functions of the various phage particle genes are according to J. Levy (in preparation). The replication origin was located by Schnös and Inman (1971), and the location of the deletions in vir_{22} and Del_1 by Chattoraj and Inman (1972). The deletion in Del_2 has been located by Chattoraj (Pers. Comm.). Phenotypically, vir_{22} is $\underline{att}^-$, $\underline{int}^-$, and C^- (Bertani, Pers. Comm., Calendar, Pers. Comm.). Del_1 is heat resistant and $\underline{old}^-$ (Bertani, Pers. Comm.). Del_2 is $\underline{fun}^-$ (Chattoraj, Pers. Comm.). The "early" gene nutants in A make no phage DNA, those in B, very little (Lindqvist, 1971). The function of genes A and B with respect to "late" mRNA is inferred rather than proved (Linqvist, in preparation, this work)

Genetic and Physical Map of Phage P2



show nearly absolute clustering of genes for each type of function: In the case of λ, the repressor gene, CI is flanked on both sides by early genes. On the left, and proceeding left-ward is the N gene, the CIII gene (involved in lysogeny), a group of genes involved in recombination and turn-off of host functions, and two genes involved in the recombination events which insert into and excise from the bacterial genome. Immediately after these latter two is the site at which they operate, the attachment site, followed by the b2 region whose function is not known and which becomes severed from the rest of the early genes upon insertion.

On the right, and proceeding right-ward are the cro gene, regulating repressor, and other early gene production, and CII, also involved in establishing lysogeny. The DNA replication origin is next and then the O and P genes, which are necessary for replication, and then the activator gene, Q. Beyond the Q gene are two late genes R and S (concerned with cell lysis). These are connected by virtue of fusion of the cohesive ends to the late genes which appear on the left arm of the linear map. Genes A thru F are concerned with the production of phage heads, Z through J with phage tails. Gene A, which is the closest known gene to the left cohesive end, appears to code for a product which produces these ends (Wang and Kaiser, in press).

The known genetic map of P2 shows a similar type of grouping in that the int gene and the attachment site are to the immediate left of the repressor gene C. The two known "early" genes, A and B, lie to the right of C. Those genes known to be concerned with head formation are clustered in one group, those concerned with tail formation in two groups separated by two nonessential genes. One of these latter, fun, is known

to be expressed from the prophage and converts the cell to enhanced sensitivity of fluorodeoxyuridine, the other Z, affects the phage's ability to lysogenize.

C. Intramolecular heterogeneity in base composition of λ DNA

Skalka, Burgi and Hershey (1968) have mapped the λ DNA molecule with respect to its GC content. In an elegant series of experiments, they first used controlled shearing to cut the molecule into various sized pieces. Next, they took advantage of the fact that Hg^{++} ion binds preferentially to AT pairs, and separated these pieces on the basis of density in a Cs_2SO_4 gradient. Finally, by annealing the cohesive ends and sedimenting through a sucrose gradient, they were able to separate the "outside" from the "inside" pieces. The right and left halves of the molecule were placed with respect to the genetic map (Kaiser, 1962; Radding and Kaiser, 1963) by means of the helper dependent transformation assay. They determined that the DNA is heterogeneous with respect to GC content*, fairly large segments varying between 37% and 57% GC, and located these segments along its length.

Figure 1 shows the placement of these various segments with respect to the genetic map. (Distances between most genes are based on recombination frequencies. The exact placement of the b2 region and the CI gene were made by heteroduplex electron microscopic analysis (Westmoreland et al., 1969). The replication origin on the physical map was located by Inman and Schnös, 1971). There appears to be at least some correlation between GC content, the function of various genes, and the timing of their

*GC content refers to the amount of guanine plus cytosine. AT refers to the amount of adenine plus thymine.

expression in the lytic cycle. The segment with the lowest GC content is expressed early, but is dependent on N gene activation as are those genes adjacent to it. These adjacent genes, the recombination and host-turn-off functions, are part of a 43% GC segment. The N gene itself and the "immunity region" (CI, rex as determined by heteroduplex $\lambda, \lambda i^{434}$ mapping) appear to be part of the larger 48.5% GC segment though the precision of the shearing method is not good enough to fix the boundary with respect to the N gene. Those genes on the immediate right of the immunity region are also part of 48.5% segment. Those genes concerned with the construction of the phage particle, namely those on the left arm of the linear map are all contained in a segment of much higher GC content - 57%.

Perhaps of significance is the fact that those genes on the left which are expressed under the influence of the N gene are of low GC content. Those on the right are of higher GC content, approaching that of bacterial DNA (50% Handbook of Biochemistry, 1968). They are not expressed as intensively (Szybalski et al., 1970). Szybalski attributes this to lower transcription due to a weak promoter, however, so this difference in GC content may be fortuitous.

Falkow and Cowie (1968) have demonstrated that this heterogeneity in base composition is reflected in the thermal denaturation profile of the phage DNA. By plotting the increment of change in optical density at 260 nm per degree rise versus the temperature (in effect a first derivative plot), they were able to detect 4 rather distinct "regions" in both ø80 and λ. An early-melting region and therefore presumed low GC "region" was missing in the b2 deletion of λ. This mutant is known to be missing a section of DNA corresponding to the 37% GC segment described by Skalka

et al., (1968). Consequently, the existence of "regions" of differential denaturation can provide information on intra-molecular heterogeneity of base composition.

D. Events leading to lysogeny

The DNA of both phages circularizes by virtue of its cohesive ends. The circular forms thus generated are intermediates in at least the first stages of phage DNA replication (Schnös and Inman, 1971; Lindqvist, 1971; review by Kaiser, 1971). They are also necessary for insertion of the phage DNA into the bacterial chromosome (review by Gottesman and Weisberg, 1971), a necessary event in the establishment of stable lysogeny. This insertion occurs by a reciprocal recombination event occurring between the bacterial chromosome and a specific site on the phage chromosome (Gottesman and Weisberg, 1971; Calendar and Lindahl, 1969). The recombination event is mediated in both cases by a gene called int. (Also, in both cases, the product of the int gene mediates site-specific recombination in lytic multiplication (Weil and Signer, 1965; Echols et al., 1968; Lindahl, 1969b)).

Repression of lytic functions enabling the establishment of lysogeny has been shown, in the case of λ, to be dependent on the products of three genes, a mutation in any one of which produces a clear-plaque phenotype (Kaiser, 1957). That these genes code for proteins is demonstrated by the finding of nonsense mutants in all three of them. A fourth type of mutation, called cy (see Figure 1) appears to be a structural mutation since it is cis-dominant. The course of events in those cells destined for lysogeny has been described by Reichart and Kaiser (1971) and Echols and Green (1971) and appears to be the following: Transcription begins at the two promoters P_L and P_R. Leftward transcription produces the gene

N product which in turn allows transcription to proceed past itself through CIII, the recombination region (which includes int), and on into the b2 region. Rightward transcription proceeds through cro, CII, O and P. Replication of the phage DNA now begins. The products of CIII and CII act at the cy site initiating leftward transcription back through the cro gene in the opposite direction, the CI (repressor gene), and rex. This is very intensive transcription, resulting in a rapid rise in repressor concentration, and also serves to at least partially block rightward transcription through the replication genes. The concentration of repressor soon becomes high enough to block further transcription of lytic genes and replication (a genome which is bound by repressor can not replicate, (Thomas and Bertani, 1964); the product of gene Q appears to be necessary for transcription of a repressed genome - review by Dove, 1971). The repressor not only blocks transcription by binding to O_L and O_R, but also activates the promoter for its own transcription, thus allowing repressor to be made when the rest of the genome is shut off. Sufficient int product has been made by this time to induce a recombination event between the bacterial chromosome and one of the copies of the chromosome present. The rest of the copies are diluted out by subsequent cell division.

Once lysogeny has been established, the product of the CI gene is sufficient to maintain this state. Support for this comes from two sources. Ptashne (1967a) has isolated the CI repressor, and shown that it binds to two sites on λ DNA (Ptashne, 1967b); this repressor has been shown to repress λ transcription in vitro (Steinberg and Ptashne, 1971; Wu et al., 1971). In vivo transcription experiments (Taylor et al., 1967; Szybalski, 1969) have demonstrated that in a λ lysogen, only a short segment of the λ genome is transcribed, and this has been localized to the "immunity

region" comprised of CI and rex.

Very little is known about the events leading to lysogeny by P2. Replication does not seem to be necessary, since A and B amber mutants which do not replicate, do lysogenize at fairly high frequencies (Lindahl, Personal communication). Also, lysogens of Rep⁻ bacterial can be obtained. Nevertheless, lysogeny can occur after some replication has taken place (Bertani, 1962). There is some evidence that the int gene is expressed in the presence of repressor (L. E. Bertani, 1968; Sunshine, 1972). There are at least two genes which must function for lysogeny to occur with P2, the genes, C and Z. The C gene lies between the int on the left and the two early genes A and B on the right. The Z gene is located between two operons governing phage tail production. There are two genes remote from the C gene, old and fun, which are expressed from the prophage. This implies that either the repressor acts in several widely spaced places or that the lytic genes cannot be expressed without the mediation of an early gene.

The process of induction of lysogens of these phages also differs. Both phages require the action of an additional gene (xis in λ - Guarneros and Echols, 1970; and cox in P2 - Lindahl and Sunshine, 1972) for excision from the chromosome. However, P2 belongs to the non-inducible class. It cannot be induced by the action of UV or other treatments that interfere with DNA metabolism. The explanation for this phenomenon may be trivial - it may reflect a lack of an ind site on the repressor (Jacob and Campbell, 1959; Ptashne, 1967b). Nevertheless, a further difference remains. Inactivation of a thermosensitive repressor leads, in the case of λ to very efficient induction, and the production of a normal burst of phage. The same maneuver with a P2 lysogen carrying a thermosensitive repressor

produces loss of immunity and cell death, but very little phage (L. E. Bertani, 1968). Excision/induction events do occur, however, as evidenced by the presence of infections phage particles in a liquid culture of a lysogen.

L. E. Bertani (1970) has proposed that the P2 attachment site lies between the int gene and its promoter (or within the int gene). Thus, when P2 DNA is inserted into the bacterial genome, they become separated and int cannot be transcribed. On the other hand, Calendar et al., (1972) have isolated from a P2 mutant carrying a thermosensitive repressor, a second-site mutant which is capable of thermal induction. This mutant, called nip, produces int product from the prophage (Sunshine, 1972). It does form relatively stable lysogens, and a lysogen carrying it is stable at the nonpermissive temperature if it also carries a wild-type prophage. This second mutation has been tentatively mapped between int and C.

E. The course of lytic growth

The course of lytic growth of λ has been quite thoroughly mapped out,and is known to take place in several steps. The first step is the circularization of the phage DNA. This is apparently mediated by host functions since it occurs on superinfecting an immune lysogen, a situation in which practically all phage functions are repressed.

When the repressor is not present or is inactivated, the (unaltered) bacterial polymerase can begin transcribing on either side of the CI gene; leftward through N, and rightward through cro. The product of the N gene is required for full expression of all further functions. Under its influence, transcription proceeds leftward through the recombination genes, past the attachment site, and into the b2 region. Transcription also

proceeds rightward through cro, CIII, O and P. This transcription is less efficient than the leftward, and actually proceeds rightward through the entire genome. Nevertheless, replication occurs because the products of the O and P genes, the replication genes, are needed only in catalytic amounts.

The product of gene Q is necessary for the dramatic increase in transcription of the late genes (those responsible for head and tail formation and cell lysis). Mutants in Q exhibit delayed lysis and very small phage burst. There appears to be an "essential site" at which the Q product acts and which Szybalski has postulated to be a promoter. (See Szybalski, 1971; Dove, 1971 for review of above). The combination of the Q product and the multiplicity of DNA copies now available allows for the large production of protein necessary for the production of phage particles. Since the host macromolecular synthesis has now been shut off by the action of the δ, exo, β, and γ genes (Cohen and Chang, 1972), and the cro gene has shut off early gene transcription, nearly all the energies of the cell are now devoted to producing phage particles.

In summary, λ development is programmed by at least four regulatory mechanisms. Negative regulation is provided over all λ growth by the action of the repressor protein of the CI gene. If this protein is not functioning, two sequential positive regulation events occur, that mediated by the N gene followed by the action of the Q gene. It is not known how these last operate. There is some evidence that the N gene affects the bacterial RNA polymerase; bacterial nutants which oppose the action of the N gene have altered polymerase (Georgeopoulous, 1971; Glyson and Pironia, 1972). Negative regulation of early function is provided late in infection by the product of the cro gene.

Once again, very little is known about P2 lytic expression and its regulation. The gene C product is necessary for negative regulation (repression) to maintain the lysogenic state, since inactivation of it in lysogens leads to cell death. The function of the other lysogenic gene, Z, is unknown. It is probable that the repressor gene acts only in one spot since virulent mutants can be obtained with relatively high frequency and are single mutations (Bertani and Bertani, 1971; Lindahl, 1971; Sunshine, Personal communication). There is some evidence that the int gene is not under negative regulation (L. E. Bertani, 1968; Sunshine, 1972), but the significance of this is obscure.

The two genes A and B are called early simply because mutations in either prevent phage DNA replication. They are also necessary for cell killing (cells infected by B mutants do lyse eventually but it is not known whether this is because the requirement for B gene product is not absolute or that the B mutants are slightly leaky (Calendar, Personal communication)). Mutants in gene A cannot be complemented, implying that their protein product (there are amber and temperature sensitive mutants in this function) can only service the genome which coded for it. They also cannot complement any other phage mutants except those with lesions in gene B implying that they do not make significant quantities of necessary phage proteins except B gene product (Lindahl, 1970). (Two other phages have cis-acting replication genes; S13 - Tessman, 1966 - and - ØX174 - Borrias et al., 1969. Frank and Ray (1972) have found that gene 1 mutants in ØX174 do not make a specific "nick" in the replicating form I, a double-stranded covalently closed circle). Since P2's late genes, namely those concerned with particle production and cell lysis, are grouped

together in much the same way as they are in λ, it may be that they are subject to some form of positive regulation. The product of gene B seems a likely candidate for this function.

Even though P2 was discovered at about the same time as λ, the elucidation of it's functioning has lagged far behind. The major reason for this is the very low recombination frequency which P2 exhibits. It has only been in recent years that enough information has been available to construct a genetic map. Now that a fairly detailed map is available, it is possible to investigate the properties of defined genes. It was the purpose of this work to find out if P2 (a) exhibits intramolecular heterogeneity of base composition, (b) if so, whether this heterogeneity could be correlated with function, and if it exhibits the same correspondence of timing with GC content, and (c) if its transcription patterns could be demonstrated to be subject to some form of regulation.

MATERIAL AND METHODS

A. Chemicals

Tryptone broth, yeast extract, and nutrient broth were obtained from Difco.

Polyethylene glycol, MW 6000-7500 was obtained from Matheson, Colemen and Bell.

Optical grade CsCl was obtained from Schwartz/Mann

99% pure CsCl was obtained from the Rare Earth Division, American Potash and Chemical Corporation, West Chicago, Ill., 60185.

Optical grade CsCl was obtained from Schwartz/Mann.

99% pure CsCl was the Rare Earth Division, American Potash and Chemical Corporation, West Chicago, Ill., 60185.

Optical grade Cs_2SO_4 was obtained either from Schwartz/Mann or Gallard Schlesinger.

Enzymes used were obtained from Sigma.

^{3}H-uridine was obtained from Schwartz/Mann at a specific activity of 10-27 Ci/mmole.

Reagent grade phenol was distilled into distilled H_2O and was stored under the same H_2O in a dark bottle at 4°C.

All other chemicals used were reagent grade or were further purified.

All solutions were made with either singly or doubly distilled water.

B. Solutions and Media

LB is 10 g. Bacto-tryptone, 5 g. yeast extract, 10 g. NaCl, 1 g. glucose per liter, pH 7.4.

Davis-CAA is 14 g. K_2HPO_4, 4 g. KH_2PO_4, 1 g. Na_3-citrate. $5H_2O$, 8 ml.).1 M $MgSO_4$, 2 g. $(NH_4)_2SO_4$ per liter, autoclaved.

For use this is supplemented with 2 ml. 20% glucose, 2.5 ml. 10% Casamino Acids which had been filtered through activated charcoal, trace metals, and 90 ml. sterile distilled H_2O per 100.

A medium-CAA is 15 g. KH_2PO_4, 3.6 g. $(NH_4)SO_4$, ca. 65 ml 1 N NaOH (to bring final pH to 6.8) per liter, autoclaved. To this is added 16 ml. 0.1 M $MgSO_4$, 2 ml. 0.05 M $CaSO_4$, 25ml. 25% NaCl, 100 ml. 10% glucose, and 100 ml. 10% Casamino Acids.

TM is 0.01 tris-HCl (pH 7.1) + 0.01 M. $MgSO_4$.

SSC is 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0.

KAB is 0.01 M $MgCl_2$, 0.1 N NaCl, 0.005 M $CaCl_2$.

Bacterial strains were:

Cla, F^-, prototrophic, (Bertani and Bertani, 1970).

C10, F^-, endo I^-, (Hoffman-Behrling, 1968).

C1055, F^+, a multi-auxotrophic strain described by L. E. Bertani, (1968) used as indicator for P2 strains not carrying amber mutations.

C1748, F^-, (P2) from Cla by lysogenization, for growth and titering of $P4vir_1$.

C1757, F^-, arg^-, try^-, Tl^r, str^r, $SupD^+$ (Sunshine et al., 1971).

C1792, F^-, arg^-, his^-, try^-, $SupF^+$ (Sunshine et al., 1971).

Phage strains were:

P2 wild-type (Bertani and Bartani, 1971).

$P2vir_{22}$ (Lindahl, 1971; Chattoraj and Inman, 1972).

$P2vir_1$ (Lindahl, 1971).

$P2amP_{137}$ (Sunshine et al., 1971).

$P2amO_{71}$ (Sunshine et al., 1971).

$P2amF_4$ (Sunshine et al., 1971).

$P4vir_1$ (Six and Klug, in preparation).

C. Phage growth and strains used

The general techniques described by Adams (1959) were used for assays of phage and bacteria.

Phage were grown by infection of the appropriate host either in 400 ml batches of LB on a shaker bath or in 40 l batches in A medium-CAA in a biogen. Amber mutant phage stocks were grown from a single plaque and were tested for reversion by plating on a nonpermissive host. They were concentrated either by differential centrifugation (two cycles) or by precipitation with polyethylene glycol. This last procedure consisted of spinning down the bacterial debris from the lysate, making it 0.5 M in NaCl and 10% by weight with polyethylene glycol, and allowing it to stand at least overnight at 4°C. The precipitate was then spun down and was dissolved in an appropriate amount of buffer.

Phage to be used for infection were not purified further. Phage to be used for DNA preparation were further purified by at least one and often two buoyant density bandings in CsCl. It was found that extracting the phage band by inserting a hypodermic needle just below the band and withdrawing it without disturbing the rest of the gradient gave cleaner preparations than dripping through the bottom of the tube. After one DNA preparation seemed to be contaminated with bacterial DNA, those preparations used to make sheared halves were treated with 10 μg pancreatic DNAase/ml. for 10 min at 37°C before the final banding in CsCl.

D. Thermal denaturation

P2 phage which had been purified by two cycles of differential centrifugation and resuspended in TM at a concentration of 2-5 x 10^{12} p.f.u./ml. were extracted with phenol which had been redistilled, stored under distilled water at 4°C, and equilibrated with 0.2 M tris at pH 8.0 just prior to use. Extraction was done by gently inverting the rubber-capped tube by hand for 3 min at room temperature followed by brief centrifugation to break the emulsion, and was repeated three times. The aqueous layer was then dialyzed against 8-500x volumes of SSC over a period of three days at 4°C and stored at 4°C in this condition. Over the night prior to its use, it was dialyzed against one 1000x volume of SSC/10 at $O.D._{260}$ of 0.5-0.7.

The absorbance at 260 nm was measured in a Perkin-Elmer Model 202 dual beam spectrophotometer whose output was displayed on the Y axis of a Moseley Autograph Model 7000A X - Y recorder. The sensitivity of the recorder was set to produce a pen displacement of 10 inches/0.15-0.2 absorbance units.

Sample volumes were 1.4 ml. in a 1.6 ml. thermal-jacketed cell of 1 cm path length. Temperature control was maintained by circulating water from a MGW Lauda Model K-2 circulator ($\pm$ 0.2°C) through the jacket of the cell. Temperature was monitored by a thermistor press-fitted in a glass or Teflon sleeve which was inserted into the solution but out of the light beam. The thermistor comprised one leg of a Wheatstone bridge the output of which was put onto the X axis of the plotter. Temperature could be read to 0.1°C. The thermoregulator of the water bath was driven by a 1/4 RPM synchronous motor providing a continuous temperature increase of

1°C/8 min. After each run, the thermistor was calibrated against the water bath thermometer at 2°C intervals.

Analog to digital conversion was accomplished on an OSCAR F/DCF strip chart and film digitizing system, Computer Industries, Van Nuys, California, with 10-15 samplings taken per 1°C. Computer processing linearized the logarithmic temperature axis using a logarithmic interpolation between the calibration values, compensated for any recorder offsets necessary to keep the pen on scale, and calculated the first derivative of the resultant curve at each point by a linear least squares analysis of the point in question and three points on each side of it. The absorbance and derivative values were scaled between 1 and 100, and 1 and 50 respectively, and these arrays used in a plotting routine utilizing a CalComp plotter.

E. Buoyant density of DNA strands

The procedure used follows that of Hradecna and Szybalski (1967) fairly closely. This procedure was evolved to produce a minimum of strand breakage, and consists of disrupting the phage, denaturing the DNA, and complexing it with ribopolynucleotides (if present) in a single step. Phage at an $O.D._{260}$ of about 0.5 are dialyzed against a low ionic strength solvent such as 10^{-3}M EDTA or SSC/10. To a small quantity of this (typically, 0.25 ml.) is added ribopolymer if used, in an amount from 1-3x that of DNA. To this is added a few microliters of a 30% sarkosyl solution and sufficient IN NaOH to bring the pH up to 9-10. This mixture is heated at 90°C for 3 min with gentle swirling, and immediately plunged into ice. To this is added sufficient room temperature saturated CsCl solution buffered at pH 8.5 with 0.01 M tris to bring the density up to about 1.72 g/cc. It was found unnecessary to use optical-grade CsCl if the solution was first treated with activated charcoal, filtered through

paper, and then through a Millipore 0.45 micron filter. Centrifugation was in a Spinco Model E analytical ultracentrifuge, 25°C, 44,770 RPM, 20-24 hr in a 12 mm 4°C sector Kel F cell. The UV optical system was used to photograph the cell, and a Densicord Recording Electrophoresis densitometer, Photovolt Corp., New York City, with a 3/1 reduction gear adaptor and a 1 mm aperture used to trace the resulting photograph.

Using the small amount of P2 DNA which was not denatured as reference, and taking its density to be 1.7095 (Bertani et al., 1967), the densities of the single-stranded bands were calculated by the relationship:

$$\rho_{unk} = \rho_{std} + 0.0092\ (r^2_{unk} - r^2_{std})\ g/cc \text{ at } 44,770 \text{ RPM}$$

where r's are distances from the center of rotation in cm.

F. Preparative scale separation of DNA strands

It was found that poly UG gave slightly better separation of strands than did poly IG, though the difference was not great, so it was used in almost all preparative runs. It was obtained from Miles Laboratories, was stored at -20°C in solid form, and solutions of about 1 mg/ml in distilled water made up immediately prior to use. The procedure paralleled that described above except that enough phage and ribopolymer was used to provide 150-200 μg of DNA and slightly more ribopolymer per centrifuge tube. Centrifugation was done in a Spinco Model L2-65B preparative ultracentrifuge at 5°C, 30-32,000 RPM 60-90 hr, in a type 40 or type 65 angle-head rotor. The resolving power of an angle-head rotor is greater than that of a swinging bucket rotor due to the shallower gradient produced for a given rotor speed, and the amplification of this factor in parts of the tube when it is placed in an upright position (Flamm, Bond and Burr, 1966).

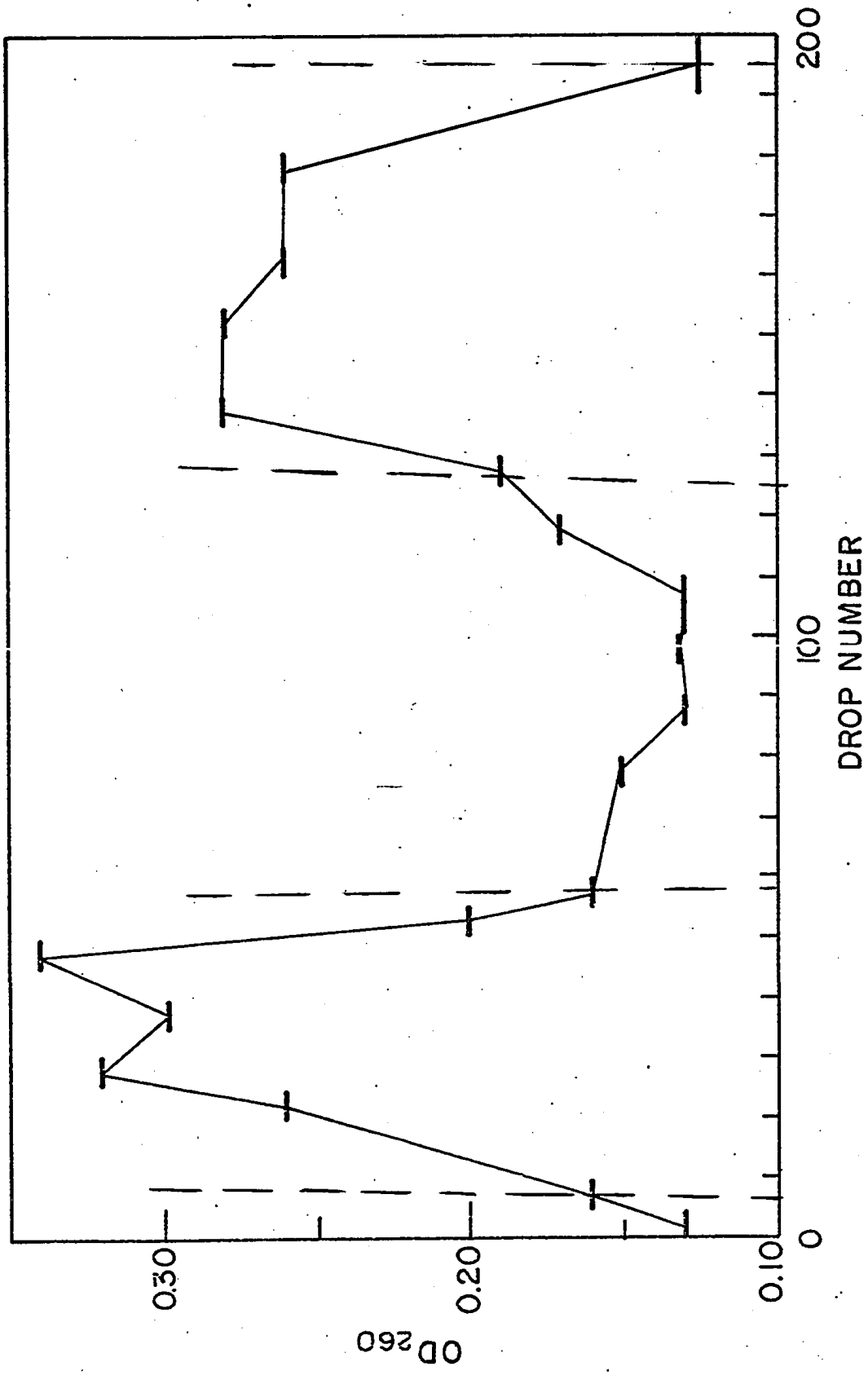
To check on the position of the bands, a small amount of every preparation was run in the analytical ultracentrifuge at 44,770 RPM 18-20 hr, 25°C. This allowed a choice in the sampling technique to be used for each run - if the bands were near the bottom of the cell, samples were taken by puncturing the bottom of the tube, and it was not necessary to take small samples throughout the entire gradient. If, on the other hand, the bands were nearer the top of the tube, sampling was done by forcing heavier CsCl solution into the bottom and collecting from the top. Some difficulty was encountered with this technique, however, due to incomplete flushing out of the mineral oil used to overlay the DNA solution and fill the tube. Generally, samples of 7-10 drops were taken in the part of the gradient containing DNA. Tubes used to collect samples were cleaned in Alconox solution, rinsed with distilled water, and baked overnight in a 120°C oven.

Several methods of assaying the $O.D._{260}$ of the resultant samples were tried all of which had some drawbacks. The most effective and least wasteful was to put 50μl in a Pyrocell microcuvette with a 2 mm path-length. This was then read in a Beckman Model DU spectrophotometer which had been fitted with a carefully positioned pin hole aperture. Since a slight gradient of ribopolymer exists in the centrifuge tube, there is no proper "blank", and the cuvettes were read against air. Generally every second or third sample was read since the peak absorbances were contained in 5-7 samples. Figure 3 is a gradient from which 5 drop samples were taken throughout and every other sample read. The samples containing the absorbance peaks were pooled in the manner indicated in Figure 3. From 3-10 samples between the peaks were discarded. The tubes for each peak were then rinsed by pouring about 0.25 ml. CsCl solution from one tube

Fig. 3

Separation of P2 DNA Strands on a Preparative Scale

Procedure as described in the text. Centrifugation at 32,000 RPM, 7° C., 64 hr. 5 drop samples were taken from the bottom of the tube. OD_{260} readings are of 1/10 dilutions of part of every other sample in a 10 mm. light path cuvette.


0.30
0.20
0.10
OD_{260}
0
100
200
DROP NUMBER

to the next, and this was added to the pooled samples.

The pooled samples were then self-annealed by heating at 65°C for 2 hrs. No attempt was made to eliminate the associated ribopolymer after treatment of the first preparation with ribonuclease and subsequent dialysis as described by Hradecna and Szybalski (1968) resulted in complete loss of DNA. The binding of ribopolymer to DNA is weak compared to that of homologous RNA, and after this initial disappointment, it was felt that any unnecessary manipulation of the rather fragile single-stranded DNA should be avoided. Bøvre and Szybalski (1971), in a chapter of Methods in Enzymology, seem to have arrived at the same conclusion. To check on cross-contamination of the individual strands, a 1/10 dilution of the final preparation in CsCl solution of the appropriate density was run in the Model E. In this case, two cells containing the individual strands were run in the rotor at the same time. One of these had a side wedge window at the bottom. Pictures of the individual cells were taken by placing a mask in the UV optical track at a point past the camera lens but in front of the long tube which comprises the major distance from the lens to its focal point where pictures are taken, in such a manner as to block off one image after the other. Great care must be taken to position this mask precisely as the dimensions at this point are small, and any incomplete masking of the unwanted image will produce spurious results. Care must also be taken to replace the dust cover flush against the tube, since light leaks here are disastrous. The separated strands in CsCl solutions were stored at -20°C. The solution does not freeze at this temperature.

G. Isolation and purification of RNA from phage infected cells

Cells of the appropriate host were grown to an $O.D._{590}$ of about 0.5 in Davis-CAA medium at 37°C with aeration. 10 ml. of this culture was

spun down in the cold, and cold phage solution plus enough cold KAB were added to make a volume of 1.5 ml. The mixture was vortexed to resuspend the cells, placed at 4°C for 5 min. to allow for absorption.

It was then placed in a 37°C incubator for 5-10 min. to allow injection to occur, and was immediately poured into 5 ml. of prewarmed medium on the 37°C shaker bath. At appropriate times, without stopping the shaker, 10 microcuries of ^{3}H-uridine were added from a syringe. The pulse was stopped 2-3 min. later by pouring the entire culture into a cold 50 ml. beaker containing 2 1/2 ml. of the same medium which had been frozen, crushed, and refrozen. The beaker was swirled until most of the frozen medium had melted, and was subsequently stored in ice.

Three or four separate cultures were pulsed in this manner, after which the cells were spun down in the cold in two 7 x 75 mm test tubes for each culture. To each tube was added 0.1 ml. 5 x 10^{-3}M K_3 citrate pH 6.0, and 5 μl 25% SDS or 10 μl of 12.5% SDS. More SDS results in a large amount of sticky material at the interface in the first phenol extraction resulting in low recovery of the aqueous layer. This was then vortexed for 7-10 min. in the cold. The tubes were then warmed to room temperature, 0.1 ml. phenol at 60°C was added, and the tubes were shaken vigorously in a 60°C water bath for 3-4 min. They were then chilled, and spun for 7 min. in the cold to break the emulsion. The aqueous layer was carefully extracted with a Pasteur pipette, and the two portions from one culture combined in a fresh tube. The phenol extraction was repeated twice. The final aqueous extract was applied to the top of a Sephadex G50 solumn and flushed through with 0.06 M KCl, 0.01 M K_3 citrate, pH 6.0. Sufficient 3 M KCl, 0.01 M K_3 citrate was added to the eluate to make it 0.5 M in KCl,and it was passed through a Millipore 0.45 micron filter with very

gentle suction to prevent bubbling. At this stage, solutions could be stored frozen at -20°C for several weeks without significant loss of acid precipitable counts.

H. Hybridization in solution

This was always carried out in vials fitted with rubber inserts in their caps to prevent evaporation at the elevated temperatures of the incubation. Duplicate samples were run for each determination. The vials were acid-cleaned and oven baked. They were then autoclaved with their caps loosely fastened. For each 0.1 ml. of DNA strands in 6 M CsCl, 0.4 ml. 0.005 M K_3 citrate and 25μl of buffer-equilibrated phenol were used. The phenol appears to facilitate hybridization in an unknown manner (Hill, 1967), and helps inactivate any RNAase or DNAase which might be present. The high salt concentration of the strand preparation should be diluted enough to attain a final Na^+, K^+, Cs^+ concentration of 0.4-0.6 M (Bøvre and Szybalski, 1971). After all additions had been made, the vials were capped tightly, gently rotated to assure good mixing, and incubated at 62° - 65°C.

After incubation, the vials were rapidly chilled to minimize non-specific DNA-RNA complexing, then 5 ml. 0.5 M KCl, 0.01 M K_3 citrate at room temperature added. 20μl of a solution containing 300 units of T1 RNAase and 1 mg pancreatic RNAase/ml were added, and the solution was allowed to stand 15 min at room temperature. It was then filtered slowly through pre-washed S&S B6 filters, washed with 5 ml. 2X SSC under slow filtration, and 40 ml. 2X SSC under rapid filtration, placed in counting vials, dried at 80°C for 1/2-1 hr.

5 ml. of scintillation fluid consisting of 4 g PPO and 0.1 g POPOP per liter of toluene were added. The vials were placed in the Packard Tri-carb liquid scintillation counter and allowed to cool at least an hour before

counting. Each vial was counted twice for 5 or 10 min and the counts averaged and computed as CPM.

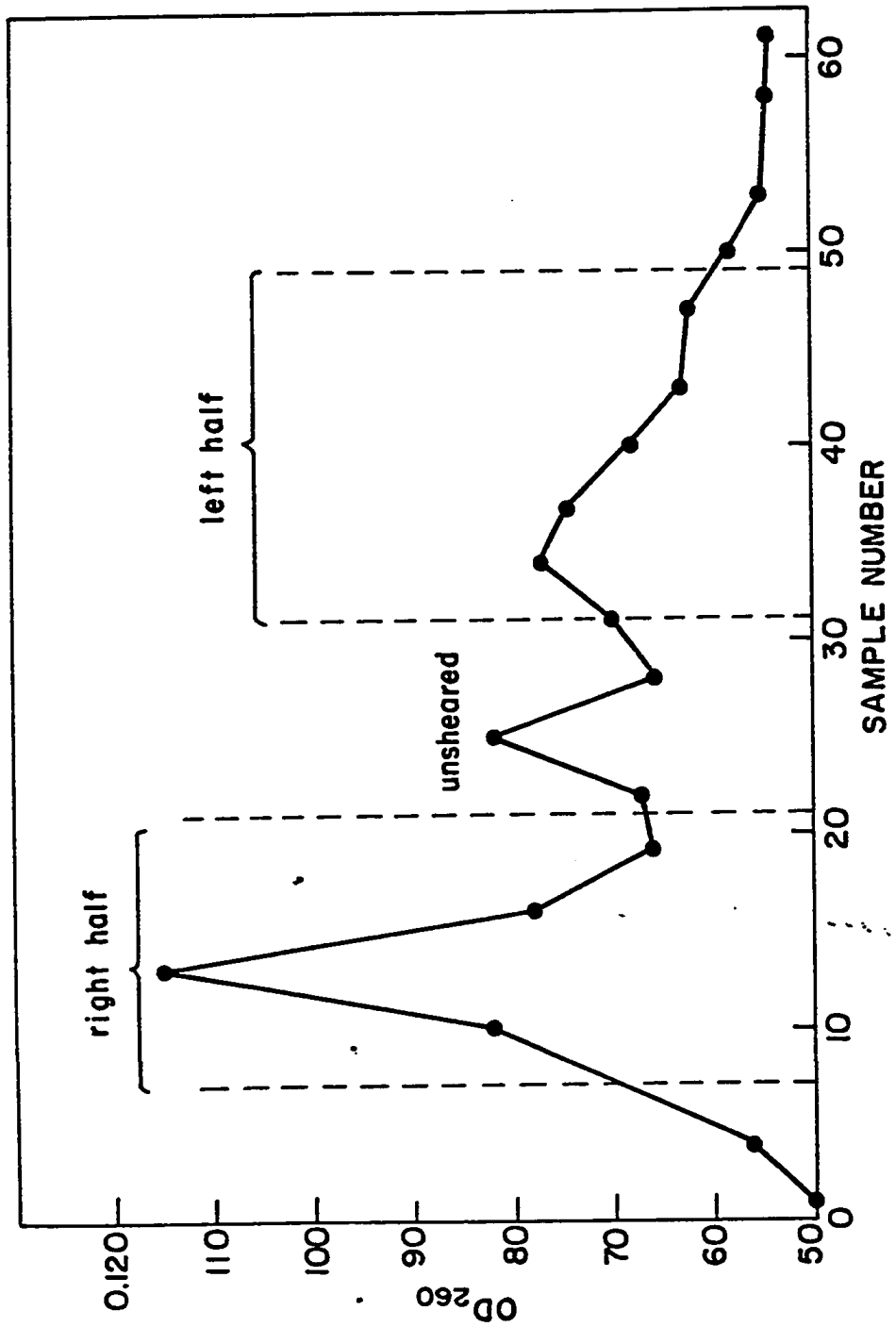
I. Preparation of sheared halves by stirring

The DNA solution at an $O.D._{260}$ 0.1–0.5 was dialyzed overnight against 1000 volumes of 0.1 M Na_2SO_4, 0.005 M $Na_3B_4O_7$, pH 9.2 at 4°C. It was then heated at 75°C for 6 min to dissociate cohesive ends and rapidly chilled in ice. 20 ml. of this solution was placed in a 20 x 70 mm pyrex tube. Stirring was accomplished with a Vir Tis 35 mm blade whose tips had been bent at 90° angle and trimmed such that it fit into the tube with about 1–2 mm clearance. A Heidolph High Torque stirrer was used at rates of 2800–3200 rpm (3200 rpm was the maximum stirring which did not produce bubbles). The rate of stirring was monitored by a General Radio Corporation Strobotac stroboscopic light. The motor was capable of maintaining its rate to ± 50 rpm. At 4°C(cold room), breakage was very nearly complete in 2 hrs. 14.3 g Cs_2SO_4 was then added to the cold solution, and after the salt was dissolved, the whole solution was poured into another container which held sufficient 0.001 M $HgCl_2$ to produce a mercury to nucleotide ratio of 0.28–0.34. The density of the solution was adjusted to 1.55 using refractive index as indicator. 0.7 ml. of this solution was run in the Model E analytical centrifuge at 44,770 rpm, 22°C, 20–24 hr to check on completeness of shearing and the position of the bands. The remainder of the solution was placed in two nitrocellulose tubes and run in a type 65 preparative rotor at 30–34,000 rpm, 7°C, 70–90 hrs. Samples were taken from the preparative run by puncture of the bottom of the tube, assayed, and subsequently pooled as shown in Figure 4.

Fig. 4

Preparative Separation of P2 DNA Halves Sheared by Stirring

Procedure as described in the text. Centrifugation at 34,000 RPM, 5° C., 70 hr. 6 drop samples taken from the bottom of the tube. OD_{260} readings are of undiluted material, every third sample, in a 2 mm. light path cuvette.


right half
unsheared
left half
OD_{260}
0.120
110
100
90
80
70
60
50
0
10
20
30
40
50
60
SAMPLE NUMBER

J. Preparation of sheared halves by forcing through a capillary

It was found that previous methods of phage DNA extraction and storage produced DNA that was nicked significantly as determined by band sedimentation through alkali. The following procedure is used in the laboratory of M. Chamberlin and was adapted here. The phage are extracted at an $O.D._{260}$ of 10-15 by phenol. This is done in specially cleaned Corex tubes with silicone rubber stoppers. The phage-phenol emulsion is gently agitated for 10 min at room temperature, and is then centrifuged to break the emulsion. The phenol is withdrawn and is replaced with clean phenol and the procedure repeated twice. The DNA is dialyzed on a rapid-dialyzer (Englander and Crowe, 1966, 1966) against two changes of appropriate buffer (in this case 0.1 M Na_2SO_4, 0.005 $Na_2B_4O_7$) in a period of two hours in a cold room. Over the next two days the dialysis fluid is changed every 8-10 hrs. Dialysis was judged complete when the dialysis fluid contained no absorbance at 270 nm. It was further checked by a detailed absorbance curve of the DNA. The DNA was stored at 0^oC in an ice bucket since freezing appeared to produce some nicks. On the evening prior to shearing, it was checked in the Model E by sedimentation in alkali in a band-forming cell. The extent of shearing was also checked in this manner.

Shearing was done in the laboratory of Dr. J. C. Wang using an apparatus designed by him. The capillary and its two reservoirs are suspended in a water bath. Nitrogen under pressure is applied alternately to one side until its reservoir is nearly empty, and is then switched to the other reservoir until it is nearly empty. 15 ml. of DNA at an $O.D._{260}$ of 0.2 in 0.1 M NaCl was sheared at a time at a temperature of 25^oC and a pressure of 50 psig. A total of 200 two-way passes were made in a period of 3 hrs.

The solution was then made up to a density of 1.546 g/cc. with optical

grade Cs_2SO_4, and sufficient HgCl added to give a molar Hg/DNA phosphate of 0.3. This was centrifuged for 78 hrs at 5-10°C, 32,000 RPM in a type 40 rotor. Part of it was also centrifuged for 20 hr in the Model E at 44,770 RPM to check on the position of the bands. Figure 5 is a Joyce-Loebel tracing of the photograph of that run. 10 drop samples were taken from the bottom of the tube, and the $O.D._{260}$ read. Figure 6 is a graph of the readings. Samples were pooled as shown in the figure. They were then dialyzed into Na_2SO_4-borate buffer and stored at 0°C.

K. Loading of sheared DNA onto filters

3 ml. of the DNA solution plus 3 ml. of 5 x SSC were treated with 0.1 ml., 10 N NaOH for 15 min in the cold. The solution was then neutralized with conc. HCl. It was found necessary to repeat this procedure twice with the right half and 3 times with the left half. The increase in O.D. at 260 nm of the neutralized solutions was 35% with the right half and 30% with the left. 42 ml. 5X SSC was then added, and 2 ml. of this solution applied to pre-soaked filters under very slow filtration. This provided 2 μg DNA/filter. The filters were then washed with 10 ml. 5X SSC, allowed to dry at room temperature overnight. They were then baked in an 80°C oven for 2 hrs, and placed in a vacuum dessicator until used.

L. Hybridizations conditions for DNA on filters

Previous experimentation had determined that 2 μg DNA was required to hybridize all of the usually applied mRNA. A salt concentration approximately that of 2X SSC gave the most efficient hybridization. At least 40 hrs at 65°C was required. The efficiency was about half of that obtained with separated strands in liquid.

Three filters (which had been labelled with pencil) were used per

Fig. 5

Analytical Ultracentrifuge Determination of Position of Separated P2 DNA halves Sheared by Forcing Through a Capillary

Procedure as described in the text. Centrifugation at 44,000 RPM, 22 hr., 24° C. Photograph of the solution traced with a Joyce-Loebl recording densitometer.

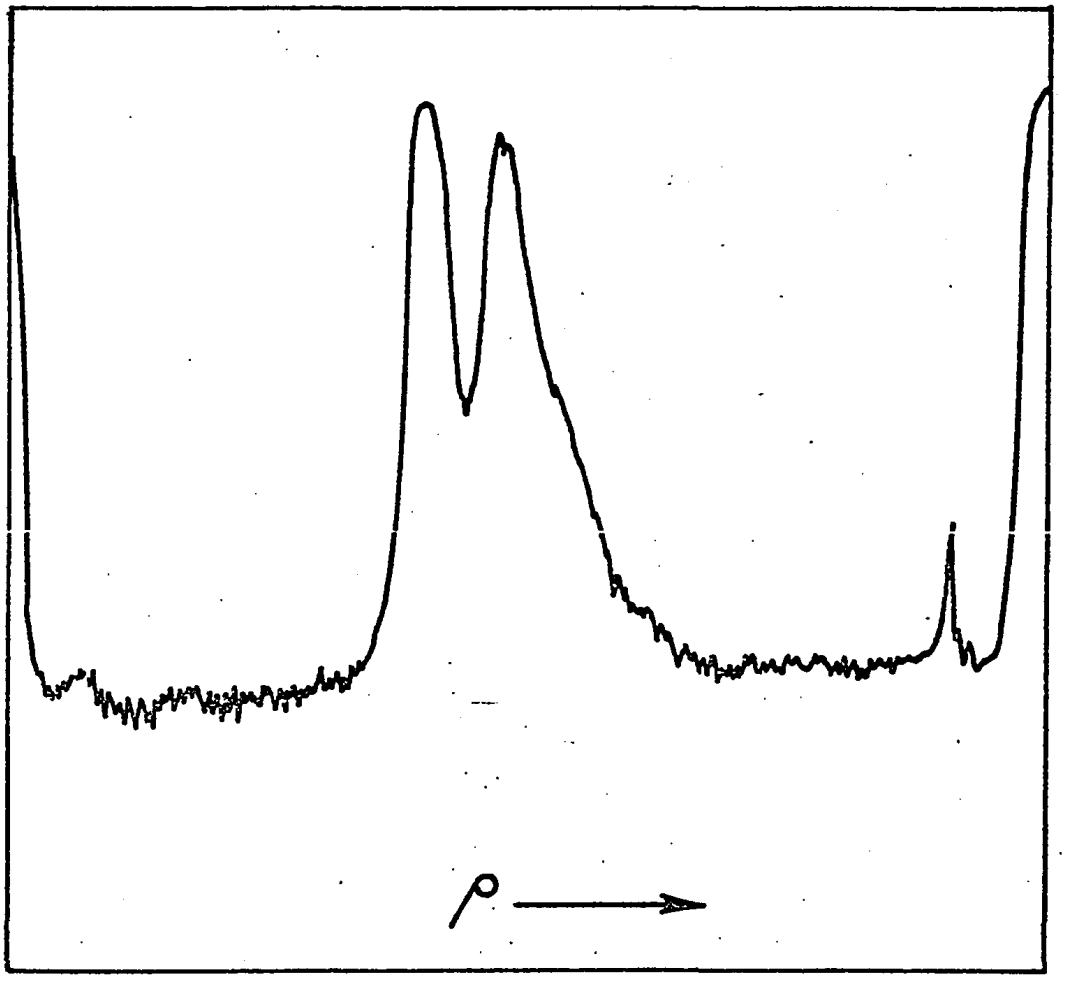
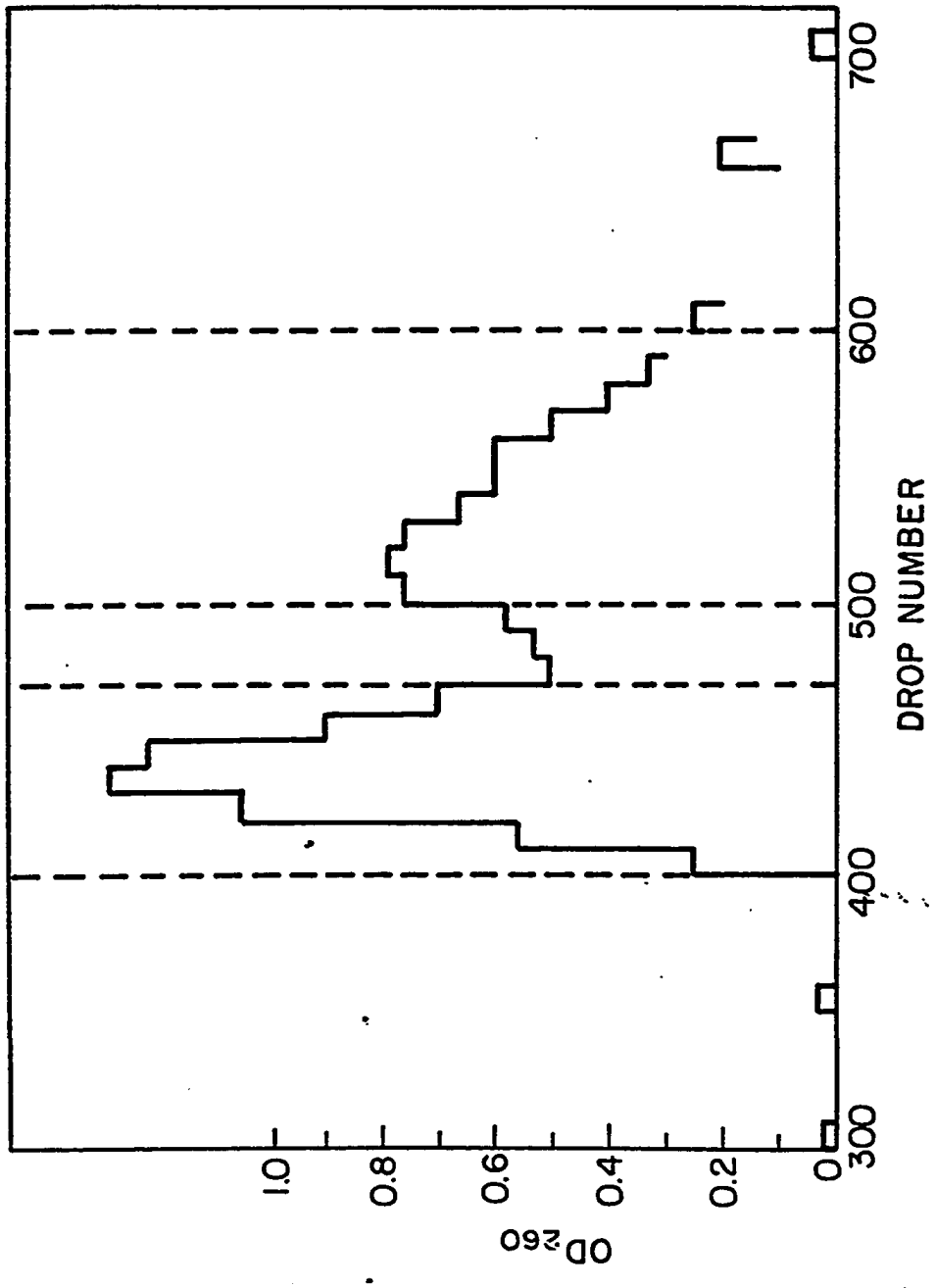

ρ

Fig. 6

Preparative Separation of P2 DNA Halves
Sheared by Forcing Through a Capillary

Same solution as in Fig. 5. Centrifugation at 32,000 RPM approximately 7°C., 90 hr. 10 drop samples taken from the bottom of the tube. $O.D._{260}$ readings are of 1/10 dilutions of part of every sample in a 10 mm. light path cuvette.


700
600
500
400
300
DROP NUMBER
1.0
0.8
0.6
0.4
0.2
0
OD260

vial; one loaded with right half DNA, one with left half DNA, and a blank. The hybridization mixture contained in addition to the labelled RNA and sufficient 2X SSC to make 0.4 ml., 20 μl of buffer-equilibrated phenol. The vials were then tightly capped and placed in a water bath at 65°C for 42-48 hr. 5 ml. of 2X SSC was added, and also 20 μl of the RNAase solution described before. They were vortexed for 10 seconds and allowed to stand for 1 hr at room temperature. The solution was then poured off, and 5 ml. 2X SSC added. This was vortexed for 30 seconds and the solution poured off. The wash was repeated 2 more times. The filters were placed in counting vials, dried at 80°C for 1/2 hr. Counting solution was then added, the vials allowed to cool for 1/2 hr in the counter, and the samples counted twice for 10 min.

M. Denaturation mapping of sheared halves

The procedure followed was that described in Schnös and Inman (1970), and was performed in the laboratory of Dr. Inman with the aid of Dr. D. K. Chattoraj. The denaturation was done at pH 11.03 for 15 min at 0°C. The electron microscope technique and computation procedures are described in detail by Schnös and Inman (1970).

It was found that the DNA had suffered considerable nicking. Figure 7a and b are histograms of the length distribution of native left half (a) and partially denatured left half (b). In both cases, the peak of the distribution is at around 6.5 microns, but the native DNA contains a larger amount of longer pieces. Figures 8a and b, the right halves show this much more dramatically.

Nevertheless, it is possible to demonstrate from the denaturation patterns that the DNA which banded at the higher density corresponds to the right half of the molecules as described by Inman and Bertani (1969),

Fig. 7

Length of Fragments in Left Half Preparation

Native a) (pH 9.89) and partially denatured b) (pH 11.03). Histogram generated as described by Schnös and Inman (1970).

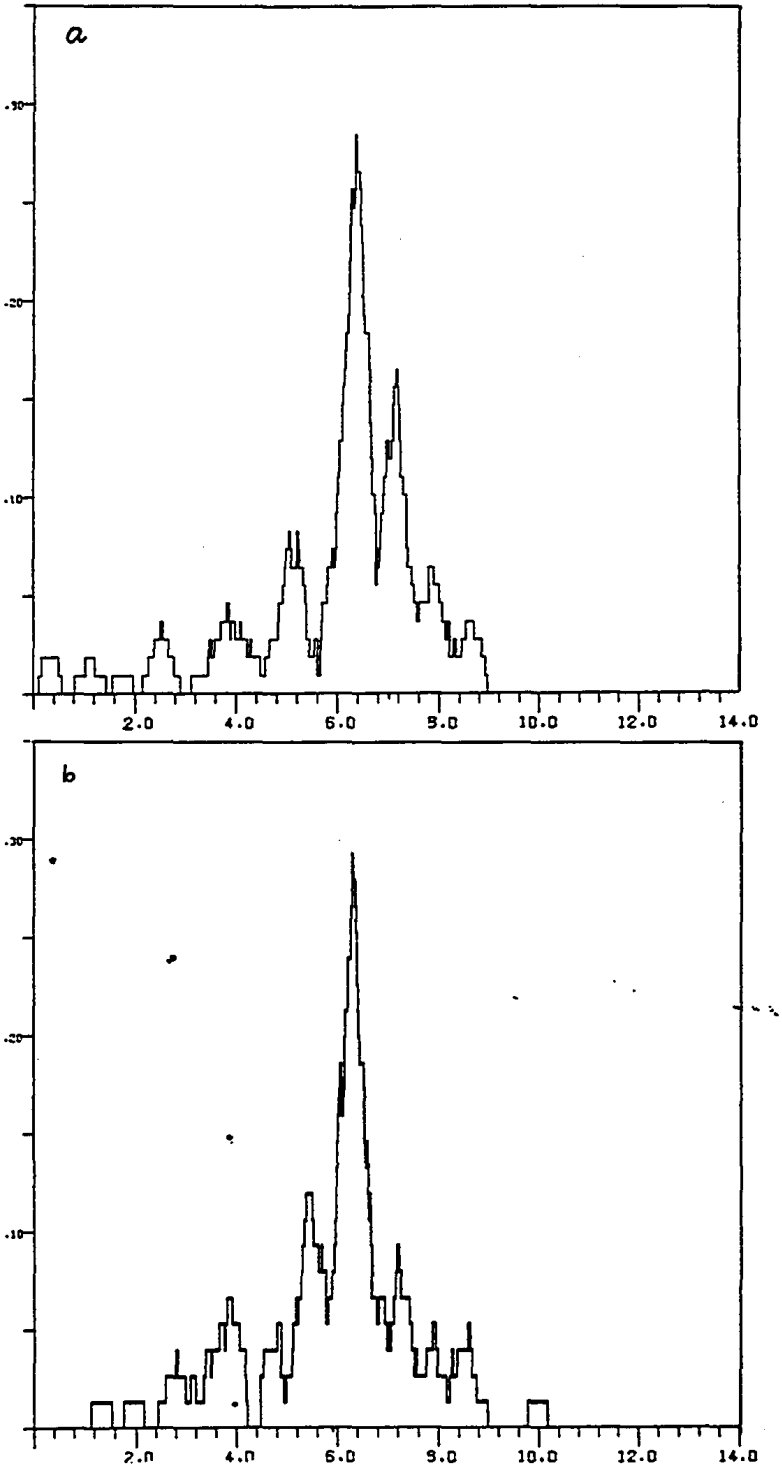
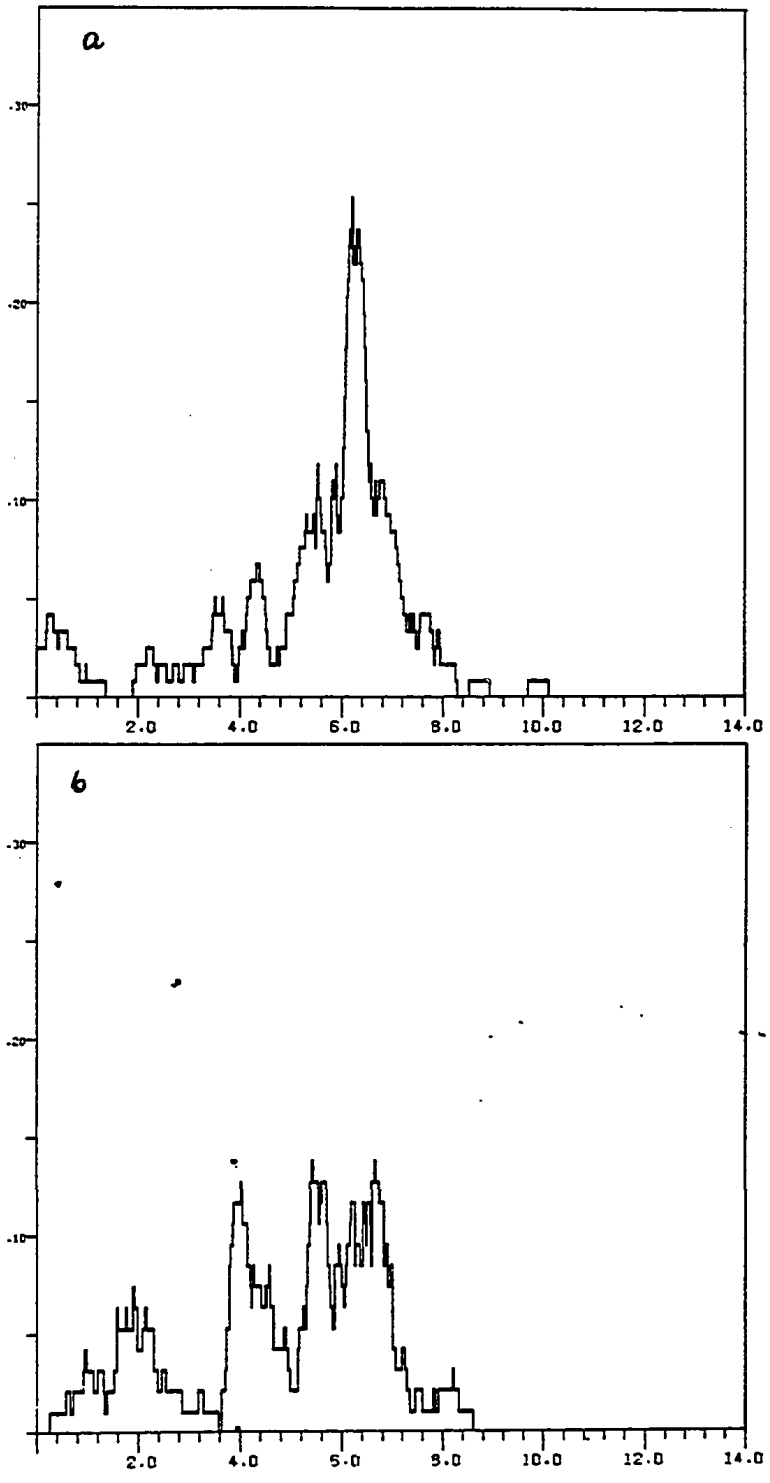

a
2.0
4.0
6.0
8.0
10.0
12.0
14.0
b
2.0
4.0
6.0
8.0
10.0
12.0
14.0

Fig. 8

Length of Fragments in Right Half Preparation

Native a) (pH 9.62) and partially denatured b) (pH 11.03) Histograms generated as described by Schnös and Inman (1970).


a
.30
.20
.10
2.0
4.0
6.0
8.0
10.0
12.0
14.0
b
.30
.20
.10
2.0
4.0
6.0
8.0
10.0
12.0
14.0

and the lighter band corresponds to the left half. (Chattoraj and Inman (1972) have attempted to demonstrate that the right half of the denaturation map corresponds to the right half of the genetic map). Figures 9a and b are weight average histograms of the denatured regions observed in the "left half" and "right half" preparations respectively. Figure 10a is a combined weight average histogram of all molecules examined. Figure 10b is a histogram of the whole P2 DNA examined by Schnös and Inman (1971). The whole P2 DNA was not quite as denatured as the sheared halves preparation, and the sheared half data is somewhat blurred due to the presence of several molecules in the preparation that were internal pieces. (All molecules in the sheared samples were either aligned at 0 or 12.5 microns). Four regions of denaturation are clearly discernable, however, and those that are on the right half of the whole molecule plus the early-melting region in the middle are present in the "right half" preparation. Conversely, the one region on the left half of the whole molecule and also the middle region are present in the "left half" preparation.

In order to obtain an estimate of the amount of cross-contamination between the two samples, the computer print-out of each molecule in the set was closely examined for each sample. Most were definitely assignable to the "half" from which they were assumed to originate. A few were obviously from the other "half". A considerable number of the smaller molecules were not assignable to either half. A lower estimate of the amount of cross-contamination can then be derived by summing the molecular length of these definitely wrong half molecules and dividing it by the total length of molecules examined. An upper estimate can be derived from the summation of the "wrongs" and those that are unassignable. Table 1a is a listing of these calculations.

Fig. 9

Denatured Sites in Left and Right Half Preparations

Weight average histograms of denatured sites of molecules in left a), and right b) half preparations partially denatured at pH 11.03. Computation and histogram generation as described by Schnös and Inman (1970).

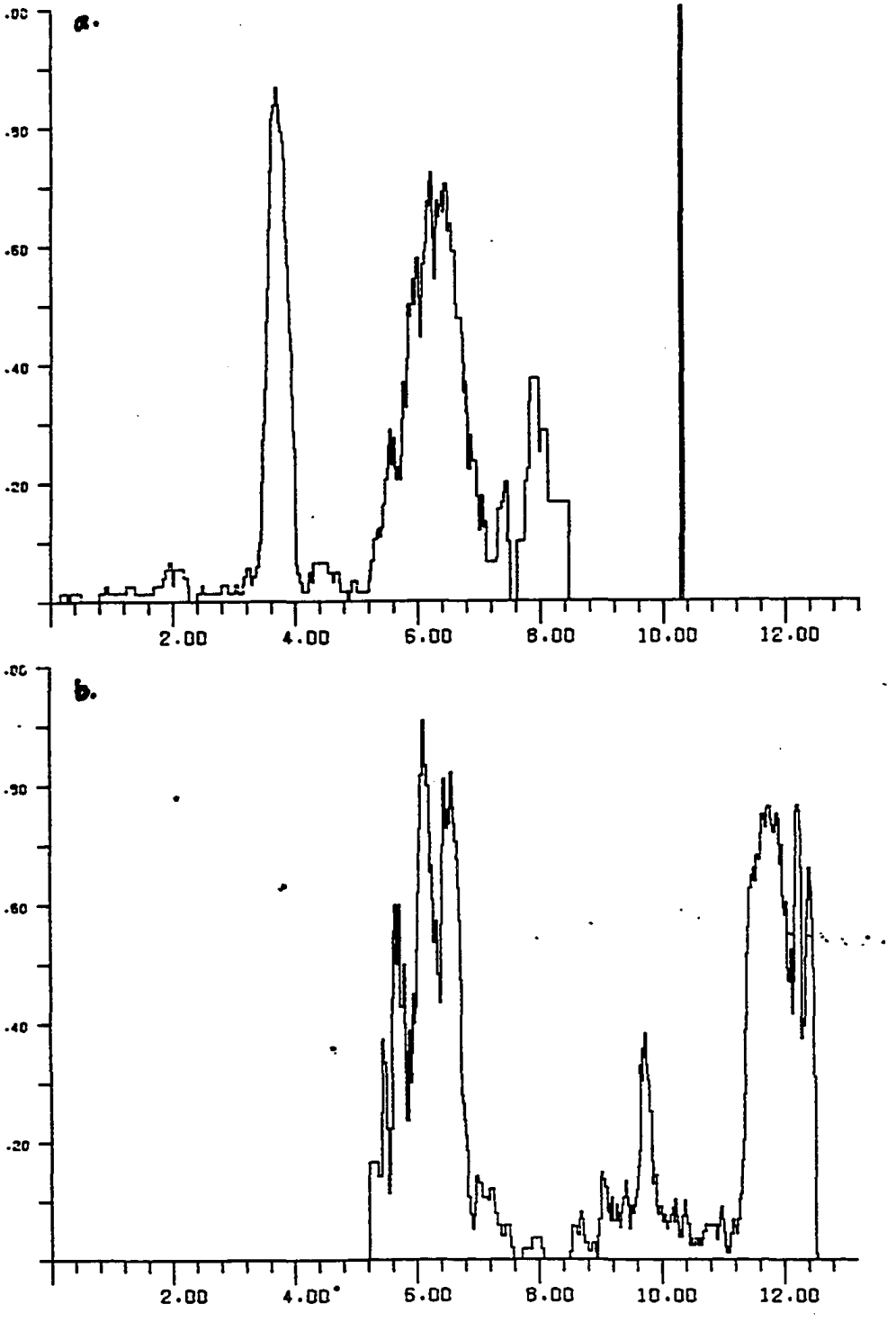
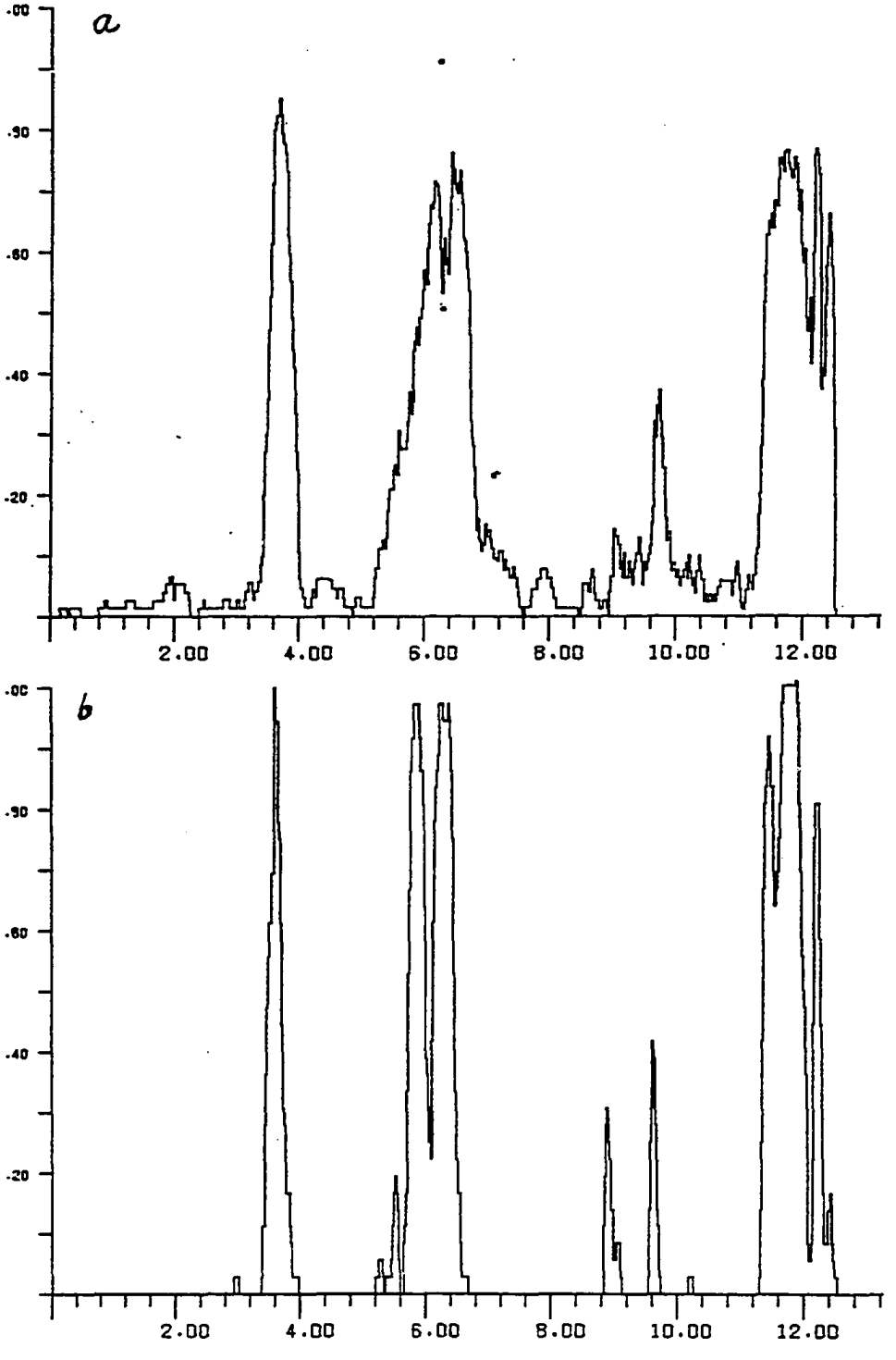

a.
.00
.80
.60
.40
.20
2.00
4.00
6.00
8.00
10.00
12.00
b.
.00
.80
.60
.40
.20
2.00
4.00
6.00
8.00
10.00
12.00

Fig. 10

Comparison of Sheared Halves with Whole Molecules

Weight average histograms of a), all molecules in left and right halves combined, and b), those whole molecules described by Schnös and Inman (1971).


a
.00
.90
.80
.40
.20
2.00
4.00
6.00
8.00
10.00
12.00
b
.00
.90
.80
.40
.20
2.00
4.00
6.00
8.00
10.00
12.00

A second source of cross-contamination is the inner ends of those molecules in the preparation which are longer than half a molecule. Since some breakage was observed in the partially denatured preparations, undenatured, or native molecules were used to estimate this error. The length of all molecules over 6.25 was summed, and from this value the total length of that many molecules of length 6.25 subtracted. Table 1b is a compilation of these calculations. It must be understood that the size of the samples used for these estimates were not large, and that they are estimates only, not quantitative assessments.

Table 1

IMPURITY ESTIMATION

	LEFT HALF	RIGHT HALF
a) Cross contamination - from partially denatured molecule data		
No. of molecules examined	75	94
Number av. molecular length (microns)	5.93	4.85
Total length of molecules examined (microns)	444.5	455.7
Total length unambiguously wrong half	23.7	17.8
Total length unassignable	12.8	32.1
Lower limit = wrong half/total length x 100	6.7%	3.9%
Upper limit = wrong+unass./total length x 100	9.6%	10.9%
b) Middle Overlap - from native molecule data		
No. of molecules examined	109	118
Number av. molecular length	5.99	5.40
Total length of molecules examined	653.3	637.7
Number of molecules greater than 6.25 microns	64	44
Total length of molecules g.t. 6.25 microns	450.8	306.4
No. of molecules g.t. 6.25 x 6.25	400.0	275.0
Total length of overlap	50.8	31.5
Length of overlap/total length x 100	8.2%	4.9%

RESULTS

A. Thermal denaturation

Figure 11 is an example of a computer plot of the melting of P2 DNA. The squares mark the absorbance curve, and it is obvious from this curve that the transition is broad and marked by steps. The "first derivative" curve amplifies the step-wise nature of the transitions by displaying the steep portions as peaks and the flat portions as valleys. At first considerable difficulty was encountered in reproducing the absolute temperatures. The span of the transition remained constant as did the fine-structure features, but the mid-point temperature varied as much as $3^{o}C$. Initially a glass sleeve was employed to house the thermistor, and was wrapped with teflon tape and then press-fitted into the cuvette. Some evaporation occurred occasionally under this condition. A teflon sleeve was then machined and was sealed onto the cuvette with silicon rubber cement. Evaporation under these conditions was minimal. Nevertheless, the mid-point temperature continued to vary. A radiograph of the sleeve with thermister inserted was taken, and it was found that the thermister was not all the way down in its hole probably because the fit was tight enough to trap a variable amount of air in the passage. This was remedied by cutting a small groove in the plastic cover around the thermistor.

While this work was in progress Inman and Bertani (1969) published data on thermal denaturation of P2 DNA. Their work was done with different apparatus and at discrete temperature intervals rather than with continuously increasing temperature. Professor Bertani was kind enough to provide his raw data and it was run through the computer program using one point on either side and the point in question to calculate slopes. Figure 12,

Fig. 11

Hyperchromic Shift of P2 DNA Solution

Procedure, computation and plotting as described in the text.

▫▫▫▫ relative optical density versus temperature _____ relative slope versus temperature.

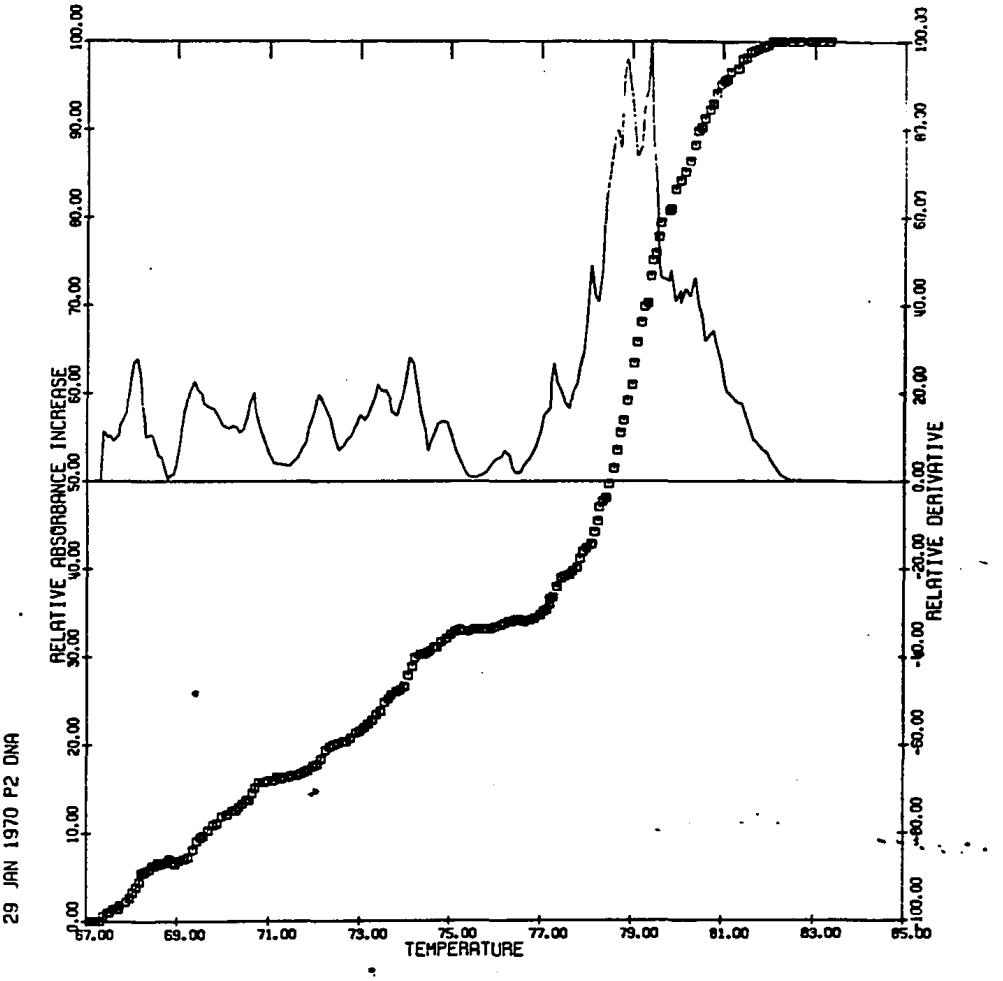

29 JAN 1970 P2 DNA
RELATIVE ABSORBANCE INCREASE
RELATIVE DERIVATIVE
TEMPERATURE
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-20.00
-40.00
-60.00
-80.00
-100.00
67.00
69.00
71.00
73.00
75.00
77.00
79.00
81.00
83.00
85.00

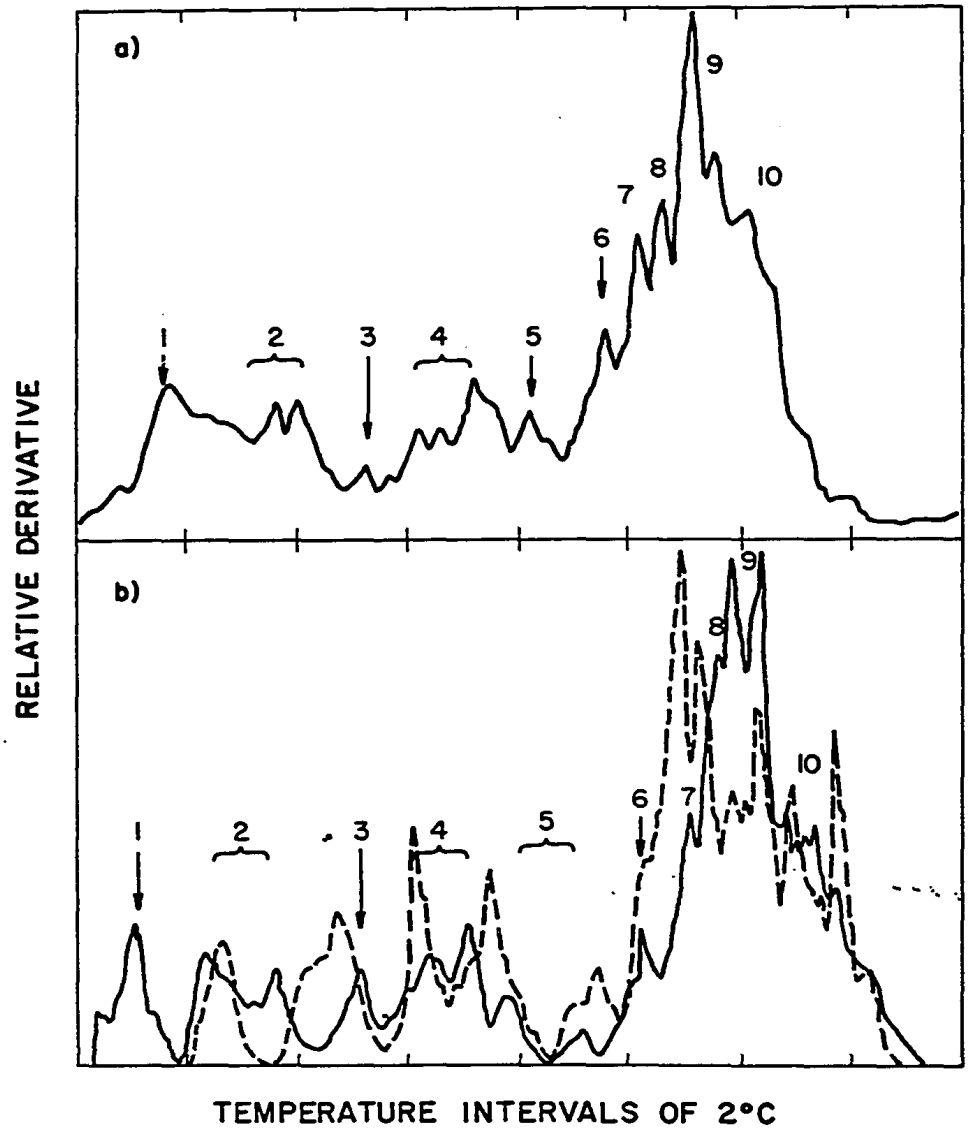
Fig. 12

Denaturation Profiles of P2 and 299 DNA

Fig. 5 of Geisselsoder and Mandel (1970).

_____ P2

- - - - - - - - 299


a)
b)
1
2
3
4
5
6
7
8
9
10
RELATIVE DERIVATIVE
TEMPERATURE INTERVALS OF 2°C

Geisselsoder and Mandel (1970), shows a comparison between the two derivative plots. Even though the two sets of data were taken in different manners and in different solvents (SSC vs SSC/10), there is considerable similarity in fine structure details. Ten groupings have been arbitrarily marked off to aid in the comparison. The dotted line in (b) of Figure 12 is the derivative curve of the phage 299. This phage is very closely related to P2, sharing all the properties of the P2 family described in the introduction, and common immunity as well. There are some phenotypic differences in host range (G. Bertani, Personal communication) and Ca^{++} requirement for adsorption. Geisselsoder and Mandel (1970), report a slightly lower molecular weight for 299 DNA than for P2 (20-21 x 10^6 vs 22 x 10^6) and a slightly higher GC content (51.7% vs 50%). A comparison of the two derivative curves shows that these properties are reflected in their thermal denaturation. Most of the structural details present in the P2 profile are also seen in the 299 profile. Conspicuously absent, however, are all of the peak designated as one on the P2 profile, and a large part of the double-peaked region designated 2. The first P2 peak comprises 6% of the total shift, and corresponds to a GC content of 35.4%. The second, 10% of the shift, and a GC content of 38.8%, 299 is missing 1/2 of this. Assuming the molecular weight of 299 DNA to be 90% of that of P2 DNA, the GC content of 299 DNA can be calculated from the following:

$$(35.4 \times 0.06) + (38.8 \times 0.05) + (GC_{299} \times 0.90) = (50.0 \times 1.00)$$

The result of this calculation is 51.7%, in exact agreement with the value obtained by Geisselsoder and Mandel (1970) from buoyant density in CsCl.

The phage 186 is also a member of the non-inducible P2 group and

its DNA has cohesive ends sufficiently similar to those of P2 to form mixed dimers with it and serve as helper for infection by DNA. It differs from P2 in several particulars including immunity and host range. Figure 13 shows the first derivative curves of P2 and 186 superimposed on each other, slightly offset on the ordinate. It can be seen that the majority of the features of the higher GC portion are present in all three DNAs, but 186 lacks even more of the low GC section than does P2. Skalka and Hanson (1972) have investigated the homologies present in the two DNAs by shearing P2 DNA into small pieces, banding it in Cs_2SO_4-Hg^{++} gradients, and hybridizing the various fractions to 186 DNA adsorbed on a nitrocellulose filter (Denhart, 1966). They find 5% of the 37% GC fraction of P2 DNA will hybridize to 186, 32% of the 49% GC will hybridize, and 62% of the 55.5% GC fraction. The same fractions hybridize to the extent of 90.9%, 83.3%, and 82.1% to P2 DNA (control to determine the efficiency of the technique). They also find that 186 DNA is 53.4% GC, a value consistent with the melting profile shown here.

Figure 14a was generated in the laboratory of Dr. Ross Inman, and is a denaturation map of 186 DNA. It was obtained from a portion of the DNA preparation used to obtain the melting profile. Figure 14b is a P2 denaturation map (Schnös and Inman, 1971) for comparison. P2 DNA has two regions that denature early, one at the extreme right end and one in the middle. 186, on the other hand, appears to have three regions that denature at approximately the same pH on the right end, and only a very small portion in the middle of the molecule of relatively low GC content. To summarize, the thermal denaturation profiles of the three phages show that all contain considerable degrees of base composition heterogeneity, otherwise stepwise profiles would not be obtained. The profiles are sufficiently detailed

Fig. 13

Denaturation Profiles of P2 and 186 DNA

First derivative curves. Procedures as described in the text. The ordinate of the 186 curve has been shifted down with respect to that of P2.

_____ P2

. 186

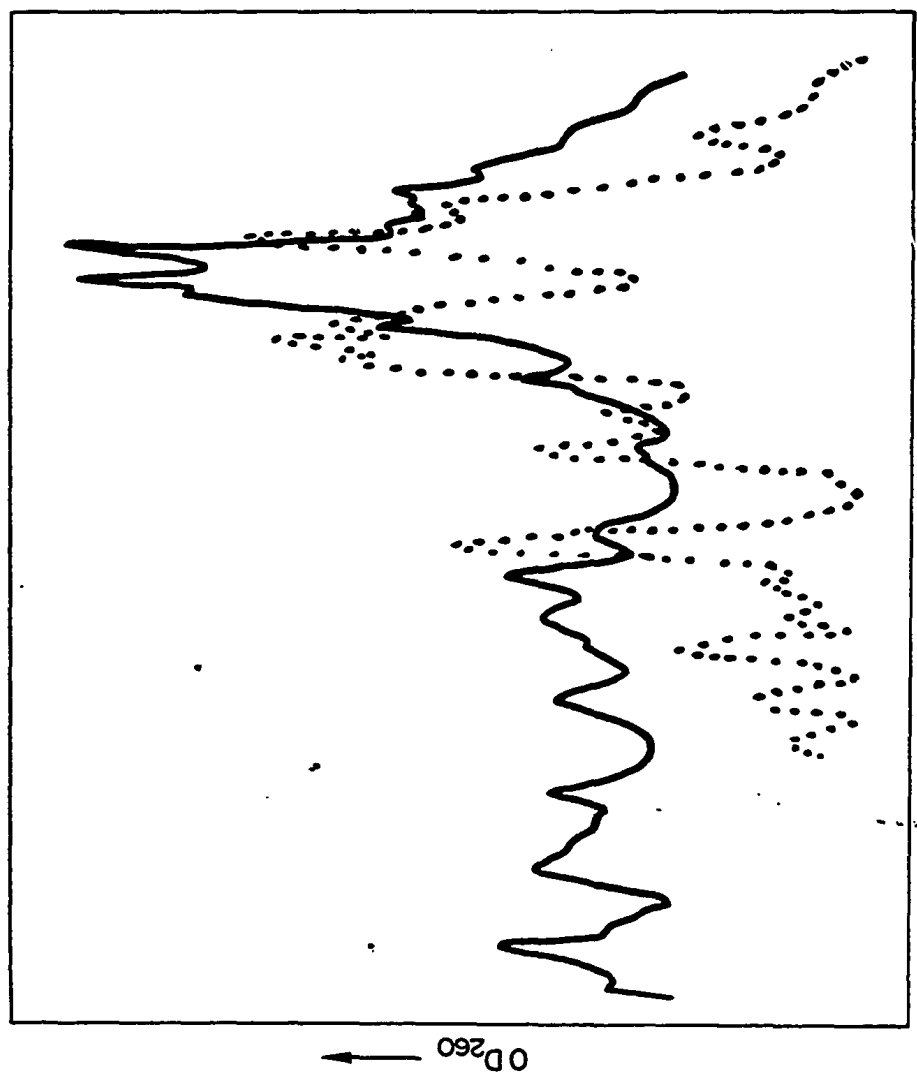
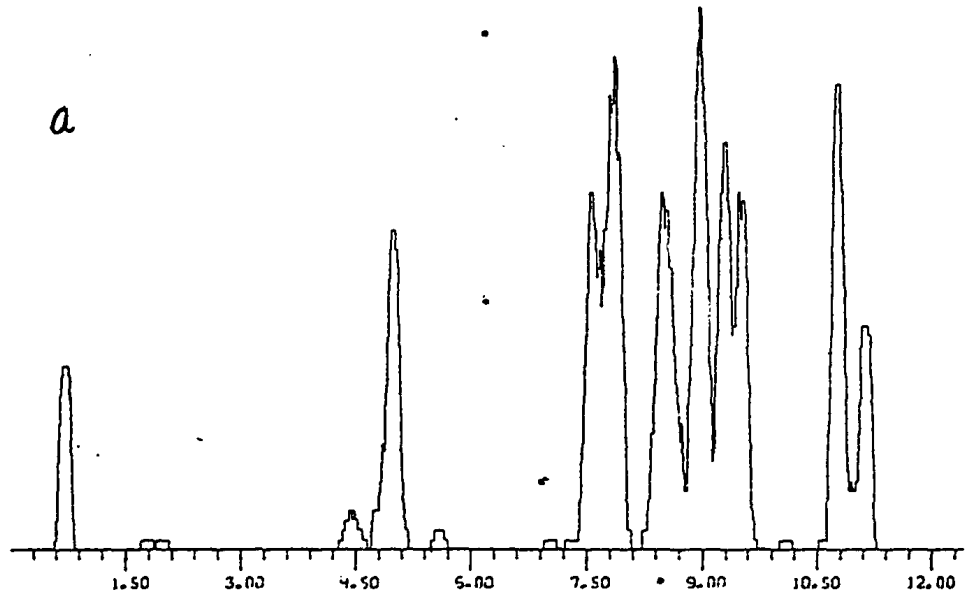
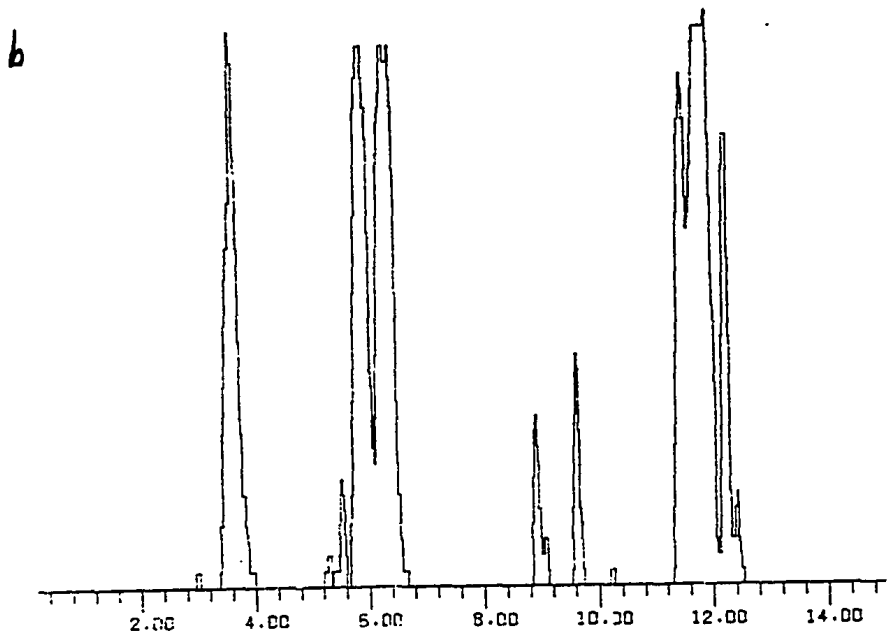

TEMPERATURE
OD_{260}

Fig. 14

Denaturation Maps of 186 and P2 DNA

a.) 186 DNA (pH 11.18), and b.) P2 DNA (pH 11.10). P2 DNA taken from Schnös and Inman (1971). Weight average histograms of denatured sites as described by Schnös and Inman (1970).


a
1.50
3.00
4.50
6.00
7.50
9.00
10.50
12.00


b
2.00
4.00
6.00
8.00
10.00
12.00
14.00

and precise to demonstrate the relationships between the two phages and point out some differences. The supporting data presented show (a) that the fraction of 186 DNA which is the least homologous to P2 DNA is indeed that of low GC content, (b) P2 DNA has distributional heterogeneity of readily denatured sites a large number of which are clustered at one end of the molecule, and (c) at least some of that portion of P2 which is not present in 186 may be in the middle of the molecule.

B. Binding of poly IG to DNA strands

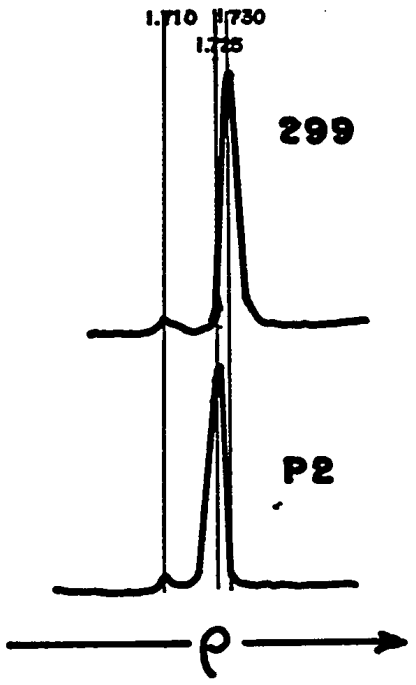
Figure 15a is a densitometer tracing of denatured P2 and 299 DNAs in CsCl spun in the Model E analytical ultracentrifuge. A small amount of undenatured P2 DNA was added to serve as a marker. The density of the P2 strands was calculated to be 1.725 g/cm^3 and that of 299 strands, 1.730. Figure 15b shows that in the presence of poly UG, the two strands of both phages are resolved due to their differences in binding the ribopolymer. In the case of P2, the density of one strand has been shifted by 0.015 g/cm^3, the other by 0.030 g/cm^2. One 299 strand, on the other hand, binds less ribopolymer, and its density is only shifted by 0.010 g/cm^3. The other or "heavy" strand appears to bind as much as its P2 counterpart, since its density is shifted by 0.031 g/cm^3. Poly UG was used in this case because Hradecna and Szybalski (1967) found that it gave slightly better separation of the two strands of λ DNA than did poly IG. They also showed that the strand that bound the most poly UG corresponded to the strand that bound the most poly IG. One of several preparative strand separations made for the present work was done with poly IG, and isolated RNA hybridized to it with the same distribution as to the poly UG preparations so the two polymers appear to have the same effect in the case of P2 as well.

Fig. 15

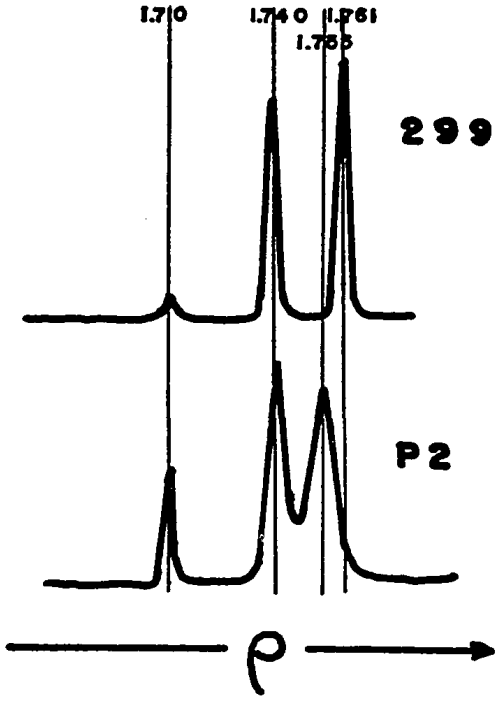
Buoyant Densities of P2 and 299 DNA Strands

Procedure as described in text. Centrifugation at 44,770 RPM, 22° C., 17-24 hr. Native DNA added as marker. a.) without added ribopolymer, and b.) with poly UG at a concentration 2x that of denatured DNA.

a



b



Summers and Szybalski (1968) have found that only one strand of phage T7 DNA binds poly IG. This strand also appears to be the only one transcribed *in vivo* since labelled RNA extracted from phage-infected cells hybridizes to it and not to the other strand. By using deletion mutants of λ, Hradecna and Szybalski (1967) have located the binding sites for poly IG along the different strands, and these appear to coincide with that portion of the molecule which is transcribed *in vivo*. The fact that both strands of P2 bind poly IG suggests that both strands are used for transcription. Since one strand binds more than the other, it probably is transcribed to a greater extent.

C. Hybridization of labelled RNA to separated strands of P2 DNA

All hybridizations to separated DNA strands were carried out with 1-3 μg of DNA, and 0.1 ml. of the isolated RNA. Assays were always done in duplicate. Under these conditions, a three-fold increase in added RNA resulted in a three-fold increase in recovered hybrid. This was true of both light and heavy strands. It was determined that for both strands, hybridization was essentially complete after 10 hrs at 62°C, so hybridization was generally done overnight.

Table 2a shows the results obtained from infection with P2vir$_{22}$. Table 2b is a repeat of that experiment. A virulent mutant was used in order to avoid the complications of lysogeny when studying the vegetative cycle, though this particular mutant was not used further since it was later found to be a deletion mutant which is missing approximately 5% of the DNA and to contain as well, 0.5% of its length as an insertion or inversion as seen by electron microscopy of heteroduplex molecules (Chattoraj and Inman, 1972). It can be seen that at 8 min post-infection, slightly

19 MAY 71 P2 VIR22 INFECT OF C10

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			8 MIN				
LIGHT			364.	4.82	271.	0.22	
HEAVY	0.	119000.	2440.	2.83	2347.	1.97	0.115
BLANK			93.	13.35			
			20 MIN				
LIGHT			3194.	1.71	2981.	2.02	
HEAVY	0.	147000.	41655.	2.46	41442.	28.19	0.071
BLANK			212.	5.79			
			UNINFECTED CONTR				
LIGHT			225.	6.74	138.	0.07	
HEAVY	0.	180000.	513.	2.96	426.	0.23	0.325
BLANK			86.	11.39			

INFECTION IN KAB, 5 MIN. IN REFRIG, 5 MIN. IN 37 INCUB. PULSE TIMES = 8 MIN.,8-10 MIN. CONT,20-22 MIN. 20 MIN,20-22 MIN. HYBRIDIZATION = 10 HR AT 60 DEGREES.

10 MARCH 1972 P2VIR22 WITH TIME

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			7 MIN				
LIGHT			570.	2.45	451.	0.17	
HEAVY	290500.	260750.	4429.	0.62	4311.	1.65	0.104
BLANK			118.	7.17			
			12 MIN				
LIGHT			2046.	1.90	1903.	0.91	
HEAVY	207500.	208750.	30163.	1.35	30020.	14.38	0.063
BLANK			143.	12.58			
			20 MIN				
LIGHT			752.	0.06	702.	1.29	
HEAVY	143050.	54113.	10362.	5.82	10312.	19.05	0.068
BLANK			50.	0.00			
			UNINFECTED CONTR				
LIGHT			222.	54.60	190.	0.39	
HEAVY	330250.	47848.	193.	5.94	161.	0.33	1.179
BLANK			32.	6.25			

INFECTION IN KAB. 5 MIN COLD. 10 MIN AT 37 DEGREES. PULSE TIMES 7 MIN = 7.0 - 9.0 MIN. 12 MIN = 12.0 - 14.0. 22 MIN = 22.0-24.0. HYBRIDIZATION FOR 14.5 HR. AT 64 DEGREES.

more than 2% of the acid-precipitable counts could be recovered as P2-specific hybrid and that both strands are transcribed. The ratio of light to heavy strand transcription is 0.115. At 20 min after infection 30% of the input RNA can be recovered as hybrid. A lower proportion of it is transcribed from the light strand. (Under these conditions, lysis begins at 30 min after infection (see methods)). In this particular experiment, the amount of input RNA hybridized from the uninfected control was higher than usual. (Usually less than 0.03% from the uninfected control hybridized to P2 DNA). This may have been due to incomplete digestion of non-specifically complexed RNA. The amount of input from vials with no DNA (blank) which stuck to the filters was rather high in this experiment also.

The satellite phage P4 has a DNA molecule about 1/3 the size of P2. It appears to code for its own replication, (Lindqvist and Six, 1971), but requires all the known late genes of its helper to assemble phage particles (Six and Lindqvist, 1970). This helper can be present as either a coinfecting phage or as a prophage in a lysogen. In order to get some of what portion of the P2 DNA coded for the light strand-specific RNA, the RNA isolated from a P4 infected lysogen of P2 was hybridized to the P2 separated strands. Since P4 does not need the early genes of P2 it was reasoned that it would only transcribe those (late) genes which it did need. Tables 3 and 4 show the results of two such experiments. (Under these conditions lysis begins at 70 min, and is complete by 90 min). It can be seen that in this case, too, transcription occurs from both strands, and the ratio of light to heavy strand specific RNA is less than 0.10. The total amount of label incorporated as measured by insolubility in cold 5% TCA decreases somewhat as the infection progresses and at the time of lysis, 70 min, the amount of P2-specific RNA has fallen to 3.5% of the

4 JUNE 71, P4 INFECT, C10(P2AMK)

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			50 MIN				
LIGHT			107.	2.04	58.	0.34	
HEAVY	68700.	16641.	1183.	0.07	1133.	6.81	0.051
BLANK			49.	2.41			
			70 MIN				
LIGHT			63.	3.79	13.	0.13	
HEAVY	86300.	9906.	403.	1.44	354.	3.57	0.038
BLANK			49.	10.28			
			INFEC NONLYSOGEN				
LIGHT			59.	2.03	7.	0.00	
HEAVY	245626.	86300.	56.	1.32	4.	0.00	1.489
BLANK			51.	1.83			

INFECTION IN KAB, 5 MIN IN REFRIG, 10 MIN IN 37 INCUB. PULSE TIMES = 50 MIN,52-54 MIN, CONTR,57-59 MIN, 70 MIN,72-74 MIN. HYBRIDIZATION = 15.5 HR AT 62 DEGREES.

11 JUNE 71 P4 INFECT, C10(P2AMK)

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			30 MIN				
LIGHT			703.	1.90	654.	1.04	
HEAVY	148534.	62511.	8971.	2.50	8922.	14.27	0.073
BLANK			49.	2.44			
			50 MIN				
LIGHT			535.	3.49	492.	0.56	
HEAVY	86784.	86537.	10776.	0.30	10733.	12.40	0.045
BLANK			42.	9.02			
			UNINF LYSOGEN				
LIGHT			133.	21.35	74.	0.10	
HEAVY	155589.	68640.	73.	16.90	14.	0.02	5.192
BLANK			58.	6.55			

INFECTION IN KAB, 5 MIN COLD,10 MIN WARM. PULSE TIMES = 30 MIN,30-32 MIN, CONTR,40-42 MIN, 50 MIN,50-52 MIN. HYBRIDIZATION = 17 HR AT 62 DEGREES.

input. (it was found that under the conditions used here, 97% of the bacteria in the culture were "infective centers", that is produced plaques when plated on a lawn of sensitive cells). It would seem that the phage began to slow down its RNA production without affecting the bacterial RNA synthesis to the same extent.

P2 has two "early" genes A and B, both of which are necessary for phage DNA replication (Lindahl, 1970). It was reasoned that if one of these genes were responsible for early transcription, it could block replication in that manner since the replication of several organisms appears to require a prior transcription event: e. g. (Dove et al., 1969; Dove et al., 1971), M 13 (Brutlag et al., 1971; Wickner et al., 1972), E. coli (Lark, 1972), plasmid 35 in Salmonella pullorum (Kline, 1972). Table 5 shows the results of one experiment designed to test this. It can be seen that both mutants make significant quantities of P2 specific RNA. In both cases the amount is reduced by a factor of 30, and the light to heavy strand ratio is slightly greater than that of the control.

Bjorn Lindqvist, working with Kjel Bøvre in Norway, has also worked on P2-specific transcription. His results in general verify some of the results obtained here though his methods of isolation and purification of RNA is quite different. Also, his hybridization efficiencies are 2 - 3 times lower than mine. The reason for this last discrepancy has not been determined as yet.

Lindqvist (Lindqvist and Bøvre, submitted to Virology) did a detailed time-course study of infection by P2$vir_1$1 (This mutant was also used in the present study after the initial experiments with P2vir_{22}. The vir_1 marker is a point mutation in the gene coding for the immunity repressor).

21 JUNE 71 P2 A&B AMBER MUTANTS

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID.	PCT INPUT IN HYBRID	L/H RATIO
			VIR1A81				
LIGHT			309.	3.52	234.	0.07	
HEAVY	254100.	324393.	3577.	1.65	3503.	1.07	0.066
BLANK			74.	7.57			
			VIR1B118				
LIGHT			875.	2.94	677.	0.12	
HEAVY	266600.	531200.	6509.	1.11	6311.	1.18	0.107
BLANK			198.	1.15			
			CONTROL P2VIR1				
LIGHT			6274.	0.05	6007.	1.77	
HEAVY	193600.	339099.	108607.	0.99	108340.	31.94	0.055
BLANK			267.	0.33			

INFECTION IN KAB, 5 MIN COLD, 10 MIN WARM. PULSE TIMES = AMA,12-15 MIN, AMB,12.5-15.5 MIN, VIR1,13-16 MIN. HYBRIDIZATION = 18 HR AT 62 DEGREES.

He administered 2 min pulses at 8 different times during the course of infection and found that the percent of input RNA hybridizing to the heavy strand rose from 1.6% in the 0-2 min interval to around 9% from the 12-14 min interval until lysis some 18 min later. The ratio of light strand to heavy strand hybrid remained essentially constant throughout the course of the infection. This is an interesting observation. If the analogy with λ holds, (clustering of genes for different functions, different clusters expressed at different times, early genes having a lower GC content than late genes), P2 must be expressing some functions from the light strand early in infection and some others at a different place at a later time. The λ late genes, on the other hand, are all transcribed from the heavy strand. Light strand transcription is primarily from regions of relatively low GC content. Inman and Bertani (1969) found one region of early denaturing material at approximately 47 - 53% from the right-hand end of P2.

There are some amber mutants of P2 which are strongly polar over several other genes among those responsible for head and tail formation. One of these is am137, a mutation in gene P which is polar on gene Q. Another is am71, a mutation in gene O which is polar on genes N, M, L, K, R and S (see Figure 2 for genetic map and polarity groups). One of the characteristics of a polar mutation is that recoverable mRNA downstream from the mutation is reduced (Imanoto and Yanofsky, 1967a, and b; Contesse et al., 1966). In order to ascertain which strand was being transcribed to produce the products of the genes mentioned, the RNA isolated from non-permissive cells infected with these mutants was studied. (Both mutants also carried the vir_1 marker to reduce complications due to the lysogenic response). Table 6 shows the results of one experiment. Three

15 JULY 71 P2 VIR1,AMS 071, P137

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			VIR1				
LIGHT			2810.	2.21	2612.	1.55	
HEAVY	267771.	168238.	47199.	3.12	47000.	27.93	0.055
BLANK			198.	0.28			
			VIR1 AMO				
LIGHT			6557.	2.17	6290.	3.32	
HEAVY	241060.	189321.	60931.	0.98	60665.	32.04	0.103
BLANK			266.	1.71			
			VIR1 AMP				
LIGHT			2318.	4.36	2044.	1.09	
HEAVY	247649.	186600.	76732.	3.48	76459.	40.97	0.026
BLANK			273.	12.83			

INFECTION IN KAB, 5 MIN COLD, 10 MIN WARM. PULSE TIMES = VIR 1 = 12.0-15.0 MIN. AMO = 12.5-15.5 MIN. AMP = 13.0-16.0 MIN. HYBRIDIZATION TIME = 19 HR AT 62 DEGREES.

others were done. The average light to heavy ratio exhibited by the vir_1 control was 0.046 with a spread of from 0.042-0.055. The average ratio obtained from the amber mutant in P was 0.026 with a spread of 0.018-0.033. The reduction in light strand hybrid with the P mutant indicates that P and Q are transcribed from this strand. The light to heavy ratio found with the O mutant, on the other hand, was 0.092 with a spread of 0.087-0.103. The reduction in heavy strand hybrid relative to light strand hybrid indicates that the O operon is transcribed from the heavy strand. The fact that the shift is greater in the case of the O mutant probably reflects the fact that the operon appears to be longer.

In order to gain more information on the location of possible "early" light strand material, a deletion mutant of P2 was obtained from Professor G. Bertani. This mutant lacks 8% of the wild-type DNA from the extreme right end of the molecule, though the cohesive ends are preserved (Chattoraj and Inman, 1972). It was reasoned that since the light strand material comprises only5% of the total hybridizeable RNA, it could all be contained in the deleted section of the Del_1 mutant. Tables 7a and 7b show that light strand material relative to heavy strand is lightly higher at 7 min after infection than in the case of the vir_1 mutant (taken to be the control). The slight relative increase could well be due to the absence of some heavy strand material normally present. Figure 16 shows the position of both the vir_{22} deletion and the Del_1 deletion. Recalling that the vir_{22} deletion also gave a higher light to heavy ratio at the early time, it may now be stated that the material which is transcribed from the light strand at early times after infection does not come from the C region (vir_{22} is known to delete gene C and to be pheno-typically $\underline{int}^-$ - G. Bertani, Personal communication), nor from the far right end of the DNA

14 MAR 72 P2VIRIDEL1 WITH TIME

	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			8 MIN				
LIGHT			729.	10.21	515.	0.47	
HEAVY	251500.	108000.	10108.	13.90	9894.	9.16	0.052
BLANK			214.	2.09			
			13 MIN				
LIGHT			2183.	3.02	1940.	2.15	
HEAVY	190000.	89954.	31653.	0.80	31410.	34.91	0.061
BLANK			243.	4.52			
			22 MIN				
LIGHT			491.	1.62	355.	1.01	
HEAVY	111500.	34978.	7396.	0.79	7260.	20.75	0.048
BLANK			136.	16.17			
			UNINFECTED CONTR				
LIGHT			97.	3.09	69.	0.23	
HEAVY	297000.	29438.	223.	8.27	195.	0.66	0.352
BLANK			28.	3.57			

INFECTION IN KAB. 5 MIN COLD. 10 MIN AT 37 DEGREES. PULSE TIMES = 8 MIN=8.0 - 10.0. 13 MIN=13.0 - 15.0. 22 MIN=22.0 - 24.0 MIN AFTER DILUTION. HYBRIDIZATION FOR 16.0 HR AT 64 DEGREES

24 MAR 72 P2VIR1DEL1 WITH TIME

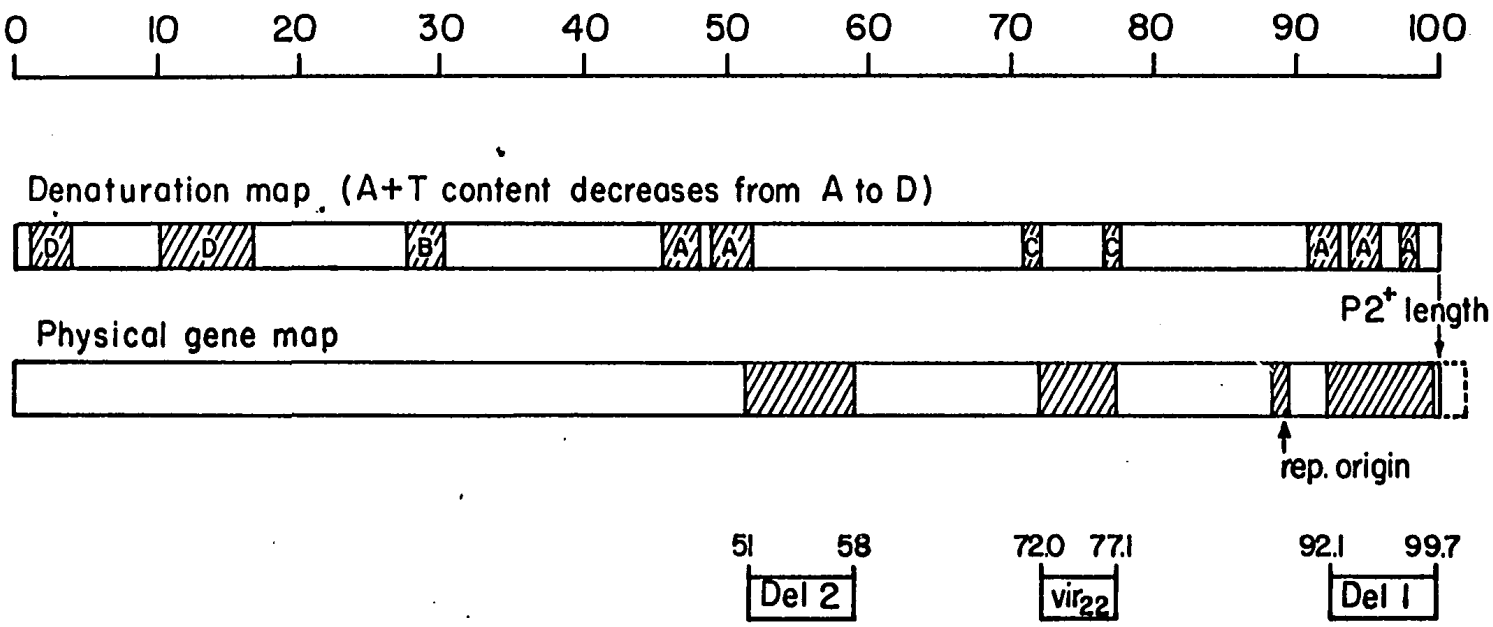
	CPM INCORP	CPM IN MESSAGE	CPM ON FILTER	PCT SPRD OF DUPS	CPM IN HYBRID	PCT INPUT IN HYBRID	L/H RATIO
			7 MIN				
LIGHT			296.	2.52	245.	0.15	
HEAVY	244000.	157500.	3255.	2.28	3204.	2.03	0.076
BLANK			51.	0.97			
			12 MIN				
LIGHT			1199.	3.25	1147.	1.10	
HEAVY	167000.	104000.	18998.	5.22	18946.	18.21	0.060
BLANK			51.	6.79			
			22 MIN				
LIGHT			255.	7.63	222.	1.10	
HEAVY	57275.	20110.	3740.	4.81	3706.	18.43	0.059
BLANK			33.	7.46			
			UNINFECTED CONTR				
LIGHT			79.	3.79	49.	0.14	
HEAVY	223000.	34695.	186.	0.53	156.	0.45	0.316
BLANK			29.	8.47			

INFECTION IN KAB. 5 MIN COLD. 10 MIN AT 37 DEGREES. PULSE TIMES= 7 MIN=7.0 - 9.0. 12 MIN=12.0 - 14.0. 22 MIN=22.0 - 24.0 MIN AFTER DILUTION. HYBRIDIZATION FOR 15.5 HR AT 64 DEGREES.

Fig. 16

Physical Map of P2 DNA

Top line represents location in per cent of distance from left end. Middle line represents characteristic denaturation sites as described by Inman and Bertani (1969). Bottom line marks off that DNA deleted in the vir_{22} and Del_1 mutants (Chattoraj and Inman, 1972), and the Del_3 mutant (Chattoraj, Pers. Comm.).


0
10
20
30
40
50
60
70
80
90
100
Denaturation map (A+T content decreases from A to D)
D
D
B
A
A
C
C
A
A
A
P2+ length
Physical gene map
rep. origin
51
58
Del 2
72.0
77.1
vir22
92.1
99.7
Del 1

molecule.

D. Hybridization of mRNA to sheared, separated halves of P2 DNA

P2vir$_1$ was used to infect non-lysogenic cells. At 7, 12, and 22 min after growth was started by diluting into pre-warmed medium, 2 min pulses of ^{3}H-uridine were administered, after which growth was terminated. The recovered RNA was hybridized to sheared separated halves as described in the Methods section. The results are shown in Table 8. It can be seen that transcription originates primarily from the right half of the molecule early in infection. Some transcription appears to originate from the left half, though, since the upper limit of cross-contamination estimated does not exceed the amount of the discrepancy, and it would have to compete with the much larger amount of homologous DNA on the right half filter.

As the infection procedes, more RNA hybridizes to the P2 DNA, and the bias shifts towards more transcription from the left half. Finally, late in infection (22-24 min under conditions in which lysis begins at 30 min), most transcription originates from the left half. This would be the expected course of events if the genetic and physical maps were correctly oriented and those genes called "early" were truly early. No evidence of a shut-off of "early" genes can be gleaned from this data, however, since the absolute amount of input RNA which hybridizes to the right half increases during infection. There is some evidence that the products of the genes A and B function throughout infection (Lindahl, in preparation), though the synthesis of B gene product declines and does not appear to be made late (J. Levy in preparation).

In order to more closely determine the amount of transcription late in infection which is attributable to genes other than those responsible

Table 8

Time Course of Right and Left Half Transcription

After $P2vir_1$ Infection

Time, min.	% Input in Right Half	% Input in Left Half	Right/Left
7	0.64	0.12	5.2
12	1.7	1.4	1.2
22	3.1	9.0	0.30

(average of 2 experiments)

for particle construction, cells were infected with P2vir$_1$amF$_4$ and were pulsed from 20–22 min after infection. This phage bears an amber mutation in gene F which is polar over the entire FETUD operon. There exists a deletion mutant, Del$_2$ which is deleted from 51–58% from the left end of the physical map. Genetically, it is fun$^-$ (Chattoraj, Personal communication). Since the fun gene maps between the WJHG operon and the FETUD operon, FETUD must be on the right half of the DNA molecule. (The fact that the ratio of light strand to heavy strand transcription in this case was 0.090 vs. 0.063 for the P2vir$_1$ control demonstrates that the mutation does eliminate some hybridizeable heavy strand RNA). Care was taken to ensure that the pulses were administered to the mutant and control-infected cells at exactly the same time after growth was started. The results are given in Table 9. In the amF$_4$ infection, 28% of the total hybridizeable RNA is right half specific. In the vir$_1$control, 37% of the total hybridizes to the right half. If there is a maximum of 10.9% contamination of the right half sample with left half material, and a 4.9% overlap due to the presence of long molecules (see Methods, p.31), then there is at most 16% left half material on the right half filter. This must compete with an amount of DNA on the left half filter which equals that on the right half filter, and so could be expected to hybridize with 16% as much left half message as the left half filter. If the amber mutation reduces recoverable (FETUD) transcription to less than 10% of its normal quantity, and the amount of left half specific material on the right half filter is 16% of that on the left half filter, then (477–0.66 x 1210/1687 x 100) or 17% of the total P2 specific RNA must come from right half not part of the FETUD operon at this late stage of infection.

Table 9

Hybridization to Separated Halves

20 May 72 P2amF4, P2vir$_1$

	Input CPM x 10^{-5}	Left Half	Right Half	Blank	net L	Net R	R/L
amF4	0.36	1250	517	40	1210	477	0.39
vir$_1$	0.17	814	490	33	781	457	0.59
unif. cont.	0.60	47	44	61	-	-	-

Infection at a m.o.i. of 7 followed the usual protocol. The cells were pulsed for 2 min. starting exactly 20 min. after dilution into warm medium. Values listed are CPM and are the averages of 2 10 min. counts of duplicate samples. Maximum spread between duplicates was 10%.

In order to ascertain whether the products of the genes A and/or B were necessary for the shift from early to late gene transcription, cells were infected with those amber A and B mutants which had been used previously. They were pulsed for three minutes with ^{3}H-uridine at 22 min. after dilution into warm medium. Unfortunately, some contaminant was present: after 42 hr. at 64°C., the hybridization fluid was bright yellow. Hybridization efficiency was very low. (Table 10, experiment 1) (Recoverable hybrid would have been low in any case, since A and B mutants do not make much P2 specific mRNA - see p. 39, this work). One of the controls in this experiment was P2vir$_1$ infected cells, and it can be seen (Table 10, line 3) that despite the low hybridization efficiency, the shift from right to left half transcription had taken place. Neither the A mutant nor the B mutant appears to effect this shift though the low hybridization efficiency and consequent low number of counts above background precludes any further quantitation.

All solutions were discarded, new solutions made up and autoclaved in the usual fashion. This time cells infected with the mutants were pulsed for three minutes starting at 22 minutes and then 57 minutes (different culture) after dilution into warm medium. This was done to check the possibility that the mutants did have the capability of shifting transcription, but that the shift was delayed. Once again the hybridization fluid was bright yellow. This time the counts on the filters were higher, but the blank filters also retained a large number of counts. This high blank could have been due to inefficient digestion of non-hybridized RNA, so the filters were removed from the counting vials, blotted on filter paper, and treated again with the usual amount of RNAase (from a different container) for the usual amount of time. (the counting fluid

Table 10

Left and Right Half Transcription
by Mutants in Genes A and B

PHAGE	INPUT	LEFT	RIGHT	BLANK	net L	net R	R/L
			Experiment 1.				
amA81	0.75	94	162	56	38	106	2.8
amB116	0.59	198	554	54	144	500	3.5
vir_1	0.19	723	438	35	688	403	0.6
uninf.	0.45	54	47	48	-	-	-
			Experiment 2, first count				
A22min	0.90	637	1006	371	256	635	2.5
B22min	1.18	1700	3381	974	726	2407	3.3
A57min	1.74	839	1389	510	329	879	2.7
B57min	0.87	2338	3162	172	2166	2990	1.4
			Experiment 2, second count				
A22min		333	509	218	115	290	2.5
B22min		701	1906	589	112	1317	11.8
A57min		430	737	274	156	463	3.0
B57min		1218	1168	107	1111	1061	1.0

INPUT is expressed in CPM x 10^{-5}. Others are in CPM, from 10 min. counts of duplicate samples.

in which they had originally been counted was recounted to ensure that none of the radioactivity had eluted into it. None had.) Table 10, Experiment 2, second count shows the effect of this treatment. It did indeed lower the counts on the blank filters, but not to their usual level, and it did not drastically change the right to left ratio except in the case of the B mutant at 22 minutes. What is apparent from the results in the second experiment is that even at 57 minutes after infection, the A mutants do not seem to effect the shift to the left half. Apparently some of the left half is transcribed at all times, though the level of counting on those filters is so low that it could be said to be insignificant. The B mutants, on the other hand, do appear to show a tendency towards a shift: At 22 minutes, the ratio of right to left half transcription is around 3 (ignoring the second count on the 22 minute point in the second experiment - even after a second RNAase digestion, the counts on the blank filters are abnormally high). After 57 minutes the ratio has declined to approximately one.

The interpretation of these results is not simple. The B mutant shift in transcription at 57 minutes could have been due to a slight leakiness of the mutant. If this is true, the evidence would point to the B gene product as the agent responsible for effecting the shift. On the other hand, Lindahl (1970) has demonstrated that gene A mutants are able to complement gene B mutants under some conditions implying that they do produce some B gene product. Why then can they not effect the shift? Perhaps B product cannot act on a genome that is not replicating or has not been replicated, so that even if B product were present, it could not be used. Gene B mutants do replicate slightly (Lindqvist, 1971) but not as actively as phage not mutated in this gene. It may be that

the B product serves a dual function in that its presence in some way stimulates replication as well as late gene transcription. This stimulation may be the "transcription activation" described by Dove et al., (1971), and the B gene product is capable of mediating more efficient "activating" transcription near the replication origin than the bacterial transcription system.

CONCLUSIONS

The three questions posed at the end of the Introduction have been answered at least in a general way. P2 and its relatives do exhibit intramolecular heterogeneity. Thermal denaturation profiles (first derivative curves of hyperchromic shift with increasing temperature) of the three phages examined show very similar profiles at the high GC end, and a lack on the part of 299 and, more particularly 186, of some low GC material. This is consistent with their measured GC composition and with Hanson and Skalka's data on 186 which shows that it is the low GC P2 material which exhibits the least homology with 186.

With respect to correlation of GC content to timing and function of expression, it can be said that late genes are phage particle genes, and these are of high GC content:

a) Schnös and Inman (1971) have placed the replication origin with respect to their denaturation maps at 90% from the left end of the molecule. The origin has not been mapped, but one would expect it to be near the genes concerned with replication, A and B.

b) Chattoraj and Inman have also located the three deletions with respect to one end of the molecule.

c) The data presented here shows that there is a shift in transcription during the course of infection from DNA on the right half to DNA on the left half of the physical map, and

d) The existence of the Del_2 mutant which is <u>fun</u>$^-$ places the FETUD operon on the right half of the genetic map. This work demonstrates that FETUD-specific RNA is transcribed from the right half of the physical map, thus completing the correlation between the genetic and physical maps.

The genes A and B are probably not in a segment with the lowest GC content since they are present in the Del_1 mutant which deletes a segment at that end of the molecule near the vir_{22} mutant which is deleted for C, int, and at least part of att (Calendar, pers. comm.). One of the regions which denatures first is one the right end of the physical map. The Del_2 mutant deletes material in the middle of the molecule. One of the two early-denaturing regions is also in the middle of the molecule though the overlap is not complete. This mutant is phenotypically fun$^-$, the Del_1 mutant is deleted for that gene which restricts the growth of the phage λ. Both of these genes are expressed from the prophage, hence it can be said that those parts of the P2 DNA which are of lowest GC content are transcribed by the (unaltered) bacterial polymerase from a repressed molecule.

There must be some form of regulation of transcriptional expression utilized by P2. Initially, transcription originates primarily from the right half of the molecule. As infection proceeds, more RNA originates from the left half until at 22 minutes, most is coming from the left half. The genes A and B both appear to have an effect on producing this shift since mutations in either gene block it. Both are also involved in replication since mutations in either of these genes induce defective replication.

The data presented by Franke and Ray (1972) demonstrate that the cis-acting gene 1 in ØX174 produces a strand-specific nick in the RFIDNA which is necessary for replication to proceed. If this is the function of the cis-acting gene A in P2, and gene A mutants can supply at least some gene B product but no other phage gene product (Lindahl, 1971), then perhaps the action of these proteins is in some way coupled. It may be

that B product is indeed the transcription activator but it cannot act on a genome that is not replicating or has not been replicated except on a site at or near the replication origin. Or perhaps it cannot begin without the nick produced by gene A, and those replication events which follow cannot take place without a prior transcription event.

The site from which early light strand-specific RNA is made has not been located. It is not in those segments deleted in the vir_{22} and Del_1 mutants. It is probably also not in genes A and B since virulent mutants map between C and B, and probably mark the site of repressor binding. Perhaps a leftward transcription event across the replication origin is the act which triggers replication.

Finally, if gene B product is the transcription activator, it cannot act in the way λ's gene N product appears to; as an anti-terminator. If it did, the genes P and Q would be read from the wrong strand. It seems most likely that it operates at several places, namely at the beginning of each operon in the set coding for the construction of phage particles, and also perhaps at the replication origin.

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